Molecular mechanisms regulating Golgi architecture during the mammalian cell division cycle

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A thesis submitted for the degree of Doctor of Philosophy at the University of London

February 2000

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Abstract

During mammalian M phase vesicular transport ceases and the stacked Golgi ribbon is converted into a disseminated array of tubulovesicular clusters, as part of the process that ensures Golgi inheritance through successive generations. At telophase these tubulovesicular clusters are remodelled to regenerate the Golgi stack. In this thesis, an established cell free system that recreates many of these events has been used to probe the molecular workings of this process.

During disassembly Golgi cisternae unstack, while coat protein I (COPI) vesicle budding in the absence of fusion consumes the cisternal rims, and a COPI independent pathway consumes the cisternal cores. Release of p115, a vesicle tethering protein, from the Golgi membrane at mitosis is shown to contribute to the unstacking process. Rab GTPases are shown to be required for correct disassembly. An assay is established to study the COPI independent pathway.

The regeneration of Golgi cisternae at telophase proceeds via two intersecting pathways controlled by the N-ethylmaleimide sensitive factor (NSF) and p97 ATPases. Stacking of these reassembling cisternae requires Golgi ReAssembly Stacking Protein (GRASP) 65. During reassembly, p115 in conjunction with its two Golgi receptors, GM130 and giantin, is required to stack nascent cisternae, at a stage upstream of GRASP65. The activity of a G protein is also required coincident or downstream of p115. A novel GRASP, GRASP55, also plays a role in cisternal stacking. Phosphorylation of p115 by a casein kinase II like activity is essential for NSF catalyzed cisternal regrowth, but not cisternal stacking, and may strengthen the giantin-p115-GM130 complex. The first coiled-coil domain of p115 can interact with SNARE molecules and is required for NSF catalyzed cisternal regrowth. NSF catalyzed cisternal regrowth does not require the ATPase activity of NSF. Finally, a novel p97/Ufd1p/Npl4p complex is unable to catalyze cisternal regrowth.
For my friends and family (you know who you are).
I was tired again. I tried again.

Stephen Morrissey
Acknowledgements

First of all I would like to thank Graham Warren who has been a constant source of enthusiasm, drive, intellectual challenge and scientific rigour throughout this time. I am also very grateful to Francis Barr for support at crucial times and for perpetual fierce intellectual debate. Barbara Dirac-Svejstrup for support, encouragement and team spirit. Rose Watson and Eija Jokitalo for abundant assistance with EM. Then Joyce Müller, Laurence Pelletier, Hemmo Meyer, Joachim Seemann, Giovanni Lesa, Martin Lowe, Birte Sønnichsen, Carlos Fernández, Nobuhiro Nakamura and Dave Shima for always making the Warren lab an interesting, funny and usually bizarrely existential place to be and for always being up for a pint or eight. The Golgi All-Stars for evolving into the meanest 6-a-side team in ICRF. Then Dave Shima, Giampietro Schiavo and Sharon Tooze for always constructively challenging my work.

Then I need to thank Anna Abbott for help when I first moved up to London. Dougal Woodland, Phil Seagrave, Matty Davis, Nicky Brandon, Benny Maiden, Max Miskella, Sammy Arie, Shona Osborne, Andster Reynolds, Diane Maurice and Jyoti Srivastava for always being able to make me laugh when I needed to.

Paula Hawkins for love and support.

Tanya Carus for love and laughter.

Andrew Balls for unflagging support, pool and friendship during good and (mostly) bad times.

Then finally my parents and sister, who are just brilliant.

Hope to see you all in the colonies.
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Abbreviations

26mer: C-terminal 26 amino acids of p115 peptide
75mer: C-terminal 75 amino acids of p115 peptide
AAA: ATPases associated with diverse cellular activities
AMBA: Acrylamide, bis-Acrylamide (30:0.8)
AP: Adaptor protein
APS: Ammonium persulphate
ARF1: ADP ribosylation factor 1
BAPTA: O, O’-Bis(2-aminophenyl) ethyleneglycol-N, N, N’, N’- tetraacetic acid
BARS-50: BFA-induced ADP-ribosylated substrate of 50kDa
BFA: Brefeldin A
BiP: Binding protein
BSA: Bovine serum albumin
CoA: Coenzyme A
CHO: Chinese hamster ovary
CKII: Casein kinase II
COP: Coat protein
CGN: cis-Golgi network
DMSO: Dimethylsulphoxide
DRB: 5,6-Dichloro-1-D-ribofuranosylbenzimidazole
DTT: Dithiothreitol
ECL: Enhanced Chemi-Luminescence
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethylene glycol bis (β-aminoethyl ether) N, N, N’, N’-tetraacetic acid
EEA1: Early endosome antigen 1
EM: Electron microscopy
ER: Endoplasmic reticulum
ERK: Extracellular signal regulated kinase
ERGIC: ER-Golgi intermediate compartment
EST: Expressed sequence tag
FRAP: Fluorescence recovery after photobleaching
GalNAc: N-acetylgalatosamine
GalNAc-T: N-acetylgalactosamine:polypeptide N-acetylgalactosaminytransferase
GalT: β-1,4-galactosyltransferase
GAP: GTPase activating protein
GDI: Guanine nucleotide dissociation inhibitor
GDF: GDI displacement factor
GEF: Guanine nucleotide exchange factor
GEF-CK: Golgi-enriched-fraction casein kinase
GFP: Green fluorescent protein
GlcNAc: N-Acetylglucosamine
GM130: Golgi matrix protein of 130kDa
GMAP-210: Golgi microtubule-associated protein of 210kDa
GOS28: Golgi SNARE of 28kDa
GRASP55: Golgi ReAssembly Stacking Protein of 55kDa
GRASP65: Golgi ReAssembly Stacking Protein of 65kDa
GSK3: Glycogen synthase kinase 3
GST: Glutathione S-transferase
Gtn448: N-terminal 448 amino acids of giantin
Gtn1967-2541: Amino acids 1967-2541 of giantin
H: Head domain of p115
Hepes: N-[Hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid]
HRP: Horseradish peroxidase
HTA: Head, tail and acidic domain of p115.
IQ: Illimaquinone
LMA1: Low molecular weight activity 1
LPA: Lysophosphatidic acid
mAb: Monoclonal antibody
Man: Mannose
Mann I: α-1,2-mannosidase I
Mann II: α-1,3-1,6-mannosidase II
MAP: Microtubule associated protein
MEK1: MAP kinase/ERK kinase 1
MGF: Mitotic Golgi fragments
MW: Molecular weight
N73pep: N-terminal 73 amino acids of GM130 peptide
NADH: Nicotinamide adenine dinucleotide
Abbreviations

NAGTI: β-1,2-N-acetylglucosaminyltransferase I
NEM: N-ethylmaleimide
NSF: NEM sensitive factor
Npl4p: Nuclear protein localization protein 4
NRK: Normal rat kidney
p115 728-765: peptide comprising amino acids 728-765 of p115
p115 788-827: peptide comprising amino acids 788-827 of p115
p115 843-930: peptide comprising amino acids 843-930 of p115
PA: Phosphatidic acid
PBS: Phosphate buffered saline
PC: Phosphatidylcholine
PC12: Rat Pheochromocytoma
PDI: Protein disulphide isomerase
PH: Pleckstrin homology
PI-4-OH: Phosphatidylinositol-4-OH
PIP₂: Phosphatidylinositol bisphosphate
PITP: Phosphatidylinositol transfer protein
PKD: Protein kinase D
PLD: Phospholipase D
PP1: Protein phosphatase 1
PP2A: Protein phosphatase 2A
PTA: Phosphotungstic acid
RACE: Rapid amplification of cDNA ends
rER: Rough endoplasmic reticulum
RLG: Rat liver Golgi membranes
SDS: Sodium dodecyl sulphate
SDS-PAGE: SDS-polyacrylamide gel electrophoresis
SEM: Standard error of the mean
sER: Smooth endoplasmic reticulum
sHeLa: Spinner HeLa
SialylT: α-2,6-Sialyltransferase
SNAP: Soluble NSF attachment protein
SNARE: SNAP receptor
SRP: Signal recognition particle

t-SNARE: target SNARE

TA: Coiled coil Tail and acidic region of p115

TCA: Trichloroacetic acid

TEMED: N, N, N', N'-Tetramethyl-ethylenediamine

TGN: *trans*-Golgi network

TIP47: Tail interacting protein of 47 kDa

TMD: Transmembrane domain

TRAPP: Transport protein particle

Tris: 2-Amino-2-(hydroxymethyl)-1,3-propanediol

TX-100: Triton X-100

UfdIp: Ubiquitin fusion degradation protein 1

VAMP: Vesicle associated membrane protein

v-SNARE: vesicle SNARE

VSVG: Vesicular stomatis virus G-protein

VTCs: Vesicular tubular clusters
Publications

Some of the findings presented in this thesis have been published or submitted for publication.


Chapter 1

Introduction
1.1 The Secretory pathway.

1.1.1 The division of labour in eukaryotic cells.

The quintessence of all eukaryotic life is the complex compartmentalization of the cell into a series of highly specialized machines that exist as discrete membrane bound organelles (Alberts et al., 1994). This organization represents a co-operative division of labour, a trait common to many of the major evolutionary transitions (Szathmáry and Maynard Smith, 1995). Such compartmentalization has enabled the creation of highly interdependent, yet diverse and discrete microenvironments within the cell, which are optimized for the specific biochemical reactions which must occur there. The specialized microenvironments of organelles are established and maintained by the flux of ions and small molecules across the demarcating semi-permeable organelle membrane. Coupled to this, vesicle transfers between compartments and signal dependent import systems also serve to regulate organelar microenvironments (Blobel, 1980). Each organelle possesses its own characteristic architecture and unique complement of lipids, proteins and co-factors that enable the achievement of specific functions. The unique and complex architecture of individual organelles is usually a highly conserved phenotype. However, the precise role it plays in the functioning of these highly sophisticated intracellular devices is still unclear. This may be partially due to poor definition of the molecular components and mechanisms responsible for generating and maintaining organelar architecture. Just as structure dictates function at the organismal and multicellular level, so too organelar form and structure must bear upon cellular function. A detailed molecular knowledge of how organelle architecture is maintained will enable precise manipulation of the components that underpin it, and allow one to test functional predictions concerning the significance of architectural phenotypes. The primary aim of this thesis has been to attempt to dissect molecules and molecular mechanisms that underpin the architecture of one particular organelle, the Golgi apparatus.

The approach adopted in this study has been to take advantage of the dramatic changes in Golgi morphology that occur during mammalian M-phase as a part of the process that ensures Golgi inheritance through successive generations (Cabrera-Poch et al.,
1998; Shima and Warren, 1998; Lowe et al., 1998a; Linstedt, 1999). By using a cell free system that recreates many of these events it is possible to biochemically manipulate this process and document the morphological consequences of such manipulations by quantitative electron microscopy (EM; Misteli and Warren, 1994, 1995a; Rabouille et al., 1995b, c, 1998; Barr et al., 1997; Shorter and Warren, 1999; Shorter et al., 1999). As a prelude to this subject I shall first of all outline the structure and functions of the early organelles of the secretory pathway and how the Golgi apparatus fits into this organization (Section 1.1). The principles that govern the secretory pathway and organelle identity within the secretory pathway will then be considered (Section 1.2). Central to this is the process of vesicle transport and the current molecular view of this process will be presented (Section 1.3). I shall then give a more detailed description of the structure of the Golgi apparatus, how this may relate to its function, and candidate molecules that may be responsible for this architecture (Section 1.4). Finally, I shall arrive at the attempts that have been made to delineate the molecular mechanisms that are responsible for the maintenance and establishment of this morphology. Culminating with what is known so far about the mammalian mitotic processes of Golgi disassembly and reassembly as revealed by in vivo studies and the cell free system (Section 1.5).

1.1.2 Organelles of the secretory pathway.

The intracellular route typically followed by newly synthesized proteins in the lumen of the endoplasmic reticulum (ER), to their ultimate cellular destination is referred to as the classical secretory pathway. This route was first charted by the pioneering studies of Caro and Palade who utilized autoradiography and EM to track the progress of metabolically labelled proteins from the ER to the lumen of the acini where they were secreted (Caro and Palade, 1964). The secretory pathway comprises a series of functionally distinct organelles that sort, distribute and post-translationally modify secretory proteins in transit (Figure 1.1). Membrane flow between these compartments proceeds via transport vesicles, which bud from a donor compartment, and are targeted to a specific acceptor compartment with which they fuse and release their contents (Rothman, 1994). The journey begins in the ER where newly synthesized protein is
Figure 1.1 Organelles and events of the exocytic secretory pathway.
Secretory cargo is translocated into the ER and modified and folded correctly prior to export via COPII vesicles, which subsequently fuse to generate VTCs. VTCs deliver cargo to the cis-Golgi network (CGN), and cargo then proceeds through the stack of cisternae where it matures, receiving extensive post-translational modification. Upon arrival at the trans-Golgi network (TGN) cargo is sorted to either endosomes, lysosomes, plasma membrane or secretory granules as is appropriate. Retrograde transport via COPI vesicles salvages ER residents and recycles components of the transport machinery. Adapted from Mellman and Warren (2000).
inserted into the membrane or translocated into the lumen, folded and oligomerized correctly (Helenius, 1994), and then rapidly exported (Quinn et al., 1984). After export, secretory cargo is sorted and concentrated in vesicular-tubular clusters (VTCs) that migrate toward the juxtanuclear region in a microtubule dependent manner (Scales et al., 1997; Presley et al., 1997). Then at the heart of the secretory pathway lies the edifice of the Golgi apparatus, the organelle of focus in this thesis. The Golgi apparatus receives virtually the entire output of de novo synthesized polypeptides from VTCs at the cis-Golgi network (CGN), and functions to post-translationally modify them in the stack, and sort them to their final cellular destinations at the trans-Golgi network (TGN), which may be endosomes, lysosomes, secretory granules or the plasma membrane.

1.1.3 The endoplasmic reticulum (ER).

The ER exists as a complex, three dimensional mesh like labyrinth of branching tubules and occasional cisternae that are in continuity with the nuclear envelope and extend throughout the entire cytoplasm (Alberts et al., 1994). In a typical animal cell the ER is the largest organelle and typically contributes over 50% of the total cellular membrane (Weibel et al., 1969). This reticular network of branching three-way junctions is highly dependent on microtubules (Terasaki et al., 1986; Lee et al., 1989), which may be anchored directly to the ER membrane by the integral ER membrane protein p63 (Klopfenstein et al., 1998). Furthermore, visualization of ER membrane dynamics in real time illustrates that this steady state ER architecture is actually maintained by a dynamic balance between tubule extension, fusion and fission (Lee and Chen, 1988; Dabora and Sheetz, 1988; Allan, 1995; Lane and Allan, 1999). ER tubule motility seems to be regulated by both microtubule motors and dynamics (Waterman-Storer et al., 1995; Waterman-Storer and Salmon, 1998).

The ER may be subdivided into two functionally distinct domains: the rough ER (rER) and smooth ER (sER). The surface of the rER is studded with ribosomes, which are absent from the sER, which is also more tubular in nature. However, these domains are physically connected as revealed by the ability of the transmembrane vesicular
stomatis virus G-protein (VSVG) and the soluble ER resident BiP to freely diffuse between them (Bergmann and Fusco, 1990). Other proteins are more restricted to either the rough or the smooth domains. For instance, ribophorin I, II and the signal recognition particle (SRP) are found in the rER (Hortsch and Meyer, 1985), while epoxide hydrolase is predominantly found in the sER (Galteau et al., 1985). Certain drugs also reveal the distinct nature of the sER relative to the rER. For example, ilimaquinone (IQ), a sea sponge metabolite which induces Golgi vesiculation (Takizawa et al., 1993; Section 1.4.7), also dismantles the sER, but has no obvious effect on rER morphology (Wang et al., 1997).

The rER is the site of entry for proteins into the secretory pathway. The vast majority of proteins are inserted or translocated across the ER membrane through an aqueous ER translocon pore complex co-translationally in SRP dependent style (Blobel and Dobberstein, 1975; Walter and Johnson, 1994). Capture of nascent polypeptides by SRP requires a signal sequence, which classically is located at the N-terminus of proteins and contains at least six core hydrophobic amino acids (Nothwehr and Gordon, 1990). This sequence may be cleaved off after translocation or may serve to anchor proteins in the membrane. Alternatively, the cytosolic chaperones Hsp70 and eukaryotic DnaJ homologues may deliver completely synthesized proteins to the ER after their release from ribosomes (Hansen et al., 1986; Waters and Blobel, 1986). These two pathways are functionally redundant, and as a consequence provide a fail-safe mechanism of translocation, in case one pathway is compromised (Hann and Walter, 1991; Ogg et al., 1992). Once in the lumen of the ER, the high concentration of molecular chaperones ensures newly synthesized secretory and membrane proteins are deterministically folded, post-translationally modified and oligomerized correctly prior to shipping out of the ER (Stevens and Argon, 1999). The folding of proteins can be extremely rapid, molten globule intermediates can be achieved in milliseconds for some proteins, but post-translational modification and further folding can take several minutes or more.
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Misfolded or unassembled proteins are retained in the ER or a pre-Golgi compartment and are eliminated by a process which requires their retrograde translocation through the aqueous ER translocon pore complex from the ER lumen to the cytosol, followed by their hydrolysis by the ubiquitin-proteasome machinery (Plemper and Wolf, 1999). Such a quality control system prevents a lethal aggregation of misfolded protein. Once correctly folded, proteins are rapidly exported from the ER (Quinn et al., 1984), yet examples of proteins are known which continue to fold and assemble in more distal compartments. For instance, connexin 43 oligomerizes in the Golgi apparatus (Musil and Goodenough, 1993), while hepatitis B surface antigen assembles in the VTCs (Section 1.1.4; Huovila et al., 1992).

The sER in the majority of cells is scant, and usually comprises only a small region of the ER, termed the transitional elements, where the secretory cargo is packaged into coat protein (COP) II coated vesicles. The transitional elements appear as an assembly of blind ending tubules extending from the ER from which COPII vesicles bud (Palade, 1975; Saraste and Kuusmanen, 1984; Bannykh et al., 1996; Bannykh and Balch, 1997). The sER is extremely prominent in certain specialized cells where it performs additional functions. For example, as the sarcoplasmic reticulum of muscle cells (Alberts et al., 1994) or as the site of very low density lipoprotein synthesis in hepatocytes (Alexander et al., 1976).

1.1.4 The ER-Golgi intermediate compartment (ERGIC) or vesicular tubular clusters (VTCs).

Export of secretory cargo occurs at the transitional elements of the sER from which COPII vesicles bud and then coalesce to evolve pleiomorphic tubulovesicular clusters (Saraste and Svensson, 1991; Plutner et al., 1992; Krijnse-Locker et al., 1994; Stinchcombe et al., 1995). These ER exit sites occur in direct apposition to the cis-Golgi as well as in the cell periphery (Whaley, 1975; Farquhar and Palade, 1981; Scales et al., 1997). Ultrastructurally these clusters are particularly prominent in a number of glandular cells such as the pancreatic acinar cells (Merisko et al., 1986), plasma cells (Rambourg et al., 1989), prolactin cells (Rambourg et al., 1992), and mucous cells of
Brunner’s gland (Rambourg et al., 1987). They appear as a collection of small vesicles, and tubules, as well as polygonal meshes of tubular network, and are found between ER exit sites and the cis-aspect of the Golgi apparatus. Transport of these relatively large clusters from the cell periphery to the cis-Golgi occurs by a saltatory microtubule motor dependent process (Presley et al., 1997; Scales et al., 1997). Collectively, these membranes have been termed the ER-Golgi intermediate compartment (ERGIC; Hauri and Schweizer, 1992), intermediate compartment (Lotti et al., 1992), salvage compartment (Warren, 1987), tubulovesicular transport complexes (TCs; Scales et al., 1997; Shima et al., 1999) or vesicular tubular clusters (VTCs; Balch et al., 1994), and represent a pre-Golgi compartment proposed to concentrate and sort secretory cargo (Balch et al., 1994; Martinez-Menárguez et al., 1999; Shima et al., 1999; Warren and Mellman, 1999). The term VTC will be used in this thesis. VTCs accumulate and increase in size in response to viral infection or reduced temperatures (e.g. 15°C), presumably due to a rate limiting step in membrane transport through these intermediates (Saraste and Kuismanen, 1984; Lotti et al., 1992; Krinse-Locker et al., 1994; Bannykh et al., 1996). Other proposed functions for VTCs include the formation of disulphide bonds required for correct protein folding (Huovila et al., 1992), antigen attachment to MHC class I (Kleijmeer et al., 1992), protein palmitoylation (Bonatti et al., 1989), and the initial steps of O-glycosylation (Tooze et al., 1988; Krijnse-Locker et al., 1994).

Biochemically, VTCs have a distinctive protein composition relative to the ER and Golgi, and can be separated from them by isopycnic centrifugation (Schweizer et al., 1991, 1994; Lahtinen et al., 1992; Hammond and Helenius, 1994). Characteristic VTC markers include: the lectin ERGIC53 (Schweizer et al., 1988) which functions as a cargo transport receptor (Moussalli et al., 1999; Appenzeller et al., 1999); the KDEL-receptor (Tang et al., 1993; Griffiths et al., 1994) which functions to retrieve soluble escaped ER residents which contain a C-terminal KDEL sequence (Semenza et al., 1990; Lewis et al., 1992); Rab1a (Tisdale et al., 1992) which may be required for a late targeting/fusion step controlling the delivery of cargo to Golgi compartments (Pind et al., 1994); Rab2 (Chavrier et al., 1990) which may regulate COPI association and
vesicle budding from VTCs (Tisdale and Balch, 1996; Tisdale and Jackson, 1998; Tisdale, 1999); the 42kDa form of syntaxin-5 (Hui et al., 1997; Hay et al., 1998), a t-SNARE; p115, a vesicle tethering protein (Nelson et al., 1998; Alvarez et al., 1999); COPI coat complex at a late VTC stage (Scales et al., 1997; Shima et al., 1999); and the COPII coat complex at an early VTC stage (Scales et al., 1997). However, it is important to note that possibly due to the extensive bidirectional vesicle flux between the VTC and both the ER and Golgi (Aridor et al., 1995) none of these markers is unique to the VTC, but may also be found associated with either the Golgi apparatus and/or the ER. As a result of this the membrane boundaries between ER, VTCs and cis-Golgi are often blurred, and so whether the VTC represents a bona fide compartment remains controversial. This is part of a general problem in defining organelle boundaries in the secretory pathway since it may only be possible to define compartments as intermediates in a continuous phenomenon of transformation or maturation (Helenius et al., 1983; Glick and Malhotra, 1998). To illustrate this EM has revealed direct connections between VTCs and the ER (Hauri and Schweizer, 1992; Clermont et al., 1994; Krijnse-Locker et al., 1994; Stinchcombe et al., 1995) as well direct connections to the cis-Golgi (Tang et al., 1993; Griffiths et al., 1994), suggesting VTCs may represent specialized subdomains of the ER and/or the cis-Golgi, or a transport intermediate that emerges from the ER and later fuses with the cis-Golgi (Saraste and Svensson, 1991; Scales et al., 1997).

Visualization of VTC dynamics in real time in living mammalian cells has greatly advanced our understanding of this compartment (Presley et al., 1997; Scales et al., 1997; Shima et al., 1999), especially in parallel with high resolution quantitative EM studies (Martinez-Menarguez et al., 1999). Tracking of a chimera of a temperature sensitive form of VSVG and green fluorescent protein (ts-VSVG-GFP) reveals two distinct stages in the lifetime of a VTC. In the first stage newly synthesized ts-VSVG-GFP concentrates in COPII coated structures in the ER, which coalesce to form larger VTC structures that hover as though subject to Brownian motion (Scales et al., 1997). In the second stage, COPI associates with these structures, gradually replacing COPII, and this replacement facilitates an association with microtubules (Scales et al., 1997).
These structures, coated solely with COPI, then proceed *en masse* towards the Golgi apparatus, utilizing microtubules and the minus-end directed motor complex of dynein/dynactin (Scales et al., 1997; Presley et al., 1997). COPI is progressively segregated into domains of the VTC rich in retrograde cargo (e.g. KDEL receptor and Bet1; Martinez-Menarguez et al., 1999; Shima et al., 1999), and in so doing couples the sorting of ER recycling components to the concentration of anterograde cargo in non-COPI coated domains of the VTC (Martinez-Menarguez et al., 1999; Warren and Mellman, 1999). COPI is then most likely to be involved in the retrieval of earlier acting factors to the ER (e.g. chaperones, v-SNAREs, COPII associated proteins, ERGIC-53), from these anterograde moving structures, and this is consistent with the fact that retrieval of ERGIC53 and the KDEL-receptor is blocked by microinjection of Fab fragments of anti-COPI antibodies (anti-EAGE) which block COPI function (Girod et al., 1999; Majoul et al., 1998; Pepperkok et al., 1993). Verification of this will require the covisualization of retrograde cargo and COPI in living cells. Viewed in this light the VTC, rather than being a *bona fide* stable compartment, is more a transient transport intermediate that forms by the coalescence and fusion of COPII vesicles, and once formed requires a COPI step that performs functions crucial for Golgi delivery. Whether VTCs fuse to generate the *cis*-Golgi network, which then matures into a Golgi cisterna as a result of retrieval of components by COPI vesicles, remains unclear.

1.1.5 The Golgi apparatus.

After the VTC stage of the secretory pathway comes the Golgi apparatus, the current subject of diverse and multiple polemics (Glick and Malhotra, 1998; Roth, 1999). The Golgi apparatus was first visualized under the light microscope by application of a specific black reaction to neuronal cells (3-4% copper acetate/sulphate for 1-2 days, followed by 3% potassium bichromate for several days and enhanced by the chromium-silver reaction) devised by Camillo Golgi (Golgi, 1898). This revealed an elaborate reticular structure, which is now eponymous with its discoverer, and whose function Camillo Golgi categorically and very wisely refused to speculate upon (Golgi, 1898). However, this method of staining gave diverse results between cell types, and the structure of the Golgi apparatus was only truly appreciated with the advent of the
electron microscope in the 1950s (Dalton and Felix, 1954), and the formulation of more reliable staining techniques (Novikoff and Goldfischer, 1961).

The Golgi apparatus of mammalian cells is a single copy organelle, which exists as a compact juxtanuclear reticulum. This reticulum consists of a series of discrete, parallel arrays of disk shaped membranes, termed cisternae, that form a stack. These discrete stacks are linked by more tubular connections to generate an extensive ribbon structure. The Golgi stacks are bound on either face by the extensive tubulovesicular networks of the CGN and TGN. Since the architecture of the Golgi apparatus, and in particular the stack of Golgi cisternae, is the immediate subject of this study, a more detailed description is given in Section 1.4.

Biochemically the Golgi apparatus is characterized by its exclusive resident enzymes that catalyze a series of reactions that include: terminal N-glycosylation of glycoproteins (trimming of mannoses and addition of N-acetyl glucosamine, galactose, fucose, and sialic acid) and glycolipids (Kornfeld and Kornfeld, 1985); O-glycosylation of glycoproteins (Clausen and Bennett, 1996); addition of mannose-6-phosphate as a marker for lysosomal enzymes (Pfeffer, 1991); sulphation of proteoglycans/glycosaminoglycans (Silbert, 1996) and tyrosine (Niehrs et al., 1994); proteolytic processing of pro-proteins (Schnabel et al., 1989; Lepage-Lezin et al., 1991; Jung et al., 1993); lipoprotein processing (Kendrick et al., 1998); and sphingolipid biosynthesis (Allan and Kallen, 1993). The extensive glycosylation reactions that occur in the Golgi apparatus necessitates the presence of a range of nucleotide sugar, nucleotide sulphate, and ATP transporters in the Golgi membrane (Hirschberg, 1997). Many of the glycosylation enzymes have been used as general Golgi markers and to define Golgi subcompartments both biochemically and immunocytochemically. However, rigid classification of Golgi subcompartments has proven difficult owing to the considerable overlapping distributions of Golgi enzymes in different cisternae of the stack, coupled to cell type specific variations in the location of a given enzyme within the stack (Roth et al., 1986; Velasco et al., 1993; Nilsson et al., 1993a; Rabouille et al., 1995a; Rabouille and Nilsson 1995; Prescott et al., 1997; Lovelock et al., 1998;
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Röttger et al., 1998). This again probably reflects blurred compartmental boundaries due to considerable vesicle flux between compartments (Glick et al., 1997; Glick and Malhotra, 1998) which itself may differ from cell to cell depending on secretory demand.

However, in the simplest sense it is a possible to view the Golgi apparatus functionally in terms of three compartments (Mellman and Simons, 1992). First, is the cis-Golgi network, which at a minimum is the entry point into the Golgi apparatus for proteins arriving from the ER via VTCs. Second, is the Golgi stack, where the majority of post-translational modifications occur. Third, is the trans-Golgi network, or exit point from the Golgi apparatus, where completely post-translationally modified molecules must be sorted and packaged into appropriate carriers which shuttle them on to their terminal destination.

1.1.6 The cis-Golgi network (CGN).

The CGN probably receives the entire output of de novo synthesized polypeptides from the ER (but see Kunau and Erdmann, 1998) and it may even be that the fusion of incoming VTCs generates this structure (Presley et al., 1997; Pelham, 1997). The CGN is the last major quality control step for misfolded proteins and sorting station for escaped soluble ER residents (Mellman and Simons, 1992). Misfolded ts-VSVG is retrieved from the CGN by virtue of interaction with the molecular chaperone BiP, and does not proceed into the Golgi stack (Hammond and Helenius, 1994). This retrieval is mediated by an interaction between BiP and the KDEL receptor. However, retrieval of KDEL-ligands is also possible from later Golgi compartments (Miesenböck and Rothman, 1995). This is likely to be a rarer event since the KDEL receptor is more concentrated at the cis-side of the stack, but can adopt a more trans-localization in response to cellular stress (e.g. viral infection or heat shock; Griffiths et al., 1994).

The CGN constitutes the cis-most cisterna associated with a diverse array of tubular networks, and is selectively stained by prolonged osmication (Friend and Murray, 1965; Lucocq et al., 1989; Rambourg and Clermont, 1997). The tubular elements of the
CGN are not always in close contact with the whole surface of the Golgi ribbon (Rambourg and Clermont, 1997). However, a recent three dimensional reconstruction of portions of the Golgi apparatus in fast frozen, freeze-substituted NRK cells by dual axis, high voltage EM tomography at 7nm resolution revealed the cis-Golgi to be much less reticular in nature, and more similar to cisternae deeper into the stack (Ladinsky et al., 1999). This discrepancy may be due to previous fixation procedures that induce reticulation of the extreme aspects of the Golgi apparatus by extracting and destroying Golgi membrane proteins (Cunningham et al., 1974; Ladinsky et al., 1999). However, the discrepancy may also be due to the small number of cells sampled in the tomographic study (Ladinsky et al., 1999).

The distinct compartmental nature and membrane dynamics of the CGN relative to the rest of the Golgi stack is also revealed by ATP depletion and brefeldin A (BFA) treatment (del Valle et al., 1999; Lippincott-Schwartz et al., 1989, 1990). Upon depletion of cellular ATP pools by 75-85% the CGN is specifically disassembled and redistributes to the ER via tubular intermediates, while the Golgi stack and TGN remain intact (del Valle et al., 1999). While upon BFA treatment CGN markers, in contrast to most other Golgi enzymes, do not completely redistribute to the ER (Lippincott-Schwartz et al., 1989, 1990), but rather adopt a more ERGIC53, VTC-like localization (e.g. GOS-28 [Subramaniam et al., 1995]; syntaxin 5 and GM130 [Nakamura et al., 1995]; p115 (Nelson et al., 1998); gp74 [Alcalde et al., 1994]; GMAP-210 [Rios et al., 1994; Infante et al., 1999]). These data suggest that many of the components of the CGN may constitutively cycle through this VTC compartment, and possibly the ER as well.

In biochemical terms the CGN can be defined as the first compartment where α-1,2-mannosidase I (Mann I) functions (Balch and Keller, 1986), although Mann I can also be found in later Golgi compartments (Velasco et al., 1993). Similarly, O-glycosylation may commence in the CGN with the action of UDP-N-acetylglactosamine:polypeptide N-acetylglactosaminyltransferases (GalNAc-T) (Roth et al., 1994; Schweizer et al., 1994). However, the site of initiation of O-
glycosylation has also proven controversial, since the GalNAc-T activity of transferring GalNAc to serine and threonine residues, corresponds to a family of at least six different enzymes (Clausen and Bennett, 1996). Three of these enzymes have been cloned, and GalNAc-T1, -T2 are widely expressed while GalNAc-T3 is more restricted to pancreas and testis (Bennett et al., 1996). Each enzyme has a similar, but subtly different substrate specificity and are localized throughout the Golgi stack (Röttger et al., 1998), suggesting O-glycosylation may be initiated at multiple specific sites, which depends on the precise nature of the substrate. Another cis-Golgi modification may be the addition of mannose 6-phosphate to enzymes destined for a lysosomal localization. This reaction proceeds in two steps, first a phosphotransferase transfers N-acetylglucosamine 1-phosphate to one or more mannose residues on lysosomal enzymes, and this phosphodiester is then cleaved by a glycosidase to reveal the mannose 6-phosphate sorting signal (Kornfeld and Mellman, 1989). Although these enzymes have yet to be localized by immuno-EM, fractionation studies suggest that they occur in cis-Golgi compartment rather than in the VTC (Schweizer et al., 1991).

1.1.7 The Golgi stack.

Once correctly folded proteins proceed to the Golgi stack, which can be seen as a post-translational modification device. The Golgi stack harbours the majority of enzymes that catalyze the post-translational modifications of proteins as they move en passant through the Golgi stack. The Golgi stack may then represent a single, functionally continuous compartment irrespective of the number of cisternae per stack which can vary widely from cell to cell and from organism to organism. Consistent with this is the observation that the spatial organization of the glycosylation enzymes is not required for the correct execution of the sequential addition of sugar residues, since redistribution of these enzymes to the ER by BFA treatment does not affect the nature of the final glycosylation product (Doms et al., 1989). However, the modification enzymes are usually found distributed through the stack in the order in which they act on their substrates. Early acting enzymes having a cis-localization and late acting enzymes having a trans-localization. This concentration of enzymes involved in sequential reactions in sequential compartments may enhance the efficiency of the
glycosylation process (Farquhar, 1985). Furthermore, immuno-EM of epitope tagged versions of the Golgi enzymes β-1,2-N-acetylglucosaminyltransferase I (NAGT I), β-1,4-galactosyltransferase (GaIT), α-1,3,1,6-mannosidase II (Mann II), and α-2,6-Sialyltransferase (SialylT) reveals that their distributions overlap within the stack, in that they are all found in two or more adjacent cisternae rather than in a single one (Nilsson et al., 1993a; Rabouille et al., 1995a). However, each enzyme does have its own unique gradient through the stack. Therefore, each Golgi cisterna has a unique mixture of enzymes rather than a unique set, and as a consequence a biochemical compartment may not necessarily be synonymous with a morphological compartment.

1.1.8 The trans-Golgi network (TGN).

The TGN is regarded as a specialized compartment involved in the sorting of proteins and lipids to their final destinations, and is the final exit point from the Golgi apparatus (Griffiths and Simons, 1986; Simons and van Meer, 1988). The TGN was originally defined as the compartment in which VSVG accumulated in a sialylated form on reducing the temperature to 20°C (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Griffiths et al., 1985). The TGN harbours the last two enzymes of the N-linked glycosylation pathway GaIT and SialylT (Roth et al., 1982, 1985; Rabouille et al., 1995a), and the integral type I membrane protein TGN38 (Luzio et al., 1990), whose function remains obscure. However, in contrast to GaIT and SialylT which are also present in the trans most cisterna of the Golgi stack as well as the TGN, TGN38 is exclusive to the TGN. Tyrosine sulphation (Niehrs et al., 1994) and proteolytic cleavage of viral glycoproteins and cellular protein precursors also occurs in the TGN (Sossin et al., 1990). The TGN may have a lower pH than preceding compartments in order to better facilitate these reactions (Anderson and Pathak, 1985; Seksek et al., 1995; Kim et al., 1996; Llopis et al., 1998).

The TGN can be readily visualized by EM by labelling with thiamine pyrophosphatase (Novikoff, 1967), cytidine monophosphatase (Novikoff and Novikoff, 1977), and NBD-ceramide (Pagano et al., 1989, 1991) and exhibits a complex three dimensional tubulo-reticular morphology at the trans-face of the Golgi stack. The
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TGN can be composed of as many as 1-6 superposed cisternal elements, which often do not remain strictly parallel to each other, but often 'peel off' from the Golgi ribbon and are continuous at their ends with tubules and condensing vacuoles (Ladinsky et al., 1994; Rambourg and Clermont, 1997). As with the CGN, a recent high voltage EM tomographic study on fast frozen, freeze-substituted NRK cells revealed the TGN not to be so reticular in nature, but more cisternal, suggesting that this morphology may be partly an artefact of fixation (Ladinsky et al., 1999). This study also revealed extremely close apposition of the TGN with the rER, which even wraps around trans-Golgi elements, with the ribosomes always on the side that is distal to the Golgi. This region of ER lacks vesicle buds suggesting there is no direct vesicular transfer between the compartments. The close apposition may facilitate the exchange of lipids between membranes by a nonvesicular mechanism (Ladinsky et al., 1999). Similar appositions between the ER and mitochondria are also seen and proposed to have this same function (Ardail et al., 1993).

The TGN displays a distinct response to BFA treatment relative to other Golgi compartments, in that it fails to redistribute to the ER and rather collapses onto the centrosome (Reaves and Banting, 1992) and even shows increased continuity with the plasma membrane (Lippincott-Schwartz et al., 1991). This emphasizes the discrete compartmental nature and membrane dynamics of the TGN relative to the rest of the Golgi apparatus.

The TGN serves as a sorting station interface of membrane flow between the Golgi apparatus, the plasma membrane and the endosome/lysosome system and can be seen as a directional valve between these systems (Mellman and Simons, 1992). Consistent with this is the fact that the TGN undergoes dramatic alterations in size and morphology contingent on the volume of membrane flow going through this compartment (Griffiths, 1989). The TGN is unique to the Golgi apparatus as it is the only site from which clathrin coated vesicles bud (Orci et al., 1984; Griffiths et al., 1985), destined for either the plasma membrane or the endosomal system (Schmid, 1997). Mannose 6-phosphate receptors concentrate lysosomal destined hydrolases and
shuttle between the TGN and endosomes using clathrin coated vesicles in the TGN to endosome direction (Kornfeld, 1992), and a novel class of coated vesicle containing TIP47 as a cargo selection device in the endosome to TGN direction (Diaz and Pfeffer, 1998). Secretory granules also form in neuroendocrine cells at the TGN (Tooze, 1998). A mysterious third population of vesicles with a 'lace-like' coat have also been observed budding from the TGN (Ladinsky et al., 1994). The sorting function of the TGN is perhaps best illustrated in polarized epithelial cells where the TGN must direct plasma membrane molecules to either the apical or basolateral domains of the plasma membrane. Basolateral targeting is often dependent on a signal in the cytoplasmic domain of the protein, and may be facilitated by a novel clathrin adaptor complex (Fölsch et al., 1999). In contrast, apical targeting is thought to require segregation into glycolipid rafts which are selectively incorporated into apically destined transport vehicles (Simons and Ikonen, 1997).

Live cell studies that track the path of VSVG-GFP to the plasma membrane from the TGN have revealed that the primary vehicles responsible for this transfer were large (up to 1.5\(\mu\)m in length) pleiomorphic tubulovesicular structures which bud as entire domains from the \textit{trans}-aspect of the Golgi apparatus (Hirschberg et al., 1998; Toomre et al., 1999; Polishchuk et al., 2000). These structures might be considered analogous to the VTCs that act as transport vehicles between the ER and CGN. They too undergo complex fusion and fission dynamics, and seem to move along microtubules (Hirschberg et al., 1998; Toomre et al., 1999; Polishchuk et al., 2000). Clathrin coated vesicles may even act to retrieve escaped TGN residents from these carriers, in a manner similar to the proposed retrieval of furin to the TGN from immature secretory granules (Dittié et al., 1997).

1.2 Principles of the secretory pathway.

1.2.1 Bulk flow and receptor mediated export.

Protein transport from the ER to the plasma membrane requires the sorting of cargo molecules away from those intended for other destinations and from resident components of the compartments through which they move. The initial step of this
process occurs in the exit from the ER. The protein concentration in the lumen of the ER has been estimated to be as high as 100mg/ml and as much as 95% of this protein is involved in the translocation and deterministic folding/post-translational processes that must occur there. The remaining few percent is the secretory cargo (Quinn et al., 1984) that must be selectively extracted from this solution and shuttled to the Golgi apparatus. Two contrasting models have been put forward to explain how this may be achieved, and it seems likely that the luminal concentration of the cargo in question determines which pathway is used (Warren and Mellman, 1999).

The bulk flow model suggests that transport along the secretory pathway occurs in the absence of positive transport signals and that signals exist to divert proteins from their default destination, the plasma membrane (Wieland et al., 1987; Pfeffer and Rothman, 1987). Such signals would include the mannose 6-phosphate signal indicating a lysosomal destination (Dahms et al., 1989), or the –KDEL/KKXX signals that induce ER retrieval (Munro and Pelham, 1987; Jackson et al., 1990). Similarly, retention signals would keep resident proteins in their own compartments, and secretory cargo would simply pass them by default. This model provides a parsimonious solution to the problem, as the thousands of secretory proteins need not all have individual export signals, and is consistent with the efficient secretion of small peptides too short to contain a transport signal (Wieland et al., 1987; Vanleyen et al., 1994) and the secretion of prokaryotic proteins that are unlikely to contain eukaryotic transport signals (Wiedmann et al., 1984). The bulk flow model predicts that secretory cargo will not be concentrated in COPII vesicles leaving the ER, but will be present at the prevailing ER luminal concentration. This prediction has been tested using quantitative immunocytochemical techniques and has been verified for both amylase and chymotrypsinogen in rat pancreatic acinar cells (Martinez-Menárguez at al., 1999). Instead, these two zymogens were concentrated in the non-COP I coated tubular regions of the VTC, and this occurred concomitant with removal of membrane from the VTC by COPI vesicles. Such concentration in the tubular regions necessitates the fusion of COPII vesicles to generate the VTC. The zymogens must also be excluded from COPI vesicles, which possibly proceeds by some preliminary condensation of
these molecules in the VTC lumen, as has been reported to occur under some conditions (Tooze et al., 1989). Such bulk flow sorting of zymogens may be possible due to their sheer abundance, suggesting they may be already sufficiently concentrated in the ER lumen, and would not be more efficiently sorted by a receptor mediated process.

Receptor mediated ER export proposes that cargo molecules contain signals that mediate their incorporation into nascent COPIII vesicles. A prediction arising from this is that cargo will be concentrated at ER exit sites, and this has been found for serum albumin (Mizuno and Singer, 1993) and VSVG (Balch et al., 1994), but has been challenged on technical grounds (Griffiths et al., 1995). However, receptor mediated export is likely to increase the rate and efficiency of export of less abundant cargo from the ER. ER export signals have been elucidated in VSVG (Nishimura and Balch, 1997; Sevier et al., 2000), yeast α mating factor (Kuehn et al., 1998), Sed5p (Peng et al., 1999), MannII, and NAGTI (Dominguez et al., 1998) which may selectively bind components of the COPIII coat. The v-SNAREs required for ER to Golgi transport may also be concentrated into COPIII vesicles by interaction with COPIII coat components, and may actually function in marking the sites on the ER from which COPIII vesicles will bud (Matsuoka et al., 1998a; Springer and Schekman, 1998). The p24 family of coat-binding proteins also act as cargo receptors (Schimmöller et al., 1995; Belden and Barlowe, 1996), and contain a double phenylalanine (FF) motif in their cytoplasmic domain essential for ER export (Fiedler et al., 1996) and binding to the COPIII coat subunit Sec23p (Dominguez et al., 1998). Similarly, the mannose specific membrane lectin ERGIC53 functions also contains this COPIII binding FF motif (Kappeler et al., 1997) and acts as a cargo receptor for glycoproteins (e.g. cathepsin Z; Appenzeller et al., 1999). Ligand binding is carbohydrate and Ca\(^{2+}\) dependent, and release occurs in the VTC lumen, possibly due to a decrease in lumenal [Ca\(^{2+}\)] (Appenzeller et al., 1999). ERGIC53 may also serve as a cargo receptor for coagulation factors V and VIII, and disruption of this interaction may underlie certain types of classic haemophilia (Moussalli et al., 1999; Nichols et al., 1998). The
identification of such cargo receptors provides suggestive evidence for the receptor mediated export model.

Finally, it is worth noting that bulk flow and receptor mediated export need not be mutually exclusive, and it may be that receptor mediated export increases the efficiency of export of a subset of proteins that would still be secreted/exported anyway by a background rate of bulk flow.

### 1.2.2 Compartmental identity and membrane flux

The overall direction of transport of secretory cargo along the secretory pathway is vectorial towards the plasma membrane, i.e. is in an anterograde or forward direction. However, there must also be a counterbalancing flow of material in the direction of previous compartments and the ER, i.e. in a retrograde or backward direction, in order to maintain the surface area of previous compartments and the ER (Griffiths et al., 1984; Wieland et al., 1987), ensure that escaped ER residents return to the ER (Pelham, 1995), and for the reutilization of the transport machinery required for anterograde transport (Wooding and Pelham, 1998). This antagonistic anterograde/retrograde membrane flux establishes a finely tuned equilibrium between the acquisition and removal of membrane from any organelle within the secretory pathway, and so by extension can affect the very identity of these organelles (Lippincott-Schwartz et al., 1998). Thus, compartmental identity and function is highly dependent on specific membrane fusion events. First, the transfer of transport vesicles between different compartments (termed heterotypic fusion), and second, the maintenance of the delimiting membrane of a compartment by fusion with identical copies (termed homotypic fusion). In the case of ER and Golgi membranes ATPases Associated with diverse cellular Activities (AAA) proteins (Patel and Latterich, 1998) govern these events: NSF in the case of heterotypic fusion (Section 1.3.5), and p97 in the case of homotypic fusion (Section 1.5.6). Provided each enveloping membrane has markers that determine specificity or identity the differential content of compartments naturally follows. Definition of compartmental identity may be achieved by the SNARE proteins (Rothman, 1994; Rothman and Warren, 1994; Section 1.3.3, 1.3.4)
1.2.3 Retention.

Organelle identity is maintained in the secretory pathway despite the extensive bidirectional anterograde and retrograde membrane flux. This requires that proteins involved in post-translational modifications such as molecular chaperones and glycosylation enzymes must not be transported to cellular destinations other than their resident compartment, and on arrival at their resident compartment must be retained there. Two general principles have been elucidated as to how this is achieved: retention and retrieval (see Section 1.2.4), and both mechanisms tend to co-operate to maintain the localization of any resident protein. A retention mechanism acts to actively maintain proteins in their target compartment, possibly by excluding them from transport vesicles (Nilsson and Warren, 1994). A corollary of this is that the retention signal must act in, but not before or after the correct compartment. It must therefore allow anterograde transport along the secretory pathway until arrival at the destination compartment.

The localization of lumenal Golgi enzymes is achieved in part by retention mechanisms, which operate through combinatorial protein-protein and protein-lipid interactions. Two models have been proposed to explain how resident proteins in the Golgi stack are retained. The 'bilayer thickness' or lipid sorting model (Bretscher and Munro, 1993; Munro, 1991, 1995, 1998) proposes that the length of the transmembrane domain (TMD) is the pivotal factor in sorting of Golgi resident proteins. This proposal is based upon the observation that a cholesterol gradient exists across the Golgi stack, whereby the cholesterol concentration increases on moving from cis- to trans-compartments (Orci et al., 1981), and as a consequence so too does the bilayer thickness. Coupled to this is the observation that endogenous Golgi enzymes have shorter TMDs (c. 17 amino acids) than plasma membrane (which has an even higher cholesterol content) proteins (c. 21-22 amino acids). Early Golgi residents are therefore excluded from upstream compartments because their TMD is too short to allow thermodynamically stable incorporation into thicker bilayers. This proposal was supported by the finding that the TMD of SialylT can be replaced by 17 leucine residues, indicating that the primary sequence per se is not essential for retention, and
that increasing the length of the SialylT TMD to 23 leucines resulted in a plasma membrane localization (Munro, 1991, 1995; Dahdal and Colley, 1993). The TMD has also been shown to be sufficient to localize GalT and NAGTI to the trans- and medial-Golgi and grafting of these TMDs on to reporter molecules induces their localization to the appropriate Golgi compartment (Nilsson et al., 1991; Teasdale et al., 1992; Burke et al., 1992; Tang et al., 1992). However, it is not clear in these cases whether these effects are due to the biophysical features of the primary sequence or the length of the TMD. Furthermore, hydrophilic residues flanking the TMD may also be required for Golgi localization (Munro, 1991). The bilayer thickness model is challenged by observations that the TMDs of GalNAc-T1 and T3 are 25 and 18 amino acids respectively (Bennett et al., 1996), suggesting a plasma membrane localization for GalNAc-T1 and a trans-Golgi localization for GalNAc-T3. This is totally inconsistent with the available localization data, which reveals that GalNAc-T1 is found throughout the Golgi stack, and GalNAc-T3 in the medial Golgi cisternae (Röttger et al., 1998). The length of the TMD of GalT is also insufficient for Golgi retention (Masibay et al., 1993).

The alternative model proposes that luminal Golgi proteins are retained by the formation of oligomeric complexes that is induced by the prevailing microenvironmental conditions of the appropriate compartment and prevents access to transport vesicles (Swift and Machamer, 1991; Weisz et al., 1993). A related concept entitled ‘kin recognition’ assumes that resident Golgi enzymes are incorporated into large pre-existing hetero-oligomers upon ingress to their specific compartment (Nilsson et al., 1993b). Distinct Golgi enzymes which reside in the same compartment are termed ‘kin’ and are thought to interact via their luminal stalk domains, as has been demonstrated for MannII and NAGTI (Nilsson et al., 1993a, 1994, 1996). Consistent with this is that retention of NAGTI in the ER also results in the accumulation of MannII in the ER and vice versa (Nilsson et al., 1994). Detergent extraction of purified rat liver Golgi membranes reveals large oligomeric complexes containing MannII and NAGTI that bind an intercisternal matrix (Slusarewicz et al., 1994; Hui, 1997). This ‘kin recognition’
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may also reflect the formation of highly specific multienzyme complexes, which have also been found in yeast (Jungmann and Munro, 1998).

The existence of these large oligomeric complexes is difficult to equate with the high diffusional mobility of Golgi enzymes tagged with GFP in the Golgi membranes of living cells, as revealed by fluorescence recovery after photobleaching (FRAP) experiments (Cole et al., 1996b). Although these observations cannot exclude the possibility that oligomers of a few hundred molecules exist, they are inconsistent with the presence of larger oligomers that are anchored to an intercisternal matrix (Munro, 1998; Slusarewicz et al., 1994). However, other studies suggest that this high diffusional mobility may be due to the transient expression system used. When similar experiments are performed in cell lines stably expressing GFP tagged Golgi enzymes at endogenous levels, no such high diffusional mobility is observed in Golgi membranes, but only upon redistributing these enzymes to the ER by BFA treatment (George Banting and Dave Shima, personal communication). It is also unclear whether the GFP tag affects oligomer formation. However, the kin recognition model does not seem to apply to GalT, SialylT (Munro, 1995), GalNAc-T1, -T2 nor -T3 (Röttger et al., 1998). It therefore seems likely that both features of the TMD and kin recognition are likely to play a role in retention.

Peripheral Golgi membrane proteins must also be targeted to the correct cisternae, and this may be achieved in part by binding specifically to the cytoplasmic tails of specific luminal residents, which would then act as membrane receptors. N-terminal fatty acylation has been determined to be important for a number of proteins (e.g. endothelial nitric oxide synthase [Liu et al., 1997], SCG10 [DiPaulo et al., 1997], glutamate decarboxylase [Solimena et al., 1994], GRASP65 [Barr et al., 1997] and GRASP55 [Shorter et al., 1999]), but how this imparts specificity to targeting remains unclear, and may require additional signals (McCabe and Berthiaume, 1999). Other proteins such as GM130, Golgin-245 and Golgin-97 may ‘piggy back’ target to their compartment via such acylated proteins (Barr et al., 1997; Barr, 1999). Certain pleckstrin homology (PH) domains may also impart Golgi targeting specificity (Levine
and Munro, 1998). The distribution of peripheral membrane proteins could also be biased by how easily they are incorporated into COPI vesicles (Linstedt, 1999), as has been proposed for Golgi enzymes (Glick et al., 1997).

1.2.4 Retrieval and recycling.

Retention mechanisms are usually reinforced by retrieval mechanisms, which ensure that if a resident does escape from its compartment of residence it is recognised and returned. Such retrieval requires retrograde vehicles, which are most likely COPI vesicles (Cosson and Letourneur, 1994; Letourneur et al., 1994; Cosson et al., 1996; Spang and Schekman, 1998; Girod et al., 1999). Retrieval has been most clearly demonstrated for ER residents, and was initially shown for the soluble ER residents BiP and PDI (Munro and Pelham, 1987; Pelham, 1988). Deletion of the four C-terminal amino acids (KDEL) from either protein induced their gradual secretion. Furthermore, grafting this C-terminal KDEL sequence onto a lysosomal resident, cathepsin, induced its accumulation in the ER. Cathepsin received early Golgi sugar modifications, suggesting that it left the ER only to be retrieved from an early Golgi compartment. Other C-terminal tetrapeptides induce ER retrieval most notably HNEL in mammals (Bu et al., 1995, 1997), HDEL in Saccharomyces cerevisiae (Pelham et al., 1988), and DDEL in Kluyveromyces lactis (Lewis et al., 1990). This retrieval is accomplished by interaction with a KDEL receptor (Semenza et al., 1990; Lewis and Pelham, 1992) which exists at steady state in the CGN and cis-Golgi (Griffiths et al., 1994). Interaction between receptor and ligand occurs specifically in the Golgi because of the lower pH of this compartment relative to the ER (Wilson et al., 1993). On binding KDEL ligand the KDEL receptor oligomerizes and is packaged into retrograde moving COPI vesicles (Majoul et al., 1998), and returned to the ER (Townsley et al., 1993). On reaching the ER, the more alkaline pH induces ligand release and return of monomeric, unoccupied receptor to the Golgi (Townsley et al., 1993). This retrieval pathway may operate as distal from the ER as the TGN or plasma membrane (Miesenböck and Rothman, 1995; Majoul et al., 1996).
ER residents with type I membrane topology are retrieved by virtue of a C-terminal KKXX or KXXX (where X is any amino acid) motif in their cytoplasmic domain (Nilsson et al., 1989; Jackson et al., 1990). In type II proteins the retrieval signal consists of two crucial arginine (RR) residues which must reside within the first five N-terminal amino acids (Schutze et al., 1994). Grafting these signals onto reporter molecules results in an ER localization, but the molecules obtain Golgi sugar modifications indicative of retrieval (Jackson et al., 1993; Schutze et al., 1994). KKXX containing molecules interact directly with coatamer, and this interaction is essential for retrieval, suggesting COPI vesicles are the retrograde vehicle (Cosson and Letourneur, 1994; Letourneur et al., 1994; Dominguez et al., 1998). Furthermore, this interaction between a KKXX bearing molecule and coatamer can actually promote COPI vesicle formation, at least from liposomes in a purified system (Bremser et al., 1999).

A retrieval mechanism has also recently been proposed to be important for maintaining the distribution of Golgi enzymes within the Golgi stack (Glick et al., 1997), and such a mechanism may co-operate with the retention mechanisms outlined above. This model is contingent upon a cisternal maturation (Section 1.2.5) view of Golgi dynamics and constructs a mathematical tautology to explain the asymmetric localization of Golgi enzymes within the stack. In this model cis-localized Golgi enzymes are more likely to enter retrograde moving COPI vesicles than trans-localized enzymes, and this enhanced retrieval maintains the observed asymmetric enzyme distribution despite the anterograde motion of maturing cisternae which bear the secretory cargo (Glick et al., 1997). Enzyme oligomers may still be incorporated into this model, in that they form and exclude enzymes from vesicles, until the cisternae reaches a certain age. In support of this the S. cerevisiae cis-Golgi mannosyltransferase Och1p was found to obtain TGN modifications suggesting its cis-Golgi localization is maintained by retrieval (Harris and Waters, 1996) and similar movements have been reported for GPP130 (Linstedt et al., 1997) and mammalian NAGTI although with slower kinetics (Hoe et al., 1995). Whether Golgi enzymes actually enter COPI vesicles is a highly contentious issue, and although present, they do not appear to be enriched in COPI vesicles isolated in the presence of the non-hydrolyzable GTP analogue GTPγS (Sonnichsen et al., 1996). However, a steady state population of vesicles can be isolated from tissue
culture cells which contain high amounts of Golgi glycosylation enzymes (Love et al., 1998), but the precise nature of these vesicles is unclear. Recently GTP hydrolysis by ADP-ribosylation factor (ARF) 1 has been shown to affect the content of COPI vesicles both \textit{in vitro} and \textit{in vivo} (Nickel et al., 1998; Lanoix et al., 1999; Pepperkok et al., 2000), and in one such study Golgi enzymes were found to be efficiently packaged into putative COPI vesicles (Lanoix et al., 1999). Furthermore, NAGTI a medial-Golgi enzyme was found to be incorporated into COPI vesicles in preference to GalT a trans-Golgi enzyme in accordance with the proposal of Glick and colleagues (Lanoix et al., 1999; Glick et al., 1997). The cytoplasmic tail of NAGTI has also been found to interact weakly with coatomer suggesting a mechanism of incorporation (Dominguez et al., 1998). However, whether the vesicles isolated by Nilsson and colleagues truly are COPI vesicles is not entirely clear. The ultimate test of this model will come by determining whether Golgi enzymes can be detected in COPI vesicles \textit{in vivo} by quantitative immuno-EM. This approach has demonstrated the presence of both anterograde and retrograde moving cargo in distinct populations of COPI vesicles associated with the Golgi stack (Orci et al., 1997).

Retrieval of Golgi enzymes may also operate through non-COPI coated vehicles, as demonstrated in a recent studies that reveal a Rab6 dependent, COPI independent ER-Golgi recycling pathway for glycosylation enzymes, which is hijacked by the Shiga toxin (Girod et al., 1999; White et al., 1999). However, such recycling to the ER of Golgi enzymes may reflect the need to degrade or ‘fix up’ functionally expired transmembrane proteins.

The concepts of retention and retrieval are perhaps most clearly unified for the TGN resident TGN38. TGN38 is predominantly a TGN marker but cycles to the plasma membrane via an endosomal compartment (Luzio et al., 1990). Internalization and retrieval from the plasma membrane is induced by a \textit{YQRL} signal in the TGN38 cytoplasmic tail (Bos et al., 1993). Additionally, the TMD of TGN38 represents a retention signal, as its transplantation onto reporter molecules is sufficient to localize them to the TGN (Ponnambalam et al., 1994). Thus, TGN38 contains two non-
overlapping localization signals that must co-operate in order to maintain TGN38 in the TGN. The presence of multiple localization signals may be a general trait of resident proteins.

1.2.5 Cisternal maturation and vesicle transport.

Perhaps the most fiercely discussed issue in the Golgi field at the current time is whether transport of secretory cargo proceeds through the Golgi stack by cisternal maturation or anterograde COPI vesicle transport (Figure 1.2; Pelham 1998; Glick and Malhotra 1998). The cisternal maturation model postulates that secretory cargo is transported in the anterograde direction by maturing cistemae that move in a cis-trans direction (Figure 1.2A). The asymmetric distribution of cisternae specific molecules, such as the glycosylation enzymes, is maintained by their continuous retrieval by retrograde moving COPI vesicles (Schnepf, 1993; Glick et al., 1997). In contrast, the anterograde COPI vesicle transport model postulates that each cisterna represents a stable compartment harbouring a distinct complement of specific enzymes, which are excluded from anterograde moving COPI vesicles, that transport secretory cargo to each cisterna in turn (Figure 1.2B; Palade, 1975; Rothman, 1994; Rothman and Wieland, 1996).

The cisternal maturation model, although initially derived from morphological observations (Grasse, 1957; Beams and Kessel, 1968; Morré et al., 1971, 1979), lost favour since it was unable to explain the asymmetric distribution of Golgi enzymes throughout the Golgi stack (Farquhar and Palade, 1981). However, the realization that COPI vesicles can be retrograde vehicles (Cosson and Letourneur, 1994; Letourneur et al., 1994; Cosson et al., 1996) and such models as put forward by Glick and colleagues (Glick et al., 1997), could explain this distribution and have revived the theory. Although as outlined in the previous section there is as yet no unequivocal evidence that Golgi enzymes enter COPI vesicles in vivo, as revealed by immuno-EM, which is not the case for anterograde cargo (Orci et al., 1997; Schekman and Mellman, 1997). Such evidence would go a long way towards verifying the cisternal maturation concept.
Figure 1.2 Cisternal maturation and anterograde vesicle transport.

(A) Cisternal maturation, where each cisterna bearing anterograde cargo matures by the retrograde transport of Golgi enzymes in COPI vesicles.

(B) Anterograde COPI vesicle transport where Golgi enzymes are resident and cargo is transferred between adjacent cisternae by anterograde moving COPI vesicles. Adapted from Warren and Malhotra (1998).
Despite the absence of this crucial piece of data the cisternal maturation model is currently enjoying a renaissance period. This is mainly due to the contention that large multimeric protein complexes such as: algal scales (Brown, 1971; Becker and Melkonian, 1996), procollagen (Leblond, 1989; Bonfanti et al., 1998), and casein submicelles (Clermont et al., 1993b) are too large to enter conventional transport vesicles and so may be transported by the cisternae themselves. The algal scale evidence has usually been dismissed as an algal peculiarity, since the scales may simply be deforming transport vesicles such that they resemble cisternae (Mellman and Simons, 1992; Warren and Malhotra, 1998). By using a drug that reversibly blocks procollagen assembly in the ER, Luini and co-workers were able to follow a synchronized wave of procollagen movement across the stack, and found that procollagen seemed to traverse the stack without leaving the Golgi lumen (Bonfanti et al., 1998). However, since the procollagen did not seem to leave the ER in conventional COPII vesicles, the possibility remains that the aggregates were deforming vesicle carriers. Were the procollagen being transferred by deformed COPI vesicles in the Golgi apparatus, they might always appear to be in continuity with a cisterna owing to their large size.

Pelham has argued with zeal that the number of SNAREs encoded by the yeast genome is insufficient to account for multiple vesicle transport steps that occur through the Golgi apparatus, and that this implies a maturation mechanism of transport (Pelham, 1998). However, post-translational SNARE modification, the extent of SNARE homooligomerization or combinatorial SNARE associations could serve to boost this number and distinguish between compartments. Furthermore, although it is likely that all the Q-SNAREs have been identified in yeast, the presence of additional R-SNAREs and distant members of the SNARE family cannot be formally ruled out (Jahn and Südhof, 1999).

Finally, FRAP experiments with VSVG-GFP in the Golgi region gives the very strong visual impression that VTCs fuse to form the CGN, which would be the first stage of the maturation process (Presley et al., 1997; Lippincott-Schwartz et al., 1998).
Similarly, whole domains of the TGN seem to peel off heading towards the plasma membrane, which would represent the final stage of maturation (Hirschberg et al., 1998; Toomre et al., 1999). Attempts are surely being made to visualize cisternal maturation utilizing GFP technology, perhaps by labelling early and late Golgi markers with spectral variants of GFP (Wooding and Pelham, 1998), a cisterna would then change colour as it matures. However, the ability to track the motion of a single cisterna is currently beyond the resolution of contemporary fluorescence microscopy.

The lack of unequivocal evidence that proves the model of Glick and colleagues (Glick et al., 1997) means that the fixed and discrete locations of Golgi enzymes still seems to better support the model of anterograde COPI vesicle transport (Farquhar and Palade, 1981). Much of the evidence that COPI vesicles were involved in anterograde transport, could also be explained if they were functioning to recycle essential components required for anterograde movement to earlier compartments, and so would only function indirectly in anterograde transport (Letourneur et al., 1994; Lewis and Pelham, 1996). However, strong evidence for the anterograde COPI vesicle transport model comes from the fact that two populations of COPI vesicles can be identified by immuno-EM carrying either retrograde (KDEL receptor) or anterograde (pro-insulin) cargo in vivo (Orci et al., 1997). Of course, the direction of movement of these vesicles cannot be determined directly from such static images.

The p24 family of COP/cargo receptors may even determine the direction of COPI vesicle movement since they contain information in their cytoplasmic tails that specify anterograde (FF motif) or retrograde (KKXX motif) movement within the Golgi stack (Fielder et al., 1996). These signals differ in regard to which coatomer subunits they interact with (Fiedler et al., 1996; Sohn et al., 1996; Fiedler et al., 1997). Furthermore, some members of the p24 family have both motifs, while some have only the anterograde (FF) motif (Fiedler et al., 1996). However, these results were not reproduced in a study by Nilsson and colleagues who found that p24 family members containing only the FF motif bound COPII but not COPI (Domínguez et al., 1998). The orientation of COPI vesicle tethers (see Section 1.3.2) may also specify the
directionality vector of COPI vesicle movement within the Golgi stack (Shorter and Warren, 1999).

COPI vesicles containing anterograde cargo will fuse with anterograde target Golgi membranes \textit{in vitro} (Orci et al., 1989; Ostermann et al., 1993), but vesicles containing Golgi enzymes can also fuse with secretory cargo bearing cisternae (Love et al., 1998; Lanoix et al., 1999). \textit{In vitro} intra-Golgi transport can also be supported by tubular connections (Orci et al., 1991; Elazar et al., 1994b; Happe et al., 1998), and such tubules have also been postulated to be the anterograde vehicle (Mironov et al., 1997b). Such tubular connections between neighbouring cisternae have also been observed \textit{in vivo} (Rambourg and Clermont, 1997), but are extremely rare in comparison to the plethora of coated vesicles and buds that encompass the dilated cisternal rims.

There is clearly considerable conflicting evidence supporting both models, and it may be that there is truth in them both. The apparent controversy may then be false since the models are not necessarily mutually exclusive. Synthetic models that incorporate anterograde vesicle movement, transient tubular connections and cisternal maturation are rarely proposed (Mironov et al., 1998). However, if the possibility is considered, it may be that the vehicle used (i.e. cisterna or anterograde COPI vesicle) is dependent on the rate at which transport of the cargo molecule is required. Anterograde COPI vesicles may be used for rapid transit through the stack, whereas cisternae are used for more constitutive transit (Mironov et al., 1998; Glick and Malhotra, 1998).

1.3 Vesicle transport.

Whatever the resolution of the current conflict both models have a requirement for the transfer of vesicles between compartments and adjacent cisternae in the stack. Vesicle transfer between compartments requires the formation of a vesicle by a donor compartment and its fusion with a specific acceptor compartment. This process entails a series of discrete and highly regulated sequential subreactions which comprise vesicle biogenesis, tethering, docking, fusion and priming (Figure 1.3). Each subreaction must
Figure 1.3 Life cycle of a transport vesicle
Stepwise assembly of coat subunits deform the membrane of the donor compartment into a bud, and incorporates occupied cargo receptors. Following release from the membrane the vesicle uncoats so as to recycle coat subunits. Vesicles are then tethered to acceptor membranes by fibrous proteins in conjunction with Rab GTPases. This leads to the v-SNARE on the vesicle binding to its cognate t-SNARE on the target membrane, and this docking leads to membrane fusion, possibly with the aid of downstream factors. Cargo is then released into the acceptor compartment, and vacant cargo receptors can be returned to the donor compartment. The v-t-SNARE pair are separated by the action of NSF and SNAPs which may prime the t-SNARE and allow recycling of the v-SNARE to the donor compartment. Adapted from Mellman and Warren (2000).
be completed per vesicle life cycle and each requires a characteristic and highly conserved molecular machinery as outlined below.

1.3.1 Vesicle Biogenesis

A number of classes of vesicle, defined by their coat proteins, are known to exist in eukaryotic cells and each mediates a distinctive transport step or set of transport steps. The coat proteins serve to assemble on a donor membrane and deform it into a bud. COPII vesicles export cargo from the ER (Barlowe et al., 1994; Schekman and Orci, 1996), COPI vesicles carry material between the ER and the Golgi (Pepperkok et al., 1993; Scales et al., 1997; Majoul et al., 1998; Spang and Schekman, 1998; Girod et al., 1999), and through the Golgi apparatus in probably both anterograde and retrograde directions (Sönnichsen et al., 1996; Orci et al., 1997; Love et al., 1998; Lanoix et al., 1999), while clathrin coated vesicles mediate transport from the TGN and endocytic transport from the plasma membrane (Le Borgne and Hoflack, 1998). The clathrin and the COPI/II coats can be distinguished by their morphological appearance (Orci et al., 1986). The clathrin coat appears as a hexagonal-pentagonal lattice, whereas the COPI/II coats are embodied by a ‘fuzzy’, electron dense material about 10-15nm thick that surrounds the vesicle (Orci et al., 1986; Barlowe et al., 1994). Other classes of coated vesicles may be identified in the future for other transport steps (Ladinsky et al., 1994; Diaz and Pfeffer, 1998).

Vesicle formation can be dissected into three stages (Nickel and Wieland, 1997; Wieland and Harter, 1999). First, a member of the Ras superfamily of GTPases is recruited to the donor membrane from the cytosol by the catalyzed exchange of GDP for GTP by a membrane associated guanine nucleotide exchange factor (GEF). Second, is the formation of a vesicle bud which occurs concomitant with the GTPase mediated recruitment of hetero-oligomeric coat protein complexes to the donor membrane. Finally, the coated bud must pinch off from its donor compartment and become a discrete entity. This event has been termed fission, scission or periplasmic fusion and requires some form of membrane fusion event, initiated by the lumenal membrane leaflets, at the constricted neck of the vesicle bud. Layered upon this biomechanical
process, are regulatory mechanisms that are in place to ensure that the correct coat complex is recruited to the correct donor membrane and that the appropriate cargo molecules are selectively incorporated into nascent vesicles (Springer et al., 1999). Finally, a high fidelity proof reading mechanism must be in place to ensure that a vesicle will not form unless it contains all the necessary membrane components required for later steps in the transport pathway (e.g. v-SNAREs). Such a mechanism ensures that non-functional, fusion incompetent vesicles which would endanger organelle identity, do not form (Springer et al., 1999), and may involve at some level the Rab GTPases (Pfeffer et al., 1994; Schimmöller et al., 1998; Section 3.3.1).

The understanding of the biomechanics of vesicle formation has been greatly enhanced by the ability to reconstitute this process from either purified membranes or liposomes by the addition of a minimal set of purified soluble factors (Orci et al., 1993a; Ostermann, et al., 1993; Barlowe et al., 1994; Matsuoka et al., 1998b; Spang et al., 1998; Bremser et al., 1999). Reconstitution of COPII vesicles requires the soluble factors: Sarlp (Nakano and Muramatsu, 1989), Secl3p complex (Salama et al., 1993) and Sec23p complex (Hicke et al., 1992; Table 1.1). Formation of COPI vesicles requires the soluble proteins coatamer (a heptameric protein complex; Waters et al., 1991) and ARF1 (Kahn and Gilman, 1984; Table 1.1).

Vesicle formation is initiated by the recruitment of Sarlp (COPII vesicles) or ARF1 (COPI vesicles) to the membrane from the cytosol. These GTPases exist in the GDP bound form in the cytosol and on recruitment to the membrane GDP is exchanged for GTP in a GEF catalyzed process. The integral membrane protein Sec12p (Barlowe and Schekman, 1993) is the Sarlp-GEF and interacts closely with Sec16p and Sed4p (Gimeno et al., 1995). The localization of Sec12p ensures COPII vesicle formation is restricted to the ER (Sato et al., 1996). Numerous ARF-GEFs have been characterized which may be recruited to the membrane from the cytosol (Chardin et al., 1996; Rosa et al., 1996; Schimmöller et al., 1997). Mammalian cells contain at least three classes of ARF-GEF, with variable sensitivity to the macrocyclic antibiotic BFA. The inhibition of ARF-GEF activity by BFA (Helms and Rothman, 1992), prevents ARF binding to
membranes, and may be partly responsible for the dramatic effects it has on Golgi architecture. The precise identity of the ARF-GEFs affected in mammalian cells remains obscure (Chardin and McCormick, 1999). However, in yeast the ARF-GEFs Gea1p, Gea2p and Sec7p are likely to be responsible, each of which contains a Sec7 domain which confers the GEF activity (Schimmoller et al., 1997). BFA is an uncompetitive inhibitor of the Sec7 domain and stabilizes an abortive ARF-GDP: BFA: Sec7 domain complex, which blocks the activation of other ARF1 molecules (Peyroche et al., 1999). The exchange of GDP for GTP induces a conformational change in the ARF1 protein exposing a N-terminal myristoyl group which allows the ARF1 to stably attach to the membrane (Goldberg, 1998).
Activation of the small GTPases is followed by coat protein recruitment. In the case of COPII the Sec23p complex binds followed by the Sec13p complex. The Sar1p/Sed4p/Sec16p complex serves as a docking pad for the Sec23p complex. Sec23p interacts directly with the C-terminus of Sec16p, a large peripheral membrane protein, which may also bind cargo and v-SNAREs at its N-terminus (Espenshade et al., 1995). Sec23p and Sec24p then cluster SNAREs (e.g. Bet1p, Bos1p, Sed5p) and cargo receptors and cargo (e.g. p24, ERGIC53, VSVG, MannII, NAGTI) into the bud site by direct binding (Table 1.1), thereby ensuring cargo uptake and fusion competence for the future COPII vesicle. The selective incorporation of other essential v-SNAREs (e.g. Sec22p) into COPII vesicles, may be by virtue of interaction with these coat binding SNAREs, and may be controlled by a Rab protein. Ypt1p stimulates Bos1p/Sec22p interaction (Lian et al., 1994), and in so doing ensures the nascent vesicle contains Sec22p. In addition, Sec24p serves as the binding site for the Sec13p complex. Subsequent polymerization of the Sec13p complex then further clusters activated cargo and targeting molecules by crosslinking the Sec23p complexes and induces bud formation (Springer et al., 1999).

In COPI vesicle formation, coatomer binds to the membrane en bloc (Orci et al., 1993a, b). This entails a direct interaction between ARF-GTP and the β- and γ-COP subunits (Zhao et al., 1997, 1999). Coat protein assembly mechanically induces the deformation of the donor membrane into a coated bud, and this is sufficient to generate COPI vesicles from acidic liposomes (Spang et al., 1998). ARF-GTP also stimulates phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (Brown et al., 1993). This change in the lipid composition has been shown to enhance coatomer binding to the membrane (Ktistakis et al., 1995, 1996). Furthermore, membranes with high endogenous PLD activity did not seem to require ARF1 for COPI vesicle formation (Ktistakis et al., 1996). However, this seems to have been due to a failure in detecting membrane associated ARF1 and that in fact ARF1 is always present in COPI vesicles in a molar excess over coatomer (Serafini et al., 1991; Stamnes et al., 1998). The involvement of PLD seems less likely given the
direct interaction of ARF1 with the coatamer subunits (Zhao et al., 1997; Nickel and Wieland, 1997) and the ability to form COPI vesicles from liposomes in the absence of PLD (Spang et al., 1998; Bremser et al., 1999).

γ-COP interacts directly with the KKXX motif which is found on the cytoplasmic tails of escaped ER residents and the p24 family of proteins (Harter and Wieland, 1998). Other coatamer subunits can also bind the KKXX motif, but only upon coatamer dissociation from the membrane (Harter and Wieland, 1998). Interestingly, the p24 family have a propensity to form large hetero-oligomers (Dominguez et al., 1998; Fullekrug et al., 1999; Gommel et al., 1999), and so may mark sites from which COPI vesicles are destined to bud. This provides a mechanism for ensuring cargo enters COPI vesicles. Both p23 and p24 (p24 family members) are found in COPI vesicles at concentrations similar to ARF1 and coatamer suggesting they may be actively involved in vesicle formation. Binding of coatamer to the KKXX motif of p23, but not the KKXX motif of an ER resident, induces a conformational change in coatamer that may promote its polymerization and may be sufficient to drive membrane deformation during bud formation (Reinhard et al., 1999). Furthermore, this interaction was shown to be essential to drive COPI vesicle formation from liposomes which had a composition more akin to Golgi membranes (Bremser et al., 1999). However, how SNAREs are incorporated into COPI vesicles, and so how nascent COPI vesicles are ensured fusion competence remains unclear.

An interesting alternative hypothesis to how COPI/II binds to membranes comes from the fact the amino acid sequences of α-COP, β’-COP, Sec13p, and Sec31p reveal several WD-40 repeat motifs (van der Voorn and Ploegh, 1992). The precise function of these motifs remains unknown, but they are found in a number of different proteins, including the β-subunits of heterotrimeric G proteins. Perhaps the WD-40 repeats in the two coatamer subunits mediate an interaction of coatamer with a membrane bound α-subunit of a heterotrimeric G protein (Harrison-Lavoie et al., 1993). Regulation of coatamer binding to membranes is known to be affected by the [AlF₄]⁻ which locks G α-subunits in the active form (Donaldson et al., 1991).
Once a coated bud has formed it must be pinched off the membrane to generate a free vesicle. This event is termed fission and for COPII vesicles seems to require no additional soluble factors (Bednarek et al., 1995; Matsuoka et al., 1998b). COPI vesicle fission also requires no additional factors when formed from chemically defined liposomes (Spang et al., 1998; Bremser et al., 1999). However, COPI vesicle fission from Golgi membranes requires ATP and the transfer of palmitate from palmitoyl CoA to an unidentified acceptor molecule (Pfanner et al., 1989; Ostermann et al., 1993). Phosphatidylinositol transfer protein (PITP) has also been implicated in the COPI vesicle fission event, but its mechanism of action is unclear (Simon et al., 1998). Recently, BARS-50 a member of a group of proteins thought to function in transcriptional repression (Nibu et al., 1998), was found to cause the non-specific fission of Golgi membranes (Weigert et al., 1999; Barr and Shorter, 2000). However, whether this is coupled to COPI vesicle formation per se remains undetermined.

BARS-50 seemed to act by transferring a range of fatty acids (including palmitate) from CoA to lysophosphatidic acid (LPA) to generate PA (Weigert et al., 1999; Barr and Shorter, 2000). This event represents a change in shape of lipid, since LPA has an inverted-cone shape, while PA is thought to be cone shaped (Chemomordik et al., 1995). Production of a cone shaped lipid in the cytoplasmic leaflet of the bilayer would induce an inward distortion of the membrane, and if concentrated at the bud neck would promote a membrane curvature conducive to the fusion of the lumenal leaflets and subsequent membrane fission (Scales and Scheller, 1999). This mode of action was similar to one proposed for how endothilin-I (Ringstad et al., 1997, 1999) at the vesicle bud neck might co-operate with dynamin, a GTP driven garrotte (Hinshaw and Semid, 1995; Takei et al., 1995; Jones et al., 1998; Sever et al., 1999; Stowell et al., 1999), to drive synaptic like microvesicle or clathrin coated vesicle formation (Schmidt et al., 1999; Scales and Scheller, 1999; Simpson et al., 1999). That clathrin coated vesicles have an elaborate fission machinery and COPII vesicles seem to have none, may reflect the biophysical properties of the membranes from which they form (Bednarek et al., 1996). The higher cholesterol content at the Golgi and plasma membrane compared to
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the ER (Orci et al., 1981) may confer greater membrane rigidity, and make coated vesicle formation a more energy demanding process, requiring a specialized fission machinery.

GTP hydrolysis by Sar1p/ARF1 causes their release from the membrane and subsequent uncoating of the COPII or COPI vesicle (Barlowe et al., 1994; Tanigawa et al., 1993). Consistent with this a GTPase defective ARF1 mutant and GTPγS cause COPI vesicles to accumulate (Ostermann et al., 1993; Tanigawa et al., 1993; Pepperkok et al., 2000). Similarly, GMP-PNP causes COPII vesicles to accumulate (Barlowe et al., 1994). Since both Sar1p and ARF1 hydrolyze GTP at a slow rate, a GTPase activating protein (GAP) is thought to stimulate their GTP hydrolysis. In the case of Sar1p the GAP is Sec23p, a component of the COPII coat (Yoshihisa et al., 1993). In the case of ARF1, the GAP is a zinc finger containing protein (Cukierman et al., 1995) and is recruited to the nascent COPI vesicle by the KDEL receptor (with KDEL ligand bound) which also binds ARF1 (Aoe et al., 1997, 1998). This again confers functionality to the budding vesicle. The ARF-GAP may then be incorporated into the COPI coat, as is Glo3p in S. cerevisiae (Poon et al., 1999). In fact, in S. cerevisiae retrograde transport from the Golgi to the ER requires two ARF GAP proteins, Glo3p and Gcs1p (Poon et al., 1999). Furthermore, coatomer can act on the ARF GAP itself to stimulate GTP hydrolysis by ARF (Goldberg, 1999). Vesicle uncoating is probably essential to reveal the v-SNARE machinery, an essential component for the subsequent docking and probably fusion of the vesicle with its acceptor compartment (Section 1.3.3, 1.3.4).

1.3.2 Vesicle Tethering

Once a vesicle has formed it must reach its specific target acceptor membrane. This targeting specificity operates through several layers of protein-protein interactions. If the acceptor membrane is distant from the donor membrane (e.g. in peripheral ER to Golgi transport) this will initially require molecular motors and the actin/microtubule system to deliver the vesicle to its target (Scales et al., 1997; Echard et al., 1998; Itin et al., 1999). Fibrous tethering proteins then function to collect and restrain vesicles at the
target membrane. Within the Golgi stack tethers may act more coincident with vesicle budding, such that a vesicle is already attached to its target membrane before budding is complete (Sönntichsen et al., 1998; Shorter and Warren, 1999). In this way tethers would increase the efficiency of transport by removing the need for a vesicle to diffuse to its target membrane, and also reduce the chance of losing a vesicle in the surrounding cytoplasm (Orci et al., 1998). Tethering factors (Table 1.2) are frequently long (often greater than the diameter of a transport vesicle, i.e. 50-70nm), flexible coiled-coil, dimeric molecules that act to bind membranes together before trans-SNARE pairing (Section 1.3.3 and 1.3.4). Such tethers likely impart vesicle targeting specificity, and seem to act in concert with the Rab GTPases (Waters and Pfeffer, 1999; Pfeffer, 1999).

Table 1.2 Proteins implicated in membrane tethering.

<table>
<thead>
<tr>
<th>Transport process</th>
<th>GTPase</th>
<th>Tethering factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-Golgi</td>
<td>Ypt1p</td>
<td>Uso1p*</td>
<td>Cao et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sec34p</td>
<td>Van Rheenen et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sec35p</td>
<td>Kim et al., 1999</td>
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<tr>
<td></td>
<td></td>
<td>TRAPP</td>
<td>Sacher et al., 1998</td>
</tr>
<tr>
<td>Intra-Golgi</td>
<td>Rab1</td>
<td>Giantin*</td>
<td>Sönntichsen et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p115*</td>
<td>Shorter and Warren, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GM130*</td>
<td>Seemann et al., 2000</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Lesa et al., 2000</td>
</tr>
<tr>
<td>Endosome-Golgi</td>
<td>Ypt6p</td>
<td>Imh1p*</td>
<td>Tsukada et al., 1999</td>
</tr>
<tr>
<td>Golgi to plasma membrane</td>
<td>Sec4p</td>
<td>Exocyst</td>
<td>Guo et al., 1999</td>
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<td>Endosome-endosome</td>
<td>Rab5</td>
<td>EEA1*</td>
<td>Simonsen et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rabaptin-5*</td>
<td>Mills et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Christoforidis et al., 1999a</td>
</tr>
<tr>
<td>Vacuole-vacuole</td>
<td>Ypt7p</td>
<td>?</td>
<td>Ungermann et al., 1998b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sato and Wickner, 1998</td>
</tr>
</tbody>
</table>

*Indicates protein is extensively coiled-coil. Adapted from Waters and Pfeffer, 1999.
Perhaps the best characterized fibrous tethering molecules are those required for COPI vesicle tethering in the mammalian Golgi apparatus, i.e. p115, giantin and GM130. p115 is required for intra-Golgi transport \textit{in vitro and in vivo} (Waters et al., 1992b, Seemann et al., 2000), ER-Golgi transport (Alvarez et al., 1999), and is capable of tethering COPI vesicles to Golgi membranes \textit{in vitro} (Sönニック森 et al., 1998). The yeast homologue of p115, Uso1p, is required for ER-Golgi transport (Nakajima et al., 1991; Seog et al., 1994; Lupashin et al., 1996; Barlowe, 1997) and tethers COPII vesicles to Golgi membranes (Sapperstein et al., 1996; Cao et al., 1998). Both Uso1p and p115 exist as myosin II shaped parallel homodimers, with two N-terminal globular heads, a coiled-coil tail, and a short acidic domain at the extreme C-terminus (Sapperstein et al., 1995; Yamakawa et al., 1996). p115 acts to cross link the acceptor membrane to the incoming COPI vesicle by simultaneously binding its two receptors on Golgi membranes: GM130 on the acceptor membrane and giantin on the COPI vesicle (Sönニック森 et al., 1998).

GM130 was first identified as a highly immunogenic component of a detergent insoluble Golgi matrix (Nakamura et al., 1995). GM130 is an extensively coiled-coil, dimeric, rod-like peripheral membrane protein which may have some degree of flexibility and is tightly anchored to the Golgi membrane at its C-terminus by the N-terminally myristoylated Golgi ReAssembly Stacking Protein (GRASP) 65 (Barr et al., 1998). Giantin was first identified using a mAb raised against Golgi membranes (Linstedt and Hauri, 1993). It is also predicted to be an extensively coiled-coil, dimeric, rod like protein, and is a type II Golgi membrane protein with most of its mass projecting into the cytoplasm (Linstedt and Hauri, 1993). In contradistinction to GM130, giantin is incorporated into \textit{in vitro} generated COPI vesicles in the presence of GTPγS at the prevailing donor membrane concentration, whereas GM130 is largely excluded (Sönニック森 et al., 1998). The acidic C-terminus of p115 is thought to link the membrane distal N-terminus of giantin on the COPI vesicle to the membrane distal N-terminus of GM130 on the acceptor membrane (Nakamura et al., 1997; Nelson et al., 1998; Lesa et al., 2000; Dirac-Svejstrup et al., 2000). This hetero-ternary complex is then a SNARE independent tethering device and may be regulated by the direct
phosphorylation of p115 by a Golgi associated kinase (Sohda et al., 1998; Dirac-Svejstrup et al., 2000; Chapter 6). Given the extended, flexible rod like structure of these molecules they are excellent candidates for the ‘strings’ proposed to be important for processive intra-Golgi transport that can be visualized by EM (Orci et al., 1998; Section 1.4.1).

The yeast homologues or analogues of GM130 and giantin remain to be identified but Uso1p mediated tethering seems to be regulated by the GTPase Ypt1p (Rab1) with which it interacts genetically (Sapperstein et al., 1996; Cao et al., 1998). COPII vesicle tethering also requires a protein complex of ten highly conserved subunits, called the transport protein particle (TRAPP; Sacher et al., 1998) and the predominantly soluble protein Sec35p (Van Rheenen et al., 1998). TRAPP localizes to the cis-Golgi and may capture incoming COPII vesicles. Sec35p binds Sec34p, another predominantly soluble protein, and Sec34p interacts genetically with Grp1p, a Golgi associated, Golgin-160 related protein (Kim et al., 1999). This Sec34p/Sec35p interaction is also proposed to important for tethering. The orientation of these molecules in the tether, or how they contribute to tethering remains unclear. Similarly, the mode of action of mammalian p115 on COPII vesicles and VTCs remains unclear, but does not seem to require giantin or GM130 (Nelson et al., 1998; Alvarez et al., 1999; Seemann et al., 2000; Lesa et al., 2000).

Another well defined tethering system is in homotypic endosome fusion, which relies upon early endosome antigen 1 (EEA1) to tether membranes together (Simonsen et al., 1998; Mills et al., 1998; Christoforidis et al., 1999a). EEA1 is a large predominantly coiled-coil molecule (Mu et al., 1995) with a zinc-binding ‘FYVE’ finger (Stenmark et al., 1996), and interacts with phosphatidylinositol-3-phosphate (Patki et al., 1997) and Rab5-GTP (Simonsen et al., 1998). EEA1 may serve in principle to tether endosomes together via Rab5-GTP, since each EEA1 molecule contains two Rab5-GTP binding sites, one at the C-terminus and the other at the N-terminus. However, high levels of EEA1 can tether endosomes in the absence of Rab5 function, suggesting Rab5 may act upstream of EEA1. EEA1 is also found to interact with the SNARE machinery, and so
may act in the transition from tethering to downstream docking and fusion events (McBride et al., 1999; Simonsen et al., 1999). Rabaptin-5 occurs in a complex with Rabex-5 (a Rab5 nucleotide exchange factor) and functions to stabilize Rab5-GTP (Rybin et al., 1996), and contains two Rab5 binding sites, that could also in principle tether endosomes together (Stenmark et al., 1995; Vitale et al., 1998; Gournier et al., 1998).

A feature common to tethering reactions is that many of the molecules involved seem to be extensively coiled-coil dimers and events seem to be co-ordinated by Rab GTPases (Waters and Pfeffer, 1999; Pfeffer, 1999). The Rab proteins are ubiquitous, small GTPases possessing 30% homology to the oncogene product Ras and seem to act as molecular switches in proof-reading steps at multiple stages of vesicle transport (Pfeffer et al., 1994; Novick and Zerial, 1997; Schimmoller et al., 1998; Pfeffer, 1999). Each Rab protein seems to have multiple effector proteins and the precise step at which a given Rab acts may depend upon the precise effector molecule it is associated with at a given time (Bean and Scheller, 1997; Christoforidis et al., 1999a, b). More than 40 mammalian Rab proteins have been identified so far (Novick and Zerial, 1997). Like Ras, Rabs cycle between an active, membrane bound GTP bound state and an inactive, soluble GDP bound state. The GTP-bound form may accompany transport vesicles to their target membrane (Salminen and Novick, 1987; Goud et al, 1988; Lian and Ferro-Novick, 1993), and following vesicle fusion, a GAP stimulates Rab GTP hydrolysis converting them to the GDP bound form (Strom et al., 1993; Fukui et al., 1997; Albert and Gallwitz, 1999). Rab Guanine nucleotide Dissociation Inhibitor (GDI) then extracts GDP-bound Rabs from their fusion targets and selectively delivers them back to their membrane of origin (Soldati et al., 1993; Ullrich et al., 1993, 1994; Dirac-Svejstrup et al., 1994). GDI returns the Rab to its original membrane in its GDP bound state, where it is reactivated by a process that requires two components. First, a Rab-GDI Displacement Factor (GDF) separates the Rab from GDI (Dirac-Svejstrup et al., 1997), a GEF then stimulates the exchange of GDP for GTP (Walch-Solimena et al., 1997; Horiuchi et al., 1997), after which the Rab is stably bound to the membrane by
virtue of its geranylgeranyl group(s) present on (usually) two C-terminal cysteines (Casey and Seabra, 1996), and is ready for another round of transport.

Active Rab-GTP molecules if present on nascent transport vesicles could serve to recruit tethering factors. COP II vesicle tethering is inhibited by GDI, which extracts both Ypt1p (Rab1) and Usolp from membranes, suggesting Ypt1p may regulate Usolp binding (Cao et al., 1998). Ypt6p co-operates closely with Imh1p/Sys3p, a Usolp like molecule, in vesicle tethering in an endosome to Golgi transport event (Tsukada et al., 1999). Similarly, Rab5-GTP binds to the tethering factors EEA1 and Rabaptin-5 in endosome fusion. Another example is the Exocyst which is a highly extendable (c. 30nm in length) 19.5S complex containing Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p, and is required for exocytosis in yeast (TerBush et al., 1996). Sec15p associates with the Rab Sec4p-GTP on Golgi-plasma membrane vesicles, and Sec4p-GTP affects the integration of Sec3p into the Exocyst (Guo et al., 1999). Sec3p is localized to the plasma membrane and is thought to mark the sites at which exocytosis will occur (Finger et al., 1998). Therefore, Sec4p co-ordinates the construction of the Exocyst complex, and in so doing links the transport vesicle to the plasma membrane, ready for trans-SNARE pairing (Guo et al., 1999). The general theme here is that Rab proteins seem to co-ordinate tether formation. Rabs may also act in the transport of vesicles along microtubules, since a Rab6 effector is a kinesin like molecule (Echard et al., 1998), and seems to function in an ER-Golgi transport pathway (Girod et al., 1999; White et al., 1999). Rabs have been heavily implicated in SNARE interactions as well (Søgaard et al., 1994; Brenwald et al., 1994; Lian et al., 1994), and it may be that they regulate the transition from tethering to docking and fusion (see next section).

1.3.3 Vesicle Docking

Once a vesicle is tethered to a membrane the flexibility of the tether may permit the vesicle to sample the membrane for the cognate SNARE to facilitate vesicle docking. SNAREs are members of a superfamily of highly conserved proteins (Weimbs et al., 1997, 1998) that reside on vesicles (v-SNAREs) and target membranes (t-SNAREs;
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The central tenet of the SNARE hypothesis, originally formulated with brio and verve by Rothman (Rothman, 1994), is that the v-SNARE on the vesicle binds to its cognate t-SNARE on the target membrane to form a trans-SNARE pair (trans- denotes that the SNAREs reside in opposite membranes; Bennett et al., 1993a; Söllner et al., 1993b; Protopopov et al., 1993; Pevsner et al., 1994a; Sogaard et al., 1994). This event confers targeting specificity to a transport reaction and is termed vesicle docking. Vesicle docking is distinct from tethering as it involves SNAREs, and brings the membranes into very close apposition.

Tethering may be considered to involve links that extend over distances of more than half the diameter of a transport vesicle (>25nm). Whereas, docking brings membranes to within a bilayer’s distance from one another (<5-10nm; Pfeffer, 1999). Targeting specificity is ensured by the cognate v-/t-SNARE pair, which can be seen as a ‘lock and key’ device to discrete compartments, and a multitude of v-SNARE and t-SNAREs have been identified in multiple steps of the secretory pathway from yeast to mammals (Bock and Scheller, 1999; Jahn and Südhof, 1999; Table 1.3).

The syntaxin family of t-SNAREs and VAMP family of v-SNAREs are type II membrane proteins containing a C-terminal signal anchor, and with most of the mass projecting into the cytoplasm. The SNAREs have been grouped in a superfamily on the basis of having either one or two homologous coiled-coil domains of c. 60 amino acids, termed the SNARE motif (Weimbs et al., 1997, 1998; Jahn and Südhof, 1999). This motif is the defining feature of SNAREs and functions in the associations between cognate SNAREs to form the ‘core complex’ that is essential for their function in vesicle docking and fusion (Fiebig et al., 1999; Parlati et al., 1999). SNARE subfamilies can be defined by whether a SNARE has one or two SNARE motifs, and on the sequences that flank these SNARE motifs. The majority of SNAREs contain one SNARE motif preceded by a variable N-terminal domain which may contain other coiled-coil regions important for inter- and intra-molecular interactions, as in the syntaxins (Hanson et al., 1995). The SNARE motif is then proceeded by a basic domain followed by a C-terminal TMD (Weimbs et al., 1998). This organization is typical of the syntaxin family of t-SNAREs and the VAMP and Bet1p/Bos1p v-
Table 1.3 Localization and classification of SNAREs.

<table>
<thead>
<tr>
<th>SNARE</th>
<th>Organism</th>
<th>Type</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep12p</td>
<td>Yeast</td>
<td>Q, t-SNARE</td>
<td>Golgi-vacuole/lysosome</td>
</tr>
<tr>
<td>Vam3p</td>
<td>Yeast</td>
<td>Q, t-SNARE</td>
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<td>Yeast</td>
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</tr>
<tr>
<td>Sso2p</td>
<td>Yeast</td>
<td>Q, t-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Tlglp</td>
<td>Yeast</td>
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<tr>
<td>Snc2p</td>
<td>Yeast</td>
<td>R, v-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Syntaxin-1</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Plasma membrane (neurons)</td>
</tr>
<tr>
<td>Syntaxin-2</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Syntaxin-3</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Transport vesicles, plasma membrane</td>
</tr>
<tr>
<td>Syntaxin-4</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Syntaxin-5</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Golgi, VTCs, ER, COP coated vesicles</td>
</tr>
<tr>
<td>Syntaxin-6</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>TGN-endsomes</td>
</tr>
<tr>
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<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Golgi/lysosomes</td>
</tr>
<tr>
<td>Syntaxin-8</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>ER, endosomes</td>
</tr>
<tr>
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<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>TGN</td>
</tr>
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<td>Syntaxin-12</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Endosomes</td>
</tr>
<tr>
<td>Syntaxin-16</td>
<td>Mammals</td>
<td>Q, t-SNARE</td>
<td>Golgi</td>
</tr>
<tr>
<td>rBet1</td>
<td>Mammals</td>
<td>Q, v-SNARE</td>
<td>Golgi, VTCs, ER, COP coated vesicles</td>
</tr>
<tr>
<td>GS15</td>
<td>Mammals</td>
<td>Q, v-SNARE</td>
<td>Golgi</td>
</tr>
<tr>
<td>GOS-28</td>
<td>Mammals</td>
<td>Q, v-SNARE</td>
<td>Golgi, COP coated vesicles</td>
</tr>
<tr>
<td>Membrin</td>
<td>Mammals</td>
<td>T, v-SNARE</td>
<td>ER/Golgi, COP coated vesicles</td>
</tr>
<tr>
<td>rSec22</td>
<td>Mammals</td>
<td>R, v-SNARE</td>
<td>ER/Golgi, COP coated vesicles</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Mammals</td>
<td>2Q, t-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>SNAP-23</td>
<td>Mammals</td>
<td>2Q, t-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>SNAP-29</td>
<td>Mammals</td>
<td>2Q, t-SNARE</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>VAMP1</td>
<td>Mammals</td>
<td>R, v-SNARE</td>
<td>Synaptic vesicles</td>
</tr>
<tr>
<td>VAMP2</td>
<td>Mammals</td>
<td>R, v-SNARE</td>
<td>Synaptic/clathrin coated vesicles</td>
</tr>
<tr>
<td>VAMP3</td>
<td>Mammals</td>
<td>R, v-SNARE</td>
<td>Clathrin coated vesicles</td>
</tr>
<tr>
<td>VAMP5/6</td>
<td>Mammals</td>
<td>R, v-SNARE</td>
<td>Plasma membrane and intravesicular structures (skeletal muscle and heart)</td>
</tr>
<tr>
<td>Endobrevin</td>
<td>Mammals</td>
<td>R, v-SNARE</td>
<td>Endosomes</td>
</tr>
</tbody>
</table>

Adapted from Jahn and Südhof, 1999.

SNARE families. Alternatively, SNAREs of the SNAP-25/Sec9 family are attached to the membrane by lipid modifications. For example, SNAP-25 contains two SNARE motifs that surround a palmitoylated cysteine rich region (Oyler et al., 1989; Hess et
al., 1992). Similarly, the ER v-SNARE Ykt6p may be inserted into the membrane by farnesylation at a C-terminal CAAX box (Søgaard et al., 1994). Recently, SNAREs have been classified as Q- or R-SNAREs on the basis that a central residue in the SNARE motif is either an arginine or a glutamine (Fasshauer et al., 1998). In the SNARE core complex these residues form an ionic layer in which one R-SNARE binds to three Q-SNAREs (Sutton et al., 1998). Some authors have proposed that the Q-/R-SNARE terminology is preferable to the v-/t-SNARE terminology since the localization of a given SNARE to a vesicle or target membrane does not necessarily correlate with the structurally defined SNARE subfamilies (Fasshauer et al., 1998).

A recent flurry of papers have revealed that, at least in biochemical reactions carried out to equilibrium, SNARE interactions are more promiscuous than would be predicted by the SNARE hypothesis (Rothman, 1994; Fasshauer et al., 1999; Yang et al., 1999; Grote and Novick, 1999; Tsui and Banfield, 2000). This may not be so surprising given the high homology of the SNARE motif between SNAREs, and it may be that trafficking specificity is conferred by the more variable N-terminal domain of SNAREs (Jahn and Südhof, 1999). However, it is important to note that these non-cognate SNARE interactions have not been demonstrated to be able to support any function. Thus, it is unclear whether they can support vesicle docking, and/or fusion, in any biological context, and so may be irrelevant biochemical anecdotes. It may be that such non-cognate SNARE pairs simply do not form in vivo, due to the myriad of protein-protein interactions that must occur prior to trans-SNARE pairing, and that in fact each level of these protein-protein interactions confers overall targeting specificity to each vesicle transport step (Pfeffer, 1999).

Vesicle docking must be a highly regulated process, were cognate v-/t-SNAREs always able to pair this might lead to all the organelles in the cytoplasm becoming docked, and clustered together, and could even compromise organelle identity (Pfeffer, 1999). In yeast vacuole docking, trans-SNARE pairing requires vacuole acidification, suggesting a highly regulated process (Ungermann et al., 1999b). The Sec1/Munc18 family of proteins also serve to regulate t-SNARE accessibility, and have been termed t-SNARE
protectors (Pfeffer, 1999). These are soluble molecules of c. 65kD that bind to the N-terminus of Q-SNAREs of the syntaxin family. Since a t-SNARE cannot bind a Sec1/Munc18 protein and a v-SNARE simultaneously, it has been proposed that these complexes form sequentially (Pevsner et al., 1994a, b; Lupashin and Waters, 1997). t-SNARE protectors may block trans-SNARE pairing by sterically hindering access to the SNARE motif. Alternatively, the Sec1/Munc18 molecule may bind to a t-SNARE conformation that interacts only poorly with a v-SNARE. Such a conformation occurs shortly after the action of NSF and α-SNAP (see Section 1.3.5) to dissociate cis-SNARE pairs (cis- denotes the SNAREs reside in the same membrane; Hanson et al., 1995), and may involve an intramolecular interaction between the N-terminal and C-terminal coiled-coil domains of the syntaxin (Hanson et al., 1995; Kosodo et al., 1998). The Sec1/Munc18 protein may break up this intramolecular interaction, and in so doing serve to signal that this SNARE is ready or primed for another round of transport. In this way the Sec1/Munc18 proteins may act to positively regulate t-SNARE function (Dascher and Balch, 1996), rather than just playing a negative role in preventing v-SNARE association.

The v-SNARE on a vesicle must gain access to its cognate t-SNARE to enable vesicle docking and fusion, and this involves displacement of the t-SNARE protector from the t-SNARE. Such t-SNARE deprotection may be tightly coupled to the vesicle tethering machinery (Pfeffer, 1999). In particular, the yeast Rab1 homologue Ypt1p has been shown to be required for trans-SNARE pairing between the t-SNARE Sed5p and the v-SNARE Bos1p by transiently interacting with Sed5p and displacing the Sec1/Munc18 molecule Sly1p from it (Lupashin and Waters, 1997; Rothman and Söllner, 1997). Ypt1p then rapidly dissociates from Sed5p and allows Bos1p to bind. This requires Ypt1p-GTP, which is then strictly a catalyst in trans-SNARE pairing, as it is not consumed by the reaction. Furthermore, the Sec1/Munc18 protein may enhance the reactivity of the cognate t-SNARE for specific Rab-GTP and in so doing regulate trans-SNARE pairing (Lupashin and Waters, 1997). In addition, since GTP hydrolysis by the Rab is not required for docking or fusion per se GTP hydrolysis may serve as a timer that determines the frequency of membrane docking events (Rybin et al., 1996).
Transport vesicles may then contain proteins that maintain Rabs in the active GTP bound state such that tethering and docking may occur (Pfeffer, 1999). The Rabs then serve primarily to regulate the events of vesicle tethering and docking (Pfeffer, 1999). However, they may also be involved in long range movements of vesicles via the cytoskeleton (Echard et al., 1998; White et al., 1999) and also in ensuring vesicles contain the correct complement of v-SNAREs (Lian et al., 1994). This diverse array of functions may be due to a heterogeneous collection of Rab effectors, whose point of action may be spatially and temporally regulated contingent upon the life cycle stage of the transport vesicle.

1.3.4 Vesicle Fusion

Once trans-SNARE pairing has docked a vesicle, it must then fuse with its acceptor membrane and deliver its cargo. Increasing evidence suggest that the SNARE molecules themselves represent the minimal membrane fusion machinery (Bock and Scheller, 1999). When cognate v- (VAMP1) and t-SNAREs (syntaxin-1 and SNAP-25) are reconstituted into separate liposome populations, these liposomes are able to dock and fuse with physiological kinetics resulting in mixture of both membrane and lumenal contents (Weber et al., 1998; McNew et al., 1999; Nickel et al., 1999; Parlati et al., 1999). This minimal system has enabled definition of functionally important parts of the neuronal SNARE molecules. The N-terminal domain of syntaxin-1 reduces the kinetics of liposome fusion dramatically (Weber et al., 1998; Parlati et al., 1999), and this is consistent with the fact that it may reduce the assembly of SNARE complexes by c. 2,000 fold (Nicholson et al., 1998). This may be due to an intramolecular interaction between the N-terminal domain and the membrane proximal coiled-coil SNARE motif (Hanson et al., 1995). The length of the region between the SNARE motif and the TMD is also a crucial determinant of SNARE activity (McNew et al., 1999). Other combinations of cognate v-/t-SNAREs have yet to be tested in this assay.

The neuronal v- and t-SNAREs form an extraordinarily stable core complex that is stable in 0.1% SDS (Hayashi et al., 1994) and at 90°C (Fasshauer et al., 1997). This core complex consists of a parallel four-helix bundle containing the two coiled-coil
SNARE motifs of SNAP-25, and the coiled-coil SNARE motifs of VAMP1 and syntaxin-1 (Hanson et al., 1997; Lin and Scheller, 1997; Hohl et al., 1998; Sutton et al., 1998; Fiebig et al., 1999). The Q-SNAREs SNAP-25 and syntaxin-1 contribute three helices, and the R-SNARE VAMP1 one, and this may be a feature of all cognate SNARE complexes (Jahn and Südhof, 1999). This has led to the proposal that free energy gained from formation of such a stable complex may be transformed, in concert with close membrane apposition, to drive the membrane fusion process. This energy may be harnessed to remove boundary layers of water that would hinder fusion, or in forcefully rearranging adjacent lipid bilayers to overcome the activation energy barrier to fusion (Weber, et al., 1998). An analogous hairpin-like helical bundle has been isolated from proteolytic fragments of viral fusion proteins (Skehel and Wiley, 1998), and may represent a general feature of biological membrane fusion machineries. Such pins may serve to forcefully mix adjacent membranes into which they are simultaneously inserted.

This view is challenged by data from Nilsson and colleagues who show that the ATPases NSF (Section 1.3.5) and its homologue p97 (Section 1.5.6) are also able to catalyze the fusion of donor liposomes to Golgi membranes and the fusion of donor liposomes with acceptor liposomes in a SNARE independent manner (Otter-Nilsson et al., 1999). This activity was optimized in the presence of the corresponding co-factors α-SNAP and p47, when present at a molar half ratio relative to the ATPase. At this level the ATPase activity of the complex in solution was found to be at its lowest. ATP hydrolysis was required for the fusion event, since it was inhibited by ATPγS, so it was proposed that the structural conformational change that occurs upon ATP hydrolysis is that which drives the fusion process. The soluble co-factors p47 and α-SNAP then serve to maintain the ATPase in the otherwise unstable ATP bound state. The authors then proposed that SNAREs provide a specific platform for recruitment of p97/NSF to the membrane mediated by p47/α-SNAP. Co-factor release would then allow ATP hydrolysis and catalyze membrane fusion (Otter-Nilsson et al., 1999).
This proposal rests uneasily in the context of the rest of the field, since the point of action of NSF catalyzed ATP hydrolysis (and so NSF and α-SNAP release from the membrane) is now more commonly thought to be in the untangling of cis-SNARE pairs (Section 1.3.5) prior to docking and fusion (Mayer et al., 1996a; Sato and Wickner, 1998; Ungermann et al., 1998a, b). However, NSF ATPase activity is not required for cisternal regrowth in the reassembly assay (Müller et al., 1999; Chapter 7), suggesting NSF does play other roles in the membrane fusion process that is distinct from its SNARE unpairing activity. Caution is required in interpreting these data from liposome systems as a number of proteins have been shown to mediate liposome fusion in vitro which would be ineffective on biomembranes. For example: glyceraldehyde 3-phosphate dehydrogenase (Morero et al., 1985), clathrin (Blumenthal et al., 1983), phospholipase C (Nieva et al., 1993), PLD (Blackwood et al., 1997), and annexins (Blackwood and Ernst, 1990) can all promote liposome fusion. However, given the available data that SNAREs act at or very close to the point of fusion, this makes them better candidates than NSF/p97 for the minimal fusion machinery (Bock and Scheller, 1999).

Further evidence that the SNAREs function upstream of fusion comes from Wickner and colleagues using a homotypic vacuole fusion assay. In this assay trans-SNARE pairing involving three v-SNAREs (Nyvlp, Vti1p, Ykt6p) and two t-SNAREs (Vam3p, Vam7p) is crucial for vacuole docking (Ungermann et al., 1999a), but can be uncoupled from fusion itself (Ungermann et al., 1998b). These workers claim that the trans-SNARE pairing only serves as a transient signal to downstream effectors, and so SNAREs act only indirectly in fusion. The trans-SNARE pair may invoke the release of Ca\textsuperscript{2+} from the vacuole lumen, which in turn recruits calmodulin to the membrane which triggers bilayer mixing, by an uncharacterized mechanism (Peters and Mayer, 1998; Schekman, 1998). Protein phosphatase I is also essential for the vacuole homotypic fusion process at a post trans-SNARE pairing stage, but again its target(s) or mode of action are entirely incognizant (Peters et al., 1999). Similarly this step of the reaction is also sensitive to GTP\textgamma;S and mastoparan suggesting the involvement of a G-protein (Conradt et al., 1994; Haas et al., 1994). Consistent with a fusion machinery
downstream of SNAREs is the Ca\(^{2+}\) triggered homotypic fusion of cortical vesicles from sea urchin eggs which has no requirement for the formation, presence nor disruption of trans-SNARE pairs (Coorssen et al., 1998; Tahara et al., 1998). Such localized bursts of Ca\(^{2+}\) are known to be important in neurons for neurotransmitter release, possibly due to the synaptotagmin (a Ca\(^{2+}\) sensor) mediated regulation of the neuronal SNARE core complex (Schiavo et al., 1995, 1997, 1998). However, whether they are required for all constitutive membrane fusion events remains unclear. In support of SNARE mediated fusion Scheller and co-workers working on the neuronal SNARE complex found that stable trans-SNARE complexes formed only after Ca\(^{2+}\) was present, and this occurs at the point of membrane fusion (Chen et al., 1999). Such contradictory findings are difficult to rationalize, and it may be that the findings from the minimal liposome system are not translatable to biomembranes or events \textit{in vivo}. Resolution of these differences between hypotheses will require much further work, but they will surely be unified since evolutionarily it would seem unlikely that distinct membrane fusion processes occur by radically different mechanisms.

1.3.5 Priming

After vesicle fusion has occurred the v- and t-SNAREs are complexed in the same membrane, i.e. are now a cis-SNARE pair. This complex must be unravelled to release free SNAREs in an active state ready for subsequent rounds of transport, and this is achieved by the actions of the highly conserved molecules: NSF and \(\alpha\)-SNAP (Rothman, 1994). This process is termed priming (Mayer et al., 1996a; Ungermann et al., 1998a). NSF was first characterized as a component necessary for intra-Golgi transport (Block et al., 1988; Wilson et al., 1989; Malhotra et al., 1988), and is a member of the AAA family of ATPases (Patel and Latterich, 1998). NSF exists as barrel-shaped hexamer with an outer diameter of 13-17nm and a typical height of 9-10nm with a small channel at the centre (Hanson et al., 1997; Fleming et al., 1998). Each monomer contributes one stave of the barrel and consists of a N-terminal domain, followed by two ATPase cassettes (the D1 and D2 domains) of approximately 200-230 amino acids, containing classical Walker A and B motifs required for ATP binding and hydrolysis respectively (Tagaya et al., 1993). The N-terminal domain interacts
with SNAPs and SNAREs, while the D1 domain acts upon these SNAREs, and the D2 domain contributes to the hexamerization process (Nagiec et al., 1995; Whiteheart et al., 1994).

NSF is recruited to cis-SNARE complexes (Ungermann et al., 1998a, b; Swanton et al., 1998; Littleton et al., 1998), via α-SNAP, and this binding may be enhanced by γ-SNAP (Clary et al., 1990). This complex sediments at 20S and is disassembled in the presence of Mg-ATP (Söllner et al., 1993a), probably due to NSF catalyzed ATP hydrolysis, which is stimulated by SNAPs and SNAREs (Morgan et al., 1994; Haynes et al., 1998; Matveeva and Whiteheart, 1998). α-SNAP is composed of an N-terminal twisted sheet of alpha-helical hairpins and a C-terminal alpha-helical bundle and may provide the leverage to dissociate cis-SNARE complexes in conjunction with conformational changes in NSF (Rice and Brunger, 1999). On hydrolysis of ATP the diameter of the NSF hexamer is reduced (Hanson et al., 1997; Müller et al., 1999b), and this may impart a rotational force onto the bound α-SNAP which would then unwind the cis-SNARE pair in manner analogous to a hand drill (Owen and Schiavo, 1999). This disassembly emancipates individual SNAREs for engagement in subsequent fusion reactions. In the case of yeast vacuole fusion this unpairing is coupled to the transfer of LMA1, to the t-SNARE Vam3p (Xu et al., 1998). LMA1 is a heterodimer of thioredoxin and proteinase B inhibitor (Xu et al., 1997; Slusarewicz et al., 1997), and also plays a role in yeast ER-Golgi transport (Barlowe, 1997). LMA1 is postulated to trap the t-SNARE in an otherwise unstable active state, and in so doing acts as a molecular chaperone that keeps the t-SNARE active, in preparation for trans-pairing with a cognate v-SNARE (Xu et al., 1998). Precisely how LMA1 achieves this function remains to be determined. A similar function in activating a t-SNARE ready for subsequent rounds of fusion may be carried out by the Sec1/Munc18 family of proteins (Dascher and Balch, 1996; Haynes et al., 1999).

The point of action of NSF was originally thought to be at the point of membrane fusion, and that this act of unpairing the SNAREs drove the membrane fusion process (Söllner et al., 1993a, b; Rothman, 1994). This view has become increasingly untenable.
with the findings that NSF and α-SNAP act a significant time before the fusion event to dissociate cis-SNARE complexes, rather than trans-SNARE complexes (Mayer et al., 1996a; Nichols et al., 1997; Ungermann et al., 1998a). Furthermore, the SNARE disassembling activity of NSF was not required for the liposome fusion assay of Rothman and colleagues (Weber et al., 1998). Two recent studies suggest that NSF carries out other functions in the membrane fusion process that may be distinct from its ATPase dependent SNARE disassembly (Otter-Nilsson et al., 1999; Müller et al., 1999b; Schwarz, 1999; Chapter 7). It is clear from these studies that NSF will continue to attract considerable attention.

1.4 Morphological organization of the Golgi apparatus.

1.4.1 Organization and conservation.

The Golgi apparatus exists as a series of flattened disk shaped membranes, termed cisternae, that are tightly aligned in parallel to one another to form a stack (Figure 1.4; Tanaka et al., 1991; Rambourg and Clermont, 1997; Ladinsky et al., 1999). The number of cisternae per stack can vary from a few to as many as 40 (e.g. in certain Euglenoids [Becker and Melkonian, 1996] and the nurse cells of the insect Oniscus [Fawcett, 1981]), although within a particular cell type the number appears to be constant (Rambourg and Clermont, 1997). Cisternae are approximately 20-30nm in cross-sectional width and are separated from each other by a 10-15nm intercisternal gap. Each cisterna can be divided into two morphological domains, a central flattened core region without fenestrations that likely harbours the Golgi enzymes and a fenestrated peripheral rim from which vesicles appear to bud (Figure 1.5A; Weidman et al., 1993). Transport vesicles are closely associated, often by fibrous attachments (Figure 1.5B; Weidman et al., 1993; Orci et al., 1998) with these fenestrated cisternal rims at every layer of the stack. In fact, such fenestrations can be found to precisely align between adjacent cisternae through the entire Golgi stack and are then termed wells (Rambourg and Clermont, 1997; Ladinsky et al., 1999). Transport vesicles appear to transfer between cisternae within these wells, budding from the internal edge of one fenestration and fusing with the internal edge of the fenestration of the adjacent cisterna (Ladinsky et al., 1999). Such wells may serve to increase the surface area available for vesicle
Figure 1.4 The Golgi apparatus of mammalian cells.
(A) Transmission electron micrograph of a NRK cell. Note the distinct profiles of interconnected stacks (arrows) and frequent transport vesicles (arrowheads). M, mitochondrion; N, nucleus. (B) Scanning electron micrograph of rat lacrimal gland cell. Note the reticular structure of the Golgi apparatus. Arrows denote stacks and arrowheads transport vesicles. Bars, 0.5 \mu m. Courtesy of Eija Jokitalo (A) and K. Tanaka (B).
Figure 1.5 Cisternal domains and fibrous tethers.

(A) Golgi membranes immobilized on glass coverslips and incubated with 2mg/ml cytosol for 10min at 37°C in the presence of 20μM GTPγS, and then processed for freeze etch EM. Note two clear cisternal domains, the unfenestrated cisternal core (marked by an asterisk) which probably harbours the glycosylation enzymes, and the fenestrated peripheral rim from which vesicles bud (arrows). Bar, 250nm. From Weidman et al. (1993). (B) At higher magnification numerous fibrous tethers or strings can be seen to link vesicles to each other and to adjacent cisternae. Bar, 50nm. Courtesy of John Heuser.
transfers at the rims of cisternae.

This unique architecture is highly conserved throughout eukaryotic evolution, and is even found in the diplomonad *Giardia lamblia*, one of the most primitive extant eukaryotes (Reiner et al., 1990; McCaffery and Gillin, 1994; Luján et al., 1995; Lanfredi-Rangel et al., 1999). Although most organisms have stacks of cisternae there are a few exceptions. For instance, the ancient eukaryotic phyla of archamoebae (e.g. *Pelomyxa* species) and microsporidia seem to lack a Golgi stack, but this may be due to lack of precise ultrastructural examination (Becker and Melkonian, 1996). Similarly, the human malaria parasite *Plasmodium falciparum* seems to lack Golgi cisternae and stacked membranes, especially at the ring and trophozoite life cycle stages (Haldar, 1998). Instead the parasite possesses separated tubulovesicular membranes that contain cis- and trans-Golgi markers which fulfil Golgi function, and may represent a primitive form of the Golgi apparatus (VanWye et al., 1996; Haldar, 1998). However, perhaps the most notable exception is the budding yeast, *S. cerevisiae* where the cisternae are mostly discrete, although up to 40% exist as stacks of two or even three cisternae (Preuss et al., 1992) implying that the Golgi stacks of this organism may not be as stable as in others. This is not a universal feature of yeasts as *Schizosaccharomyces pombe* and *Pichia pastoris* possess clear stacks of Golgi cisternae (Ayscough et al., 1993; Rossanese et al., 1999). In plants and fungi, the Golgi stacks are discrete and multiple copies are dispersed throughout the cytoplasm, apparently at random (Driouich and Staehelin, 1997). In mammalian cells, the Golgi apparatus can be seen as a single copy organelle as the discrete Golgi stacks are linked to form a tight, twisting ribbon (Figure 1.4; Tanaka et al., 1991; Shima et al., 1997; Rambourg and Clermont, 1997; Ladinsky et al., 1999). The linkages between discrete Golgi stacks are usually tubulovesicular in nature, and link equivalent cisternae in adjacent stacks. These regions of the ribbon are referred to as the non-compact zone, whereas the stacked regions are referred to as the compact zone (Rambourg and Clermont, 1997). The Golgi stack is bounded on either face by the extensive tubulovesicular networks of the CGN and TGN.
1.4.2 Position in the cell.

The Golgi apparatus typically occupies a juxtanuclear, usually pericentriolar position in most mammalian cells. Which side of the nucleus Golgi apparatus resides can reflect which area of the cell surface exocytosis is directed towards. For example, the Golgi apparatus is orientated on the side of the nucleus proximal to the leading edge of a migrating cell (Nobes and Hall, 1999). Similarly, when a cytotoxic lymphocyte binds to an antigenic cell, there is a reorientation of the Golgi apparatus to the side of the nucleus proximal to the offending cell and a directed exocytosis of cytoplasmic granules toward it (Atkinson and Bleackley, 1995). This long range ordering of Golgi structure is dependent on interactions with the cytoskeleton. That Golgi apparatus positioning is dependent on microtubules is confirmed by observations that microinjection of antitubulin antibodies (Wehland and Willingham, 1983) or depolymerization of microtubules with nocodazole (Sandoval et al., 1984) fragments the Golgi apparatus into a series of disseminated, discrete mini-stacks (Cole et al., 1996a; Shima et al., 1998; Storrie et al., 1998). These mini-stacks may represent the discrete stacked units of the Golgi ribbon and remain functional in that nocodazole treatment has no effect on the secretion or sialylation of VSVG (Featherstone et al., 1985; Rogalski et al., 1984). In contrast, disruption of microtubules in S. pombe, using thiabendazole or in a cold sensitive mutant cell line, resulted in unstacking of Golgi cisternae (Ayscough et al., 1993). Nocodazole induced dispersal of Golgi mini-stacks appears to be a kinesin driven process (Minin, 1997), and is currently a controversial subject. This is because some authors propose that nocodazole induced dispersal involves transit through the ER (Cole et al., 1996a; Storrie et al., 1998), while others suggest that this is not required (Shima et al., 1998). Contradictory results have been obtained whereby use of a dominant negative mutant Sar1p that blocks ER exit, either accumulates Golgi residents in the ER (Storrie et al., 1998) or does not (Shima et al., 1998) upon nocodazole treatment. The reasons for such discrepancies remain unclear, but may have to do with the different incubation times used.

Recovery from nocodazole also involves microtubule dependent saltatory movements which cluster Golgi mini-stacks in the pericentriolar region (Kreis et al., 1988; Ho et al.,
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1989). Such movements are consistent with a role for dynein in moving the Golgi apparatus to the pericentriolar region, and this has been confirmed in an assay where broken CHO cells 'capture' purified Golgi membranes and return them to the centrosomal region (Corthésy-Theulaz et al., 1992). This process can be accelerated by mapmodulin, a protein that may clear microtubule tracks of obstructing microtubule associated proteins (MAPs), so accelerating dynein mediated organelle movement (Ulitzur et al., 1997a, b). The precise mode of linkage of Golgi membranes to microtubules remains unclear, but may involve linkage via dynactin to dynein (Burkhardt et al., 1997; Harada et al., 1998; Ma et al., 1999) and an extensively coiled coil peripheral membrane Golgin GMAP-210 (Infante et al., 1999). In support of the latter, the C-terminal microtubule-binding domain of GMAP-210 when tagged with GFP and transfected into cells localized to the centrosome (Infante et al., 1999).

With the exception of the unstacking data from S. pombe (Ayscough et al., 1993), cytoskeletal proteins seem to be more responsible for the long range order of Golgi architecture, rather than the structure of individual stacks. Cytochalasin D treatment to disrupt actin based microfilaments or microinjection of antibodies specific for intermediate filaments has no effect on Golgi morphology (Ho et al., 1989). However, interactions with the actin cytoskeleton may affect the position of the Golgi in mammalian cells (di Campli et al., 1999), and may be more important for Golgi stack movements towards ER exit sites and sites of cell wall synthesis in plant cells (Nebenfuhr et al., 1999).

1.4.3 Stacking.

Since Golgi membranes can be isolated from tissues as stacks (Morré and Mollenhauer, 1964; Morré et al., 1970; Fleischer and Fleischer, 1970; Leelavathi et al., 1970; Hino et al., 1978; Hui et al., 1998) it would appear likely that the stack is a stable structure. In vivo the Golgi apparatus is embedded in an electron dense 'zone of exclusion', which represents a fibrous matrix and is so-called because it excludes structures the size of ribosomes and above (Morré and Ovtracht, 1977; Mollenhauer and Morré, 1978). Close inspection of cisternae by EM reveals the presence of fibrillar structures running
parallel to cisternae (Mollenhauer, 1965; Amos and Grimstone, 1968; Staehelin et al., 1990) and electron dense proteinaceous material that bridges the intercisternal space (Franke et al., 1972; Cluett and Brown, 1992). Such intercisternal crossbridges are c. 8.5nm in width and 11nm in height and recur at 10nm intervals along individual cisternae (Cluett and Brown, 1992). These structures can be degraded by proteolysis which also unstacks Golgi cisternae (Mollenhauer et al., 1973; Cluett and Brown, 1992). Such structures constitute a cisternal exoskeleton or matrix that probably acts to maintain the Golgi stack. Such an exoskeleton may also co-operate with a cisternal endoskeleton to maintain Golgi architecture. Consistent with this, electron dense intracisternal crossbridges can also be visualized by EM, that span the cross-sectional width of cisternal lumen and are sometimes continuous with intercisternal crossbridges (Franke et al., 1972). The fact that single cisternae liberated by proteolysis maintain their disk shaped morphology indicates that this Golgi endoskeleton or luminal matrix maintains the cisternal shape. It may be that the large oligomeric structures formed by Golgi enzymes, such as MannII and NAGTI, function to maintain the characteristic disk shape of cisternae (Nilsson et al., 1994, 1996). Consistent with this possibility is the observation that the induction of the glycosylation enzymes in G. lamblia during trophozoite encystation correlates with the formation of prominent, flattened cisternal membranes (Luján et al., 1995). Furthermore, the absence of disk shaped cisternae in P. falciparum may be due to the lack of glycosylation enzymes in this organism (Haldar, 1998).

Attempts have been made to isolate these matrix like structures by detergent extraction of purified Golgi membranes in a manner similar to that used in the identification of the nuclear lamina (Dwyer and Blobel, 1976). Extraction of purified Golgi stacks with detergent at low salt reveals fibrous structures highly reminiscent of stacked cisternae (Slusarewicz, 1994; Slusarewicz et al., 1994; Fath et al., 1997). Furthermore, this Golgi matrix was found to bind to the medial-Golgi enzymes NAGTI and MannII with high affinity (Slusarewicz et al., 1994). This in itself suggests a stacking mechanism for the Golgi apparatus since these enzymes have been localized to both medial- and transcisternae within the Golgi stack (Nilsson et al., 1994; Rabouille et al., 1995a). Were the
cytoplasmic tails of the NAGTI and MannII in adjacent cisternae to interact with the intercisternal matrix, this would anchor cisternae together. However, the identity of matrix proteins that interact with only the cytoplasmic tails of enzymes has so far proven elusive. Furthermore, peptides representing the cytoplasmic tails of MannII and NAGTI were unable to compete for binding of the enzymes to the matrix. This coupled to the fact the lumenal domains of the enzymes could also bind to the matrix suggest that a fraction of the matrix may be composed of lumenal proteins (Slusarewicz et al., 1994; Slusarewicz, 1994). However, consistent with the importance of Golgi enzymes in maintaining stacked structure is the observation that mutation of the TMD of NAGTI to a series of leucine residues resulted in unstacking of cisternae, and loss of cisternal shape (Nilsson et al., 1996). The conformational change this mutation induced in NAGTI may have disrupted inter- and intracisternal interactions essential for maintenance of Golgi morphology (Rabouille and Nilsson, 1995).

Fractionation of the Golgi matrix by SDS-PAGE revealed the presence of 12-15 major proteins and numerous minor proteins (Slusarewicz et al., 1994; Slusarewicz, 1994; Hui, 1997). Amongst these proteins were GM130 (Nakamura et al., 1995), GRASP65 (Francis Barr, personal communication), giantin and p115 (Nobuhiro Nakamura, personal communication) the COPI vesicle tethering machinery (Sonnichsen et al., 1998; Section 1.3.3). This hinted at another mechanism by which the Golgi stack may be maintained or established, if p115 were to simultaneously bind GM130 and giantin in adjacent cisternae (See Chapter 4). Furthermore, GRASP65 has been implicated in stacking Golgi cisternae (Barr et al., 1997, 1998; Shorter and Warren, 1999; Shorter et al., 1999; See Section 1.5.7; Chapter 4 and 5). Other components of the Golgi matrix were identified as actin, cytokeratins 8 and 18 (Hui, 1997), suggesting the Golgi matrix may be linked to elements of the cytoskeleton. A number of other bands were subjected to peptide-mass fingerprinting analysis, but were not found in the database (Pappin et al., 1993; Hui, 1997).

There are now a number of other proteins that are candidates for components of the Golgi matrix. One group of proteins, the Golgins, represent a large family of long, rod-
like coiled coil proteins which often contain one or more flexible hinges (Chan and Fritzler, 1998). The Golgins are either peripherally or integrally associated with the Golgi apparatus with the C-terminus anchored at the Golgi membrane and N-terminus projecting out into the cytoplasm to capture interacting molecules. They were initially identified using sera from patients with auto-immune diseases such as Sjögren’s syndrome and rheumatoid arthritis (Chan and Fritzler, 1998; Table 1.4). A number of Golgins still remain to be identified: Golgins 35kDa to 260kDa (14 proteins), of which only Golgin-245 is sequenced so far (Kooy, 1994).

Table 1.4 The Golgins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>Interactions</th>
<th>Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golgin-67</td>
<td>67kDa</td>
<td>?</td>
<td>Cdc2 and Src kinase phosphorylation motifs</td>
<td>Jakymiw et al., 2000</td>
</tr>
<tr>
<td>Golgin-84</td>
<td>84kDa</td>
<td>?</td>
<td>Transport vesicle associated.</td>
<td>Bascom et al., 1999</td>
</tr>
<tr>
<td>Golgin-95</td>
<td>95kDa</td>
<td>?</td>
<td>A shorter form of GM130.</td>
<td>Fritzler et al., 1993</td>
</tr>
<tr>
<td>Golgin-97</td>
<td>97kDa</td>
<td>Rab6</td>
<td>60nm in length</td>
<td>Barr, 1999</td>
</tr>
<tr>
<td>Golgin-160</td>
<td>160kDa</td>
<td>Sec34p, Sec35p</td>
<td>COPII vesicle tether? Microtubule binding?</td>
<td>Kim et al., 1999; Misumi et al., 1997</td>
</tr>
<tr>
<td>GM130</td>
<td>130kDa</td>
<td>p115 GRASP65</td>
<td>Part of COPI vesicle tether. 80nm in length</td>
<td>Nakamura et al., 1995; Barr et al., 1997; Sönничsen et al., 1998</td>
</tr>
<tr>
<td>GMAP-210</td>
<td>210kDa</td>
<td>Minus ends of microtubules</td>
<td>May localize Golgi to centrosome.</td>
<td>Rios et al., 1994; Infante et al., 1999</td>
</tr>
<tr>
<td>Golgin-245</td>
<td>245kDa</td>
<td>Rab6</td>
<td>Associated with non-clathrin coated vesicles. Granin signature. 150nm in length</td>
<td>Barr, 1999; Fritzler et al., 1995; Erlich et al., 1996; Gleeson et al., 1996</td>
</tr>
<tr>
<td>Giantin</td>
<td>372kDa</td>
<td>p115</td>
<td>Part of COPI vesicle tether. 250nm in length.</td>
<td>Linstedt and Hauri, 1993; Sönничsen et al., 1998</td>
</tr>
</tbody>
</table>
The best characterized Golgins are GM130 and giantin (Nakamura et al., 1995; Linstedt and Hauri, 1993), and function with p115 as components of a heteroternary COPI vesicle tether (Section 1.3.2; Sönichsen et al., 1998; Shorter and Warren, 1999; Lesa et al., 2000; Dirac-Svejstrup et al., 2000). By analogy the other Golgins have been proposed to be involved in the tethering of vesicles to cisternae or cisternae to each other, however, this has not been so clearly demonstrated as for GM130 and giantin. The yeast homologue of Golgin-160 Grp1p interacts with Sec34p, a COPII vesicle tethering protein, and has been proposed to be important for the maintenance of Golgi structure (Kim et al., 1999). Golgins -97 and -245 are targeted to the Golgi apparatus by a highly conserved C-terminal GRIP domain of approximately 50 amino acids (Munro and Nichols, 1999; Kjer-Nielsen et al., 1999; Barr, 1999) and this seems to involve an interaction with Rab6 (Barr, 1999). Rab6 has been implicated in intra-Golgi transport (Martínez et al., 1994, 1997; Mayer et al., 1996b), endosome to Golgi transport (Tsukada et al., 1999) as well as ER-Golgi retrograde transport (Echard et al., 1998; Giraud et al., 1999; White et al., 1999) suggesting a possible role for these Golgins in a vesicle tethering reaction. The Golgins may also serve to anchor the Golgi matrix to the surrounding cytoskeleton. Both GMAP-210 and Golgin-160 have been implicated in microtubule binding (Infante et al., 1999; Misumi et al., 1997), and so may contribute to the position of the Golgi apparatus within the cell. Furthermore, isolated Golgi matrices are able to move along microtubule tracks in the presence of the molecular motor dynein (Fath et al., 1997).

The similarities between Golgins and SNAREs are also intriguing. Both are coiled-coil proteins with the same membrane orientation and predicted rod-like structure. Golgins are generally much longer than SNAREs so they could act at a greater distance from the membrane. Coupled with their flexibility they may permit the vesicle to sample a target membrane for a cognate SNARE, or simply increase the efficiency of transport by preventing vesicles from diffusing away. Similarly in Golgi stacking Golgins may act to bring nascent cisternae together in the initial stages of stacking, and then hand over to the SNAREs to complete the reaction. The Gradgrindian view of science: facts come first, ideas later, is very rarely true. This is clearly demonstrated by one of the
extensions of the SNARE hypothesis, that states that the stacking of Golgi cisternae may be mediated by cognate v-/t-SNARE pairs (Rothman and Warren, 1994). Cisternal stacking would then reflect a specialized form of SNARE mediated docking (Section 1.3.3), and would differ in that the docking state must be frozen, and not be allowed to proceed on to the fusion step. This may be mediated by a hypothetical fusion clamp, or there may be SNARE isoforms that are only able to dock and not fuse membranes. Such a SNARE mediated stacking mechanism would be consistent with the ordered stacking (i.e. medial cisternae stack/dock with cis cisternae, and trans cisternae stack/dock with medial cisternae), close apposition and constant spacing of stacked cisternae (Rothman and Warren, 1994).

Another group of proteins, more commonly thought of as components of the actin cytoskeleton, may contribute to Golgi architecture, and these are the Golgi localized isoforms of ankyrin and spectrin (Beck et al., 1994, 1997; Devarajan et al., 1996; Stankewich et al., 1998; Beck and Nelson, 1998; De Matteis and Morrow, 1998). Given the importance of spectrin and ankyrin in maintaining erythrocyte plasma membrane structure (Marchesi and Steers, 1968; Morrow et al., 1997), it would seem likely that spectrin could play an analogous role in maintaining Golgi architecture. Spectrin forms a long, thin flexible rod of c. 100nm in length and exists as a heterodimer of homologous but distinct subunits, termed α and β, arranged in a head to tail orientation. Spectrin heterodimers can self-associate end to end to form linear oligomers that act as a minimal structural unit of a membrane skeleton (Shotton et al., 1979). βIII spectrin is a ubiquitously expressed spectrin isoform and localizes with the Golgi and unidentified cytoplasmic vesicles (Stankewich et al., 1998). As yet no Golgi associated α-spectrin has been detected and it may be that βIII spectrin forms homodimers (De Matteis and Morrow, 1998). Two isoforms of Golgi ankyrin have been identified a 119kDa form and a 195kDa form, and these may serve to link the spectrin lattice to the Golgi membrane (Beck and Nelson, 1998). Golgi localized actin or centractin have been hypothesized to crosslink linear spectrin oligomers to generate a two dimensional lattice on the cytoplasmic face of the Golgi membrane (Beck and Nelson, 1998). These Golgi spectrin and ankyrin isoforms form part of the detergent insoluble Golgi matrix
(Beck et al., 1997), but it is as yet unclear whether the putative spectrin skeleton is found in the intercisternal space or restricted to the cis- or trans-aspects of the Golgi stack (De Matteis and Morrow, 1998).

Assembly of the spectrin/ankyrin Golgi matrix is regulated by the small GTPase ARF1, and is dependent on ARF1 stimulated phosphatidylinositol bisphosphate (PIP₂) synthesis, which binds a PH domain in Golgi spectrin (Godi et al., 1998). ARF1 acts to a recruit phosphatidylinositol-4-OH kinase-β (PI-4-OH kinase) to the Golgi membrane and in so doing stimulate PIP₂ synthesis (Godi et al., 1999). Disruption of the spectrin/ankyrin Golgi matrix by transfecting cells with truncated forms of Golgi spectrin inhibits ER-Golgi transport of VSVG and Na⁺/K⁺-ATPase, but not that of E-cadherin (Devarajan et al., 1997). However, disruption of the matrix did not seem to perturb Golgi morphology (Devarajan et al., 1997).

1.4.4 Function of stack.

Despite the evolutionary conservation of this elaborate stacked cisternal organization, its functional significance remains unclear and moot. However, a number of hypotheses have been proposed to explain the necessity of stacked, cisternal architecture.

The flattened disk shape of cisternal membranes has been suggested to contribute to the efficiency of glycosylation events that occur within the stack, by enhancing the surface area to lumenal volume ratio of the compartment. This would be advantageous if the glycosylation enzymes acted at a rate that was more rapid than the rate of sugar nucleotide import into cisternae. Increasing the surface area of the compartment relative to volume would allow these two processes to keep the same pace (Mellman and Simons, 1992). Alternatively, the high surface area to volume ratio of cisternae may serve to keep secretory cargo concentrated to enable efficient glycosylation.

With regard to why these cisternae are stacked a number of hypotheses have been suggested. The first suggests the stack may be analogous to distillation tower, distilling secretory cargo away from escaped ER residents by an iterative process (Rothman,
1981; Rothman and Wieland, 1996). Since it is possible to retrieve ER residents even at the most trans-pole of the Golgi stack the sorting of ER residents from secretory proteins may occur in a multistage process akin to fractional distillation (Rothman, 1981). Evidence for such a distillation process is provided by the fact that the cis-Golgi cisternae have a higher freeze-fracture particle density than trans-cisternae (Orci et al., 1998). However, since resident ER proteins rarely travel beyond the first two cisternae of the stack (Pelham, 1995), it would seem improbable that cells would require extended stacks just for the purpose of retrieving escaped ER residents. The extended stack may then be more relevant for the purposes of lipid sorting, which may be more difficult than protein sorting and so require multiple steps. Consistent with this is that cis-Golgi (more ER like lipid composition) to trans-Golgi (more plasma membrane like lipid composition) lipid gradients are observed (Orci et al., 1981; Van Meer, 1998). This would particularly apply to cholesterol, which must be rapidly pumped from its site of synthesis in the ER to the plasma membrane where it functions. Pumping the cholesterol out of the ER may be essential because high cholesterol levels may interfere with the translocation of nascent proteins across the ER membrane (Bretscher and Munro, 1993).

The stack may simply be an inescapable consequence of the mode of transport through it and so represents a device that ensures rapid, efficient, processive vesicular transfers between adjacent cisternae due to their close proximity (Palade, 1975; Orci et al., 1998; Sönichsen et al., 1998; Shorter and Warren, 1999). However, this would not appear to be the case in yeasts. Firstly the majority of cisternae in S. cerevisiae are single (Preuss et al., 1992) suggesting that permanently stacked cisternae are not essential for efficient exocytic transport. However, stacks accumulate in certain mutant cells suggesting a stacking mechanism does exist (Novick et al., 1981; Benli et al., 1996). It may be that stacking is more transient in S. cerevisiae, enabling vesicle transfers only at specific times, or that vesicles are readily diffusible. Similarly, although S. pombe possesses a highly stacked Golgi apparatus, unstacking these cisternae using microtubule interfering agents, has no discernible effect on the rate of exocytic transport (Ayscough et al., 1993). However, the stacking mechanism in S. pombe may be distinct to that of
mammals in that microtubules are not required to maintain the mammalian Golgi stack,
and unlike \textit{S. pombe} the mammalian Golgi apparatus is not disrupted by inhibitors of
protein synthesis (Ayscough and Warren, 1994). So far it has not been possible to
unstack the mammalian Golgi apparatus, without inducing its vesiculation or
tubulation, so similar manipulations of the Golgi have not been possible in mammalian
cells. Until this is possible it is uncertain whether the Golgi stack contributes to
exocytic efficiency \textit{per se}.

Finally an ordered stack of cisternae may be necessary to perform efficient, sequential
post-translational modifications of the secretory cargo as it moves \textit{en passant} from
cisterna to cisterna. This is best illustrated by the distribution of N-glycosylation
enzymes. These enzymes are distributed across the stack in the order in which they
operate on the cargo passing through it, and can be considered analogous to an
assembly line (Farquhar, 1985). Although this spatial organization of the glycosylation
enzymes is not required for the correct execution of the sequential addition of sugar
residues \textit{per se} (Doms et al., 1989), it may well increase the efficiency of the process.
Furthermore, if one adopts a cisternal maturation standpoint, one can then view the
Golgi stack as a ‘delay timer’. By forcing a cisterna to progress through the entire stack
may ensure secretory cargo is exposed to the glycosylation machinery for sufficient
time to allow correct modification with high fidelity (Glick and Malhotra, 1998). This
may also explain the polymorphism of the number of cisternae per stack that exists
between cell types in the same organism and between organisms. For it may be that the
extent and complexity of the post-translational modification that must occur in the
stack correlates positively with the number of cisternae per stack (Becker and
Melkonian, 1996). For example, organisms such as ciliates which conduct little protein
glycosylation and do not manufacture complex polysaccharides, tend to have few
cisternae per stack. Whereas, the multifarious algal phyla synthesize massive quantities
of highly complex carbohydrates and possess large numbers of cisternae per stack
(Becker and Melkonian, 1996).
Testing these hypotheses has proven extremely difficult since it is not yet possible to precisely manipulate Golgi architecture, therefore evidence supporting such hypotheses tends to be correlative rather than definitive. Manipulation of Golgi architecture requires a better understanding of the structural elements that underlie it.

1.4.5 Golgi structure reflects a dynamic equilibrium.

The Golgi apparatus is a highly dynamic organelle and this has become especially clear with the advent of GFP technology to monitor Golgi behaviour in real time (Scales et al., 1997; Presley et al., 1997, 1998; Sciaky et al., 1997; Cole et al., 1996b, 1998; Shima et al., 1997, 1998, 1999; Storrie et al., 1998; Wooding and Pelham, 1998; Zaal et al., 1999). Assuming only anterograde transport and a linear ER-TGN-plasma membrane transport pathway the flux of membrane through the Golgi has been estimated to be c. 3µm² membrane/min (Griffiths et al., 1989; Johansen et al., 1984). Golgi compartments increase or decrease in surface and volume in precise accordance with the amount of membrane flux through them (Griffiths et al., 1989; Rambourg et al., 1993; Clermont et al., 1993a; Buccione et al., 1996). Since the Golgi apparatus appears to be in a dynamic equilibrium of membrane acquisition and removal, perturbations in this balance may be expected to have morphological implications. The intimate structure/function relationship of the Golgi apparatus becomes particularly apparent upon interference with vesicle transport processes. This is especially clear in mitosis where membrane traffic ceases (see Section 1.5.2; Warren, 1993). Similarly, microinjection of anti-p115 antibodies, expression of a truncated forms of p115 lacking regions of the globular head, or the GM130/giantin binding domains and expression of a GM130 mutant lacking the p115 binding domain all disrupt Golgi morphology (Alvarez et al., 1999; Nelson et al., 1998; Seemann et al., 2000). This suggests that correct p115 interactions facilitating COPI vesicle tethering are required to maintain Golgi architecture. Microinjection of a dominant negative form of Rab1a restricted to GDP binding results in vesiculation of the Golgi apparatus, presumably due to an inhibition of a tethering/docking event required for ER-Golgi transport (Wilson et al., 1994; Pind et al., 1994; Nuoffer et al., 1994). Similarly, overexpression of wild type Rab6 or a GTP restricted Rab6 mutant invokes the redistribution of Golgi markers to the ER (Martinez et al., 1997), possibly
by stimulating a COPI independent Rab6 controlled retrograde pathway (Echard et al., 1998; Girod et al., 1999; White et al., 1999). Inhibition of NSF activity with NEM also leads to vesiculation of Golgi membranes (Orci et al., 1989).

The importance of coat proteins in maintaining Golgi architecture becomes apparent upon microinjection of GTPyS and expression of a GTP restricted ARF1 mutant which results in extensive vesiculation of the Golgi apparatus (Dascher and Balch, 1994; Pepperkok et al., 1998, 2000). This is likely due to COPI vesicles being unable to uncoat and so fuse with their target membrane and this would promote their accumulation at the expense of cisternal membrane. In other words, the Golgi apparatus would bud itself to extinction. Similarly, prevention of coatomer binding to membranes causes the conversion of Golgi stacks to extensive tubular networks. In vitro and in vivo addition of BFA (Lippincott-Schwartz et al., 1989; Orci et al., 1991), low cellular ATP levels (Cluett et al., 1993) and in vitro depletion of coatomer from cytosol induced tubular network formation (Misteli and Warren, 1994). These data indicate in the absence of vesicle fusion Golgi membranes tend to vesiculate, and in the absence of vesicle formation membranes fuse promiscuously to generate tubular networks (Elazar et al., 1994b).

In order to delineate the molecular mechanisms responsible for the maintenance and establishment of Golgi architecture many investigators have taken advantage of agents that reversibly disrupt Golgi structure. Removal of such agents may then allow the reformation of Golgi structure, and allow attempts to dissect the molecular workings of this process. The approach used in this thesis to study Golgi architecture has taken advantage of the dramatic alterations that occur in Golgi morphology during the mammalian cell division cycle as part of the Golgi inheritance process (Section 1.5). However, another approach is the use of drugs that selectively and reversibly dismantle the Golgi apparatus. A number of such drugs have been identified and include: okadaic acid (Lucocq et al., 1991, 1995; Pryde et al., 1998), nordihydroguaiaretic acid (Yamaguchi et al., 1997; Fujiwara et al., 1998; Drecktrah et al., 1998), BFA (Section
1.4.5), and IQ (Section 1.4.6). BFA and IQ are perhaps the best characterized and have been used to probe the molecules that may regulate Golgi structure.

**1.4.6 Brefeldin A**

BFA is a fungal metabolite, and acts to prevent the recruitment of ARF1 to the Golgi membrane (Helms and Rothman, 1992; Donaldson et al., 1992) by inducing the formation of an abortive ARF-GDP: BFA: ARF-GEF complex (Peyroche et al., 1999). ARF1 then accumulates in the cytosol and COPI is unable to bind to Golgi membranes (Klausner et al., 1992). Similarly, the absence of ARF1 from Golgi membranes will alter the PLD and PI-4-OH kinase activities of the membranes and this may also have some effect on Golgi morphology (Ktistakis et al., 1995; Godi et al., 1999). 

*In vitro* BFA induces the formation of tubular networks by the promiscuous fusion of cisternae, possibly due to the lack of coat proteins meaning the membrane machinery is inappropriately exposed (Orci et al., 1991). This fusion activity requires the action of NSF (Orci et al., 1991). *In vivo*, in addition to this tubular-reticulum formation (Fukunaga et al., 1998), Golgi residents are redistributed to the ER, possibly due to BFA induced enhancement of a tubular retrograde Golgi-ER transport pathway (Lippincott-Schwartz, 1993). This redistribution to the ER can be inhibited by locking COPI onto Golgi membranes with either GTPγS or with the anti-β-COP antibody EAGE (Scheel et al., 1997). Locking on COPI coats also prevents the promiscuous fusion that leads to tubular network formation *in vitro* (Orci et al., 1991). BFA also induces the ADP ribosylation of two proteins: BARS-50 and glyceraldehyde-3-phosphate dehydrogenase and inhibition of these ADP ribosylation events circumvents the effects of BFA on Golgi morphology even though ARF1 is still dissociated from the Golgi membrane (De Matteis et al., 1994; Mironov et al., 1997a). It has been proposed that BARS-50 somehow negatively regulates tubule formation, and that ADP-ribosylation of BARS-50 allows tubules to form (Mironov et al., 1997a). Recently BARS-50 has been shown to be an acyltransferase that induces Golgi membrane fission, and this activity is inhibited by ADP-ribosylation (Weigert et al., 1999). How inhibition of the BARS-50 fission activity by ADP-ribosylation might
induce the BFA transformation of Golgi stacks to tubular networks remains unresolved.

*In vitro*, removal of BFA and incubation of membranes with cytosol and ATP leads to the reformation of Golgi stacks (Cluett et al., 1993). *In vivo*, removal of BFA facilitates the COPII dependent emergence of Golgi residents from the ER (Shima et al., 1998) and the rebuilding of the Golgi stack. BFA recovery displays similar requirements to the transport of VTCs to the Golgi region (Scales et al., 1997; Presley et al., 1997), and requires p115 (Alvarez et al., 1999) and Rab1a function (Wilson et al., 1994). However, this pathway has not been probed with regard to Golgi structural elements.

1.4.7 IQ

IQ is a drug extracted from the sea sponge *Hippospongia metachromia* and induces extensive tubulation and vesiculation of Golgi membranes (Takizawa et al., 1993). IQ inhibits intra-Golgi transport and prevents ARF1 and COPI associating with Golgi membranes, but unlike BFA this does not induce redistribution of Golgi markers to the ER (Takizawa et al., 1993). Such COPI independent Golgi vesiculation may be due to IQ mediated stimulation of the membrane fission machinery. This disassembly process is microtubule independent (Veit et al., 1993) and may require activation of heterotrimeric G-proteins, whose free βγ subunit somehow triggers Golgi vesiculation (Jamora et al., 1997), possibly by activation of protein kinase D (Jamora et al., 1999). However, the downstream targets of PKD remain to be identified, but may provide clues as to how IQ stimulates this COPI independent Golgi fragmentation.

Removal of IQ facilitates the reassembly of the Golgi apparatus from this population of tubules and 60nm vesicles. Using a semi-permeabilized cell system reassembly was found to be cytosol, ATP, and temperature dependent and seems to proceed in three main stages (Veit et al., 1993; Acharya et al., 1995a, b). The first stage involves the fusion of the small 60nm vesicles to form larger vesicles of 200-300nm in diameter, and this step requires the activity of NSF (Acharya et al., 1995a, b). These large vesicles then extend into tubular and eventually stacked cisternal elements and requires the
activity of p97 (Section 1.5.6; Koller and Brownstein, 1987; Peters et al., 1990, 1992; Acharya et al., 1995a, b). After stacks have formed they are transported to the perinuclear region to re-establish the Golgi ribbon, and this is a microtubule dependent process (Veit et al., 1993). In contrast to intra-Golgi transport the reassembly process is insensitive to Rab-GDI and GTPyS (Acharya et al., 1995a). This sequence of events provides an initial framework to further probe for molecules required for the establishment of Golgi structure.

1.5 Inheritance of the Golgi apparatus.

1.5.1 Organelle inheritance.

Non-quiescent cells periodically undergo a strict sequence of events culminating in cell division. This sequence is termed the cell cycle (Nurse, 2000), and passage through the eukaryotic cell cycle requires the sequential activation of distinct cyclin dependent kinases (Nigg, 1995). The G2 to M phase transition is regulated by Cdc2 kinase, which forms complexes with A and B type cyclins. Cdc2 kinase controls much of the cellular reorganization that occurs at the onset of mitosis including mitotic spindle formation, chromosome condensation and nuclear envelope disassembly (Nigg, 1995). The most important events of the cell cycle are those governing the copying and partitioning of the hereditary material, i.e. replicating chromosomal DNA during S phase and partitioning replicated chromosomes during mitosis. However, cellular mechanisms are also in place to ensure the accurate inheritance of the cytoplasm and organelles (Warren and Wickner, 1996; Shima and Warren, 1998).

The importance of organelle inheritance stems from the fact that the immensely elaborate organelle architecture means that they cannot be readily synthesized de novo (Flickinger, 1968; Zorn et al., 1979; Maniotis and Schliwa, 1991). In fact de novo synthesis may simply be impossible as in the case of mitochondria and plastids which harbour their own genetic material which is crucial for their correct functioning. Similarly, no obvious mechanism exists to generate ER membranes de novo. This suggests that there must have been a selection pressure for accurate organelle inheritance, since organisms that were unable to do so would either be inviable or have
Chapter 1

Introduction

to spend inordinate time and energy generating new organelles, and so be at a
disadvantage in a competitive population. Therefore, all the information required to
efficiently construct an organelle may not reside solely in the genetic material. Rather,
within each organelle resides epigenetic information, or a template, that pre-exists in the
organelle membrane, and which may represent the site for the deposition of newly
synthesized material. It may even be that components of the Golgi matrix (Section
1.4.3) represent such a template for the Golgi apparatus. Transmission of such a
preformed structure to progeny is achieved by inheritance of the organelle itself
(Warren and Wickner, 1996; Shima and Warren, 1998). It may even be that the
conformation of a protein/set of proteins is the inherited element. Organelle inheritance
may then be likened to extensions of the prion hypothesis, as a means of transmission
from generation to generation through functional self-perpetuating changes in the
conformational state of proteins, with no underlying change in nucleic acid (Lindquist,
1997).

During the cell cycle organelles must grow and double in mass in preparation for cell
division. Organelles tend to adopt one of two inheritance strategies: stochastic or
ordered partitioning. These strategies need not be mutually exclusive, and which is used
may vary in a cell type or organism dependent manner (Warren and Wickner, 1996).
Ordered partitioning involves a number of disparate mechanisms, but is exemplified by
the use of the mitotic spindle to accurately partition chromosomes. Stochastic
partitioning relies on the organelle being present in multiple copies dispersed randomly
throughout the cytoplasm. Such a distribution would provide each daughter with an
equal share of the organelles provided sufficient copies of the organelle are present, the
more copies the more accurate is partitioning, as governed by the binomial theorem
(Birky, 1983; Warren, 1993). Organelles such as mitochondria may adopt such a
strategy in certain cell types (Birky, 1983; Rizzuto et al., 1995). Similarly the
pervasiveness of the ER throughout the cytoplasm ensures that it too is partitioned
using a stochastic mechanism (Zeligs and Wollman, 1979).
1.5.2 Golgi apparatus at mitosis.

The Golgi apparatus of mammalian cells is a single copy organelle, and usually occupies a restricted juxtanuclear locale (Figure 1.6A). To ensure the Golgi apparatus is accurately partitioned between daughter cells through successive generations this structure must fragment, and become dispersed throughout the cytoplasm prior to cytokinesis. Such fragmentation is tightly coupled to the fact that transport of vesicles between organelles ceases at mitosis (Warren, 1993). This is true for all vesicle transport steps studied so far including ER-Golgi transport (Featherstone et al., 1985; Farmaki et al., 1999), Golgi-ER transport (Jesch and Linstedt, 1998), intra-Golgi transport (Collins and Warren, 1992; Stuart et al., 1993; Fernández and Warren, 1998), Golgi to plasma membrane (Kreiner and Moore, 1990), secretory granule to plasma membrane (Hesketh et al., 1984), and a number of events associated with endocytosis (Fawcett, 1965; Pyapaert et al., 1987, 1991; Raucher and Scheetz, 1999; Woodman et al., 1993; Warren et al., 1984). Were vesicles to continue budding in the absence of fusion during mitosis, organelles would fragment (Warren, 1985), and this phenomena appears to account for a large part of Golgi fragmentation (Misteli and Warren, 1994, 1995a; Sönnichsen et al., 1996; Section 1.5.4).

The morphogenetic changes that occur during the Golgi inheritance process have been extensively characterized in tissue culture cells by both quantitative EM (Lucocq et al., 1987, 1989; Lucocq and Warren, 1987; Pyapaert et al., 1993; Souter et al., 1993; Misteli and Warren, 1995b) and more recently with live cell imaging utilizing GFP technology (Shima et al., 1997, 1998; Zaal et al., 1999). The consensus sequence of events obtained from quantitative EM studies (Lucocq et al., 1987, 1989; Lucocq and Warren, 1987; Pyapaert et al., 1993; Souter et al., 1993; Misteli and Warren, 1995b) and live cell imaging of carefully characterized cell lines stably expressing NAGTI-GFP (Shima et al., 1997, 1998) is outlined below. The juxtanuclear ribbon of linked Golgi stacks is first of all converted into its constituent mini-stacks during early prophase (Colman et al., 1985; Misteli and Warren, 1995b; Shima et al., 1998). These mini-stacks become evenly distributed around the nucleus during prophase (Misteli and Warren, 1995b; Shima et al., 1998). Very little is known about the molecular events that are responsible...
Figure 1.6 Mammalian Golgi apparatus during interphase and mitosis. 
(A) A typical juxtanuclear collection of interconnected Golgi stacks (arrows) of an interphase NRK cell. M, mitochondrion; N, nucleus. (B) The morphology of the Golgi apparatus of a PtK1 cell during mitosis. Numerous vesicles and tubules constitute the mitotic Golgi cluster (arrows) which occur close to the spindle pole (SP) and microtubules (MT). Bar, 0.3μm. 
Courtesy of Eija Jokitalo and Dave Shima.
for these events, but the effect of ribbon breakdown into its component mini-stacks is reminiscent of the effects of microtubule disruption by nocodazole. Therefore, it seems likely that alterations in microtubule organization that accompany centrosome duplication/migration may be responsible in some way. Centrosome movements correlate very closely with mitotic Golgi movements, and perturbations of the former using anti-HsEg5 (a BimC kinesin like molecule) antibodies causes abnormal mitotic Golgi localization (Whitehead and Rattner, 1998).

At the onset of metaphase these mini-stacks fragment into a disseminated array of tubulovesicular clusters, termed mitotic Golgi clusters (Figure 1.6B). This dramatic morphological change occurs coincident with the peak of Cde2 kinase activity that also triggers nuclear envelope disassembly and microtubule rearrangements (Simos and Georgatos, 1992; Jackman et al., 1995; Macauley et al., 1995). The exact morphological composition of mitotic Golgi clusters appears to be cell type and organism specific (Warren, 1993; Stanley et al., 1997). For example, in HeLa cells (Lucocq et al., 1987; Lucocq and Warren, 1987), PtK-1 cells (Schroeter et al., 1985), parotid acinar cells (Tamaki and Yamashina, 1991), NRK cells (Burke et al., 1982) and thyroid epithelia (Zeligs and Wollman, 1979) these clusters are composed of 50-70nm diameter vesicles, larger vesicles, short tubules and tubular network. Whereas in L929 fibroblasts (Moskalewski and Thyberg, 1990), melanoma cells (Maul and Brinkley, 1970) and chondrocytes (Moskalewski et al., 1977) the clusters are more tubular and even cisternal in nature. Mitotic cells typically contain 100-150 mitotic Golgi clusters, and the precise number correlates positively with cell size (Shima et al., 1997). Mitotic Golgi clusters may continue to shed vesicles into the surrounding cytoplasm (Lucocq et al., 1989; Jesch and Linstedt, 1998), and in fact the volume of Golgi membrane occupied by clusters and that occupied by vesicles displays an inverse relationship (Lucocq et al., 1989). The extent of this further vesiculation is cell type specific, but it seems that clusters persist throughout mitosis in all cell types (Shima et al., 1997).

Mitotic Golgi clusters contain all the Golgi resident enzymes and Golgi peripheral membrane proteins so far tested, with the notable exceptions of p115 (Shima et al.,
1997; Lowe et al., 2000), βIII spectrin (Beck et al., 1994), and Golgi ankyrin (Beck et al., 1997), and represent the elements of Golgi inheritance. Remarkably, mitotic Golgi clusters maintain the cis- to trans-polarity of markers observed in the starting Golgi stack (Shima et al., 1997). Such maintenance of Golgi polarity despite the extensive morphological changes, and absence of a stack, suggests the existence of a structural template onto which the Golgi stack is reorganized during telophase. The electron dense Golgi matrix (Section 1.4.3) is still apparent in mitotic Golgi clusters (Zeligs and Wollman, 1977), and it may be that this structure maintains the observed cis to trans polarity. Thus, such a template or matrix may potentially exist independently of Golgi membrane morphology, and could serve as a blueprint which seeds the reassembly of Golgi stacks. Interestingly, many of the components of the Golgi matrix (Section 1.4.3) are not completely redistributed to the ER upon BFA treatment (Nakamura et al., 1995; Rios et al., 1994), but may exist associated with the tubulovesicular Golgi BFA remnants (Ulmer and Palade, 1991; Hendricks et al., 1992; Hidalgo et al., 1992; Orci et al., 1993c). Furthermore, such structures resemble mitotic Golgi clusters morphologically and may also serve as a template for Golgi architecture on the re-emergence of Golgi residents from the ER after removal of BFA.

The partitioning of these mitotic Golgi clusters between nascent daughter cells was found to be more accurate than predicted by a binomial theorem governed stochastic process (Shima et al., 1997). The reason for this enhanced accuracy of partitioning is likely due to an interaction between the mitotic Golgi clusters and the spindle pole and aster microtubules (Shima et al., 1998). At metaphase, coincident with the abrupt depolymerization and rearrangement of microtubules to form the mitotic spindle (Zhai et al., 1996), the originally perinuclear mitotic Golgi clusters move in a centripetal manner, concentrating in radial arrays surrounding opposite spindle poles (Shima et al., 1998). These mitotic Golgi clusters appear associated with the aster microtubules proximal to the spindle, and another subset of mitotic Golgi clusters are dispersed in the cell periphery closely associated with the spindle distal ends of the aster microtubules (Shima et al., 1998). This reorganization occurs by movements too slow to be mediated by molecular motors, which are inactive at mitosis (Allan and Vale,
1991), yet is also incompatible with simple cluster diffusion. Therefore, it appears likely that the dynamics of microtubules themselves mediate this cluster reorganization, possibly by polar ejection forces (Rieder and Salmon, 1994), or association with growing/shrinking microtubule ends (Waterman-Storer et al., 1995; Waterman-Storer and Salmon, 1998). This organization of mitotic Golgi clusters persists through anaphase, and into telophase (Shima et al., 1998; Lowe et al., 2000).

Previously, the complete vesiculation of the mitotic Golgi clusters was thought to ensure incredibly accurate stochastic partitioning of the Golgi apparatus (Lucocq et al., 1989). It may be that the cell uses both this mechanism and attachment of clusters to aster microtubules to ensure the Golgi apparatus is inherited. These two modes of inheritance may then be functionally redundant, but as a consequence provide a fail-safe mechanism for Golgi inheritance, in the event of one of the pathways becoming compromised. However, fragmentation need not be essential for Golgi inheritance per se. For instance, plants and fungi inherit their Golgi as dispersed mini-stacks (Kanbe et al., 1989; Rossanese et al., 1999; Griffing, 1991). Plants and fungi do, however, constantly secrete material through mitosis in order to maintain the cell wall (Makarow, 1988; Samuels et al., 1995). Fragmentation in mammalian cells may then be simply a consequence of the mitotic inhibition of vesicle traffic which may serve to conserve ATP solely for the purposes of cell division. However, the dramatic changes in Golgi morphology may have additional functions, for example the release of mitotic factors that are usually sequestered in the Golgi stack. It is then of interest to note that cyclin B2, TSG101, and the RIIα subunit of protein kinase A are associated with the Golgi apparatus during interphase, but are released during mitosis to occupy distinct subcellular positions (Jackman et al., 1995; Xie et al., 1998; Keryer et al., 1998).

At telophase, within minutes of a decrease in the histone kinase activity of the mitotic kinase Cdc2 (Souter et al., 1993), the mitotic Golgi clusters are transformed into discrete stacks of cisternae in a 10min period (Souter et al., 1993). These discrete Golgi stacks then coalesce in the pericentriolar, juxtanuclear region utilizing microtubules (Shima et al., 1998; Whitehead and Rattner, 1998) to reform the Golgi ribbon (Lucocq
et al., 1989; Shima et al., 1997). This process entails the extension of tubules which link the discrete stacks to recreate a ribbon like structure (Shima et al., 1997). Inhibition of centrosome migration to the juxtanuclear region by microinjection of anti-HsEg5 antibodies inhibits the coalescence of the discrete Golgi mini-stacks at this stage (Whitehead and Rattner, 1998).

This view of mitotic Golgi membrane dynamics has recently been challenged by Lippincott-Schwartz and colleagues (Lippincott-Schwartz and Smith, 1997; Zaal et al., 1999; Roth, 1999). These workers suggest that Golgi inheritance is achieved by the retrograde transport of Golgi residents to the ER which occurs at the prophase to metaphase transition (Zaal et al., 1999). Golgi residents are held in the ER until telophase owing to a mitotic inhibition of COPII vesicle formation. COPII vesicle formation may be blocked at mitosis since Sec13p is unable to associate with ER membranes at this time (Farmaki et al., 1999). At telophase Golgi residents emerge from the ER and reform the Golgi in a manner akin to recovery from BFA. Since the ER is extremely well dispersed throughout the cell during mitosis (Ellenberg et al., 1997) such relocation of Golgi residents would ensure accurate Golgi inheritance as a consequence of stochastic ER inheritance.

Consistent with this one study found that MannII could be found in the ER and mitotic Golgi clusters of metaphase cells, but this could be due to the accumulation of newly synthesized material (Thyberg and Moskalewski, 1992; Farmaki et al., 1999). Subsequently, Lippincott-Schwartz and colleagues monitored mitotic Golgi dynamics using predominantly transient expression of the Golgi enzymes GalT and MannII whose luminal domains were tagged with a triple GFP concatamer (Zaal et al., 1999). At steady state interphase conditions c. 30% of this GalT-GFP was found in the ER, suggesting that the triple GFP concatamer may be causing misfolding or mislocalization of the enzyme (Zaal et al., 1999). All previous studies have found only trace GalT in the ER, unless the enzyme is highly overexpressed (Nilsson et al., 1991, 1993a; Rabouille et al., 1995a; Prescott et al., 1997; Yamamoto et al., 1999). Despite this aberrant localization the dynamics of GalT-GFP was monitored through mitosis and
the Golgi fragmented as described previously in prophase (Shima et al., 1997, 1998), but then displayed a diffuse staining pattern from metaphase through to telophase which the authors claimed to be an ER staining. Astonishingly, no colocalization with an ER marker was demonstrated by immunofluorescence (Zaal et al., 1999). Instead anecdotal, qualitative EM evidence and FRAP was used to suggest that the diffuse staining seen at metaphase through to telophase represented an ER localization (Zaal et al., 1999). However, it may be that the ER was misidentified by EM since these workers did not use antibodies against ER marker proteins to verify the profiles were actually ER tubules. Although this study provides an interesting alternative hypothesis to Golgi inheritance, more experiments are required to determine whether the system of transient expression and the GFP chimeras used reflect the true sequence of events in wild type cells.

Several lines of evidence strongly argue against a role for ER recycling of Golgi residents at mitosis. First, BFA does not redistribute Golgi residents to the ER during mitosis suggesting Golgi to ER transport, like all other transport steps studied so far is inhibited at mitosis (Jesch and Linstedt, 1998; Dave Shima, personal communication). Second, ER and Golgi markers remain independent through mitosis as determined by biochemical fractionation (Jesch and Linstedt, 1998). Third, the trace presence of Golgi enzymes in ER at mitosis can be totally accounted for by the arrival of newly synthesized enzymes in the ER, since they are undetectable in the presence of cycloheximide (Farmaki et al., 1999). Fourth, microinjection of a dominant negative form of Sar1p that blocks COPII vesicle formation, does not cause accumulation of Golgi residents in the ER during mitosis (Shima et al., 1998). Finally, in no other studies have significant quantities of Golgi residents been found in the ER at mitosis using endogenous Golgi markers, or cell lines stably expressing tagged Golgi markers that are correctly localized (Lucocq et al., 1997, 1989; Pypaert et al., 1993; Shima et al., 1998, 1999; Farmaki et al., 1999; Jesch and Linstedt, 1998; Eija Jokitalo, Tommy Nilsson, Dave Shima personal communication).
1.5.3 Cell free system.
The dramatic transformation in morphology that the Golgi apparatus undergoes during mammalian cell division as part of the inheritance process provides a natural tool with which one can attempt to elucidate determinants that are required for the maintenance and establishment of the unique Golgi architecture. Although, the above *in vivo* ultrastructural studies provided an initial understanding of mitotic Golgi behaviour a more reductionist approach was required to get a molecular handle on these processes. To this end, a cell free system has been established that approximates many of the phenomena of mitotic Golgi membrane dynamics (Misteli and Warren, 1994, 1995a; Rabouille et al., 1995b, c, 1998; Barr et al., 1997; Shorter and Warren, 1999; Shorter et al., 1999). The selected approach has been one of complex reconstitution, in that highly purified rat liver Golgi membranes are incubated in mitotic cytosol to generate mitotic Golgi fragments. These fragments on incubation with interphase cytosol or purified cytosolic fusion machinery will reassemble into Golgi stacks. This provides a readily manipulatable biochemical system, within which sequential morphological events can be precisely followed by quantitative EM.

1.5.4 COPI dependent pathway
From the cell-free system mitotic disassembly appears to proceed via two independent, concurrent pathways (Misteli and Warren, 1994, 1995a; Misteli, 1994). The COPI dependent pathway proceeds as COPI vesicles continue to bud, but are unable to tether and so fuse with their target membrane due to a Cdc2 kinase mediated event (Misteli and Warren, 1994; Nakamura et al., 1997; Lowe et al., 1998b). This pathway likely consumes the peripheral rims of cisternae, and accounts for up to 65% of the total cisternal membrane (Misteli and Warren, 1994, 1995a; Sønnichsen et al., 1996). An ill characterized COPI independent pathway is thought to convert the flattened cisternal cores in to a heterogeneous array of tubulovesicular profiles (Section 1.5.5; Misteli and Warren, 1995b).

A possible molecular explanation for the accumulation of COPI vesicles at mitosis lies in the fact that the binding of p115 to Golgi membranes is significantly inhibited at
mitosis (Figure 1.7; Levine et al., 1996; Sohda et al., 1998). At mitosis the extreme basic N-terminus of GM130, comprising the p115 binding site (Nakamura et al., 1997) is directly phosphorylated by cyclin B-Cdc2 kinase on serine 25 (Lowe et al., 1998b) with the effect of potently inhibiting p115 binding. The introduction of this phosphate moiety may break up an electrostatic interaction, since it is the extreme acidic C-terminus of p115 that binds to the extreme basic N-terminus of GM130 (Nelson et al., 1998; Dirac-Svejstrup et al., 2000). So although p115 can still bind giantin (Sønnichsen et al., 1998), it is no longer able to cross link to GM130. As a result COPI vesicles accumulate as they are unable to tether and so fuse, and intra-Golgi transport is inhibited (Collins and Warren, 1992; Stuart et al., 1993; Misteli and Warren 1994; Nakamura et al., 1997).

In vivo GM130 is phosphorylated during prophase as the Golgi apparatus starts to fragment as revealed by an antibody that specifically recognises GM130 phosphorylated on serine 25 (Lowe et al., 2000). GM130 remains phosphorylated through metaphase and anaphase as the Golgi apparatus is fragmented further. In telophase, GM130 is dephosphorylated by PP2A containing the Bα regulatory subunit (Lowe et al., 2000). The timing of this phosphorylation and dephosphorylation of GM130 is precisely synchronous with the dissociation and reassociation of p115 with Golgi membranes (Lowe et al., 2000), strongly suggesting that these are the events which trigger Golgi disassembly and reassembly.

The requirement for cyclinB-Cdc2 kinase activity for mitotic Golgi fragmentation is in contrast to results obtained in a semi-permeabilized cell system which requires MEK1 activity and not Cdc2 activity (Acharya et al., 1998). MEK1 seems to activate a novel Golgi associated ERK activity which is essential for Golgi fragmentation (Acharya et al., 1998). The identity of this novel ERK and its downstream targets remain to be identified. However, lack of EM studies in this system raised doubts as to whether a bona fide mitotic fragmentation process was being observed (Lowe et al., 1998b). Furthermore, the fact that our cell free system has no requirement for MEK1 for mitotic Golgi fragmentation and is absolutely dependent on cyclinB-Cdc2 is given
Figure 1.7 Model for the mitotic fragmentation of the Golgi apparatus.
At interphase the vesicle tethering protein, p115, crosslinks giantin on the COPI vesicle to GM130 on the acceptor membrane. This tethering event facilitates trans-SNARE pairing and vesicle fusion. At mitosis Cyclin B-Cdc2 kinase mediated phosphorylation of GM130 inhibits p115 binding and as a result COPI vesicles are no longer able to tether and so fuse. Continued budding then converts cisternae into collections of vesicles and tubular remnants. Mitotic phosphorylation of GRASP65, the GM130 receptor, may cause mitotic cisternal unstacking.
greater credence by the fact that MEK1 activity is not required for mitotic Golgi fragmentation \textit{in vivo} (Lowe et al., 1998b). Inhibition of MEK1 activity \textit{in vivo} with either the specific inhibitor PD98059 or with the anthrax toxin which selectively proteolyzes and inactivates MEK1 (Duesbery et al., 1998) had no effect on mitotic progression or Golgi fragmentation (Lowe et al., 1998b).

1.5.5 COPI independent pathway

The COPI independent pathway of disassembly converts the cisternal cores into a series of tubular networks, tubules and heterogeneously sized vesicles (Misteli and Warren, 1995a). This is clearly an important part of the disassembly process as it consumes 40-50\% of the Golgi cisternae (Misteli and Warren 1994, 1995a), and the hallmark tubular network formation that indicates COPI independent fragmentation is also readily identifiable \textit{in vivo} (Misteli and Warren, 1995b). It is hypothesized that this pathway may involve a membrane fission activity, that is inescapably required to generate any discrete membrane profile from another, and ultimately entails the inner lumenal leaflets of the membrane bilayer coming into apposition and fusing (Rothman and Warren, 1994; Warren, 1995; Misteli, 1996). Relaxation of the Golgi scaffolding meshwork or matrix (Lorra and Huttner, 1999) at mitosis may allow more random collisions between the inner leaflets of cisternae, and may generate fenestration in the mid-regions of the cisternae, and tubules/vesicles at the periphery. It may be that the recently identified Golgi membrane fission activity of BARS-50 and/or PITP is important in this process (Weigert et al., 1999; Simon et al., 1998). The functional significance of the COPI independent pathway remains unclear. However, since similar Golgi structures are seen upon ATP depletion (Cluett et al., 1993; del Valle et al., 1999), it may be that this morphology represents a low energy form of the Golgi apparatus, and is created at mitosis as the cell diverts ATP solely for the purposes of cell division.

During interphase, tubules and tubular networks are striking morphological features of the Golgi apparatus both \textit{in vitro} and \textit{in vivo} (Weidman et al., 1993; Sciaky et al., 1997; Rambourg and Clermont, 1997; Ladinsky et al., 1994, 1999) and has led many to
speculate they may be involved in transport processes (Sciaky et al., 1997; Mironov et al., 1997b). That cell free intra-Golgi transport can still occur under certain conditions where COPI vesicle formation is blocked, has led to the suggestion that tubules may be able to substitute as the transport vehicle (Elazar et al., 1994b; Taylor et al., 1994; Happe et al., 1998). A COPI independent retrograde transport pathway has recently been uncovered that transports Golgi glycosylation enzymes from the Golgi to the ER and may utilize tubules as the transport vector (Girod et al., 1999). Certain conditions accentuate tubule formation such as osmotic shock (Lee and Linstedt, 1999) and BFA (Orci et al., 1991). Despite this, molecular mechanisms that are involved in tubule formation are extremely incognizant, although preliminary studies suggest cytosol and ATP are required (Orci et al., 1991; Banta et al., 1995; although just buffer and ATP in Weidman et al., 1993). Tubule formation may also require in some way ADP ribosylation substrates (Mironov et al., 1997a; Weigert et al., 1999), and/or calmodulin, and/or cytosolic phospholipase A\(_2\) (de Figueiredo and Brown, 1995; de Figueiredo et al, 1998, 1999). The study of the COPI independent pathway of fragmentation \textit{in vitro} may then lend some insight into the mechanisms that underlie the process of membrane tubulation.

\subsection*{1.5.6 Cisternal regrowth.}

As cells exit M-phase, mitotic phosphorylations are reversed by protein phosphatases facilitating the reversion of mitotic Golgi fragments (MGF) to their original morphology. The emerging molecular picture of this process is as follows. The first phase of reassembly entails the regeneration of single cisternae (Rabouille et al., 1995c). This process requires interplay between soluble factors and SNAREs via two intersecting pathways controlled by the two AAA proteins NSF and p97 (Rabouille et al., 1995b).

NSF catalyzes cisternal regrowth in conjunction with its co-factors \(\alpha\)-SNAP, \(\gamma\)-SNAP and p115 (Rabouille et al., 1995b) and requires the Golgi v-SNARE GOS-28 (Subramaniam et al., 1996; Nagahama et al., 1996; Hay et al., 1998) and t-SNARE syntaxin-5 (Rabouille et al., 1998). These SNAREs are also required for intra-Golgi
Chapter 1

Introduction

transport in vitro (Nagahama et al., 1996; Fernández and Warren, 1998). Therefore, NSF is thought to be important for the catalysis of heterotypic fusion events between COPI vesicles and their target membranes.

p97 was originally identified as a precursor protein harbouring valosin, a biologically active peptide (Koller and Brownstein, 1987), and was later purified on the basis of its large size and abundance (Peters et al., 1990, 1992). p97 also functions to catalyze the fusion of ER membranes (Latterich et al., 1995; Patel et al., 1998; León and McKearin, 1999) and of Golgi membranes fragmented by IQ (Acharya et al., 1995a). p97 is extraordinarily conserved as it even has archaebacterial homologues in Sulfobus acidocaldarius (Confalonieri et al., 1994) and Thermoplasma acidophilum (Pamnami et al., 1997; Rockel et al., 1999). Since archaebacteria lack internal membranes it has been suggested that p97 may be required for the cytoplasmic fusion event at cytokinesis that leads to cell separation (Kondo et al., 1997). Like NSF, p97 exists as barrel-shaped hexamer with an outer diameter of 13-17nm and a typical height of 9-10nm with a small channel at the centre (Peters et al., 1990, 1992; Coles et al., 1999). Each monomer contributes one stave of the barrel and consists of a N-terminal domain, followed by two ATPase cassettes (the D1 and D2 domains). Like NSF, p97 is inactivated by NEM (Rabouille et al., 1995b, 1998).

The mode of action of p97 is thought by analogy to be very similar to that of NSF, except the substrate for p97 is a t-/t-SNARE complex instead of a v-/t-SNARE complex. This is most firmly established in ER homotypic fusion in yeast, which requires only the t-SNARE, Ufe1p, and none of the other known ER-Golgi v- or t-SNAREs (Patel et al., 1998). Furthermore, in a temperature sensitive Cdc48 (p97) mutant at the restrictive temperature the proportion of SDS soluble Ufe1p is significantly reduced, possibly implying an accumulation of t-/t-SNARE complexes (Patel et al., 1998). In the post-mitotic reassembly of Golgi membranes, p97 catalyzed cisternal regrowth requires the t-SNARE, syntaxin-5, but not the Golgi v-SNARE GOS-28 (Rabouille et al., 1998), although other Golgi v-SNAREs such as membrin (Hay et al., 1998) have not yet been excluded. p97 catalyzed cisternal regrowth may
then be considered important for homotypic fusion, i.e. the fusion of membranes of the same compartment (e.g. cis-cisterna with cis-cisterna). p97 fusion activity requires its co-factor p47, which exists as a trimeric complex that binds at one end of the p97 hexameric barrel (Kondo et al., 1997). p47 is analogous to α-SNAP relative to NSF in that it enables p97 to bind to syntaxin-5 (Rabouille et al., 1998).

The competition for syntaxin-5 binding between α-SNAP and p47 may explain why the NSF and p97 pathways of Golgi reassembly contribute non-additively to cisternal regrowth (Rabouille et al., 1998). p47 binding to p97 also regulates p97 ATPase activity, but in contrast to α-SNAP and NSF, acts to inhibit the ATPase activity (Meyer et al., 1998; Otter-Nilsson et al., 1999), which possibly suggests a different mode of action of p97 relative to NSF. Where NSF and p97 act in the reassembly process relative to SNARE mediated docking remains to be determined. However, it is of interest to note that the ATPase activity of NSF, and so its cis-SNARE pair disassembling ability, does not appear to be crucial for reassembly (Müller et al., 1999b; Chapter 7).

NSF and p97 generate cisternae with different morphologies. NSF generates fenestrated cisternae with dilated rims, and closely associated vesicles. In contrast, p97 generates blunt ended cisternae with little fenestration and few associated vesicles (Rabouille et al., 1995b; Chapter 4). When NSF and p97 are combined in the reaction the cisternae generated displayed characteristics of both machineries, and in addition a small population (c. 10% of the total cisternae) of very long cisternae were formed (Rabouille et al., 1995b). As a consequence of these different morphologies it has been suggested that p97 acts to regenerate the cisternal cores from the COPI independent fragmentation products, and the NSF pathway regenerates the cisternal rims from COPI vesicles and cisternae (Rabouille et al., 1995b). The precise balance between these two pathways may then be modulated by the cell to generate different cisternal architecture according to cellular needs (Rabouille et al., 1998).
1.5.7 Cisternal stacking.

As single cisternae begin to form they align and dock to form stacks. This stacking process is stimulated by GTP\textgamma{}S and inhibited by microcystin, but the targets of these molecules remains unknown (Rabouille et al., 1995c). So far the only factor shown to be required for this event is an NEM sensitive membrane bound component (Rabouille et al., 1995c; Barr et al., 1997; Shorter and Warren, 1999; Shorter et al., 1999). Treatment of MGF with NEM precluded cisternal stacking but not cisternal regrowth during reassembly. Using a biotinylated analogue of NEM it was found that only three proteins are modified by NEM specifically on MGF. Two factors were identified by utilizing this alkylation by the biotinylated NEM analogue as a marker for their chromatographic behaviour, and were found to be GM130 and GRASP65, the receptor for GM130 (Barr et al., 1997, 1998). The third protein of c. 160kDa remains unidentified. GRASP65 binds to the extreme C-terminus of GM130 via a PDZ like domain (Barr et al., 1998). Typically, PDZ domains function as adaptors in the assembly of multifunctional protein complexes involved in signalling processes at membrane surfaces (Ponting et al., 1997).

Soluble GRASP65 and affinity purified antibodies raised against the region containing a conserved cysteine blocked the stacking process without affecting cisternal regrowth (Barr et al., 1997; Shorter and Warren, 1999; Shorter et al., 1999). GRASP65 is highly conserved from yeast to man, N-myristoylated and is the major mitotic Golgi phosphoprotein (Barr et al., 1997). It may be that this mitotic phosphorylation of GRASP65 is important for the observed unstacking of cisternae that occurs at mitosis (Figure 1.7). Precisely how GRASP65 functions to stack Golgi cisternae represents an epistemological gap. A major aim of this thesis was to further elucidate molecules required for this stacking process.

1.6 Purpose of study.

The definition of molecular mechanisms that underlie the processes of mitotic Golgi disassembly and reassembly are far from complete. The aim of this thesis was to further elucidate molecules and their mode of action in these processes utilizing the cell-
free system. In studying the disassembly process I found an apparent role for the Rab GTPases in the context of vesicle/tubule formation and the release of p115 in the unstacking of Golgi cisternae (Chapter 3). This latter observation led me to investigate whether p115 had any role in the stacking of cisternae during reassembly (Chapter 4). The reassembly assay then became the focus of the study and was used to further probe the stacking pathway, and revealed a role for a novel GRASP, GRASP55, in stack formation (Chapter 5). To more finely understand p115 mediated tethering the role of p115 phosphorylation was investigated (Chapter 6). Finally, the importance of NSF ATPase activity and a novel p97 containing complex in reassembly were investigated (Chapter 7).
Chapter 2

Materials and Methods
Chapter 2  

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All reagents were of analytical grade or higher and were purchased from Sigma Chemical Co., Boehringer Mannheim, BDH Chemicals Ltd., Pierce, Bio-Rad, Calbiochem, Fisher scientific, Merck, Pharmacia, New England Biolabs and Qiagen unless otherwise stated. PBS, distilled water and tissue culture media were provided by ICRF central services.

2.1.2 Antibodies

The antibodies used in this study are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised against</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>4H1 (mc)</td>
<td>p115</td>
<td>M.G. Waters</td>
</tr>
<tr>
<td>8A6 (mc)</td>
<td>p115</td>
<td>M.G. Waters</td>
</tr>
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<td>p97</td>
<td>J.M. Peters</td>
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<td>2E5 (mc)</td>
<td>NSF</td>
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<tr>
<td>FBA19 (pc)</td>
<td>GRASP65 + GRASP55</td>
<td>F. Barr</td>
</tr>
<tr>
<td>FBA30 (pc)</td>
<td>GRASP65</td>
<td>F. Barr</td>
</tr>
<tr>
<td>FBA31 (pc)</td>
<td>GRASP65</td>
<td>F. Barr</td>
</tr>
<tr>
<td>FBA32 (pc)</td>
<td>GRASP55</td>
<td>F. Barr</td>
</tr>
<tr>
<td>FBA34 (pc)</td>
<td>GRASP55</td>
<td>F. Barr</td>
</tr>
<tr>
<td>7E10 (mc)</td>
<td>GRASP65</td>
<td>F. Barr</td>
</tr>
<tr>
<td>sc-310 (pc)</td>
<td>Rab6</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>sc-311 (pc)</td>
<td>Rab1a</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>G-20 (pc)</td>
<td>Rab1b</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>M3A5 (mc)</td>
<td>β-COP</td>
<td>Sigma</td>
</tr>
<tr>
<td>CM1A10 (mc)</td>
<td>coatamer</td>
<td>J. Rothman</td>
</tr>
<tr>
<td>Anti-CKIIα (mc)</td>
<td>CKII α-subunit</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Anti-CKIIβ (mc)</td>
<td>CKII β-subunit</td>
<td>Calbiochem</td>
</tr>
<tr>
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<td>RGS-His</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Anti-GFP (pc)</td>
<td>GFP</td>
<td>K. Sawin</td>
</tr>
<tr>
<td>H-7</td>
<td>HA-epitope</td>
<td>Santa Cruz</td>
</tr>
<tr>
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<td>Mouse IgG</td>
<td>TAGO</td>
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<td>TAGO</td>
</tr>
<tr>
<td>HRP-Anti-Sheep IgG</td>
<td>Sheep IgG</td>
<td>TAGO</td>
</tr>
</tbody>
</table>

Table 2.1 Antibodies. pc=polyclonal, mc=monoclonal
2.1.3 Proteins and peptides.
Rat liver p97, His-tagged p47 (Kondo et al., 1997), recombinant His-tagged p97, Npl4p, and Ufd1p (Meyer et al., 2000) were obtained from Hemmo Meyer. His-tagged NSF, NSF (E329Q), and NSF (D604Q; Müller et al., 1999b), were obtained from Joyce Müller. His-tagged α-SNAP and γ-SNAP (Rabouille et al., 1995b), were obtained from Catherine Rabouille. His-tagged soluble GRASP65 and soluble GRASP55 (Shorter et al., 1999), purified rat liver coatamer (Waters et al., 1992a) and His-tagged GDI-α were obtained from Francis Barr. His-tagged TA, TA (S941A), and TA (S941D) were obtained from Barbara Dirac-Svejstrup (Dirac-Svejstrup et al., 2000). His-tagged giantin fragments Gtn448 and Gtn1967-2541 (Lesa et al., 2000) were obtained from Giovanni Lesa. Recombinant human CKII was from Calbiochem.

The C-terminal p115 peptides were as follows: 75mer (LQNEKNKLEVDITDSKKEQDDLLVLLADQDQKIFSLKNKLKELGHPVEEDES*GDQDDEDDEDEDDGKEEQGHI), 26mer (EDELES*GDQDDEDEDEDDGKEQGHI), and were either phosphorylated or not on the serine marked (*). The coiled coil p115 peptides were: p115 637-699 (KEE EVKKTLEQHNDIVTHYKNVIREQDLQLEELKQQVSTLKCQNEQLQTA VTQQASQIQQ), p115 728-765 (QPEEISRLREEIEELRSHQVLLQSQ LAEKDTVIENLRS), p115 788-827 (DAEQVAILKQELSALKCSQSLESITRL Q TENRELQGRAETLAK), p115 843-930 (TTDVEGRALQLQE KT KNEI K ALS EERTSIQKQLDSSNSTIAILQTEKDLKLYEVTDSKKEQDDLLVLLA DQDKILS LKS KLKD). The N-terminal GM130 peptide (N73pep; SEETRQSKLAAAKKLR EYQQKNS*PGVPAGAKKKKKIKNGHSPECTSASDCQSAENVPTDHTAPPPST AAAT) and a mutant peptide (S25D N73pep) where the serine marked (*) was mutated to aspartate. The CKII peptide substrate (RRRDDDS*DDDDD) with or without phosphorylation of the marked serine (*). All peptides contained an N-terminal biotin tag and were synthesized by the Peptide Synthesis laboratory at ICRF.

2.2 Methods
2.2.1 Purification of Rat liver Golgi membranes (RLG)
Rat liver Golgi membranes (RLG) were purified as described in Hui et al. (1998) with the following modifications. Six female Sprague-Dawley rats were starved for 24h prior to sacrifice. Livers were rapidly excised, immersed in 200ml ice cold buffer C (0.1M potassium phosphate, pH 6.7, 5mM MgCl₂, 0.5M sucrose, 5μM pepstatin A, 1
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Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml) and swirled and squeezed to expel blood and quicken cooling. The following steps were performed in the cold room. 32-44g of liver was placed into fresh buffer C, excess buffer decanted to leave a volume of c. 50ml, and livers minced into small pieces with sharp scissors. This material was rapidly homogenized by gently forcing through a 150μm-mesh steel sieve (Endecotts Ltd.), with the bottom of a 250ml conical flask. This gentle homogenization reduces the risk of disrupting Golgi architecture due to mechanical shear. The homogenate was pooled and made up to 48ml with buffer C.

4ml of this homogenate was overlaid onto 6.5ml buffer D (0.1M potassium phosphate, pH 6.7, 5mM MgCl₂, 0.86M sucrose, 5μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml) in Beckman SW-40 Ultraclear rotor tubes. This layer was then overlaid with 2.5ml buffer B (0.1M potassium phosphate, pH 6.7, 5mM MgCl₂, 0.25M sucrose, 5μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml). 12 such discontinuous sucrose gradients were prepared and centrifuged in a L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) using a SW-40 rotor at 29,000 rpm for 1h at 4°C. A 200μl aliquot of homogenate was kept on ice for enzyme assays.

After centrifugation, the lipid infranatant was removed by aspiration. The Golgi membrane fraction at the 0.5M/0.86M sucrose interface was then collected using a glass Pasteur pipette (approximately 0.8ml per gradient). The blood red 0.5M sucrose layer was also collected and used to prepare rat liver cytosol (Section 2.2.2). The membranes were then pooled and adjusted to 9% (w/w) sucrose using buffer A (0.1M potassium phosphate, pH 6.7, 5mM MgCl₂, 5μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml) and the refractive index was monitored using a 0-50% Delta refractometer (Bellingham and Stanley Ltd., Tunbridge Wells, UK). The volume was then made up to 24ml with buffer B. This was the split between two SW-40 tubes, and underlaid with 500μl buffer E (0.1M potassium phosphate, pH 6.7, 5mM MgCl₂, 1.3M sucrose, 5μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml). These samples were centrifuged at 6,000 rpm for 20min at 4°C.
The supernatant was removed by aspiration, and the final membrane felt collected with a P200 Gilson pipette. This yielded 1-1.5ml of Golgi membranes (termed RLG) which were divided into 100μl aliquots, and snap frozen in liquid nitrogen and stored at –80°C. 20μg RLG were routinely fixed with 100μl of 2% glutaraldehyde (Fluka; in PBS/0.2M sucrose) for 30min at room temperature, and processed for EM (Section 2.2.12). Another aliquot was kept for enzyme assays.

### 2.2.2 GalT activity

RLG were routinely assayed for GalT activity (Bretz and Staubli, 1977), a trans-Golgi enzyme, to ensure biochemical purity. GalT catalyzes the addition of galactose onto N-linked oligosaccharides bearing the GlcNAc₂-Man₃-GlcNAc₂ motif. Ovomucoid possesses this incompletely processed structure and serves as an acceptor molecule. An assay mixture was made up comprising: 100μl 0.4M sodium cacodylate pH 6.6, 100μl 175mg/ml ovomucoid, 3μl β-mercaptoethanol, 20μl 10mM UDP-galactose, 20μl 10% (w/v) TX-100, 10μl 0.2M ATP, 20μl 2M MnCl₂ 5μl 100mCi/ml UDP-[³H] galactose, and 520μl distilled water. 80μl assay mix was added to each 20μl sample (1:20 dilution of RLG and homogenate, to ensure within the linear range [Slusarewicz et al., 1994]) and to 20μl distilled water to serve as a blank, and the samples incubated at 37°C for 30min. Reactions were stopped by addition of 1ml ice cold 1% phosphotungstic acid/0.5M HCl (PTA/HCl) to precipitate proteins. Samples were left on ice for 10min and then centrifuged at 14,000rpm for 7sec on a bench top Eppendorf centrifuge. The supernatants were discarded and the pellets resuspended in 1ml fresh ice cold PTA/HCl, and centrifuged as before. This was repeated twice more, and then the pellets resuspended in 1ml ice cold 95% ethanol, and centrifuged as before. The ethanol was then removed and the pellet dissolved in 50μl 2M Tris (unbuffered) and 200μl 5% (w/v) SDS by shaking at room temperature for 5min. 1ml of liquid scintillant was then added, the samples vortexed and counted with a scintillation counter. The DPM value of the blank was subtracted from the sample values.

To determine the concentration of GalT in any given sample, a 10μl aliquot of assay mixture, mixed with 40μl 2M Tris (unbuffered) and 200μl 5% SDS was also counted to enable calculation of the specific activity (S.A.) of the UDP-galactose in the reaction. The specific activity of the UDP-galactose in DPM/nmol was calculated as below:
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The concentration of GalT activity in nmol/hr/ml was then calculated as below:

\[ [\text{GalT}] = \frac{1000 \times (\text{DPM of sample} - \text{DPM of blank})}{S.A. \text{ UDP-Galactose}} \]

2.2.3 NAGTI activity

Occasionally Golgi preparations were assayed for NAGTI activity, a medial-Golgi enzyme, which catalyzes the addition of N-acetylglucosamine onto Man\textsubscript{3}-GlcNAc\textsubscript{2} N-glycans. Ovalbumin contains such an incompletely processed structure and serves as an acceptor molecule (Vischer and Hughes, 1981). An assay mixture was made up comprising: 100µl 0.5M Tris/maleate pH 6.9, 100µl 200mg/ml ovalbumin, 10µl 1M KCl, 20µl UDP-GlcNAc, 10µl 10% (w/v) TX-100, 2.5µl 2M MnCl\textsubscript{2}, 2.5µl MgCl\textsubscript{2}, 10µl 100µCi/ml UDP-[\textsuperscript{3}H] GlcNAc, and 544µl distilled water. 80µl of this assay mix was added to 20µl of sample (1:20 dilution of RLG or homogenate to ensure within the linear range [Slusarewicz et al., 1994]) or 20µl distilled water as a blank, and incubated for 2.5h at 37°C. The reaction was stopped, processed and counted as in the GalT assay above. The main difference was that the SDS-solubilization took 1hr of shaking at room temperature. Purification tables were produced similar to those for GalT.

2.2.4 Protein concentration

Sample protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturers instructions. 200µl reagent was mixed with 800µl of distilled water plus sample to complete a 1ml final volume. The reaction is instantaneous, and must not be carried out for longer than 5min as precipitates will
then begin to form. The absorbance at 595nm was measured using a spectrophotometer. Protein concentration was calculated from standard curves constructed by using a 2mg/ml solution of highly purified bovine serum albumin (Pierce) as a standard. The assay was routinely carried out in the linear range of 0-8μg BSA per 1ml reaction.

2.2.5 Mitotic and Interphase cytosols

Rat liver interphase cytosol was prepared from the blood red 0.5M sucrose layer from the first sucrose gradient of the RLG preparation (Section 2.2.1; Rabouille et al., 1995c). Fractions were pooled and centrifuged at 45,000rpm for 60min at 4°C in a Ti50.2 rotor (Beckman), to remove any particulate material. NaEDTA was added to the supernatant to 1mM (from a 200mM, pH 7.0 stock). Ammonium sulphate was then gradually added to the supernatant to 40% saturation at 0°C (0.229g/ml) while stirring gently on ice, keeping frothing to a minimum. Stirring was continued for 30min on ice after dissolution, and the mixture then centrifuged in a CR 422 centrifuge (Jouan, St-Herblain Cedex, France) at 4,000rpm for 30min at 4°C. The supernatant was discarded and the pellets resuspended by gentle pipetting in KHM (60mM KCl, 25mM Hepes-KOH, pH 7.3, 5mM magnesium acetate, 1mM glutathione, 5μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml). The resuspended pellet was desalted into the same buffer containing 2mM ATP and 1mM GTP by dialysing against a 50-fold excess of buffer using Snakeskin pleated dialysis tubing with a 3,500 MW cut off (Pierce, IL). After at least 2h at 4°C, dialysis was continued against fresh buffer for 4-12h at 4°C. The dialyzed material was collected and clarified by centrifugation at 45,000rpm for 30min at 4°C in the Ti50.2 rotor (Beckman). The supernatant was collected and termed rat liver cytosol. Alternatively, if only a small volume (<1.5ml) was being desalted P-6 DG columns (Bio-Rad) or HiTrap desalting columns (Pharmacia) were used (see below).

Mitotic and interphase cytosols were prepared from spinner HeLa (sHeLa) cells (HeLa S3; American Tissue Culture Catalogue; Stuart et al., 1993; Sönningchen et al., 1996), which were grown at 37°C in RPMI (ICRF Cell Production services) with 0.2% bicarbonate, supplemented with 12.5% foetal bovine serum, 200mM glutamine, 50IU/ml penicillin, 50mg/ml streptomycin, and 1% non-essential amino acids in an
atmosphere of 5% CO\textsubscript{2}/95% air. Cells were grown in 150cm\textsuperscript{2} plastic flasks and split every 48h for up to 30-40 passages. The mean doubling time was c. 24h.

8-10 150cm\textsuperscript{2} flasks of sHeLa cells were grown to confluence and transferred to a 1l spinner culture flask as a single cell suspension. After 24h at 37°C, cells were counted in a Neubauer chamber and, if required, the cell density was adjusted to 2-2.5 x 10\textsuperscript{5} cells/ml. For interphase cytosol, the cells were then allowed to grow for a further 20-26h at 37°C. For mitotic cytosol cells were then pelleted under sterile conditions at 2,000rpm for 2min at 4°C in the CR 422 centrifuge. Pellets were resuspended in 5ml RPMI media, containing 500μl 0.2mg/ml nocodazole (in DMSO; giving a final concentration of c. 20μg/ml), and resuspended by pipetting up and down in a 10ml pipette for 10min. This mixture was then returned to the spinner flask and grown for a further 20-26h at 37°C.

At 20-26h the mitotic index of the cells was determined. A 1ml sample of culture from the spinner flask was taken and centrifuged at 4,000rpm for 1min at room temperature in a bench top Eppendorf centrifuge. The medium was discarded and the cell pellet resuspended in 20μl of 3% paraformaldehyde, 0.2% TX-100, 2mg/ml Höchst 33258 and left on ice for 2min. A drop of solution was spotted on a microscope slide, covered with a coverslip and viewed by fluorescence microscopy using the FT510 filter and a 20x oil-immersion lens on a Zeiss Axiophot microscope. Mitotic cells were easily distinguishable from interphase cells, since they contained condensed chromatin rather than a diffusely stained nucleus (Chaly and Brown, 1988). The percentage of total cells that were mitotic (mitotic index) was determined by counting 100-200 cells. Only populations with a mitotic index greater than 90% were used to make mitotic cytosol.

Cells were harvested by centrifugation at 2,000rpm for 10min at 4°C in the CR 422 centrifuge. The media was discarded and the pellets resuspended in 50ml ice cold 33.3% EBS (6.67mM EGTA, 26.7mM β-glycerophosphate, 0.033M sucrose, 5mM MgCl\textsubscript{2}, pH 7.3, 2mM ATP, 1mM glutathione, 5μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50ml) and left on ice for 15min. Cells were then recovered by centrifugation at 2,000rpm for 2min at 4°C in the CR 422 centrifuge, and were resuspended in 10ml ice cold 100% EBS (80mM β-glycerophosphate, 20mM EGTA, 15mM MgCl\textsubscript{2}, 0.1M sucrose, pH 7.3, 2mM ATP,
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1 mM glutathione, 5 μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50 ml, and recovered as before. The supernatant was removed and the cells resuspended in a 0.6x cell volume of fresh ice cold 100% EBS.

Cells were then vortexed for 3 min and homogenized with a 8,020 ball bearing homogenizer (EMBL, Heidelberg, Germany) using a ball bearing leaving 10 μm clearance. Ideally the breakage was aimed to just puncture the plasma membrane and not to rupture the nucleus of interphase cells so as to avoid mixing nucleoplasm/DNA with cytoplasm. Breakage was assessed by trypan blue staining and c. 30-40% of cells would appear blue to yield a cytosol with a protein concentration of c. 10 mg/ml. Typically 5-7 passages were required for mitotic cells, and 10-15 for interphase cells. The homogenate was then centrifuged at 100,000 rpm for 5 min at 4°C in a Beckman TL-100 bench top centrifuge. The supernatant was then taken with a hypodermic needle (avoiding the lipid infranatant) and centrifuged at 100,000 rpm for 30 min at 4°C in a Beckman TL-100 benchtop centrifuge. The supernatant was again collected using a hypodermic needle, and this fraction was termed cytosol, and was aliquoted and flash frozen in liquid nitrogen. Such aliquots were stored at -80°C for a maximum of 2 months prior to use.

Gel filtration spin columns were prepared to transfer the cytosolic proteins from EBS to a suitable buffer: MEB (10 mM MgCl₂, 15 mM EGTA, 20 mM β-glycerophosphate, 50 mM KCl, 50 mM Tris-Cl pH 7.3, 0.2 M sucrose, 2 mM ATP, 1 mM GTP, 1 mM glutathione, 5 μM pepstatin A, 1 Complete EDTA free protease inhibitor tablet [Boehringer Mannheim]/50 ml) for mitotic cytosol and KHM (with 2 mM ATP, 1 mM GTP) for interphase cytosol (SHHeLa and rat liver). A bed of glass wool was deposited at the end of a 2 ml syringe (without the plunger) and 2 ml of 10% (w/v) Bio-Gel P-6 DG (Bio-Rad) added. The syringe was then placed in a 15 ml Falcon tube, and centrifuged at 2,000 rpm for 2 min at 4°C to pack the resin. This was repeated until 2 ml of resin was packed into the syringe. Care was taken to ensure all the liquid was removed from the column to avoid diluting the cytosol. The column was then equilibrated with 10 ml buffer in several consecutive centrifugation runs. After equilibration, up to 400 μl cytosol was loaded onto the column and the centrifugation performed as before. Typically c. 95% of the cytosol sample volume was recovered.
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with a 5-10% decrease in protein concentration. Alternatively, 5ml HiTrap desalting columns (Pharmacia) were used according to the manufacturer's instructions.

2.2.6 Histone kinase assay

Cytosols were routinely checked for their histone kinase assay (Felix et al., 1989) to ensure they reflected the mitotic or interphase state. Samples were diluted with distilled water such that the protein concentration was 0.1mg/ml. 2μl sample was added to 18μl reaction mix (5mg/ml histone, 25mM KCl, 5mM EGTA, 25mM Hepes-KOH, pH 7.2, 107mM β-glycerophosphate, 67mM NaF, 1.33mM MgCl₂, 1.33mM ATP, 20μCi [γ-³²P]-ATP) and incubated for 20min at 37°C. The reactions were quenched by transferring them to ice. 12μl of each reaction was spotted onto 1.5 x 1.5 cm grid squares of P81, phosphocellulose paper (Whatman). Once dry the P81 phosphocellulose paper was washed 5 x 5min with 150mM phosphoric acid, soaked briefly in 95% ethanol and dried at 37°C in an oven. The samples were then immersed in 4ml liquid scintillant and counted. Assays were performed in duplicate and calculations performed using mean values. For determination of background histone independent counts, histones were omitted from the reaction mix. The specific activity of [γ-³²P]-ATP was determined in a separate sample.

Specific histone H1 activity was calculated as follows:

\[
\text{Activity of ATP (cpm/pmol ATP)} = \frac{\text{average cpm}}{20/18 \times 2 \times 1000}
\]

\[
\text{Activity of sample (pmol/sample)} = (\text{sample cpm} - \text{background cpm}) \times \frac{\text{Activity of ATP}}{\text{Activity of sample}}
\]

\[
\text{Specific activity per sample (pmol ATP/min/mg protein)} = \frac{\text{Activity of sample} \times 20/12 \times 1/\text{sample volume (μl)} \times 1/\text{assay time (min)} \times 1000/\text{[cytosol] (mg/ml)}}{1000/\text{[cytosol] (mg/ml)}}.
\]

2.2.7 Depletion of p115 from cytosol

p115 was depleted from cytosol using either the mAb 4H1 or a biotinylated peptide comprising the N-terminal 73 amino acids of GM130 (N73pep) which binds p115 (Nakamura et al., 1997). 4H1 was coupled to Affigel-10 (Bio-Rad) according to manufacturer's instructions to obtain 0.72mg 4H1/ml resin. Beads were washed thrice with KHM and made up as a 1:1 slurry in that buffer. 200μl slurry was dried using a
Hamilton syringe and added to 800µl rat liver cytosol (c. 20mg/ml) or sHeLa interphase cytosol (c. 10mg/ml) and incubated for 1 hour with rotation at 4°C. The beads were then recovered by centrifugation at 14,000rpm for 1min in an Eppendorf microfuge, the supernatant removed and added to fresh beads. This was repeated 4 times.

1ml biotinylated N73pep (10mg/ml in distilled water) was coupled to 2ml Neutravidin beads (Pierce, IL) according to the manufacturer’s instructions. After coupling, beads were blocked with 10mg/ml soybean trypsin inhibitor. Beads were then packed into a 0.7x10cm Econo-column (Bio-Rad), and the column was equilibrated with 20ml KHM. 2ml rat liver cytosol was loaded onto the column and allowed to interact with the resin for 15 min. The column was then eluted with KHM.

In both cases the mock depletions were made with the same blocked beads without antibody or peptide coupled. 20µg cytosolic proteins were separated on a 7.5% SDS-polyacrylamide gel (Section 2.2.17) and transferred to nitrocellulose (Hybond C, Amersham, UK; Section 2.2.18). Increasing amounts of purified rat liver p115 (0.025-0.2µg) were also separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose to construct a p115 standard curve. Blots were probed with 8A6 and compared to the p115 standard curve to determine the extent of p115 depletion. From this p115 was estimated to be present at 3-4ng/µg cytosol, and in add back experiments was added to this level.

2.2.8 Depletion of coatomer from mitotic cytosol
Coatomer was depleted from mitotic cytosol as according to Misteli and Warren (1994). 2ml CM1A10 ascites were incubated overnight at 4°C with rotation in the presence of 0.2% TX-100 and 1ml 50% slurry protein G-agarose beads in PBS. The beads were then recovered by centrifugation at 14,000rpm for 1min on a bench top Eppendorf centrifuge, the supernatant removed and the beads washed in 10ml 0.2M sodium borate (pH 9). This step was repeated four times, and the beads were finally resuspended in 12ml 0.2M sodium borate (pH 9) plus 20mM dimethylsuberimidate and incubated at room temperature with rotation for 30min to cross link the antibody to the protein G. Beads were then pelleted as above, and washed over 2h at 4°C with three changes of 10ml 0.2M ethanolamine (pH 8). Beads were then washed three times
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with 10x the bead volume of MEB (-nucleotides), before they were dried with a Hamilton syringe and added to mitotic cytosol. 750μl mitotic cytosol (c. 10mg/ml) was supplemented with an ATP regeneration system (Section 2.2.10) and incubated in a boro-silicate glass tube with 40μl of 50% CM1A10 coupled protein G-agarose beads. Sequential incubations with changes of beads were performed for 6h, 12h and 6h at 4°C with rotation. At the end of each incubation the beads were removed by centrifugation as above.

A mock depletion was made with just protein G-agarose beads. 20μg cytosolic proteins were separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Increasing amounts of purified rat liver coatomer (0.01-0.4μg) were also separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose to construct a coatomer standard curve. Blots were probed with M3A5 and compared to the coatomer standard curve to determine the extent of coatomer depletion. From this coatomer was estimated to be present at c. 5ng/μg cytosol, and in add back experiments was added to this level.

2.2.9 Purification of p115 from Rat liver cytosol

p115 was purified from rat liver cytosol in a four step purification. First a 40% ammonium sulphate cut was taken, followed by a Q-sepharose step (Levine et al., 1996), then an affinity purification step using N73pep as bait, and finally a Superose 6 molecular sieving step. A 40% ammonium sulphate cut of 100-400ml of the blood red 0.5M sucrose layer from the first sucrose gradient of the RLG preparation was taken (Section 2.2.5). The resulting pellets were resuspended in ice cold Q0 (25mM Tris-HCl, pH 7.3, 1mM DTT) to achieve the conductivity of Q200 (25mM Tris-HCl, pH 7.3, 200mM KCl, 1mM DTT). The conductivity was measured while stirring the solution gently in beaker on ice. 1 Complete EDTA free protease inhibitor tablet (Boehringer Mannheim) was added for every 50ml solution. The solution was centrifuged at 4,000rpm for 10min at 4°C in the CR 422 centrifuge (Jouan) in 50ml Falcon tubes to remove any particulates. This solution was termed the ammonium sulphate cut.

The clarified solution was loaded at 4ml/min onto a 75ml fast flow Q sepharose column pre-equilibrated with 375ml Q200. The column was then washed with 375ml
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Q200 and eluted with a 1.1mM/ml KCl gradient (i.e. a 750ml gradient from 25mM Tris-HCl, pH 7.3, 200mM KCl, 1mM DTT to 25mM Tris-HCl, pH 7.3, 1M KCl, 1mM DTT), collecting 52 14.5ml fractions. 10µl of alternate fractions were fractionated by SDS-PAGE using a 7.5% gel, transferred to nitrocellulose and probed with 8A6 to determine which fractions contained p115. p115 was typically found to peak from 470 to 535mM KCl. These fractions were pooled and termed the Q-pool. The Q-pool was dialyzed against a 50-fold excess of HK100 (20mM Hepes-KOH, pH 7.3, 100mM KCl, 1mM EDTA, 10% glycerol, 0.5mM DTT) using Snakeskin pleated dialysis tubing with a 3,500 MW cut off (Pierce, IL). After at least 2h at 4°C, dialysis was continued against fresh buffer for 4-12h at 4°C. The dialyzed Q-pool was clarified by centrifugation at 4,000rpm for 10min at 4°C in the CR 422 centrifuge (Jouan) and then loaded on to a 2ml N73pep-neutravidin column (Section 2.2.7) that had been pre-equilibrated with 20ml HK100. The flow through was collected. The load was allowed to interact with the resin for 15 min and then washed with 20ml HK100. The column was then batch eluted with 6 x 2ml HK600 (20mM Hepes-KOH, pH 7.3, 600mM KCl, 1mM EDTA, 10% glycerol, 0.5mM DTT). After elution the column was equilibrated with 20ml HK100 and the process repeated twice using the flow though from the initial loading. 10µl of each fraction was separated on 7.5% gel by SDS-PAGE for Coomassie staining and Western blotting. Virtually all the p115 comes off the column in the second fraction.

p115 could be up to 80-90% pure after this step and is relatively concentrated (c. 0.1-0.2mg/ml). When sufficiently pure this p115 was dialyzed against a 50-fold excess of HK200 (20mM Hepes-KOH, pH 7.3, 200mM KCl, 1mM EDTA, 10% glycerol, 0.5mM DTT) as above. If further purification was desirable the p115 containing fractions were pooled and concentrated using Centricon-10 (Millipore, UK) according to the manufacturer’s instructions. Typically the fractions were centrifuged in the Beckman J2 21 centrifuge at 5,000rpm for 90min at 4°C to concentrate 6ml to 600µl. The concentrated sample was then sieved through onto 24ml Superose-6 column (Pharmacia) equilibrated with 250ml HK200 at 0.15ml/min. 0.5ml fractions were collected, and 10µl aliquots analyzed by SDS-PAGE and Coomassie staining. Typically p115 peaked in fractions 21-24, corresponding to a molecular weight of c. 700kDa and an 83Å Stokes radius for a globular protein. However, this is most likely an overestimate of the true molecular weight since p115 is likely a dimer that exhibits
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a myosin II like conformation, with a globular head domain followed by an extended coiled-coil tail domain (Sapperstein et al., 1995). p115 is more than 90% homogeneous in these fractions as judged by Coomassie staining, and typically has a protein concentration of 0.05-0.15mg/ml. A typical purification is shown in Figure 2.1.

2.2.10 Disassembly reaction

The disassembly reaction was carried out as in Misteli and Warren (1994, 1995a) except that microcystin was omitted from the reaction. RLG were resuspended at 0.1mg/ml in mitotic cytosol (8-10mg/ml final concentration) in MEB buffer and supplemented with an ATP regeneration system (final concentration: 10mM creatine phosphate, 0.02mg/ml creatine kinase, 0.1mM ATP, 0.2μg/ml cytochalasin B) in a final volume of 50μl per disassembly reaction. This mix was incubated for 0-60min at 37°C as required. Reactions were terminated by transferring to ice for 2-3min followed by addition of 100μl of 2% glutaraldehyde (Fluka; in PBS/0.2M sucrose) and fixed for 30min at room temperature, and then processed for EM (Section 2.2.13).

GDI

In some experiments His-tagged GDI was included in the reaction at 1-10μM and delipidated BSA at 10μM. In order to determine whether GDI had affected the membrane association of Rab1 and Rab6 during the disassembly reaction, instead of fixing the reaction with 2% glutaraldehyde membranes were recovered by centrifugation at 15,000rpm for 20min at 4°C in a 5413 Eppendorf centrifuge fitted with a horizontal rotor. The supernatant was removed, and the pellet washed 3 x 500μl MEB to remove any contaminating cytosol. Pellets were then dissolved in SDS PAGE sample buffer and fractionated by SDS-PAGE, transferred to nitrocellulose and probed with specific antibodies against Mann-1 (to check for equal recovery of Golgi membranes), Rab6 or Rab1.

Coatomer depletion

In some experiments mitotic cytosol was replaced with coatomer depleted mitotic cytosol, mock depleted cytosol or coatomer depleted cytosol with purified rat liver coatomer added back to 50μg/ml. Coatomer depleted mitotic cytosol and RLG were also treated with NEM as follows. NEM was added at a final concentration of 1mM to RLG and coatomer depleted mitotic cytosol for 15min on ice, before quenching with
Figure 2.1 p115 purification. p115 was purified as described in the materials and methods. Rat liver cytosol (20μg), the 40% ammonium sulphate cut (20μg), the Q-sepharose pool (6μg), the N73pep-neutavidin enriched p115 fraction (1μg) and the Superose 6 p115 enriched fraction (1μg) were separated by SDS-PAGE on a 7.5% gel and stained with Coomassie. Molecular weight in kDa is shown on the left and the asterisk denotes p115.
2mM DTT for 20min on ice. NEM treated RLG and NEM treated coatamer depleted mitotic cytosol were then mixed for the disassembly reaction. In some reactions coatamer depleted cytosol was supplemented with either BAPTA (Molecular Probes, Oregon, USA) to 10mM, dibromo-BAPTA (Molecular Probes) to 10mM, 50µM AlF₄⁻ which was generated by adding KF to 5mM followed immediately by AlCl₃ to 50µM, mastoparan to 1µM or manoalide to 8µM. In each case the reagent buffer was used as the control reaction.

**Effect of KCl and N73pep extraction**
In some reactions RLG were extracted with either 60mM KCl, 1M KCl, or N73pep prior to disassembly. 50µg RLG were resuspended at 0.1mg/ml in KHM for the 60mM KCl wash or supplemented with an additional 940mM KCl for the 1M KCl extraction, or 80µM N73pep and left on ice for 15min. Membranes were then recovered onto a 2µl 2M sucrose cushion by centrifugation at 15,000rpm for 20min at 4°C in a 5413 Eppendorf centrifuge fitted with a horizontal rotor. The supernatant was then removed and proteins precipitated by the trichloroacetic acid (TCA) method (Section 2.2.14). The membranes were resuspended in 20µl KHM and the protein concentration determined. Samples of these membranes were fixed and processed for EM as for RLG (Section 2.2.1) or dissolved in SDS-PAGE sample buffer and fractionated by SDS-PAGE. In some reactions p115 was supplemented to 1M KCl and N73pep extracted membranes. 0.075µg p115 (0.37µl) was added to 5µg 1M KCl extracted RLG or N73pep extracted RLG in a final volume of 5µl (made up with KHM) and left on ice for 10min prior to the disassembly reaction.

**MGF for reassembly**
For reassembly purposes the disassembly reaction was performed as above, except the reaction time was set at 20min and the final volume of reaction was 1ml for four subsequent reassembly reactions. At the end of the disassembly reaction 250µl aliquots were transferred to fresh Sarstedt screw top tubes and underlaid with 125µl MEB containing 0.5M sucrose instead of 0.2M sucrose (or just MEB for the purpose of comparison) and a 2µl 2M sucrose cushion. The membranes were then recovered by centrifugation at 15,000rpm for 25min at 4°C in the horizontal rotor of the Eppendorf centrifuge and were termed MGF. MGF were pooled and the protein concentration
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determined. Samples of MGF isolated with or without the 0.5M sucrose cushion were
fixed as for RLG above (Section 2.2.1) and processed for EM (Section 2.2.14).

To assess the relative polypeptide composition of MGF isolated with or without the
MEB/0.5M sucrose cushion the 2μl 2M sucrose cushion was omitted, and the resulting
pellet solubilized in SDS-PAGE sample buffer, boiled for 3 min and separated on 5-
20% gradient SDS-polyacrylamide gels and either stained with Coomassie or
transferred to nitrocellulose, and probed with various specific antibodies.

2.2.11 Reassembly reaction

Reassembly with cytosol

In a typical reassembly reaction the MGF were gently resuspended (final concentration
0.75-1mg/ml) in rat liver cytosol (10mg/ml final concentration) in KHM buffer (with
2mM ATP and 1mM GTP) supplemented with an ATP regeneration system (final
concentration: 10mM creatine phosphate, 0.02mg/ml creatine kinase, 0.1mM ATP,
0.2μg/ml cytochalasin B) in a final reaction volume of 20μl, and incubated for 60min
at 37°C. Reactions were terminated by transferring to ice for 2-3min. For EM 100μl of
2% glutaraldehyde (Fluka; in PBS/0.2M sucrose) was then added and the samples
fixed for 30min at room temperature.

In comparing the MGF isolated with or without the MEB/0.5M sucrose cushion,
reassembly was conducted as above except in some reactions using just KHM buffer
instead of cytosol. Unless otherwise stated reassembly reactions in this study were
conducted using MGF isolated through the 0.5M sucrose cushion.

To optimize the above reaction it was conducted at increasing temperature, [MGF] and
with cytosol desalted into KHM with increasing [KCl]. Rat liver cytosol, p115-
depleted cytosol and p115-depleted cytosol with p115 added back were also titrated
into the above reaction. The time in the reaction at which p115 was added back to the
depleted cytosol was also varied.

GTPγS

In some reactions where indicated 1mM GTP was replaced with 0.02-0.4mM GTPγS.
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GRASP65/GRASP55

In some reactions MGF were pretreated for 15min on ice with 0-1.2μM (final concentration in the reaction) soluble GRASP65 and/or soluble GRASP55. In some reactions soluble GRASPs were first treated with NEM as follows: 50μl of GRASP (0.4mg/ml final concentration; in KHM) were treated with 1mM NEM for 15min on ice. The reaction was quenched by addition of DTT to 2mM and left on ice for 30min, prior to addition to the assay. NEM quenched with DTT was used as the as the control treatment. For antibody inhibition experiments, MGF were treated for 15min on ice with either 2μl of the appropriate pre-immune serum, 1μl of the FBA31 (anti-GRASP65) or FBA34 (anti-GRASP55) antibodies plus 1 μl of the opposing pre-immune serum, or 1μl each of FBA31 and FBA34.

Reassembly with pure components

Reassembly was carried out exactly as above except cytosol could be replaced by the purified components: NSF, α-SNAP, γ-SNAP, p115 and/or p97, p47. The optimal levels for these proteins in the reassembly assay had been determined previously (Rabouille et al., 1995b, 1998) and were used here. For a typical NSF catalyzed reaction cytosol was replaced with NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl), and p115 (30ng/µl). For a typical p97 catalyzed reaction cytosol was replaced by purified rat liver p97 or recombinant His-tagged p97 (70ng/µl) and p47 (37.5ng/µl). p97 and p47 were preincubated on ice for at least 30min prior to addition to the assay to allow formation of the p97/p47 complex (Kondo et al., 1997). In some reactions the effect of omitting each of these factors in turn was determined. For the typical NSF/p97 catalyzed reaction all these components were combined. Reactions were typically carried out for 60min at 37°C and fixed and processed for EM as for the cytosol driven reaction. In some experiments p115 was titrated into the NSF, p97 and NSF/p97 reactions from 0-30ng/µl.

Giantin, GM130, GRASP65

In some experiments the MGF were pretreated with either 1μl of the appropriate preimmune serum or 1μl anti-GM130 NN15 and/or 1μl anti-giantin for 15min on ice before resuspension for the NSF, p97 or NSF/p97 catalyzed reaction.
In some reactions MGF were pretreated for 15 min on ice with either giantin fragment buffer (20mM Hepes-KOH, pH 7.4, 100mM KCl, 1mM magnesium acetate, 1mM DTT), Gtn448 (N-terminal 448 amino acids of giantin), or Gtn1967-2541 to achieve a final concentration of Giantin fragment of 3.25 μM in the reaction. Samples were resuspended for the NSF catalyzed reaction. Gtn448 was also titrated into the NSF reaction from 0-3.25 μM.

In some experiments the MGF were pretreated for 15 min on ice with KHM (the N73pep/GRASP65 solvent), N73pep (or S25D N73pep) from 0-80 μM (final concentration in reaction) or soluble GRASP65 (or NEM treated soluble GRASP65 as above) from 0-75ng/μl (final concentration in reaction) before resuspension for the NSF, p97 or NSF/p97 catalyzed reaction. For Western blotting, completed reactions were made up to 120 μl with ice cold KHM and membranes were recovered by centrifugation at 15,000 rpm for 30 min at 4°C in the horizontal rotor of the Eppendorf centrifuge. The resulting pellet was solubilized in SDS-PAGE sample buffer and processed as for MGF as above.

The effect of N73pep and soluble GRASP65 treatment was also assessed on starting RLG. RLG at 0.75 mg/ml was treated with N73pep (80 μM) or soluble GRASP65 (75ng/μl) in KHM (with 2mM ATP and 1mM GTP) buffer and an ATP regeneration system in a final volume of 20 μl and incubated for either 15 min on ice or 60 min at 37°C. Then fixed and processed for EM.

To assess the temporal sensitivity of reassembly to N73pep and soluble GRASP65 the NSF/p97 reaction was allowed to proceed for increasing time at 37°C. At various times the reaction was transferred to ice and either fixed and processed for EM, treated with KHM, 80 μM N73pep, or 75ng/μl soluble GRASP65 for 15 min on ice. Then reincubated at 37°C for a total time of 60 min.

**p115 mutants, peptides, CKII inhibitors**

p115 was replaced in the NSF catalyzed reaction with either the 75mer, TA, TA (S941A), or TA (S941D) from 0-260 nM. In some experiments, the MGF were preincubated for 15 min on ice prior to reassembly with a 200x molar excess (over the final p115 concentration in the reaction) of 75mer, 70mers of polyaspartate,
polyglutamate, or polyglutamate/aspartate (1 glutamate:1 aspartate), 26mer/26mer-P, or a 100x molar excess (over the final pi15 concentration in the reaction) of CKII substrate/substrate-P. A 200 fold molar excess over pi15 of 26mer/26mer-P or a 100 fold molar excess of CKII substrate/substrate-P was also included in the p97 catalyzed reaction (containing 15ng/µl pi15). In other experiments, the NSF catalyzed reaction was conducted in the presence of either: 100µM Staurosporine, DRB, Chrysin, or Roscovitine, or 1µl anti-CKII-α- or 1µl anti-CKII-β-antibodies.

The NSF catalyzed reassembly reaction was conducted in the presence of a 150 fold molar excess over pi15 of the pi15 peptides: pi15 637-699, 728-765, 788-827. pi15 637-699 was titrated into the assay from 0-13µM, with the pi15 level set at 87nM.

**NSF mutants**

MGF were isolated without the 0.5M sucrose cushion and treated with 2.5mM NEM on ice for 15min and then quenched with 5mM DTT for 20min ice. NEM-MGF were resuspended with either NSF, NSF (E329Q) or NSF (D604Q) at 5ng/µl and α-SNAP (25ng/µl), γ-SNAP (25ng/µl), and pi15 (7.5ng/µl) and incubated at 37°C for 60min. In some reactions NSF and NSF mutants were treated with NEM as for MGF above, and controls were treated with NEM prequenched with DTT.

**Ufd1p and Npl4p**

MGF isolated through a 0.5M sucrose cushion were resuspended with either p97 (70ng/µl) and p47 (35ng/µl); p97 (70ng/µl), Ufd1p (35ng/µl) and Npl4p (49ng/µl); p97 (70ng/µl) and Ufd1p (35ng/µl); Ufd1p (35ng/µl) and Npl4p (49ng/µl); Ufd1p (35ng/µl); or Npl4p (49ng/µl) and incubated at 37°C for 60min. p97/p47, p97/Ufd1p/Npl4p, p97/Ufd1p and Ufd1p/Npl4p complexes were allowed to form by incubation for 30min on ice prior to addition to the reassembly reaction.

In some reactions the p97 catalyzed reaction was conducted in the presence of 0-20µM Ufd1p or Ufd1p/Npl4p.

**2.2.12 Incubation of 1M KCl extracted RLG with interphase sHeLa cytosol**

1M KCl extracted RLG (Section 2.2.10) were resuspended at 0.1mg/ml in sHeLa interphase cytosol (final concentration 8-10mg/ml) in KHM buffer (with 2mM ATP,
1mM GTP) and supplemented with an ATP regeneration system (10mM creatine phosphate, 0.02mg/ml creatine kinase, 0.1mM ATP, 0.2μg/ml cytochalasin B) in a final volume of 50μl. This mix was incubated at 37°C for 40min. Alternatively, instead of shHeLa interphase cytosol either: shHeLa interphase cytosol supplemented with N73pep to 52μM (c. 200 fold molar excess over p115); p115 depleted shHeLa interphase cytosol; or p115 depleted shHeLa interphase cytosol supplemented with p115 (3ng/μg cytosol) was used. Reactions were terminated by transferring to ice for 2-3min followed by addition of 100μl of 2% glutaraldehyde (Fluka; in PBS/0.2M sucrose) and fixed for 30min at room temperature, and then processed for EM (Section 2.2.13).

2.2.13 Electron microscopy

After samples had been fixed with glutaraldehyde for 30min at room temperature, they were centrifuged at 15,000rpm for 20min at 4°C in a 5413 Eppendorf centrifuge fitted with a horizontal rotor, and then at 14,000rpm in the vertical rotor for 10min at 4°C. The fixative was then removed and the samples washed with 5 x 1ml PBS. Samples were post-fixed for 30-120min with 1% (w/v) osmium tetroxide (Taab, UK), 1.5% (w/v) cyanoferrate in 0.1M sodium cacodylate buffer, pH 7.2. If extra contrast was required samples were then washed with 3 x 1ml 0.05M sodium cacodylate buffer, pH 7.2, and then left in the dark for 60min with 1ml 2.5% (w/v) tannic acid (Mallinckrodt Inc, Kentucky, USA). Samples were then washed for 5min with 1% (w/v) sodium sulphate (Simionescu and Simionescu, 1976). Samples were then washed with 3 x 1ml distilled water, and left in the dark for 60min with 0.5% (w/v) uranyl acetate, and then washed again with 3 x 1ml distilled water. If no extra contrast was required samples were washed with 3 x 1ml 0.1M sodium cacodylate buffer, pH 7.2.

Pellets were then dehydrated in a series of graded ethanol solutions as follows 2 x 5min with 70% ethanol, 2 x 5min 90% ethanol, 3 x 15min 100% ethanol. All the ethanol was then carefully removed and the pellets immersed in 1ml propylene oxide for 15min. Samples were then immersed in 1ml 1:1 propylene oxide/Epon 812 (Taab, UK), followed by 1ml 100% Epon 812 with 3 changes over at least 3h. Polymerization of the Epon 812 was induced by incubation at 65°C for 16h.

After polymerization the sides of the blocks were trimmed with a razor blade and the surface of the blocks flattened with a glass knife (made with a LKB knife maker 2178).
using a Reicher-Jung Ultramicrotome 2E. Transverse sections of 60-80nm nominal thickness were routinely cut through the entire pellet, to ensure the pellet was homogeneous, with a diamond microtome knife (Drukker International, Holland). Sections were always cut at the same point in the pellet and picked up on colloidal coated (Taab, UK) 100 mesh copper grids (Agar Scientific). Grids were air dried prior to staining, and were floated for 5min on a drop of 2% (w/v) uranyl acetate (unless they had already been contrasted with uranyl acetate) in 50% (v/v) ethanol, washed extensively with distilled water from a jet-stream bottle, air dried and counterstained with lead citrate for 2min in an atmosphere of NaOH. Sections were viewed at 80kV on a Philips CM10 electron microscope.

For quantitation, photographs were taken at a final magnification of 28.8K-52.5K in a systematic random fashion (Weibel, 1969). Bias was excluded by ensuring the position of the micrographs taken was fixed with respect to the squares of the supporting grid and so was independent of the membrane structures themselves. The grid was viewed at low magnification and the fluorescent screen positioned tangentially in the top left hand corner of a square of the copper grid. The magnification was then increased to the level required and the photograph taken.

2.2.14 Stereology

Stereological definitions

The stereological definitions used to define membrane profiles were based on Rabouille et al. (1995c) as follows:

**Golgi cisterna:** A membrane profile with a cross-sectional length greater than four times its width, the width being no more than 30nm. Cisternae could range from continuous to extensively fenestrated.

**Stacked region of a cisterna:** A region of a cisterna that is aligned in parallel with another cisterna for greater than 50nm and separated from it by no more than 15nm.

**Tubule:** A membrane profile with a cross-sectional length greater than 1.5 times its width, the width being greater than 30nm.

**Tubular network:** Tubular profiles with at least one branch point or, alternatively, two or more intersecting tubules.

**Vesicles:** Circular or oval membrane profiles with an average diameter of 50-150nm, where the ratio of the longest diameter of the profile to the diameter perpendicular to it
was less than or equal to 1.5. The average diameter of the profile was taken as the average of these two measurements.

All other membrane profiles were readily identifiable contaminants, and were mostly unattached lipoprotein particles, and occasionally endosomes, peroxisomes, lysosomes, mitochondria and plasma membrane sheets. These profiles generally constituted from 5-12% of the total membrane.

**Percentage total membrane present as a given profile**

To determine the percentage total membrane present as a given profile, photographs were taken at a final magnification of 28.8K or 38.8K and overlaid with a vertical line grid (5mm distance between lines) on an overhead transparency. Quantitation was performed as in Weibel et al. (1969). All intersections of membrane profiles on a photograph with the test lines were recorded. Each intersecting profile was assigned to one of the membrane categories of interest in the particular experiment and the number of intersections for each category was recorded. 100% of total membrane represented the total of all intersections ($I_t$) of membrane profiles with test lines and the percentage for each category of membrane ($P_x$) could be calculated according to:

$$P_x = \frac{I_x}{I_t} \times 100$$

where $I_x$ was the number of intersections of all membranes of category $x$ with the test lines. Routinely 5-8 pictures were quantitated per reaction.

For the reassembly reaction the crucial membrane categories were stacked regions of Golgi cisternae and Golgi cisternae. The percentage total membrane present as stacked regions of cisternae was often referred to more simply as the percentage total membrane present as stacks. To provide a comparative measure of the amount of reassembly that had occurred the amount of cisternal regrowth could be determined:

$$\text{Cisternal regrowth} = P_{\text{cisternae after incubation}} - P_{\text{cisternae before incubation}}$$
This increase in cisternal membrane was expressed as a percentage, and for the standard reassembly reaction (i.e. the untreated condition) was set to 100% for any given experiment and used to evaluate the effect of a given treatment:

\[
\text{Cisternal Regrowth } (\%) = 100 \times \frac{(P_{\text{cisterna after incubation with treatment}} - P_{\text{cisterna before incubation}})}{(P_{\text{cisterna after incubation without treatment}} - P_{\text{cisterna before incubation}})}
\]

**Cisternal cross-sectional length**

Photographs were taken at a final magnification of 52.5K. Cisternae were identified as defined above and their cross-sectional length determined by aligning a piece of thread down the centre of the cisternal profile such that it connected the two endpoints of the cisterna. The length of the thread was then measured and the cisternal cross-sectional length calculated. For any photograph the cisternae were measured in the order they appeared on moving from the left extremity to the right extremity of the photograph. Both single and stacked cisternae were measured and at least 50 cisternae were measured per reaction.

**Mean number of cisternae per stack**

Photographs were taken at a final magnification of 38.8K, and overlaid with a vertical line grid (5mm distance between lines) on an overhead transparency. The percentage total membrane present as stacked regions of cisternae was determined as above, except for each intersection with a stacked region of a cisterna it was noted whether it was part of a stack of \(x\) (\(x>1\)) cisternae. Therefore, for example, if \(x\) intersections with cisternae were part of stacks of 2 cisternae, \(y\) intersections with cisternae part of stacks of 3 cisternae, and \(z\) intersections with cisternae part of stacks of 4 cisternae the mean number of cisternae per stack was calculated as:

\[
\text{Mean number of cisternae per stack} = \frac{(2x+3y+4z)}{(x+y+z)}
\]

Strictly this is not the mean number of cisternae per stack, but rather the mean number of cisternae per stack corrected for the amount of cisternae present in that type of stack. Routinely 5-8 pictures were quantitated per reaction.
Statistical methods

Results are usually expressed as the mean of a number of experiments ± the standard error of the mean (SEM).

\[ \text{SEM} = \sqrt{\frac{\sum (x_i^2 - x^2)}{n-1}} \]

Where \( x_i \) is the measured value of one experiment, \( x \) is the mean value from all experiments and \( n \) is the number of experiments.

To determine whether frequency distributions differed in location Mann-Whitney tests were performed using the Minitab 10.5Xtra (Minitab Inc, PA) statistics package.

2.2.15 Effect of GDI on p115 binding to RLG

RLG (0.1mg/ml) were incubated at 37°C for 30min with rat liver cytosol (1mg/ml) in KH M buffer (with 2mM ATP, 1mM GTP) and an ATP regeneration system (as above) in the presence of increasing concentrations of GDI (0-0.8μM) in a final volume of 50μl. At the end of the reaction membranes were recovered by centrifugation at 15,000rpm for 20min at 4°C in the vertical rotor of the Eppendorf centrifuge. The supernatant was removed and membrane pellets were washed with 3 x 500μl KH M prior to solubilisation in SDS-PAGE sample buffer. Samples were processed for Western blotting and the amounts of Mann-I, Rab1, and p115 recovered estimated.

2.2.16 Protein precipitation with trichloroacetic acid (TCA).

If required protein was precipitated prior to electrophoresis in order to reduce the sample volume or remove reagents which would interfere with the running of gels. Samples were made up to 500μl with distilled water, supplemented with 5μg soybean trypsin inhibitor as a carrier protein, and 50μl of 0.15% (w/v) sodium deoxycholate. Samples were then vortexed and incubated at room temperature for 5min. 100μl 72% TCA was then added, samples vortexed and left on ice for 60min. Samples were then centrifuged at 14,000 rpm for 5min in a bench top microfuge and the supernatants discarded. The pellets were washed and resuspended in 1ml acetone that had been precooled on dry ice. Following a second spin, samples were washed once more in acetone and spun followed by evaporation of excess solvent for 10min at room temperature. The resulting pellets were dissolved in SDS-PAGE sample buffer and processed for electrophoresis.
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2.2.17 SDS-PAGE

One dimensional SDS-PAGE separates proteins by their relative molecular weight and differential mobility in the polyacrylamide gel. Gels consisted of either a single percentage or a linear gradient (e.g. 5-20%) of acrylamide. The resolving gel and the stacking gel were made as described in Table 2.2 and 2.3. Polymerization was initiated by addition of TEMED just before use.

Table 2.2 Solutions for Resolving gel.

For 40ml of resolving gel solution:

<table>
<thead>
<tr>
<th>Gel (%)</th>
<th>6</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>12.5</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMBA (30:0.8) (ml)</td>
<td>8</td>
<td>9.3</td>
<td>10.0</td>
<td>10.7</td>
<td>13.3</td>
<td>16.0</td>
<td>16.7</td>
<td>20.0</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>21.8</td>
<td>20.5</td>
<td>19.8</td>
<td>19.1</td>
<td>16.5</td>
<td>13.8</td>
<td>13.1</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Then add:
10ml Lower buffer (1.5M Tris, 0.4% Na SDS, pH 8.8 with HCl)
200μl 10% (w/v) ammonium persulphate (APS)
20μl TEMED

Table 2.3 Solutions for Stacking gel.

For 20ml of stacking gel solution:

<table>
<thead>
<tr>
<th>Gel (%)</th>
<th>2.5</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMBA (30:0.8) (ml)</td>
<td>1.7</td>
<td>2.0</td>
<td>2.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>13.1</td>
<td>12.8</td>
<td>12.1</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Then add:
5ml Upper buffer (0.5M Tris, 0.4% Na SDS, pH 6.8 with HCl)
200μl 10% (w/v) APS
40μl TEMED

Gels were poured at a thickness of 0.75mm or 1mm using the Bio-Rad Mini gel assembly kit. The resolving gels were overlaid with water saturated iso-butanol to ensure even polymerization. Once set the water saturated iso-butanol was removed and the gel washed once with stacking solution, before pouring the stacking gel.

Samples were dissolved in SDS-PAGE sample buffer (3% Na SDS, 62.5mM Tris, 10% glycerol, 3.33% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8 with HCl)
by shaking at room temperature for 30min. Samples were then incubated at 95°C for 5min and then cooled to room temperature. Samples were then centrifuged at 14,000rpm for 5min in a bench top microfuge, before loading onto the gel. Additionally, 0.7μl of Bio-Rad broad range molecular weight markers were processed as above and loaded onto the gel.

Gels were fitted into the apparatus and the upper and lower tanks filled with SDS-PAGE running buffer (190mM glycine, 25mM Tris, 0.1% Na SDS, pH 8.8 with HCl). Gels were run at a constant voltage of 120-160V for 30-60min.

Gels were stained by a 40s incubation in a microwave oven set at full power, with 10mg/ml Coomassie Brilliant Blue R in 10% (v/v) acetic acid / 50% (v/v) methanol in order to fix the gel by protein precipitation. Gels were then destained by extensive washing in 20% (v/v) acetic acid / 20% (v/v) isopropanol. Silver staining was performed using a kit according to manufacturer’s instructions (Daiichi Chemicals). Gels were dried at room temperature between two sheets of transparent membrane (Biosciences).

2.2.18 Western blotting
Proteins were transferred to a nitrocellulose membrane (Hybond-C, Amersham) using a semi-dry blotter. The resolving gel was removed from the stacking gel using a razor blade and then soaked in 100ml transfer buffer (20mM Tris, 150mM glycine, 20% (v/v) methanol, 0.05% (w/v) Na SDS). The nitrocellulose membrane, and six pieces of 3MM paper (Whatman) which were cut to the same size as the gel slice to be blotted were also soaked in transfer buffer. The nitrocellulose membrane and the gel were sandwiched between two sets of three sheets of 3MM paper, and placed into the apparatus with the nitrocellulose membrane facing the anode. Air bubbles were carefully removed from this sandwich prior to transfer at a constant current of 100mA for 60min. After transfer, blots were stained with Ponceau S to check the extent of transfer, and the position of molecular weight markers was marked with a pencil.

Blots were blocked overnight at 4°C with blocking buffer (10% (w/v) low fat milk powder (Marvel) in PBS). Blots were then sealed into plastic bags containing 5ml/100cm² of blocking buffer to membrane containing the appropriate dilution (from
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1:200 to 1:5000) of the appropriate primary antibody. Care was taken to exclude air bubbles from the plastic bags. Blots were then incubated on a rocker for 60min at room temperature under a bag of water. The blot was then washed twice briefly with PBS, and then for 40min with PBS and three changes of buffer. The blot was then sealed into a fresh bag containing the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (TAGO) typically at 1:1000 dilution and incubated for 60min as before. The blot was then washed with PBS as before. The blot was then immersed in 6ml of a 1:1 mixture of the ECL (Enhanced Chemi-Luminescence, Amersham) reagents for 1min. Excess solution was dried off, and the blot wrapped in Saran wrap and exposed to X-ray film (X-OMAT AR, Kodak) for periods of 1s-5min.

Western blots could be quantitated by scanning the film at 600dpi on a UMAX Powerlook II scanner to produce digital images. Pixel densities could then be determined using NIH Image 1.51, and comparisons made on the basis of appropriate standard curves.

2.2.19 Far Western blotting
Far Western blotting was as for Western blotting except that instead the primary antibody was replaced with the biotinylated p115 637-699 peptide at 5μg/ml in blocking buffer. The blot was then washed for 60min with blocking buffer, and instead of the secondary antibody, avidin-HRP (Bio-Rad) was used at a 1:1000 dilution in blocking buffer. Blots were then processed for ECL as above.

2.2.20 Cloning of GRASP55
This was performed by Francis Barr as follows:
GRASP55 was cloned using a Rapid amplification of cDNA ends (RACE) protocol and specific nested primers as follows:
TR1, GGTACCCCCATTACTCCTCTTAAGGATGGG
TR2, CAGAGGTCCAGCTGTCTTCAGTCCTCC
TR3, GGAGGACTGACTGAAGACAGCTGGACCTCTG
TR4, CCCATCCTTAAGGAGGAGTAATGGGGGTACC.

A rat testis cDNA pool was used as the template for the first round of 5' and 3' RACE, the API and AP2 cDNA adapter primers were as described in the Marathon RACE
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protocol (Clontech, UK). All reactions were performed for 25 cycles with annealing temperatures and elongation times of 60°C and 2min, respectively, and used the polymerase mix and buffer 1 from the Expand PCR system (Boehringer-Mannheim, UK). For the first round of 5' RACE primers TR3 and AP1 were used. For the second round of 5' RACE, 1μl of the first 5' RACE reaction was taken as the template, and primers TR4 and AP2 were used. The final product of 800 bp was gel purified and blunt end cloned into the EcoRV site of pBSII-KS (Stratagene) for sequencing. For the first round of 3' RACE primers TR1 and AP1 were used. For the second round of 3' RACE, 1μl of the first round 3' RACE reaction was taken as the template, and primers TR2 and AP2 were used. The final product of 1100bp was gel purified and blunt end cloned into the EcoRV site of pBSII-KS (Stratagene, UK) for sequencing. To obtain the full open reading frame of the new isoform of GRASP65 specific primers TR 12, C C G G C T G G G T C G T A C G G A T C G C C C G C G G , and TR 10, ACTTGGTGAGACAAGCCTTCAGACTC were used. The 1600bp product thus obtained was TA-cloned following the manufacturers protocol into pCRII-TOPO (Invitrogen, UK) and sequenced.

2.2.21 Northern blot

This was performed by Francis Barr as follows:

DNA probes were as follows: GRASP65 specific probe, BamHI to Smal fragment of the GRASP65 cDNA; GRASP55 specific probe, MluI to EcoRI fragment of the GRASP55 cDNA; GRASP common probe, equal amounts of the EcoRI to BamHI fragment of the GRASP65 cDNA, and the EcoRI-EcoRI fragment of the GRASP55 cDNA. All probes were labelled using the Rediprime system according to the manufacturer's instructions (Amersham Life Sciences, UK) A multiple rat tissue Northern blot (Clontech, UK) loaded with approximately 2.0µg polyA plus RNA from the tissues indicated in the appropriate figure legend, was hybridised for two hours at 65°C in rapid hybridisation buffer (Amersham Life Sciences, UK) with the appropriate DNA probes. After hybridisation, the filter was rinsed in 2x SSC plus 0.1% (w/v) SDS, washed twice for 15min in the same buffer at 42°C, rinsed in 2x SSC then exposed to film.

2.2.22 Tissue extracts

This was performed by Francis Barr as follows:
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Fresh tissue samples from one female Sprague-Dawley rat, as indicated in the appropriate figure legend, were washed three times with 10ml of ice cold PBS, then 0.6g of each was finely chopped with a razor blade. The chopped tissue was placed into a ceramic mortar and a small volume of liquid nitrogen added. Using a ceramic pestle the frozen tissue was ground to a fine powder then resuspended in 4ml of SDS-PAGE sample buffer. Samples were boiled for 5min, centrifuged at 2000g for 10min to pellet any insoluble material, then analysed by SDS-PAGE and Western blotting.

2.2.23 Transient transfection of GRASPs and immunofluorescence

This was performed by Francis Barr as follows:

HeLa cells were plated at 30% density on glass coverslips in 2cm wells and left to attach for 12h. They were transfected with the appropriate construct using lipofectin (Gibco-BRL, UK) according to the manufacturer’s instructions. After 28h, the cells were fixed in 3% paraformaldehyde and processed for immunofluorescence with the appropriate antibodies.

2.2.24 Localization of GRASP55 and GRASP65 by cryo-EM

This was performed by Rose Watson and Francis Barr as follows:

For cryo-EM, HeLa cells were plated at 30 % density in 6cm dishes, left to attach for 12h, then transfected using 2μg DNA from the appropriate construct with Effectene (Qiagen). After 18h, cells from two 6cm dishes were scraped from the dish, fixed with 0.2% glutaraldehyde and 2% paraformaldehyde in 100mM phosphate buffer pH 7.4, and prepared for cryosectioning and immunostaining (Slot et al., 1991). Immunolabelling was performed using a 1:5 dilution of the 7E10 monoclonal antibody, and a 1:50 dilution of the anti-GFP polyclonal antibody. These were detected using gold particles coupled to an anti-rabbit antibody and protein A, respectively. The distributions of GRASP55 and GRASP65 over the Golgi apparatus in single and double labelling experiments were determined as follows. For each Golgi apparatus, the cis- to trans-polarity was defined using the presence of clathrin coated buds to identify the TGN. The TGN also displayed a more peeling off configuration, and COPI coated vesicles could be seen close to the CGN (Rambourg and Clermont, 1997). Gold particles within 30nm of the membrane of a particular cisterna were assigned to that cisterna, numbered 1 to 5 or greater, from cis to trans. The number of gold particles
found over a particular cisterna was expressed as a percentage of the total number of gold particles present.

2.2.25 In vitro transcription-translation and immunoprecipitation of GRASP65, GRASP55, and GM130.

This was performed by Francis Barr as follows:

In vitro transcription-translation reactions (Promega) of 50μl were performed with the T7 polymerase for 2h at 30°C using 0.5μg plasmid DNA and methionine minus amino acid mix plus 4μl of [35S]L-methionine (typically 1400 Ci/mmol and 11 mCi/ml, ICN Pharmaceuticals). Immunoprecipitations were performed in IP buffer (20mM Hepes-KOH pH 7.3, 200mM KCl, 0.5 % (w/v) TX-100) using 4μl of NN5-1 (anti-GM130) 10μl of packed protein A-sepharose (Pharmacia). After binding for 60min at 4°C the beads were washed four times with 500 μl of IP buffer, eluted with 30 μl of SDS-PAGE sample buffer, and the eluate analysed as appropriate.

2.2.26 Immunoprecipitation of Giantin/p115/GM130.

This was performed by Barbara Dirac-Svejstrup as follows:

RLG were washed with 1M KCl, prior to extraction with 20mM Hepes-KOH pH 7.3, 200mM KCl, 1mM DTT, 1mM EDTA, 1% TX-100, 10mM MgCl2, and protease inhibitors (Nakamura et al., 1995), for 1 hour on ice. Extracts were then diluted with one volume of 20mM Hepes-KOH pH 7.3 (to yield TX-100 buffer: 20mM Hepes-KOH pH 7.3, 100mM KCl, 0.5mM DTT, 0.5mM EDTA, 0.5% TX-100, 5mM MgCl2), and clarified by centrifugation at 20,000g for 10min at 4°C.

Immunoprecipitations were carried out using these TX-100 extracts of salt-washed Golgi membranes (unless otherwise stated) which were pre-incubated with the relevant proteins. In some experiments in vitro translated and 35S-labeled proteins were used (p115-HTA, -HT, -H, -TA). In others purified rat liver p115, TA, TA (S941A), TA (S941D) and p115-26mer and -75mer peptides were used. Pre-incubations were performed in TX-100 buffer for 1h at 4°C. In experiments involving CKII (recombinant human CKII, Calbiochem), reactions were subsequently incubated at 30°C for 10min in the presence of 10μM GTP and 5μCi γ-32P-GTP. Reactions were incubated with polyclonal anti-Giantin antibodies, or anti-GM130 antibodies (3μl of the appropriate antiserum and 20μl of packed protein A beads [Pharmacia]) for 2h at
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4°C. Beads were washed with TX-100 buffer, and proteins eluted in SDS sample buffer. Samples were fractionated by SDS-PAGE and immunoprecipitated and co-immunoprecipitated proteins were detected by Western blotting using monoclonal antibodies or in experiments where $^{35}$S-labeled proteins were added by exposure to a phosphoimager.

2.2.27 In vitro protein phosphorylation

This was performed by Barbara Dirac-Svejstrup as follows:

CKII- and RLG-mediated phosphorylation of p115 was carried out by incubation of 0.2μunits of recombinant human CKII (Calbiochem), or 10μg RLG, with either p115, TA, or TA (S941A) in the presence of either 10μM GTP and 5μCi γ-$^{32}$P-GTP, or 10μM ATP and 5μCi γ-$^{32}$P-ATP in phosphorylation buffer (20mM Hepes-KOH pH 7.3, 50mM KCl, 10mM MgCl$_2$, 0.1mM DTT and 0.2M Sucrose) with added protease inhibitors (Nakamura et al., 1995). Reactions were carried out for 10min at 30°C. In some experiments reactions contained a 100x molar excess (over the final p115 concentration in the reaction) of 26mer/26mer-P or CKII substrate/substrate-P. In other experiments reactions contained either: 100μM Staurosporine, DRB, Chrysin, or Roscovitine, or 1μl anti-CKII-α or 1μl anti-CKII-β antibodies. CKII-mediated phosphorylation reactions were terminated by addition of SDS-PAGE sample buffer.

RLG-mediated phosphorylation reactions were terminated by addition of KCl to a final concentration of 1M on ice, releasing p115 from Golgi membranes. The Golgi membranes were removed by centrifugation and SDS-PAGE sample buffer added to the supernatant. The resulting samples were fractionated by SDS-PAGE, and phosphorylated p115 proteins visualised using a phosphoimager.

RLG-mediated phosphorylation of a standard CKII peptide substrate (RRRDDSDDDDDD) was carried out in the presence of 10μM GTP and 10μCi γ-$^{32}$P-GTP for 10min at 37°C. Reactions were dotted onto phosphocellulose filters (Whatman). The filters were washed in 100mM phosphoric acid followed by 100% ethanol, dried, and phosphorylation levels quantitated using a scintillation counter.

2.2.28 Affinity probing Golgi detergent extract with p115 peptides

20μg RLG were extracted with 200μl of IP buffer (Hepes-KOH, pH 7.3, 200mM KCl, 0.5% TX-100) for 15min on ice and then centrifuged at 14,000rpm for 2min in an
Eppendorf microfuge to remove any insoluble material. The supernatant was removed and added to 10μl packed neutravidin beads coupled (as according to manufacturer’s instructions) to p115 637-699 (at 2.8mg/ml beads) or mock beads and incubated for 60min at 4°C with rotation. Beads were recovered by centrifugation at 14,000rpm for 1min in an Eppendorf microfuge and washed with 4 x 1ml IP buffer. Beads were eluted by the addition of 30μl SDS-PAGE sample buffer, fractionated by SDS-PAGE, and either silver stained or transferred to nitrocellulose. Blots were probed with specific antibodies against giantin, GM130 (NN15), p115 (8A6), sly1p, syntaxin-5, α-SNAP, GOS28, Rab6 and Rab1. Alternatively, neutravidin beads were coupled (as according to the manufacturer’s instructions) to either p115 637-699, 728-765, 788-827, or 843-930 at 5μM and used to probe Golgi detergent extract as above.

TX-100 extracts of 1M KCl extracted RLG (Section 2.2.26; 400μl at 0.5mg/ml) were incubated for 60min at 4°C with either 10μl packed neutravidin beads coupled to the 26mer (2mg/ml), 26mer-P (2mg/ml) or mock beads. Beads were washed with 4 x 1ml TX-100 buffer and eluted and processed as above. Alternatively, TX-100 extracts of 1M KCl extracted RLG (40μl at 1mg/ml) were incubated for 60min at 4°C alone, or with 4μg of either the 26mer, the 75mer or their phosphorylated counterparts. The peptides were recovered on neutravidin beads and processed as above.
3.1 Introduction

The focus of this chapter is the characterization of the mitotic disassembly process in the cell free system. In particular, the characteristics of the fundamental components of the cell free system are described as well as the morphological sequence of events of the disassembly process. Finally, some factors that interfere with the disassembly process will be clarified, in particular the role of Rab GTPases, coatamer, and p115 will be probed.

3.2 The disassembly reaction

3.2.1 Rat liver Golgi membrane preparations.

Of absolute crucial importance to this whole study was the requirement for highly purified, functional Golgi membranes. A tissue source of material was preferred to the use of cultured cells, since abundant amounts of material could be readily obtained, and most importantly allowed better purification and preservation of morphology (Taylor et al., 1997). This was most likely due to the difficulty in producing an ideal homogenate, that releases organelles in suspension as discrete entities, from cultured cells due to their distinct cytoskeletal constitution that results in maintained cytoplasmic organization after homogenization (Howell, et al., 1989). In contrast, the three dimensional organization of cells within a tissue is such that gentle homogenization will still result in significant cell breakage. Rat liver was the preferred tissue for Golgi purification because hepatocytes possess a reduced amount of cytoskeleton and a minimal extracellular matrix, that facilitates the release of Golgi stacks with very mild homogenization conditions (Howell et al., 1989). A mild homogenization was preferred in order to preserve Golgi ultrastructure and to minimize any mechanical shear that would convert Golgi stacks to more microsomal like structures. The protocol used is essentially as described in Hui et al., 1998 and is derived from several earlier methods (Leelavathi et al., 1970; Fleischer and Fleischer, 1970; Hino et al., 1978). The method consisted of gentle homogenization of rat liver by pressing through a wire sieve, followed by sucrose step gradient velocity centrifugation to isolate the Golgi membrane fraction.
The quality of each Golgi preparation was routinely assessed by measuring the enrichment over the homogenate for the activity of the trans-Golgi enzyme, GalT (Bretz and Staubli, 1977; Hui et al., 1998). Typically RLG were purified 160-fold over rat liver homogenate for GalT specific activity. However, purification factors of 350-fold could be achieved, and as a general rule preparations with a purification factor of less than 75-fold were not used. Table 3.1 shows the mean GalT purification table for RLG used in this study.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[Protein] (mg/ml)</th>
<th>[GalT] (nmol/hr/ml)</th>
<th>Specific Activity (nmol/hr/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>134±14.4</td>
<td>698±66.2</td>
<td>6.33±0.8</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Golgi</td>
<td>3.00±0.4</td>
<td>2100±307</td>
<td>894±101</td>
<td>10.5±1.1</td>
<td>160±14.4</td>
</tr>
</tbody>
</table>

Table 3.1 Enrichment of a trans-Golgi marker, GalT, in rat liver Golgi compared to rat liver homogenate. This table was compiled using the results from 29 separate purifications, presented as the mean±SEM for each parameter. Note that the specific activity, yield and purification are not, therefore, arithmetically related to GalT and protein concentrations.

Purification factors were usually twofold higher when the activity of NAGTI was assessed (Vischer and Hughes, 1981). This apparent disparity may be due to some loss of the TGN during the purification procedure, which contains a large proportion of GalT, but virtually no NAGTI (Nilsson et al., 1993a). A purification table for NAGTI is shown in Table 3.2. The GalT assay was preferred for routine use as it was much more rapid than the NAGTI assay.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>[Protein] (mg/ml)</th>
<th>[NAGTI] (nmol/hr/ml)</th>
<th>Specific Activity (nmol/hr/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>143</td>
<td>76.0</td>
<td>0.56</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Golgi</td>
<td>2.40</td>
<td>445</td>
<td>187</td>
<td>16.3</td>
<td>335</td>
</tr>
</tbody>
</table>

Table 3.2 Enrichment of a medial-Golgi marker, NAGTI, in rat liver Golgi compared to rat liver homogenate. This table was compiled using the results from 2 separate purifications, presented as the mean for each parameter. Note that the specific activity, yield and purification are not, therefore, arithmetically related to NAGTI and
protein concentrations. The mean GalT purification for these two preparations was 153 fold.

Purified RLG is selectively depleted of lysosomes and ER with respect to rat liver homogenate as judged by assaying the activity of β-N-acetylhexosaminidase (Landegren, 1984) and rotenone-insensitive NADH-cytochrome c reductase (Sottocasa et al., 1967). The enrichment factor for the lysosomal marker was typically 0.4 and for the ER marker 0.8 (Slusarewicz, 1994).

The enrichment of RLG over rat liver homogenate can also be assessed by fractionation of preparations by SDS-PAGE followed by transfer to nitrocellulose and probing with specific antibodies against Golgi markers (Figure 3.1). RLG have a distinctive polypeptide composition compared to rat liver homogenate. Particularly noticeable is the enrichment for proteins that have a molecular weight greater than 200kDa. These presumably correspond to some of the high molecular weight Golgins, and giantin that are hypothesized to be important for maintenance of Golgi architecture (Section 1.4.3; Chan and Fritzler, 1998; Linstedt and Hauri, 1993). Western analysis reveals that the RLG preparation is highly enriched over rat liver homogenate for GM130, Mann I, and GRASP65 which are all cis-Golgi markers (Nakamura et al., 1995; Balch and Keller, 1986; Shorter et al., 1999). In fact, Mann I and GRASP65 appear to be undetectable in rat liver homogenate, this may be due to their being obscured by the sheer bulk of protein which seems to occur at this molecular weight. It would then seem that cis-, medial- and trans-Golgi markers are well represented in the RLG preparation, which at least suggests that the minimal three compartment requirement for a functional Golgi apparatus is met by RLG (Mellman and Simons, 1992). As well as being functional for the disassembly and reassembly reactions, these Golgi membranes are also competent for intra-Golgi transport, as measured by the synthesis of sulphated glycosaminoglycans (GAGs) onto the membrane permeable, external acceptor xyloside, a process that requires inter-compartmental transport between Golgi cisternae (Fernández and Warren, 1998).
Figure 3.1 Polypeptide composition of rat liver homogenate and rat liver Golgi. 20µg rat liver homogenate and 20µg rat liver Golgi were fractionated by SDS-PAGE using a 4-20% gradient gel and either stained with Coomassie (left panel) or transferred to nitrocellulose and probed for GM130, Mann I and GRASP65 using specific antibodies (right panel). Molecular weights in kD are shown on the left.
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The Disassembly Reaction

Since the cell free system used here relies on morphological measurements it was essential to finely determine the stereological parameters of the starting RLG. Thus, RLG preparations were routinely fixed and processed for EM, and quantitated using the stereological definitions (Rabouille et al., 1995c) described in the Materials and Methods (Section 2.2.14). RLG consisted of a population of cisternal membranes, the majority of which were present as stacks, many of which adopt a curved appearance, containing 2-4 (2.8±0.12, mean±SEM, from 5 separate preparations) cisternae per stack (Figure 3.2A, B). Close inspection of the stacks of cisternae (inset in Figure 3.2A) revealed the presence of electron dense bridges both between cisternae (intercisternal bridges) and within these cisternae (intracisternal bridges) as has been previously reported (Cluett and Brown, 1992; Franke et al., 1972). These intercisternal bridges are short structures approximately 10-15nm long, and ranging from 5-10nm in width, and appear to occur at regular intervals along adjacent cisternae. These elements are sensitive to mild proteolysis, a condition that unstacks Golgi cisternae (Cluett and Brown, 1992).

The mean RLG preparation from 29 separate purifications revealed that the percentage total membrane present as cisternae was approximately 69%, of which 36% was present as stacks and 33% present as single cisternae (Figure 3.2C). These cisternae displayed a right skew frequency distribution of cisternal cross-sectional length (Figure 3.2D), the mean length being 1.1µm, but cisternal profiles as long as 4.33µm could be found. The mean percentage total membrane present as tubules was 13% and as vesicles 10% (Figure 3.2C). The remaining 8% membrane was present as readily identifiable contaminants which were mostly unattached lipoprotein particles (4%), and occasionally endosomes (2%), peroxisomes (<1%), lysosomes (<1%), mitochondria (<1%) and plasma membrane sheets (<1%) could be found. In contrast, it was difficult to distinguish smooth ER material from vesicular Golgi material, yet since the ER contamination was considered so low as judged by assaying the activity of rotenone-insensitive NADH-cytochrome c reductase (Sottocasa et al., 1967) this was not considered a problem.
Figure 3.2 Morphology and stereological parameters of purified rat liver Golgi membranes. (A,B) Typical appearance of RLG, large arrowheads denote stacks, small arrows denote single cisternae, small arrowheads denote tubules, and large arrows denote vesicles. Bar, 0.5\( \mu m \). Inset in A is a close up of the intercisternal region of the indicated stack. a denotes an intracisternal crossbridge, b denotes the lipid bilayer, and c denotes an intercisternal crossbridge. (C) Stereological parameters of RLG, the mean total membrane present as profile (%) \( \pm \)SEM was compiled from 29 separate purifications. (D) Frequency distribution of cisternal cross-sectional length for a typical RLG preparation is presented.
The dimensions and the number of cisternae per stack of purified RLG compare well with the discrete subunit mini-stacks of the compact region of the Golgi ribbon in hepatocytes (Taylor et al., 1997; Rambourg and Clermont, 1997), or to the Golgi mini-stacks that are generated by treating interphase cells with nocodazole (Cole et al., 1996a; Storrie et al., 1998) or to those mini-stacks present at the onset of prophase (Misteli and Warren, 1995b). The RLG preparation thus has suitable morphological characteristics for the study of conversion of Golgi mini-stacks to tubulovesicular clusters, a phenomenon that occurs at pro-metaphase in vivo (Lucocq et al., 1987; Misteli and Warren, 1995b).

3.2.2 Mitotic and interphase cytosol preparations.

Cytosols were prepared as high speed supernatants from extracts of spinner HeLa (sHeLa) cells, or rat liver. Interphase cytosols were made from either unsynchronized populations of sHeLa cells or from rat liver. For mitotic cytosol, sHeLa cells were treated with the microtubule depolymerizing agent nocodazole for 20-26h. Nocodazole inhibits the formation of the mitotic spindle and consequently the cells are arrested in pro-metaphase (Zieve et al., 1981). Two measures were made to estimate the extent of the arrest. First, after arrest, cells were stained with the DNA intercalating fluorescent dye Höchst 33258, and the percentage of cells with condensed pro-metaphase chromatin was determined. This value was termed the mitotic index.

Although the mitotic index gave a measure of the effectiveness of the arrest it provided no information about the actual mitotic activity of the cytosol that had been prepared. So in order to assess this, the histone H1 kinase activity of the cytosol was also determined. Histone H1 kinase activity is elevated in mitotic cells prior to the metaphase-anaphase transition (Hunt et al., 1992). The phosphorylation state of histone H1 correlates with Cdc2 kinase activity which is required for entry of cells into mitosis (reviewed in Nigg, 1995). Since Cdc2 kinase activity is also essential for mitotic Golgi fragmentation (Misteli and Warren, 1994; Lowe et al., 1998b), it was of key importance to determine whether the histone H1 kinase activity, and so by inference the Cdc2 kinase activity, of mitotic cytosol was elevated relative to interphase cytosol.
Chapter 3 The Disassembly Reaction

The mean mitotic index, and histone H1 kinase activity of cytosols used in this study are displayed in Table 3.3.

<table>
<thead>
<tr>
<th>Cytosol</th>
<th>[Protein] (mg/ml)</th>
<th>Mitotic Index (%)</th>
<th>Histone H1 kinase activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic sHeLa</td>
<td>11.5±0.4</td>
<td>95.0±0.4</td>
<td>1760±118</td>
</tr>
<tr>
<td>Interphase sHeLa</td>
<td>10.9±0.5</td>
<td>4.0±0.1</td>
<td>64.2±21</td>
</tr>
<tr>
<td>Rat liver</td>
<td>20.2±0.7</td>
<td>-</td>
<td>39.9±19</td>
</tr>
</tbody>
</table>

Table 3.3 Characterization of cytosol preparations. Cytosols were prepared as described in Materials and Methods. The mitotic index of cells grown in suspension was measured using Höchst 33258 staining and determining the percentage of cells with condensed pro-metaphase chromatin. Histone H1 kinase activity was measured as in Felix et al. (1989). This table was compiled using the results from 56 separate mitotic sHeLa, 19 separate interphase sHeLa and 29 separate rat liver cytosol preparations, presented as the mean±SEM for each parameter.

The histone H1 kinase activity of mitotic cytosol was typically 30-fold higher than that of sHeLa interphase cytosol, and 45-fold higher than that of rat liver cytosol (Table 3.3). If the mitotic index dropped below 90%, the histone H1 kinase dropped to approximately interphase levels. Therefore, as a general rule the mitotic index had to be greater than 90% before a mitotic cytosol preparation was commenced, or could be used for a disassembly reaction.

3.2.3 Kinetic analysis of Golgi membrane disassembly.

The sequence of events in the disassembly process in the cell free system has been exhaustively characterized before (Misteli 1994; Misteli and Warren, 1994; Misteli and Warren, 1995a; Rabouille et al., 1995c). Briefly, the previous work suggests that two temporally overlapping pathways consume Golgi cisternae. The first pathway involves the continued budding of COPI vesicles in the absence of their fusion, such that these vesicles accumulate and make up to 50% of the total membrane after 60min. The second pathway involves the tubulation of cisternae to form large networks, and
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later smaller tubules, and vesicles of heterogeneous size. This accounts for the other 50% of the membrane and is a COPI independent process.

RLG were incubated at 37°C with mitotic cytosol, ATP, GTP and an ATP-regeneration system, and samples were taken at various times and processed for EM (Figure 3.3). The change in morphology of the Golgi stacks is both dramatic and rapid, after 5 min in mitotic cytosol the long stacks present in RLG have disappeared and are replaced with shorter stacks (Figure 3.3B), the mean cross sectional length of cisternae has dropped from 1.2 μm to 0.71 μm (Table 3.4). Vesicles of 50-70 nm in diameter (mean 57±5 nm, n=150), can be seen to be budding from the peripheral rims at all levels of the stack (Figure 3.3B). These most likely represent COPI vesicles (Misteli and Warren, 1994, 1995a), although most of these vesicles appeared uncoated when stained with osmium/cyanoferrate, if GTPγS was included in the reaction instead of GTP and tannic acid used to enhance contrast (Simionescu and Simionescu, 1976; Misteli and Warren, 1995a) these vesicles appeared coated (Misteli and Warren, 1994, 1995a), and also label for ε-COP by cryo-immuno-EM (Sonnichsen et al., 1996).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mean number of cisternae per stack</th>
<th>Mean cisternal length (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.92±0.070</td>
<td>1.20±0.190</td>
</tr>
<tr>
<td>5</td>
<td>2.43±0.050</td>
<td>0.71±0.10</td>
</tr>
<tr>
<td>10</td>
<td>2.21±0.040</td>
<td>0.54±0.10</td>
</tr>
<tr>
<td>20</td>
<td>*</td>
<td>0.35±0.060</td>
</tr>
<tr>
<td>30</td>
<td>*</td>
<td>0.31±0.040</td>
</tr>
<tr>
<td>60</td>
<td>*</td>
<td>0.2±0.050</td>
</tr>
</tbody>
</table>

Table 3.4 Mean number of cisternae and mean cisternal cross-sectional length during disassembly. RLG was incubated at 37°C with mitotic cytosol for increasing time, and reactions processed for EM at various times. The mean number of cisternae per stack and cisternal length±SEM is presented from five experiments. * Stacks accounted for <2% of the total membrane, and had no more than two cisternae per stack.
Figure 3.3 Time course of disassembly reaction.
RLG were incubated at 37°C in the presence of mitotic cytosol for increasing time, reactions were stopped at the indicated times, and processed for EM. (A) start of the incubation, (B) 5min, (C) 10min, (D) 20min, (E) 30min, (F) 60min. Large arrowheads denote stacks, small arrows denote single cisternae, small arrowheads denote tubules, large arrows denote vesicles, asterisks denote tubular networks, and x marks vesicles budding from tubular network. Bar, 0.5μm.
The percentage total membrane present as stacks drops from 40% to 20% in this period (Figure 3.4B), and this is matched by a concomitant increase of 10% in vesicles and a 5% rise in tubules (Figure 3.4D, E). The latter most likely represents the COPI independent fragmentation pathway (Misteli and Warren, 1994, 1995a). There was also a small increase (5%) in the percentage total membrane present as single cisternae suggesting that an unstacking process may also be occurring (Figure 3.4C, 3.5). The mean number of cisternae per stack fell from 2.92 to 2.43 in this period (Table 3.4, Figure 3.5). The next 5 min of the reaction saw further tubulation of membrane and the amount of single cisternae increase another 5% suggesting continued unstacking, the mean number of cisternae per stack decreasing to 2.21 (Figure 3.3C, 3.5; Table 3.4). The percentage total membrane present as stacks had now dropped to around 7% (Figure 3.4B). The mean cisternal cross-sectional length had dropped dramatically by c. 55% compared to the starting material to 0.54 μm (Table 3.4). The remaining population of membranes consisted of mostly single cisternae, vesicles and tubules. The tubules that had formed were either branching networks (asterisks in Figure 3.3) or unbranching, discrete tubules, that can be seen pinching off from these networks and from the ends of cisternae (Misteli and Warren, 1995a). Small putative COPI vesicles could also be seen pinching off these large tubular networks (Figure 3.3C; Misteli and Warren, 1995a).

This population of single cisternae and any remaining stacks are consumed by further vesiculation and tubulation (Figures 3.3D, E; 3.4B-E) and after 20 min of disassembly the percentage total membrane present as cisternae is approximately half that of starting RLG, and by 30 min approximately a third (Figure 3.4A). The remaining cisternae were now reduced to c. 25% of their original cross-sectional length (Table 3.4). Over the last 30 min of the incubation vesicle formation continues, and even consumes some of the larger tubular profiles that have formed. This later vesicle formation is thought to be more a COPI independent process, owing to the more irregular size of the vesicles that have formed (Misteli and Warren, 1994, 1995a). The final reaction products (Figure 3.3F) are then a population of vesicles (c. 50%), tubules (c. 30%) and short cisternae (a mean cross-sectional length of 0.2 μm, Table...
Figure 3.4 Quantitation of intermediates during disassembly reaction.
RLG were incubated at 37°C for increasing time with mitotic cytosol, reactions were stopped and processed for EM and quantitated. The percentage total membrane as (A) cisternae, (B) stacks, (C) single cisternae, (D) tubules, and (E) vesicles are shown. Results are from five experiments and values represent means±SEM.
Figure 3.5 Changes in the number of cisternae per stack during disassembly. 
RLG were incubated at 37°C in the presence of mitotic cytosol for increasing time, 
reactions were stopped at the indicated times and processed for EM. The frequency 
distribution of the number of cisternae per stack was determined at (A) start of 
incubation, (B) 5min, (C) 10min and (D) 20min. Values represent means±SEM 
(n=5).
3.4) that comprise only around 10% of the total membrane present (Figure 3.4A, D, E). In contrast to these events, replacing mitotic cytosol with in sHeLa interphase cytosol caused no dramatic alteration in Golgi morphology. In fact a 60min incubation at 37°C in sHeLa interphase cytosol yielded a population of membranes that consisted of 30%±2.5 stacks, 18%±2.1 single cisternae, 22%±3.4 vesicles and 17%±2.2 tubules (values represent means±SEM, n=4). The effects on morphology were thus specifically mitotic.

The events of the disassembly reaction just described bear very close resemblance to the already published data (Misteli and Warren, 1994, 1995a; Rabouille et al., 1995c), however, there are two subtle differences. First, the rate of the disassembly reaction is more rapid as described here. The half time of the reaction for the loss of cisternal membrane (i.e. the time it takes to consume 50% of the cisternae that are consumed in the reaction) is between 10-15min here and 20-25min as described in Misteli and Warren (1994). The rate of unstacking is also accelerated in the current system, the half time of the reaction being 5min as compared to 20min in Misteli and Warren (1994). Similarly, the rate at which cisternae shorten is also more rapid in the current study. This may reflect the fact that the starting population of cisternal membranes in this study have a longer mean cross-sectional length (1.2µm versus 0.7µm in Misteli and Warren, 1994). Consequently they may have a larger rim domain available for COPI vesicles to bud, and so are consumed more rapidly by COPI vesicles and in so doing shorten more rapidly too. Alternatively, the larger size of the cisternae may make them more prone to COPI independent membrane fission events which may cause them to divide and be split into smaller pieces.

Second, the mechanism by which cisternae unstack appears to be different. Previously, it was suggested cisternae would remain stacked and that continued vesicle budding and tubulation would decrease the length of the cisternae in the stack, without prior unstacking, until eventually the stack would disappear. This was supported as it appeared as though the cross sectional length of cisternae in the stack decreased during the disassembly whereas, the mean number of cisternae per stack decreased only very
slightly (Misteli and Warren, 1994). However, the decline in the mean number of cisternae per stack is much more appreciable in the early stages of prophase \textit{in vivo} (Misteli and Warren, 1995b). In support of this, in the current study, the percentage total membrane present as single cisternae actually rises slightly at the start of the incubation and this can be stimulated by extracting RLG with 1M KCl or N73pep prior to disassembly (see Section 3.3.3), thus suggesting unstacking. This increase in the percentage total membrane present as single cisternae is coupled to an appreciable decline in the mean number of cisternae per stack over the first 10min of the reaction (Table 3.4). A closer inspection of this reveals that at the start of the incubation the majority of stacks (59.8%; Figure 3.5A) contain 3 cisternae, and some stacks containing 4-5 cisternae (15% of the population; Figure 3.5A) can be found. After 5min virtually no stacks with 4-5 cisternae can be found, and the majority of stacks (62.8%; Figure 3.5B) now contain only 2 cisternae. A Mann-Whitney test revealed that these two distributions were significantly different in location, with \( P=0.0000 \). By 10min the proportion of stacks with 2 cisternae has risen to c. 80% (Figure 3.5C), and after 20min the only stacks that can be found contain 2 cisternae (Figure 3.5D). A Mann-Whitney test also revealed that the number of cisternae per stack distributions for 5min and 10min were significantly different in location, with a \( P \) value\( =0.0172 \). This suggests a \textit{bona fide} unstacking process.

The reason for these apparent differences between this system and that of Misteli and Warren (1994) is unclear. It may be that in the current study, changes in the methods and subsequent improvements in the purity of the RLG and in the activity of mitotic cytosol preparations means that, as a consequence, fragmentation is more rapid. The mean cross-sectional length of cisternae in RLG in this study is much longer \( (1.2 \mu m \) compared to \( 0.79 \mu m \) in Misteli and Warren, 1994). These cisternae certainly shorten dramatically in the first 5min of the reaction, but it may be that since the starting cisternae in the current study are longer, it is possible to discern an unstacking process that is occurring at the same time as the rapid shortening. This would have been difficult in the previous study since owing to their shorter cross sectional length as cisternae unstack they may be getting transformed into other structures more readily,
so making the actual unstacking of cisternae harder to resolve. Interestingly, in this study, although the cisternae decrease in cross-sectional length extremely rapidly, by c. 40% in the first 5min of the reaction, this is only coupled with a 10% increase in vesicles and a 5% increase in tubules. Thus, it may be that the stacks themselves are dividing into smaller subunits at the very start of the reaction. Such a process may be akin to the division of the Golgi ribbon into mini-stacks that occurs during early prophase in vivo (Misteli and Warren, 1995b; Shima et al., 1998). A similar process may occur during interphase in the growth and subsequent division of Golgi stacks.

Additionally, in this study different stereological definitions of a cisterna, and a stack were employed. Whereas, in the initial study a cisterna was defined as a profile with a length more than four times their width, the width not being more than 80nm (Misteli and Warren, 1994). In this study the widest a cisterna can be is 30nm in accordance with Rabouille et al. (1995c) a definition which reflects the width of cisternae in the RLG preparation and in hepatocytes (Taylor et al., 1997). The implication of this is that certain profiles would have been scored as cisternae and as stacks in Misteli and Warren (1994), which would not have been in this study. Similarly, a stack was defined as a two or more cisternae separated by a gap of no more than 15nm and overlapping by more than half their length (Misteli and Warren, 1994). Whereas, in this study the requirement for 50% overlap was omitted, and stacked regions of cisternae were scored as stacks. This may provide a more sensitive method of measuring the amount of stacking that is occurring. Furthermore, how the mean number of cisternae per stack was calculated also differs slightly as in this study a correction was made for the amount of membrane present in that type of stacked region (i.e. whether it was part of a stack of 2 or 3 cisternae; Section 2.2.14). Whereas in Misteli and Warren (1994) no correction was made for the amount of membrane present in a given type of stack, and the mean number of cisternae in 25 or more discrete stacks was determined. This may bias the calculated value by giving disproportionate value to stacks which contain very many or few cisternae per stack. Such alteration of definitions may explain why differences in the perceived mechanisms and kinetics of the process have been found.
3.3 Factors that affect the disassembly reaction.

3.3.1 GDI.

In order to test for an involvement of the Rab GTPases in disassembly the effect of excess GDI on the process was determined. GDI functions to extract Rab-GDP from the membrane and selectively recycle it to its membrane of origin (Pfeffer, 1994; Section 1.3.2). Mammalian cells express several GDI isoforms (Janoueix-Lerosey et al., 1995), the significance of which is poorly understood, yet the relative proportion of Rabs complexed to a specific GDI is cell type specific (Yang et al., 1994). Despite this, GDI-α ('GDI'; Sasaki et al., 1990) has been found to be able to interact almost indiscriminately with all Rab proteins so far tested (Ueda et al., 1991; Shisheva et al., 1994; Nishimura et al., 1994; Beranger et al., 1994; Elazar et al., 1994a), although it will preferentially target certain Rabs over others (e.g. Rab11 over Rab8 and Rab2; Chen et al., 1998). GDI has been shown to interfere with a number of transport/membrane fusion assays, and these effects correlate with the extraction of Rabs from the membranes (Elazar et al., 1994a; Peter et al., 1994; Dirac-Svejstrup et al., 1994; Haas et al., 1995; Turner et al., 1997; Urbé, 1998). Excess unoccupied GDI acts to remove Rabs from the membranes (Soldati et al., 1993) by a mass action stripping effect that moves the equilibrium from the membrane bound to the cytosolic form and by interfering with the recruitment of cytosolic Rabs complexed to GDI to the membrane (Soldati et al., 1994, 1995), possibly by competing for the membrane associated GDF (Dirac-Svejstrup et al., 1997).

Although the vast majority of evidence implicates Rabs in the tethering/docking step of vesicle transport/membrane fusion (Section 1.3.2, 1.3.3; Schimmöller et al., 1998; Novick and Zerial, 1997; Pfeffer, 1999) accumulating evidence also suggests that Rabs must be present in the active GTP-bound state on nascent transport vesicles to enable their biogenesis (Pfeffer, 1994). This does not necessarily imply that Rab proteins are required for the mechanics of vesicle formation per se, for which minimal machineries are becoming defined for COPI and COPII vesicles (Spang et al., 1998; Bremser et al., 1999; Matsuoka et al., 1998b). Rather, it may be that the Rab plays a regulatory proof-reading role in vesicle formation, such that a vesicle will not form unless it
contains the correct cargo, or components required for docking/fusion that it cannot obtain after the budding process is complete. This would ensure non-functional vesicles do not form (Schimmöller et al., 1998).

Several observations indicate that Rab proteins may be involved in vesicle budding. Rab1 and Rab9 mutants that preferentially bind GDP inhibit vesicle formation from the ER and endosome, respectively (Nuoffer et al., 1994; Riederer et al., 1994). A complex of Rab5 and GDI is required for coated pit invagination and receptor sequestration in clathrin coated vesicles (McLauchlan et al., 1998). Rab6 forms a cytosolic complex with p62, a protein essential for the budding of exocytic vesicles from the TGN (Jones et al., 1993). In addition, Rab1 coimmunoprecipitates with a fraction of coatomer (Peter et al., 1993), while Rab2 enhances coatomer recruitment (Tisdale and Jackson, 1998), and a Rab2 mutant that preferentially binds GTP stimulates COPI vesicle formation from VTCs (Tisdale, 1999). Depletion of the Rabs Ypt31p and Ypt32p in yeast induces the accumulation of stacked Golgi membranes reminiscent of those visualized in mammalian cells (Benli et al., 1996) suggesting a block in intra-Golgi transport and budding of vesicles from the trans aspect of the Golgi apparatus. Finally, GDI is a potent inhibitor of intra-Golgi transport, yet it is unclear whether this reflects a block on COPI vesicle budding, fusion or both (Elazar et al., 1994a; Peter et al., 1994).

Coupled to this is the fact that Rab6 may regulate the Golgi targeting of Golgin-97 and Golgin-245 (Barr, 1999), two putative structural components of the Golgi apparatus (Griffith et al., 1997; Fritzler et al., 1995), suggesting Rab6 may have some structural influence over the Golgi apparatus. Additionally, the membrane distribution of Rab1 is mitotically regulated. Cdc2 kinase mediated phosphorylation of Rab1 slightly increases the amount of Rab1 associated with membranes (Bailly et al., 1991). Given the central importance of COPI vesicle formation to the mitotic disassembly process, it was of interest to determine whether the Rabs were involved in this process. Excellent a priori candidates would be: Rab1, 2, 6 and 33b which all exhibit a Golgi apparatus
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localization (Saraste et al., 1995; Tisdale et al., 1992; Goud et al., 1990; Zheng et al., 1998).

To this end, RLG were incubated at 37°C in mitotic cytosol for either 20min or 60min in the presence or absence of increasing amounts of His-tagged GDI-α (‘GDI’). GDI was a potent inhibitor of disassembly (Figure 3.6). When GDI was included in the reaction at 10μM, after 20min of disassembly, numerous cisternae that remained stacked were still readily visible (Figure 3.6B) as compared to the population of short cisternae, tubules and vesicles that formed in the absence of GDI (Figure 3.6A). In fact, the percentage total membrane present as cisternae only dropped by approximately 7% in the presence of 10μM GDI, as compared to 33% in the absence of GDI (Figure 3.7A). This was matched by an inhibition of vesicle and tubule formation during the first 20min of the reaction (Figure 3.7A, B). The number of vesicles and tubules present remained very close to that of the starting RLG (Figure 3.7A, B). Given that this is the stage of the reaction where the COPI dependent pathway is most active (Misteli and Warren 1994, 1995a), it seems likely that GDI is interfering with COPI vesicle formation per se.

A very similar inhibition was found for longer disassembly reactions. When GDI was included in the reaction at 10μM, after 60min of disassembly, stacks of cisternae persisted (Figure 3.6D), as compared to a predominantly vesicular population of membranes in the absence of GDI (Figure 3.6C). In fact, the percentage total membrane present as cisternae decreased by 49% in the absence of GDI, but only 16% in the presence of 10μM GDI. Again, this appeared to be due to an inhibition of vesicle and tubule formation, since levels of vesicles and tubules remained close to their starting values in the presence of 10μM GDI (Figure 3.7E, F). This effect of GDI was dose dependent as 1.5μM GDI caused similar, but less dramatic effects (Figure 3.7).
Figure 3.6 Effect of GDI on disassembly reaction. RLG were incubated with mitotic cytosol at 37°C for either 20min or 60min in the presence or absence of increasing amounts of GDI. Samples were fixed and processed for EM, and then quantitated for the mean percentage total membrane present as cisternae±SEM (n=3). Morphology of membranes after (A) 20min-GDI, (B) 20min+10μM GDI, (C) 60min-GDI, (D) 60min+10μM GDI. Note the presence of stacked membranes in B and D (arrowheads) and vesicles in A and C (arrows). Bar, 0.5μm.
Figure 3.7 Quantitation of effect of GDI on mitotic Golgi membrane disassembly. RLG were incubated with mitotic cytosol at 37°C for either 20 min or 60 min in the presence or absence of increasing concentrations of GDI. Samples were fixed and processed for EM, and then quantitated for the percentage total membrane present as cisternae, vesicles, or tubules. (A-C) Results from 20 min disassembly reaction, bars represent mean values from 3 experiments±SEM. (D-F) Results from 60 min disassembly reaction, bars represent mean values from 3 experiments±SEM. On all graphs the horizontal line represents the amount of that type of membrane present in the starting RLG.
A concern was that the GDI may be non-specifically interacting with the membranes and so inhibiting the disassembly process. As a control for this, RLG were incubated at 37°C in mitotic cytosol for either 20min or 60min in the presence of 10µM delipidated bovine serum albumin (BSA). Delipidated BSA at this level had no effect on the disassembly reaction, the percentage total membrane present as cisternae decreased to 33% for the 20min reaction, and to 15% for the 60min reaction. Vesicle formation and tubulation were also unaffected, the percentage total membrane present as vesicles was 25% for the 20min reaction, and 43% for the 60min reaction. While the percentage total membrane present as tubules was 33% for the 20min reaction and 30% for the 60min reaction. This is consistent with the fact that delipidated BSA is unable to remove Rabs from the membrane or compete for their recruitment from cytosol (Beranger et al., 1994), and suggests the effect of GDI may be due to specific effects on Golgi apparatus associated Rab proteins (see below). However, the ideal control for this experiment would be to conduct the disassembly in the presence of a GDI mutant that is unable extract Rabs from the membrane and inhibit their recruitment from the cytosol. Aided by the crystal structure of bovine GDI-α (Schalk et al., 1996), such GDI mutants have very recently been characterized (Wu et al., 1998; Luan et al., 1999).

The inclusion of 10µM GDI altered the distribution of Rab1, but not Rab6 in the reaction (Figure 3.8). RLG were incubated at 37°C with mitotic cytosol for either 20min or 60min in the presence or absence of 10µM GDI. Membranes were then isolated, fractionated by SDS-PAGE, transferred to nitrocellulose and probed with specific antibodies against Mann-I, Rab6 or Rab1. Consistent with Bailly et al. (1991) more Rab1 was bound to MGF than to RLG, and the presence of 10µM GDI removed this Rab1 from the membrane (Figure 3.8A). Quantitation revealed that approximately twice as much Rab1 is bound to MGF generated after 20min as compared to RLG, and that MGF generated after 60min had approximately 30% more Rab1 bound than starting RLG. Why MGF generated after 20min have more Rab1 bound than MGF after 60min is not clear, but may reflect a reduction in the amount of Cdc2 kinase activity over the course of the assay, which has been reported Figure 3.8
Figure 3.8 Presence of GDI during disassembly affects Golgi membrane concentration of rab1, but not rab6. RLG were incubated with mitotic cytosol at 37°C for either 20 min or 60 min in the presence or absence of 10μM GDI. Membranes were then isolated and fractionated by SDS-PAGE, transferred to nitrocellulose, and probed for mann I and either rab1 (A) or rab6 (B) using specific antibodies. Shown are, from left to right: 10μg RLG, 10μg MGF from a 20 min incubation, 10μg MGF from a 60 min incubation, 10μg MGF from 20 min incubation with 10μM GDI, 10μg MGF from 60 min incubation with 10μM GDI. Molecular weights in kD are shown on the left.
to occur for disassembly conducted in the absence of the phosphatase inhibitor microcystin (Misteli, 1994). The presence of GDI reduced Rab1 levels by 80% relative to RLG, 90% relative to MGF generated after 20min, and 85% relative to MGF generated after 60min. This was not due to differential recoveries of membranes as the amount of Mann-I remained relatively constant under all conditions. In contrast, the levels of Rab6 associated with the Golgi membranes was unaffected by the mitotic treatment, and also the presence of GDI had no effect on the amount of membrane associated Rab6 (Figure 3.8B). Again the levels of Mann-I remained relatively constant under all conditions.

Unfortunately, antibodies against Rab2 and Rab33b were not available, so their association with the Golgi membrane could not be tested. However, a clear positive correlation is revealed whereby GDI inhibits both vesicle and tubule formation during disassembly, and Rab1 association with the Golgi membrane, possibly suggesting a regulatory role for Rab1, but not Rab6 in COPI vesicle and tubule formation during mitotic Golgi disassembly.

3.3.2 Coatomer.
In order to study COPI independent fragmentation in isolation mitotic cytosol was selectively depleted of coatomer using the monoclonal antibody CM1A10 (Orci et al., 1993b), which recognizes the native coatomer complex, as in Misteli and Warren (1994). The depletion was very effective, removing approximately 95% of coatomer from mitotic cytosol (Figure 3.9A). Purified rat liver coatomer (Waters et al., 1992a) was obtained from Dr Francis Barr to replenish this depleted cytosol, to a level of 50µg/ml coatomer (Figure 3.9A).

RLG were incubated at 37°C for 60min with either mock depleted mitotic cytosol, coatomer depleted mitotic cytosol, or coatomer depleted mitotic cytosol replenished with purified rat liver coatomer (Figure 3.9B-D). Incubation of RLG in mock depleted mitotic cytosol yielded a predominantly vesicular population of membranes very similar to that of untreated mitotic cytosol (Figures 3.9B, 3.10B). In contrast,
Figure 3.9 Mitotic Golgi fragmentation in the absence of coatomer. (A) sHeLa mitotic cytosol was depleted of coatomer as described in materials and methods using the CM1A10 mAb. Coatomer was purified from rat liver cytosol and added back to the depleted cytosol at 50μg/ml. 20μg of cytosol was fractionated by SDS-PAGE using a 7.5% gel and transferred to nitrocellulose and probed with the anti-β-COP mAb M3A5. RLG were incubated at 37°C for 60min with either, mock depleted mitotic cytosol (B), coatomer depleted mitotic cytosol (C), coatomer depleted mitotic cytosol supplemented with purified rat liver coatomer (D), or NEM treated coatomer depleted mitotic cytosol (E, RLG was also NEM treated here). Note the presence of large tubular networks in C and E (large arrows), in comparison to vesicles in B and D (small arrows). Bar, 0.5μm.
Figure 3.10 Quantitation of COPI independent Golgi fragmentation. RLG were incubated at 37°C for 60min with either mitotic cytosol, mock depleted mitotic cytosol, coatamer depleted mitotic cytosol, coatamer depleted mitotic cytosol supplemented with purified rat liver coatamer, or NEM treated coatamer depleted mitotic cytosol (RLG was also NEM treated). Samples were fixed and processed for EM, and the percentage total membrane present as cisternae (A), vesicles (B), tubules (C), and tubular networks (D) was determined. Mean values are shown from 3 experiments±SEM.
coatomer depleted mitotic cytosol generated a series of large tubular networks (Figure 3.9C) similar to those observed previously (Misteli and Warren, 1994). Quantitation revealed that these tubular networks made up c. 30% of the total membrane, and that they were much less prominent in control reactions where they made up less than 10% of the total membrane (Figure 3.10D). Usually, these networks are consumed in part by COPI vesicles (Misteli and Warren, 1995a), thus in the absence of coatomer they accumulate. This was the distinguishing feature of incubations in coatomer depleted mitotic cytosol, and was paralleled by there being very much fewer vesicular profiles (Figure 3.10B). When the total tubule population was considered the percentage total membrane present as tubules was between 28-42% for all cytosols tested, there being c. 10% more tubules present in coatomer depleted reactions. Coatomer depleted mitotic cytosol did not consume so much of the cisternae as mock depleted or control cytosols. The percentage total membrane present as cisternae at the end of the incubation was 12-15% in control reactions, as compared to 25-30% for coatomer depleted cytosol (Figure 3.10A). Replenishing depleted cytosol with purified rat liver coatomer reversed these effects (Figure 3.10A), returning the percentage total membrane present as cisternae, vesicles and tubular network to control levels (Figures 3.9D, 3.10B and D). This suggests that coatomer itself was the component responsible for these effects and not another factor that was co-depleted with coatomer.

Given the large amount of tubular network that was generated in coatomer depleted cytosol it seemed possible that this would be a good measure on which to screen for factors which may inhibit/promote this process. Similar tubular network structures had been generated in vitro previously by treating Golgi stacks with BFA and interphase cytosol (Orci et al., 1991). This process required NSF both in vitro and in vivo (Orci et al., 1991; Fukunaga et al., 1998), and so was thought to involve membrane tubules emanating from one cisterna and fusing with another cisternae to eventually create a large network. The formation of these networks then requires a cytoplasmic fusion event. To determine whether a similar process was acting to form the tubular networks formed by coatomer depleted mitotic cytosol, RLG and coatomer depleted mitotic cytosol were treated with NEM (Rabouille et al, 1995a) to inactivate any NSF or p97.
Any residual NEM was quenched with DTT and the NEM-treated membranes and cytosol were then incubated for 60 min at 37°C. In contrast to the findings of Orci et al. (1991), NEM treatment had no effect on tubular network formation in coatomer depleted mitotic cytosol (Figure 3.9D, 3.10D). In fact the treatment had no discernible effect on the coatomer depleted reaction at all (Figure 3.10). This provides suggestive evidence that the COPI independent pathway of fragmentation is not due to a cytoplasmic fusion process, but rather a periplasmic fusion or fission process (Rothman and Warren, 1994), which is NEM insensitive.

A number of other factors were screened to determine whether there was any effect on tubular network formation (Table 3.5), including BAPTA and analogues (rapid calcium chelators, capable of disrupting calcium gradients) which disrupt certain membrane fusion events (Sullivan et al., 1993; Peters and Mayer, 1999), aluminium fluoride and mastoparan that activate heterotrimeric G-proteins implicated in regulating Golgi architecture (Jamora et al., 1997, 1999), and manoalide, a phospholipase A2 inhibitor, since phospholipase A2 has been implicated in tubule formation (de Figueiredo et al, 1998, 1999). Regrettably none of these agents yielded promising results. The project was therefore discontinued owing to there being better results in competing projects. However, given the large signal in the assay (i.e. the large number of tubular networks formed), it could still prove useful in screening and identifying factors necessary for COPI independent fragmentation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total membrane present as tubular network (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>32.1±1.6</td>
</tr>
<tr>
<td>BAPTA (10 mM)</td>
<td>27.6±3.1</td>
</tr>
<tr>
<td>dibromo-BAPTA (10 mM)</td>
<td>30.2±2.9</td>
</tr>
<tr>
<td>AlF4 (50 μM)</td>
<td>26.5±3.3</td>
</tr>
<tr>
<td>Mastoparan (1 μM)</td>
<td>25.9±4.1</td>
</tr>
<tr>
<td>Manoalide (8 μM)</td>
<td>33.2±1.5</td>
</tr>
</tbody>
</table>

Table 3.5 Effect of various treatments on COPI independent tubular network formation. RLG were incubated at 37°C for 60 min with coatomer depleted mitotic cytosol. Reactions were processed for EM, and the percentage total membrane present as tubular network was determined. Values represent means±SEM (n=2).
3.3.3 p115

In mammalian cells, p115 is a COP1 vesicle tethering protein required for intra-Golgi transport \textit{in vitro} and \textit{in vivo} (Waters et al., 1992b; Sönntichsen et al., 1998; Seemann et al., 2000). p115 is thought to contribute to the tethering process by cross-linking its two Golgi receptors: GM130 on the target membrane and giantin on the COP1 vesicle. Given this apparent membrane cross-linking ability of p115, it has been tested whether p115 is capable of tethering cisternae to cisternae, thereby contributing to the stacking of Golgi cisternae, as well as playing a role in COP1 vesicle tethering (Chapter 4; Shorter and Warren, 1999). This is conceivable given that giantin is present in cisternae and COP1 vesicles at equal concentrations (Sönntichsen et al., 1998). p115 is released from Golgi membranes during mitosis (Levine et al., 1996), due to Cdc2 kinase mediated phosphorylation of GM130 (Nakamura et al., 1997; Lowe et al., 1998b), and this has been proposed to be a possible mechanism behind the accumulation of COP1 vesicles at mitosis. However, if p115 also plays a role in the stacking of cisternae, does the release of p115 from the membrane also contribute to the mitotic unstacking process? During the disassembly reaction, it takes 10 min to deplete p115 from the Golgi membrane (Levine et al., 1996). If this is the first stage of unstacking, then prior removal of p115 from the membrane, before entrance into the disassembly reaction, may accelerate the rate of unstacking.

85% of p115 can be removed from the Golgi membrane by 1M KCl extraction (Waters et al., 1992b; Levine et al., 1996; see below). RLG were then either left untreated, extracted with 60mM KCl (which does not remove p115; Waters et al., 1992b), or extracted with 1M KCl and incubated at 37°C with mitotic cytosol for increasing time. At various times the reactions were fixed and processed for EM. Removal of p115 from the Golgi membrane by 1M KCl extraction does not change the percentage total membrane present as stacks (Figure 3.11A, E and I) which remains very close to 40% for all treatments, and is consistent with previous reports (Cluett and Brown, 1992; Hui, 1997). Thus, if p115 does contribute to cisternal stacking it is certainly not the sole determinant. On incubation with mitotic cytosol, 1M KCl extracted RLG unstacked at an accelerated rate compared to untreated or 60mM KCl extracted RLG.
Figure 3.11 Effect of KCl extraction on the rate of mitotic unstacking of Golgi cisternae.
RLG were either left untreated, or extracted with 60mM KCl, or 1M KCl and then incubated at 37°C with mitotic cytosol for increasing time, reactions were stopped at the indicated times, and processed for EM. (A-D) 0-20min time course for unextracted RLG. (E-H) 0-20min time course for 60mM KCl extracted RLG. (I-L) 0-20min time course for 1M KCl extracted RLG. Large arrows denote stacks, small arrows denote single cisternae. Note that stacks disappear and single cisternae appear more rapidly for 1M KCl extracted RLG. Bar, 0.5μm.
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(Figure 3.11, 3.12A). Unstacking was complete after just 10min of the reaction for 1M KCl extracted RLG, whereas the same extent of unstacking took 20min in control reactions (Figure 3.12A). After just 5min of the reaction single cisternae were more apparent for 1M KCl extracted RLG (Figure 3.11B, F, J; 3.12B), and by 10min stacked structures were hard to find for 1M KCl extracted RLG, yet made up c. 10% of the total membrane in control reactions (Figure 3.11C, G, K; Figure 3.12B). By 20min virtually no stacks remained in any of the incubations (Figure 3.11D, H, L; Figure 3.12A). The initial rates of loss of stacks (i.e. the rate of loss of stacks for the first 5min of the reaction) were 4.9%min⁻¹ for untreated RLG, 4.8%min⁻¹ for 60mM KCl extracted RLG and 6.2%min⁻¹ for 1M KCl extracted RLG. Suggesting that the initial rate of unstacking was 25% quicker for 1M KCl extracted RLG. It was not determined whether the time point of 5min was still in the linear range, that is no earlier time points were looked at, therefore these differences in initial rate may actually be an underestimate. These differences did not appear to be due to any increase in the rate of consumption of cisternae which looked similar for all treatments (Figure 3.12C). In fact the initial rates of loss of cisternae (i.e. the rate of loss of cisternae in the first 5min of the reaction) were 3.7%min⁻¹ for untreated RLG, 2.9%min⁻¹ for 60mM KCl extracted RLG, and 2.3%min⁻¹ for 1M KCl extracted RLG. So even though the initial rate of unstacking was accelerated upon 1M KCl extraction, the initial rate of loss of cisternae was actually reduced.

Obviously, 1M KCl extraction of RLG will release numerous proteins other than pi 15 from the Golgi membrane (Figure 3.13A). In fact, 60mM KCl extraction removed numerous proteins from RLG as well, suggesting a number of loosely attached, possibly cytosolic proteins are present in the RLG preparation. However, a number of proteins Coomassie stained much stronger in the 1M KCl supernatant as compared to the 60mM KCl supernatant (arrows in Figure 3.13A), and these may be factors whose removal facilitates the more rapid unstacking. Given that pi 15 is very clearly removed from RLG by 1M KCl and not 60mM KCl (Figure 3.13B) it was decided to supplement the 1M KCl extracted RLG with purified rat liver pi 15 and determine whether this had any effect on the rate of unstacking.
Figure 3.12  Quantitation of effect of KCl extraction on the rate of mitotic unstacking of Golgi cisternae. RLG were either left untreated, or extracted with 60mM KCl, or 1M KCl and then incubated at 37°C with mitotic cytosol for increasing time, samples were processed for EM at various times and quantitated for the percentage total membrane present as stacks (A), single cisternae (B) and all cisternae (C). Values represent means±SEM (n=3).
Figure 3.13 Effect of KCl extraction on Golgi membrane polypeptide composition. 10µg RLG was either left untreated or extracted with 60mM KCl or 1M KCl. After the extraction membranes were pelleted and the supernatant (SN) removed. The membrane pellets were dissolved in SDS-PAGE sample buffer, and the polypeptides in the SN TCA precipitated, and then dissolved in SDS-PAGE sample buffer. Samples were then fractionated by SDS-PAGE using a 4-20% gradient gel, and either stained with Coomassie (A) or transferred to nitrocellulose (B) and probed for p115 using the specific mAb 8A6. Molecular weights in kD are shown on the left. Arrows in A denote bands which appear stronger in the 1M KCl SN as compared to the 60mM KCl SN. Asterisks in A denote soybean trypsin inhibitor which was used as a carrier protein for the TCA precipitation.
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Excess p115 inhibits the consumption of cisternae during the disassembly reaction (Levine et al., 1996), so it was important to add back p115 to the level which it was present on RLG prior to 1M KCl extraction. The levels of p115 on RLG, and the binding characteristics of p115 to 1M KCl extracted RLG had been very carefully determined previously (Levine et al., 1996) and 0.1-0.15μg p115 was the maximum that could bind to 10μg 1M KCl extracted RLG. Therefore, after 1M KCl extraction, 0.075μg p115 was supplemented to 5μg 1M KCl extracted RLG and allowed to bind for 10min on ice. Membranes were then incubated at 37°C with mitotic cytosol for increasing time, and at various times fixed and processed for EM. Supplementing 1M KCl extracted RLG with p115 reduced the rate of cisternal unstacking (Figure 3.14, 3.15A), and returned it to levels similar to untreated RLG. The initial rate of loss of stacks was 5.7%min⁻¹ without p115 supplementation and 4.5%min⁻¹ with p115 supplementation. Stacks still represented c. 12% of the total membrane after 10min of the reaction after p115 supplementation, but were virtually absent from unsupplemented 1M KCl extracted RLG (Figure 3.14B, E). This was reflected in the changes in the percentage of total membrane present as single cisternae, which rose very sharply for 1M KCl extracted RLG in the first 5min of the reaction, but much less so when p115 was supplemented (Figure 3.14A, D; 3.15B). Again the increase in the rate of unstacking was not due to an increase in the rate of consumption of cisternae (Figure 3.15C). In fact the initial rate of loss of cisternae was slower for 1M KCl extracted RLG being 1.8%min⁻¹ compared to 2.8%min⁻¹ for 1M KCl extracted RLG supplemented with p115. So again, even though the initial rate of unstacking was increased the initial rate of consumption of cisternae was decreased in 1M KCl extracted RLG.

p115 can also be removed from Golgi membranes by incubating in the presence of N73pep, a peptide corresponding to the N-terminal 73 amino acids of GM130, which comprises the p115 binding site on GM130 (Nakamura et al., 1997; Shorter and Warren, 1999). Incubation of RLG for 15min on ice with 80μM N73pep is sufficient to remove 85-90% of the Golgi bound p115 (Figure 3.16A), and this should represent a more specific procedure to extract p115 from the Golgi membrane as compared to
Figure 3.14 Effect of adding p115 back to 1M KCl extracted RLG on the rate of mitotic cisternal unstacking. RLG were extracted with 1M KCl and then either supplemented with purified rat liver p115 or buffer and left for 10min on ice. Membranes were then incubated at 37°C with mitotic cytosol for increasing time, reactions were stopped at the indicated times, and processed for EM. (A-C) Time course of unstacking for 1M KCl extracted RLG preincubated with buffer. (D-F) Time course of unstacking for 1M KCl extracted RLG preincubated with p115. Large arrows denote stacks, while small arrows denote single cisternae. Note that the stacks persist for longer for membranes preincubated with p115. Bar, 0.5μm.
Figure 3.15 Quantitation of effect of supplementing 1M KCl extracted RLG with p115 on the rate of mitotic cisternal unstacking. RLG were extracted with 1M KCl, and then were either supplemented with buffer or purified rat liver p115 for 10 min on ice. Membranes were then incubated at 37°C with mitotic cytosol for increasing time, reactions were stopped at the indicated times, and processed for EM. The percentage total membrane present as stacks (A), single cisternae (B) and all cisternae (C) was determined. Values represent means±SEM (n=2).
Figure 3.16 Effect of N73pep extraction and p115 add back on the rate of mitotic cisternal unstacking. (A) RLG at 0.1 mg/ml were treated with 80µM N73pep for 15min on ice, membranes were then pelleted and the supernatant (SN) removed. The pellet (N73pep extracted RLG) was dissolved in SDS-PAGE sample buffer, while the proteins in the SN were TCA precipitated, recovered and also dissolved in SDS-PAGE sample buffer. Samples were then fractionated on a 7.5% gel, and transferred to nitrocellulose, and then probed for p115 using the p115 specific mAb 8A6. (B-G) N73pep extracted RLG was supplemented with either buffer (B-D) or p115 (E-G) for 10min on ice, and then incubated at 37°C with mitotic cytosol for increasing time, reactions were stopped at the indicated times, and processed for EM. Large arrows denote stacks and small arrows denote single cisternae. Note that stacks persist for longer when p115 is supplemented to the N73pep extracted RLG. Bar, 0.5µm.
1M KCl extraction. In a similar way to 1M KCl extracted RLG, N73pep extracted RLG unstack more rapidly than untreated RLG, and this phenomenon is reversed by supplementing N73pep extracted Golgi with p115 (Figure 3.16B-G; 3.17). The initial rate of loss of stacks was 5.6%min\(^{-1}\) with N73pep extraction, and 4.2%min\(^{-1}\) when p115 was supplemented to the N73pep extracted RLG. Again, there was an increase in the number of single cisternae seen after 5min with N73pep extraction (Figure 3.17B) and the rates of loss of cisternae looked similar (Figure 3.17C). In fact the initial rate of loss of cisternae was 2.1%min\(^{-1}\) for N73pep extracted RLG and 3.8%min\(^{-1}\) for N73pep extracted RLG supplemented with p115.

Taken together these data indicate that prior removal of p115 from the Golgi membrane stimulates the rate of mitotic cisternal unstacking, the initial rate being c. 25% more rapid. This was not due to any increase in the rate of the consumption of cisternae, the initial rate of which tended to be slower under conditions where p115 had been removed from the membrane. Therefore, it may be that release of p115 from the Golgi membrane contributes to the mitotic cisternal unstacking process, and may be the first step in this process. This would in fact mirror stack formation during the reassembly process, where p115 also plays an early role (Chapter 4; Shorter and Warren, 1999).

3.4 Discussion

3.4.1. The disassembly reaction.

The cell free mitotic disassembly of the mammalian Golgi apparatus has been successfully reproduced. This system recreates morphological changes in Golgi architecture reminiscent of the changes that occur during prophase and metaphase \textit{in vivo} (Lucocq et al., 1987, 1989; Misteli and Warren, 1995b). Stacks of cisternae unstacked while vesiculation and tubulation processes were rapidly consuming them. The kinetics of the reaction described above were more rapid than had been previously described (Misteli and Warren, 1994), and this may reflect improved methods of RLG and mitotic cytosol preparation. The mechanism by which cisternae unstack also appears to be different in the current study. The percentage total membrane present as single cisternae increases during the early stages of the reaction (Figure 3.3B), and this
Figure 3.17 Quantitation of effect of N73pep extraction and p115 add back on the rate of mitotic cisternal unstacking. RLG were extracted with N73pep, and then either supplemented with buffer or p115 for 10 min on ice. Membranes were then incubated at 37°C with mitotic cytosol for increasing time, reactions were stopped at the indicated times, and processed for EM. The percentage total membrane present as stacks (A), single cisternae (B) and all cisternae (C) was determined. Values represent means±SEM (n=2).
is coupled to a decline in the mean number of cisternae per stack (Table 3.4; Figure 3.5), which is also seen in vivo (Misteli and Warren, 1995b). This suggests an actual unstacking process, where cisternae physically separate, and are then consumed. This separation of cisternae and subsequent consumption are temporally very close together, and can been also be seen to occur in vivo (Eija Jokitalo, personal communication), but is difficult to resolve as cisternae are also being consumed while still stacked. Previously, this physical separation of cisternae was hard to resolve in vitro, possibly since the starting membranes had a shorter cisternal cross-sectional length, so were more readily consumed as they unstacked (Misteli and Warren, 1994). Differences in stereological definitions between studies may also contribute to these different views.

A major challenge for the future will be to reconstitute the disassembly process using purified proteins instead of mitotic cytosol, as has been done for the reassembly reaction (Rabouille et al., 1995b, 1998; Shorter and Warren, 1999).

3.4.2 A role for Rab1 in the disassembly process.

A positive correlation was found, where excess GDI inhibits both vesicle and tubule formation during disassembly (such that very little disassembly actually occurs), as well as Rab1 association with the Golgi membrane, which is usually stimulated at mitosis (Figure 3.8A; Bailly et al., 1991). Rab6 association with the membrane was unaffected by the presence of GDI, which is in contrast to previous studies (Elazar et al., 1994a; Beranger et al., 1994), where purified bovine liver GDI or a recombinant GST-GDI were used rather than a recombinant His-GDI as in this study. However, the difference may also be that this is a mitotic system and it may be that GDI cannot affect Rab6 under mitotic conditions. GDI does undergo a phosphorylation cycle, where it is phosphorylated on serines when complexed to a Rab in the cytosol, but unphosphorylated when unoccupied or complexed to a Rab on the membrane (Steele-Mortimer et al., 1993). It may be that mitotic treatment of GDI therefore affects its Rab interacting properties. Unfortunately, owing to the unavailability of antibodies, the effect of GDI on the other prominent Golgi Rabs, Rab2 (Tisdale et al., 1992) and
Rab33b (Zheng et al., 1998), could not be determined. Rab2 would have been of interest since it has been shown to enhance the recruitment of coatamer and promote COPI vesicle formation from VTCs (Tisdale and Jackson, 1998; Tisdale, 1999). In the future it may be possible to assess precisely which Rabs are affected by GDI by a combination of two-dimensional gel electrophoresis and GTP overlay/Western blot of MGF (Huber et al., 1994).

Rab1 has been implicated in COPI vesicle formation before (Peter et al., 1993, 1994), however, since this was from ER membranes, where COPII vesicles usually form the significance of these findings is uncertain. The fact that excess unoccupied GDI prevents Rab1 from associating with the Golgi and prevents disassembly, a COPI budding dependent process, strongly suggests that Rab1 may be involved in COPI vesicle formation from the Golgi apparatus. The fact that COPI independent tubulation is also inhibited suggests that Rab1 or another Rab protein may also have a role in this process. Depletion of mitotic cytosol of Rabs complexed to GDI and resupplementation of different combinations of candidate Rabs complexed to GDI, would allow a finer characterization of the Rabs involved, as has been done in other systems (Dirac-Svejstrup et al., 1994). Equally, utilization of Rab mutants that preferentially bind GDP would allow further insights into which Rabs were involved, as this would also bias the distribution of Rab away from the membrane. However, the ultimate test of whether a certain Rab complexed to GDI can stimulate COPI vesicle formation would come by including this component in a minimal COPI vesicle formation assay (Ostermann et al., 1993). It may also be given the recent explosion of Rab interacting molecules (Christoforidis et al., 1999a, b; Cuif et al., 1999; Diaz et al., 1997; Barr, 1999; Echard et al, 1998), that depriving the Golgi membranes of Rabs at mitosis may also be depriving them of many other key components required for disassembly. Whatever the case, the data presented here is consistent with a Golgi apparatus specific Rab, and possibly Rab1, having some regulatory or other role in COPI vesicle and possibly tubule formation.
3.4.3 COPI independent fragmentation.

The COPI independent fragmentation pathway was studied in isolation by specifically depleting mitotic cytosol of coatamer using the mAb CM1A10 (Orci et al., 1993b; Misteli and Warren, 1994). Incubation of RLG in this cytosol induced an extensive fragmentation process that yielded mostly tubular network and tubular profiles. Tubular networks could actually account for up to 30% of the total membrane population, and this signal was some 3 times higher than in mitotic cytosol that contained coatamer. Given the size of this signal this assay could prove useful in screening for factors that are involved in this poorly defined fragmentation pathway. Interestingly, NEM did not inhibit the process, suggesting that COPI independent fragmentation is a periplasmic fusion or fission driven process rather than a cytoplasmic fusion driven process. This would be in contrast to BFA stimulated tubular network formation which requires cytoplasmic fusion events (Orci et al., 1991; Fukunaga et al., 1998). Recently, PITP has been shown to possess Golgi membrane fission activity (Simon et al., 1998), but this is NEM sensitive so is unlikely to be responsible for this COPI independent fragmentation. An interesting candidate for the COPI independent pathway would be BARS-50 which appears to be able to support Golgi membrane fission (Weigert et al., 1999). Regrettably, none of the other factors tested here seemed to affect the COPI independent pathway. However, given the large signal of the assay, others should be able to use it to probe for factors responsible for COPI independent fragmentation and membrane tubulation.

3.4.4 Release of p115 from the Golgi membrane stimulates mitotic cisternal unstacking.

Prior removal of p115 from the Golgi membrane by either 1M KCl or N73pep extraction lead to a small, but reproducible increased rate at which cisternae unstack during disassembly. The initial rate of reaction was 25% more rapid, although this may be an underestimate as it was not determined whether the 5min time point (the first time point measured) was still in the linear range. These results were obtained despite the fact that mitotic cytosol contains an abundant source of p115. However, it is likely that Cdc2 kinase mediated phosphorylation of GM130 occurs rapidly, so preventing
p115 in mitotic cytosol from occupying GM130 sites on the Golgi membrane. This effect could be reversed by supplementing the extracted Golgi membranes with p115 to a level that it was found prior to extraction, suggesting that p115 was the factor released that was causing this effect.

These results suggest that the release of p115 from the membrane at mitosis may be the first stage of, or contribute to, the unstacking process. p115 may act to cross-link cisternae by simultaneously binding its two Golgi receptors GM130 and giantin on adjacent cisternae (Shorter and Warren, 1999). This interaction cannot be solely responsible for stacking as removal of p115 by 1M KCl or N73pep extraction does not cause unstacking *per se* (Figure 3.111; Chapter 4), but rather an accelerated rate of unstacking during disassembly. This giantin-p115-GM130 complex may be particularly active at the peripheral rims of stacks where this heteroternary complex is involved in COPI vesicle tethering during intra-Golgi transport (Waters et al., 1992b; Sönichsen et al., 1998; Shorter and Warren, 1999). Release of p115 from the membrane by the Cdc2 kinase mediated phosphorylation of GM130, would then release COPI vesicles, and separate adjacent cisternae at the peripheral rim. This opening up of the stack may then allow the mitotic kinases access to the remaining stacking machinery, and so facilitate further unstacking. An important event in this process may be the Cdc2 kinase mediated phosphorylation of GRASP65 and GRASP55 (Barr et al., 1997; Francis Barr personal communication), two putative Golgi stacking proteins (Chapter 4 & 5; Barr et al., 1997, 1998; Shorter et al., 1999). One might also compare p115 to the A-type lamins of the nuclear lamina, since both are released in a soluble state at mitosis (Levine et al., 1996; Gerace and Blobel, 1980). This release may facilitate the breakdown of a complex meshwork (Lorra and Huttner, 1999) and enable mitotic membrane disassembly. This possible role for p115 in the mitotic unstacking process is supported by its possible role in the stacking of reassembling Golgi cisternae and maintenance of the Golgi stack under interphase conditions. These possible roles for p115 are the focus of the next chapter.
Chapter 4

A role for the vesicle tethering protein, p115, in the stacking of reassembling Golgi cisternae at the end of mitosis
4.1 Introduction

It is possible to view cisternal stacking and COPI vesicle tethering as functionally equivalent processes. Both require agents that bring and hold membranes in close proximity. The intimacy of these two processes may be reflected by the intimacy of the interaction between GM130 and GRASP65 (Barr et al., 1998), the former being required for COPI vesicle tethering, and the latter for cisternal stacking. Both proteins are phosphorylated at mitosis (Barr et al., 1997; Lowe et al., 1998b), when COPI vesicle tethering is inhibited and Golgi cisternae unstack. Given that pl15 has the apparent capacity to cross link membranes via GM130 and giantin and that giantin is present at equal concentration in Golgi cisternae as it is in COPI vesicles (Sonnichsen et al., 1998), could pl15 be involved in the tethering reaction that leads to stacking as well as the tethering reaction that leads to COPI vesicle fusion? Inter alia, can pl15 tether cisterna to cisterna as well as COPI vesicle to cisterna?

In this chapter it is examined, using a modified reassembly assay, whether pl15 plays a direct role in stacking cisternae at the end of mitosis that is distinct from its role in membrane fusion. The results suggest that pl15, in conjunction with giantin and GM130, is essential for cisternal stacking and NSF-mediated cisternal regrowth. pl15 acts most potently at an early stage in the stacking reaction, upstream of GRASP65, and may facilitate the initial tethering of Golgi cisternae that is a prerequisite for stacking. This study was also extended to probe whether pl15 plays a role in maintaining the Golgi stack under steady state interphase conditions. Finally, a role for a G-protein in the stacking process has been clarified, which seems to act at a point coincident with, or downstream of pl15 in the process. The possibility that this G-protein is a Rab GTPase is raised.

4.2 Role for pl15 in the post-mitotic stacking of reassembling Golgi cisternae.

4.2.1. MGF for the reassembly reaction.

One of the major aims of the disassembly reaction was to generate a set of MGF that was suitable to study the reassembly process. If RLG are incubated with mitotic
cytosol for too long (i.e. periods greater than 30min), the MGF generated will not reassemble when incubated with interphase cytosol (Rabouille et al., 1995c). The reasons for this are still unclear, but may reflect the loss of some component from the MGF that is essential for subsequent reassembly. Whether this component is a type of membrane structure that serves as a template for subsequent reassembly, or an essential molecule(s) that is rendered dysfunctional will require further work. Bearing this problem in mind it was deemed essential to keep the disassembly reaction as short as possible, yet to achieve the maximum fragmentation. Since the primary interest in this study was the stacking process during reassembly, the disassembly time point of 20min rather than 30min (as had been used before, Rabouille et al., 1995b, c, 1998) was chosen to study reassembly. At this time point, stacked structures had already reached their minimum level of representation in the membrane population, usually compromising less than 2% of the total membrane (Figure 3.4B). The amount of membrane present as cisternae was about half that of starting RLG, and in fact the majority of the membrane population was represented as tubules and vesicles (Figure 3.4; 4.1C, D). These MGF generated after 20min did not differ significantly in overall morphology to those used before by others to study reassembly, so one could compare between systems (Rabouille et al., 1995b, c, 1998).

In order to test whether p115 played a role in cisternal stacking during reassembly, it was necessary to alter the reassembly assay to make it dependent on added soluble factors (Shorter and Warren, 1999). Previously, MGF reassembled into stacked cisternae in buffer alone to the same extent as when cytosol was added suggesting that everything required for correct reassembly and cisternal stacking was present on the MGF (Rabouille et al., 1995b). This background fusion and stacking activity was abolished by treating the MGF with the sulphydryl modifying reagent NEM (Rabouille et al., 1995b). The irreversible chemical modifications rendered by NEM precluded study of cisternal stacking as it abolished correct GRASP65 function (Barr et al., 1997; Shorter et al., 1999). To study the importance of soluble factors for cisternal stacking it was necessary to remove this background reassembly competence without NEM treating the membranes. This was achieved by removing any cytosolic
Figure 4.1 Morphology and quantitation of MGF isolated with or without a 0.5M sucrose cushion. RLG were incubated with mitotic cytosol at 37°C for 20min, and then the membranes were isolated either with or without a 0.5M sucrose cushion, and processed for EM. (A, C) Morphology and quantitation of MGF isolated without a 0.5M sucrose cushion. (B, D) Morphology and quantitation of MGF isolated with a 0.5M sucrose cushion. Bar, 0.5μm.
contaminants from the MGF by isolating them through a 0.5M sucrose cushion, so enabling simultaneous assessment of the relative importance of added soluble factors in cisternal stacking and cisternal regrowth.

It was important to determine whether the presence of the 0.5M sucrose cushion altered the composition of the membrane population of the MGF. Thus, MGF were generated by incubating RLG with mitotic cytosol for 20 min at 37°C, and membranes were reisolated by centrifugation either in the presence or absence of a 0.5M sucrose cushion. The two populations of MGF were very similar in morphology (Figure 4.1). The percentage total membrane present as Golgi cisternae was 31% in either set of MGF (Figure 4.1C, D) and the mean cross-sectional cisternal length was 0.33μm in MGF isolated through the 0.5M sucrose cushion and 0.35μm in those isolated without the 0.5M sucrose cushion.

Analysis of the polypeptide composition of the two sets of fragments revealed that the MGF isolated through the 0.5M cushion were significantly less contaminated with cytosolic factors (Figure 4.2A). Many of the bands that are present in mitotic cytosol can be found on MGF isolated without the 0.5M sucrose cushion, whereas this is not the case when the 0.5M sucrose cushion is included (Figure 4.2A). In fact, the MGF isolated through the 0.5M cushion contained 65% less protein. This increased presence of cytosolic factors may explain why the MGF isolated without a 0.5M sucrose cushion are capable of reassembly in buffer alone (Rabouille et al., 1995b; Shorter and Warren, 1999). Western analysis revealed MGF isolated with or without the 0.5M sucrose cushion contained very similar amounts of Mann I, GM130 and GRASP65 (Figure 4.2B). Therefore, the 0.5M cushion was not affecting the amount of membranes that were recovered. However, when the MGF are compared to starting RLG virtually all the Mann I was recovered, but only 40-50% of the GM130 and GRASP65 appeared to be recovered. This may be due to a decrease in the reactivity of the antibodies against mitotically phosphorylated GM130 and GRASP65. MGF isolated with or without the 0.5M sucrose cushion had very similar levels of Rab6 and α-SNAP (Figure 4.2B).
Figure 4.2 Biochemical characterization of MGF.

10μg RLG, MGF derived from 10μg RLG isolated with or without a 0.5M sucrose cushion, and 10μg sHeLa mitotic cytosol (1% of input) were fractionated by SDS-PAGE, and either stained with Coomassie (A) or transferred to nitrocellulose (B), and probed for GM130, p115, p97, NSF, Mann I, GRASP65, α-SNAP, and Rab6 using specific antibodies. Molecular weights in kD are shown on the left. Note the shift in molecular weight of GM130 and GRASP65 in the MGF owing to mitotic phosphorylation of these two proteins.
Chapter 4

p115 in Stacking of Reassembling Golgi cisternae

The presence of the 0.5M sucrose cushion reduced the MGF p115 levels 4-5 fold, and the AAA proteins: NSF and p97 20-25 fold. The insertion of a 0.5M sucrose layer separates the mitotic cytosol (of which 1% of total input is shown in the far right lane of Figure 4.2A, B) which rests on top of this layer from the MGF which sediment through this layer on to the underlying 2M sucrose cushion. This reduces the risk of collecting contaminating cytosolic proteins on collection of the MGF. As the membranes enter the 0.5M sucrose layer there may also be some differential removal of the p115, p97 and NSF that are still loosely bound to the membranes. Since both p97 and NSF are released from the membrane upon ATP hydrolysis, and that raising the sucrose concentration stimulates the rate of ATP hydrolysis by these two proteins (Hemmo Meyer and Joyce Müller, personal communication), it may be that these proteins are released from the membranes when they enter the 0.5M sucrose layer as a result of increased ATP hydrolysis.

4.2.2 MGF isolated through 0.5M sucrose cushion require cytosolic components for cisternal regrowth and stacking.

The MGF isolated without the 0.5M sucrose cushion (Figure 4.3A) were fusion competent when incubated in KHM buffer (60mM KCl, 25mM Hepes-KOH, pH 7.3, 5mM magnesium acetate, 0.2M sucrose, 2mM ATP, 1mM GTP, 1mM glutathione) alone for 60 min at 37°C (Figure 4.3C, 4.4) as previously reported (Rabouille et al., 1995b). The percentage total membrane present as cisternae increased from 31% to 50% (Figure 4.4A) and the percentage total membrane present as stacked regions of cisternae from <1% to 7% (Figure 4.4B). The mean number of cisternae per stack was 2.3. The mean cross sectional length of these reassembled cisternae was 0.98μm. This was not the case for MGF isolated through the 0.5M sucrose cushion (Figure 4.3B), where the percentage total membrane present as cisternae showed no significant increase (Figure 4.3D, 4.4A), virtually no stacked structures could be found (Figure 4.3D, 4.4B) and the mean cross sectional length of cisternae increased from 0.33μm to 0.38μm. In fact these membranes looked very similar to those at the start of the incubation (compare Figure 4.3B and D).
Figure 4.3 MGF isolated through a 0.5M sucrose layer require cytosolic components for cisternal regrowth and stacking. MGF were generated by incubating RLG at 37°C with mitotic cytosol for 20min. MGF were then isolated without (A) or with (B) a 0.5M sucrose cushion, and processed for EM. Alternatively, MGF isolated without a 0.5M sucrose cushion were incubated at 37°C for 60min with either buffer (C) or rat liver cytosol (E). Similarly, MGF isolated with a 0.5M sucrose cushion were reassembled in buffer (D) or rat liver cytosol (F). Large arrows denote reassembled stacked cisternae and small arrows denote unfused tubulovesicular material. Note that if isolated through a 0.5M sucrose cushion the MGF will not reassemble in buffer alone (D). Bar, 0.5μm.
Figure 4.4 Stereology of RLG, MGF and Reassembled Golgi membranes. RLG were incubated at 37°C with mitotic cytosol for 20min, and isolated either with or without a 0.5M sucrose cushion, and termed MGF. MGF were then processed for EM or incubated at 37°C with either buffer or rat liver cytosol for 60min, and then processed for EM. The percentage total membrane present as cisternae (A) and stacks (B) was determined. Values represent means±SEM (n=3).
However, both sets of MGF were fusion competent when incubated in rat liver cytosol (10mg/ml) for 60 min at 37°C (Figure 4.3E, F). The percentage total membrane as cisternae rising from 31% to c. 60% for both sets of MGF (Figure 4.4A). This increase in the total membrane as cisternae can also be expressed as the amount of cisternal regrowth, which is calculated by setting the percentage total membrane present as cisternae in MGF to equal 0% cisternal regrowth, and the percentage total membrane present as cisternae at the end of successful reassembly as 100% cisternal regrowth (Section 2.2.14; Rabouille et al., 1995b). Expressed this way 100% cisternal regrowth indicates this 29% increase in the total membrane present as cisternae on going from MGF to reassembled cisternae. This way of expressing the extent of reassembly will be used in future sections.

The percentage total membrane present as stacked regions of cisternae increased from <1% to 20-25% (Figure 4.4B). The mean cross sectional length increased to 1.3µm in cisternae reassembled from MGF isolated with the 0.5M sucrose cushion and 1.2µm in cisternae reassembled from MGF isolated without the 0.5M sucrose cushion. The mean number of cisternae per stack was 2.6, although stacks with more than 3 cisternae could readily be found (Figure 4.3E, F). MGF isolated through a 0.5M sucrose have reduced levels of p115, NSF and p97. The latter two ATPases are in fact depleted 20-25 fold, and their near absence could explain why the MGF isolated through the 0.5M sucrose cushion are fusion incompetent in buffer alone. This is consistent with previous observations that NEM treatment or 0.25M KCl extraction of MGF isolated without a 0.5M sucrose cushion renders them fusion incompetent in buffer alone (Rabouille et al., 1995b).

4.2.3 Effect of temperature, salt and MGF concentration on cisternal regrowth.

In order to determine more finely the optimal conditions for reassembly the temperature, [KCl], and [MGF] were varied and the amount of cisternal regrowth determined (Figure 4.5). MGF isolated through a 0.5M sucrose cushion were incubated at 1mg/ml in rat liver cytosol (10mg/ml) for 60min at increasing temperatures (Figure 4.5A). Very little cisternal regrowth occurred at 4°C, but an appreciable amount
Figure 4.5 Effect of temperature, [KCl], and [MGF] on reassembly in rat liver cytosol. MGF isolated through a 0.5M sucrose cushion were incubated with rat liver cytosol (10mg/ml) for 60min at varying temperatures (A), [KCl] (B) and [MGF] (C). Reactions were processed for EM and the amount of cisternal regrowth determined. Values represent means±SEM (n=2).
occurred at room temperature (25°C), however, reassembly worked best at physiological temperature (37°C). The fact that activity falls at temperatures above this suggests that reassembly is an enzymatic process.

Cisternal regrowth showed a distinctive requirement for KCl (Figure 4.5B). Rat liver cytosol was desalted into KHM containing different levels of KCl, and these cytosols (10mg/ml) were incubated with MGF (1mg/ml) isolated through the 0.5M sucrose cushion for 60min at 37°C. Cisternal regrowth was optimal at 60mM KCl, and declined at salt concentrations higher than this. Interestingly, this is the concentration of KCl, for which p115 binding to Golgi membranes is optimal (Waters et al., 1992b).

The [MGF] variable had been harder to determine previously owing to greater contamination from cytosolic factors, leading to over-estimates of the amount of Golgi membrane protein present in the assay (Catherine Rabouille, personal communication). Increasing concentrations of MGF isolated through a 0.5M sucrose were incubated at 37°C for 60min with rat liver cytosol (10mg/ml). The concentration of MGF had to be relatively high to get successful reassembly, once above 0.75mg/ml cisternal regrowth was optimal (Figure 4.5C). This is in contrast to concentrations of membranes typically used in transport assays which tends to be 0.1-0.5mg/ml for Golgi membranes (Beckers and Rothman, 1992; Fernández and Warren, 1998). It may be that relatively few fusion events are required to obtain the biochemical readout of a transport reaction, so it is less sensitive to dilution. In contrast, the stereological readout of the reassembly reaction may require more fusion events to obtain an appreciable signal, and so requires a higher membrane concentration. Alternatively, it may be that membrane bound structural components are more crucial for reassembly than for transport events.

4.2.4 p115 is essential for the post-mitotic stacking of reassembling Golgi cisternae.

To assess p115 function in the reassembly process rat liver cytosol, p115-depleted cytosol, and p115-depleted cytosol with purified p115 added back were titrated in to
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the reassembly assay. p115 was depleted >95% from rat liver cytosol using either the mAb 4H1 or the N73pep (Figure 4.6). p115 was purified to near homogeneity from rat liver cytosol to add back to this depleted cytosol. MGF were resuspended in cytosol of increasing concentration and incubated for 60min at 37°C.

In rat liver cytosol cisternal regrowth was near maximal at 1mg/ml (Figures 4.7A, B, C and 4.8A), which is similar to intra-Golgi transport assays (Beckers and Rothman, 1992), and the same was true for the mock depleted cytosols (cisternal regrowth 78%±3.1 for N73pep mock depleted cytosol at 1mg/ml; 82%±0.9 for 4H1 mock depleted cytosol at 1mg/ml, values are means±SEM, n=3). At cytosol concentrations below 1mg/ml the p115-depleted cytosol supported threefold less cisternal regrowth (Figures 4.7D and 4.8A). This inhibition was reversed by adding purified p115 back to the depleted cytosol (Figures 4.7G and 4.8A). This loss of activity was then due to p115 activity itself and not the activity of another factor that may have been co-depleted from the cytosol by virtue of an interaction with p115. However, at cytosol concentrations of 1mg/ml and above p115-depleted cytosol supported full cisternal regrowth (Figures 4.7E, F and 4.8A). p115 is then not essential for this process, or a p115 independent pathway of cisternal regrowth is operating. The latter explanation is preferred as two non-additive pathways of cisternal regrowth controlled by NSF and p97 have been described previously (Rabouille et al., 1995b, 1998). The p97 pathway has no requirement for p115 for cisternal regrowth, and is presumably responsible for the complete cisternal regrowth activity of p115-depleted cytosol. A hint that this may be true comes from the morphology of the cisternae reassembled in p115-depleted cytosol, in that they are often blunt ended with few associated vesicles (compare asterisks in Figures 4.7E and H). This is the characteristic phenotype for p97 reassembled cisternae (Rabouille et al., 1995b).

The stacking process in rat liver cytosol displayed distinct properties to cisternal regrowth in that the number of stacks were still increasing at the highest cytosol concentration tested which was 10mg/ml (Figures 4.7C and 4.8B). This asymmetry may be due to an imbalance in factors required for cisternal regrowth and stacking. This
Figure 4.6 Depletion of rat liver cytosol of p115. Rat liver cytosol was depleted of p115 as described in Materials and Methods using either the anti-p115 mAb 4H1 or the N73pep. 20µg of cytosol was fractionated by SDS-PAGE using a 7.5% gel, transferred to nitrocellulose and probed with the anti-p115 mAb 8A6. Molecular weight in kD is shown on the left.
Figure 4.7 Effect of interphase cytosol depleted of p115 on the reassembly process. (A-I) MGF isolated through a 0.5M sucrose cushion were incubated for 60 min at 37°C with increasing concentrations of either rat liver cytosol (A-C), p115-depleted cytosol (D-F), or p115-depleted cytosol supplemented with purified rat liver p115 (G-I) fixed and processed for EM. Representative fields are shown. Note the presence of stacks (arrowheads) in A-C and G-I, but only single cisternae (arrows) in D-F, and the reduced number of cisternae in D suggesting poor reassembly at this concentration of p115-depleted cytosol. Note that the cisternae formed often have a wrinkled appearance (asterisks in F) in p115-depleted cytosol and are often blunt ended with few associated vesicles (compare asterisks in E and H) in contrast to when p115 is present. Bar, 0.5μm.
Figure 4.8 Quantitation of Golgi membrane reassembly in rat liver cytosol, p115-depleted cytosol, and p115-depleted cytosol supplemented with p115. MGF isolated through a 0.5M sucrose cushion were incubated for 60 min at 37°C with increasing concentrations of either rat liver cytosol, p115-depleted cytosol, or p115-depleted cytosol supplemented with purified rat liver p115, fixed and processed for EM, and quantitated as described in Materials and Methods. (A) The percentage cisternal regrowth±SEM (n=3) for each cytosol concentration tested. (B) The percentage total membrane present as stacked regions of cisternae±SEM (n=3) for each cytosol concentration tested.
mirrors the disassembly process in that low concentrations of mitotic cytosol are sufficient to inhibit transport (Stuart et al., 1993), yet do not affect Golgi structure significantly (Misteli and Warren, 1994).

The most striking effect on reassembly in p115-depleted cytosol at all concentrations tested was the virtual complete absence of stacked Golgi structures at the end of the incubation (Figures 4.7D, E, F and 4.8B). This effect could be reversed by adding purified p115 back to the depleted cytosol (Figures 4.7G, H, I and 4.8B), again suggesting that p115 itself was the active component and not another factor that had been co-depleted. Cisternal regrowth and stacking are thus separable processes. The single cisternae formed in the absence of p115 had a more wrinkled, corrugated appearance (asterisks in Figure 4.7F) suggesting an involvement of p115 in a membrane smoothing event during the reassembly process. This effect was again reversed by supplementing the depleted cytosol with purified p115. These effects of p115 depletion on reassembly were identical if sHeLa interphase cytosol was used instead of rat liver cytosol.

Kinetic analysis revealed the reassembly reaction was complete for both cisternal regrowth and stacking after 60min (Figure 4.10) in rat liver cytosol (10mg/ml). The first intermediates that formed very quickly during the first 15min of the incubation were single cisternae (Figures 4.9A, B), frequently with tubular networks at their rims (asterisks in Figure 4.9B). By 15min these intermediates had begun to dock and align to form the beginnings of stacked Golgi structure (arrowheads in Figure 4.9B). The lag in the formation of stacked structures (Figure 4.10) may then be considered due to the need to form single cisternae first. By 45min this process was well advanced and Golgi stacks with 2-3 or more cisternae per stack were prevalent and these discrete stacks were becoming linked via tubular networks (Figure 4.9C). By 60-120min these linkages had been made, the tubular networks were less apparent, and long cisternal stacks were the end product, which often adopted an approximate closed concentric circular morphology (compare Figure 4.9D and Figure 4.7C).
Figure 4.9 Kinetic analysis of Golgi membrane reassembly in rat liver cytosol and p115-depleted cytosol. (A-H) MGF isolated through a 0.5M sucrose cushion were incubated for increasing time at 37°C with either rat liver cytosol (A-D) or p115-depleted cytosol (E-H) with the cytosol concentration set at 10mg/ml, fixed and processed for EM, and quantitated as described in Materials and Methods. Representative fields are shown. In rat liver cytosol note that the first intermediate formed is the single cisterna after 5 min (arrows in A). By 15 min these single cisternae had grown in length, had tubular networks associated with their rims (asterisks in B) and had begun to align and dock to form stacks (arrowheads in B). Many discrete stacks had formed by 45 min (arrowheads in C) which had joined up by 120 min (arrowheads in D). In p115-depleted cytosol single cisternae were again present after 5 min (arrows in E), and had increased in length by 15 min (arrows in F), but were often blunt ended (asterisk in F) and were not stacked. At 45 min these cisternae remained blunt ended (asterisk in G) and unstacked. By 120 min some stacks of blunt ended cisternae had begun to form (arrowhead in H), but many single cisternae remained (arrows in H). Bar, 0.5μm.
Figure 4.10 Quantitation of time course of reassembly. The percentage total membrane present as cisternae ±SEM (n=3) and stacks ±SEM (n=3) are presented for each cytosol at every time point tested.
In p115-depleted cytosol, single cisternae formed at the start of the reaction although with a reduced initial rate (Figure 4.10 and 4.9E). Once again these cisternae were blunt ended indicating the p97 pathway of reassembly may be dominant (asterisks in Figures 4.9F and 4.9G). By 15min these single cisternae were still well separated (Figure 4.9F), and even after 45-60min single, blunt ended cisternae were the major reaction product (Figure 4.9G and 4.10). However, after 120min even though no more cisternal regrowth occurred, these single cisternae did begin to align and form stacks (Figure 4.9H and 4.9I). Even then, the level of stacking only reached approximately 50% that of rat liver cytosol (Figure 4.10) and the intercisternal distance between adjacent cisternae of the stack seemed more variable (compare Figures 4.9C and 4.9H). It appears then that the absence of p115 severely retards both the initial rate and overall extent of the stacking of Golgi cisternae during the reassembly reaction.

Analysis of the temporal changes in the mean number of cisternae per stack provides insight into how stacking proceeds in the reassembly reaction (Figure 4.11). The mean number of cisternae per stack rises very sharply and is virtually maximal after 30min of the reaction. This increase is much sharper than the increase in the percentage total membrane present as stacks during the incubation (Figure 4.10). This indicates that stacks form and then extend laterally rather than one cisterna growing, followed by the next and so on. This is similar to the situation in vivo (Souter et al., 1993). However, there is a lag in the stacking process, during the first 5min of the reaction, where few stacks are present. Thus, single cisternae must first reform, and possibly reach a minimum length before they are able to stack. This formation of stacks then lateral growth is the exact reverse of the disassembly situation where cisternae unstack and are consumed by tubulation and vesiculation.

Was the time at which p115 added back to the depleted cytosol crucial to reverse the effect on stacking? To assess this reassembly was conducted in p115-depleted cytosol and p115 was added back to the reaction at 15, 30, or 60min and the reaction was allowed to proceed for 120min. If added within the first 30min the p115 was able to restore stacking activity to the cytosol, however, if added at 60min the p115 only
Figure 4.11 Changes in the mean number of cisternae per stack during reassembly. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C with rat liver cytosol (10mg/ml) for increasing time. At various times reactions were processed for EM and the mean number of cisternae per stack was determined. Values represent means±SEM (n=3).
slightly stimulated stacking (Figure 4.12). This suggests that p115 must be present as
cisternae are reassembling for it to fulfil its stacking function. Once cisternae have
formed completely it seems p115 is no longer able to stimulate stacking.

4.2.5. Reassembly using purified cytoplasmic fusion components.

To more finely discern the role played by p115 in the p97 and NSF pathways of
reassemble and to corroborate the above findings experiments were performed using
the purified reassembly system. These two pathways can sustain maximal cisternal
regrowth individually, that is their effects are not additive, yet when all the
components of the p97 and NSF reactions are combined the cisternae that form have a
distinct morphology (Rabouille et al., 1995b). The components and levels of
components required for the NSF and p97 catalyzed reactions had been determined in
fine detail previously (Rabouille et al., 1995b, 1998). However, given the MGF used in
this study had a different biochemical composition it was necessary to confirm these
findings. To this end, MGF were incubated at 37°C for 60min with the predetermined
optimal levels of components for either the NSF or p97 catalyzed reaction, and for
each reaction, each co-factor was omitted in turn.

The NSF reaction requires NSF, α- and γ-SNAP, and p115 for cisternal regrowth
(Rabouille et al., 1995b). Inclusion of all components at maximal levels yielded
regrowth of stacks of cisternae (Figure 4.13A). This is an important advance, since
previously it was not possible to study the stacking process using the pure reassembly
system as the MGF were pretreated with NEM to eliminate background fusion
activity. This NEM pretreatment inactivates GRASP65, and possibly GRASP55,
essential components of the Golgi stacking machinery (Barr et al., 1997; Shorter et al.,
1999) so preventing stacking. Omission of NSF, α-SNAP or p115 from the reaction
drastically reduced the amount of cisternal regrowth (Figure 4.13B, C, E and F), even
though low levels of these components could be found on the MGF (Figure 4.2B).
However, omission of γ-SNAP had only a small effect on the amount of cisternal
regrowth that occurred, reducing it to approximately 80% (Figure 4.13D and F). This is
consistent with results obtained from a cell free intra-Golgi transport assay where
Figure 4.12 Effect of time of addition of p115 to p115-depleted cytosol on the amount of stack formation. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C with p115-depleted cytosol for 120min, and supplemented with p115 at various times during this 120min (time of addition of p115). Reactions were then fixed and processed for EM, and the percentage total membrane present as stacks ±SEM (n=2) are presented for each time point tested.
Figure 4.13 Effect of omission of co-factors from the NSF catalyzed reassembly reaction. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl) and p115 (30ng/µl) (A), or by having all these components but omitting NSF (B), α-SNAP (C), γ-SNAP (D), or p115 (E). Reactions were processed for EM. Large arrows denote reassembled stacks, while small arrows denote unfused tubulovesicular profiles. Note that omission of γ-SNAP has no drastic effect on reassembly. Bar, 0.5µm. (F) Reactions were quantitated for cisternal regrowth, values represent means ±SEM (n=2).
omission of γ-SNAP results in only a 30% reduction in transport (Whiteheart et al., 1993). γ-SNAP has also been shown to be dispensable for ER-Golgi transport (Peter et al., 1998). Although it has been shown that α- and γ-SNAP act synergistically to promote transport and NSF binding to Golgi membranes (Whiteheart et al., 1993; Clary et al., 1990), it is α-SNAP that makes by far the larger contribution. Therefore, γ-SNAP can be seen as a ‘fine tuning’ molecule for the NSF catalyzed reaction, and given its inclusion consistently yields c. 20% more cisternal regrowth (figure 4.13F) it was always included in NSF catalyzed reactions.

The p97 reaction requires p97 and p47 (Kondo et al., 1997; Rabouille et al., 1998). Two sources of p97 were available, one purified from rat liver, and the other a recombinant His-p97 (Meyer et al., 2000). Both these forms of p97 were able to catalyze cisternal regrowth equally well when complexed to p47 (Figure 4.14A, B, F; Meyer et al., 2000). However, when p47 was omitted from the reaction the p97 purified from rat liver was still able to support a limited amount of cisternal regrowth (c. 30%; Figure 4.14C, F), presumably due to the difficulty in completely separating p97 from p47 during the p97 purification (Kondo et al., 1997; Meyer et al., 1998). This problem was obviated in the case of recombinant His-p97, which contains no contaminating p47, and was unable to support any significant cisternal regrowth alone (Figure 4.14D, F). If p97 was omitted from the reaction cisternal regrowth was blocked (Figure 4.14E, F; Kondo et al., 1997).

The amount of cisternal regrowth using these systems was moderately better than that achieved in rat liver cytosol. In that, the NSF and p97 catalyzed reactions generated c. 70% total membrane present as cisternae from 31% in MGF after a 60 min incubation at 37°C. Thus, 100% cisternal regrowth in the purified system is more than in the cytosol driven reaction.
Figure 4.14 Effect of omission of co-factors from p97 catalyzed reassembly reaction. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with p97 (70ng/μl; of either rat liver or recombinant source) and p47 (37.5ng/μl; A,B), or with p97 alone (C,D) or with p47 alone (E). Reactions were processed for EM, large arrows denote reassembled cisternae and small arrows denote unfused tubulovesicular material. Bar, 0.5μm. (F) The amount of cisternal regrowth was determined for each reaction, values represent means±SEM (n=2).
4.2.6 The p97 pathway generates only single cisternae in the absence of p115.

To more closely assess the function of p115 in the reassembly reaction, p115 was titrated into the p97, NSF and NSF/p97 combined catalyzed reactions. Titration of p115 into the p97 reaction revealed that this fusion pathway was insensitive to added p115 (Figure 4.16A). However, a population of solely single cisternae form in the absence of p115 (Figure 4.14A, B, 4.15A). These single cisternae had a corrugated, wrinkled appearance reminiscent of those formed in p115-depleted cytosol (compare Figure 4.7F and 4.15A). On addition of p115 these cisternae had a smoother appearance and formed stacks (Figure 4.15B). However, the stacks formed rarely had more than two cisternae per stack, the mean number of cisternae per stack being 2.2 (Table 4.1, Figure 4.17B).

Titration of p115 into the NSF reaction revealed that p115 was absolutely required for both cisternal regrowth and stacking (Figure 4.15C, D, 4.16B). In the absence of p115 only unfused tubulovesicular material was visible (Figure 4.15C). Both processes were saturating, but still rising at the maximum p115 concentration tested, 30ng/μl (Figure 4.16B). The reassembling cisternae formed stacks which usually had 3 or more cisternae per stack, the mean number of cisternae per stack being 3.1 (Table 4.1, Figure 4.17C). A Mann-Whitney test revealed that the number of cisternae per stack frequency distributions of p97 (+p115) and NSF catalyzed reassembly were significantly different in location P=0.0000. The NSF reaction actually generated a small population of stacks that had more cisternae per stack than the starting RLG (Figure 4.17A, C).

When p115 was titrated into the combined NSF/p97 reaction cisternal regrowth was insensitive to added p115 (Figure 4.15E, F, 4.16C). However, once more stacking absolutely required p115. In the absence of added p115 only wrinkled single cisternae were formed. On addition of p115 the cisternae formed were smooth and formed stacks with 2 or 3 cisternae per stack similar to the number formed in rat liver cytosol (Table 4.1, Figure 4.17D, E). In fact a Mann Whitney test revealed that the number of
Figure 4.15 Titration of p115 into the p97, NSF and NSF/p97 catalyzed reassembly reactions. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60 min with (A, B) p97 (70 ng/μl) and p47 (37.5 ng/μl); (C, D) NSF (100 ng/μl), α-SNAP (25 ng/μl), γ-SNAP (25 ng/μl); (E, F) or with all these components combined with from 0-30 ng/μl p115. The two extreme p115 concentrations are shown. Note the long, single wrinkled cisternae formed by the p97 pathway in the absence of p115 (arrow in A) and the long stacks of two cisternae with maximum p115 (arrowheads in B). Note the virtual absence of cisternae for the NSF pathway in the absence of p115 (C) and the short stacks of three or more cisternae with maximum p115 (arrowheads in D). Note the long, wrinkled single cisternae formed by the NSF/p97 pathway in the absence of p115 (arrow in E) and the stacks of two or three cisternae with maximum p115 (arrowheads in F). Bar, 0.5 μm.
Figure 4.16 Quantitation of titration of p115 into the p97, NSF, NSF/p97 catalyzed reassembly reactions. Samples were fixed and processed for EM and the percentage total membrane as cisternae ±SEM (n=3) and the percentage total membrane as stacks ±SEM (n=3) was determined. (A) p97 catalyzed reaction, (B) NSF catalyzed reaction, and (C) NSF/p97 catalyzed reaction.
Figure 4.17 Number of cisternae per stack frequency distributions of reassembled Golgi. MGF were incubated at 37°C for 60 min with either: (B) p97 (70 ng/μl), p47 (37.5 ng/μl) and p115 (30 ng/μl); or (C) NSF (100 ng/μl), α-SNAP (25 ng/μl), γ-SNAP (25 ng/μl) and p115 (30 ng/μl); or (D) all these components combined; or (E) rat liver cytosol (10 mg/ml). Reactions were fixed and processed for EM, and the number of cisternae per stack frequency distribution was determined and are shown for (A) RLG. (B) p97 driven reassembly, (C) NSF driven reassembly, (D) NSF/p97 driven reassembly and (E) rat liver cytosol driven reassembly. Values represent means±SEM from three experiments.
cisternae per stack frequency distributions of NSF/p97 and rat liver cytosol catalyzed reassembly were not significantly different in location, P=0.8345. This provides more correlational evidence that the p97 and NSF pathways operate to reform cisternae in rat liver cytosol.

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Mean number of cisternae per stack</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLG</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>MGF</td>
<td>*</td>
</tr>
<tr>
<td>Rat liver cytosol reassembled</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>p97 reassembled</td>
<td>*</td>
</tr>
<tr>
<td>p97+p115 reassembled</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>NSF reassembled</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>NSF/p97 reassembled</td>
<td>2.5±0.1</td>
</tr>
</tbody>
</table>

Table 4.1 Mean number of cisternae per stack for RLG, MGF and Reassembled Golgi membranes. The mean number of cisternae per stack was determined for the membranes listed. Values represent means±SEM from 3 experiments. * Stacks accounted for <2% of the total membrane, and had no more than two cisternae per stack.

4.2.7 Cisternae regenerated by the p97 catalyzed reaction have a longer mean cross-sectional length.

The frequency distribution of cisternal cross-sectional length in RLG displays a right skew shape, with a mean cisternal cross-sectional length of 1.1μm, and with some 24% of the population having a length greater than 1.5μm (Figure 4.18A). On incubation with mitotic cytosol to generate the MGF used for reassembly purposes this distribution alters dramatically. The distribution remains right skew in shape, but is shifted much closer to the origin. The mean cisternal cross-sectional length is 0.33μm, and 98% of the population has a cisternal cross-sectional length less than 1μm (Figure 4.18B).

The cisternae generated by the NSF catalyzed reaction display a more normal distribution of cisternal cross-sectional length, with a mean of 0.79μm (Figure 4.18C). No very long cisternae are formed, only 20% of the population being over 1μm in
Figure 4.18 Cisternal cross-sectional length frequency distributions for RLG, MGF and reassembled Golgi membranes. RLG (A), were incubated at 37°C for 20min with mitotic cytosol and recovered through a 0.5M sucrose cushion to yield MGF (B), which were then incubated at 37°C for 60min with either NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl), p115 (30ng/μl; C); or p97 (70ng/μl) and p47 (37.5ng/μl; D); or all these components combined (E); or with rat liver cytosol (10mg/ml;F). Reactions were processed for EM and the cross-sectional length of cisternae was measured and the frequencies determined.
length and only 8% over 1.5μm. This is in contrast to RLG, where 24% of the population of cisternae are longer than 1.5μm. This is also in contrast to the population of cisternae generated by the p97 catalyzed reaction (Figure 4.18D). Here a population of cisternae is generated with the familiar right skew frequency distribution, and a mean length of 1.42μm. 28% of the population has a length greater than 1.5μm, and cisternae longer than 5μm can be found. The NSF and p97 cisternal length frequency distributions are significantly different in location when a Mann-Whitney test is performed, P=0.0023. This difference may reflect the different modes of fusion these two ATPases are hypothesized to catalyze. p97 generates longer cisternae, which may suggest it acts in the homotypic fusion of cisternae. NSF generates short cisternae, possibly by catalyzing the heterotypic fusion of COPI vesicles with their acceptor membrane. Once formed these cisternae may not fuse together so readily to generate long cisternae.

Incubation of the MGF for reassembly via the NSF/p97 pathway generates a population of cisternae whose cisternal length frequency distribution is very similar to when reassembly is performed with rat liver cytosol (Figure 4.18E, F). These distributions do not differ significantly in location (Mann-Whitney test, P=0.5602) and are also very similar to starting RLG. This again provides suggestive evidence that it is the NSF/p97 reactions acting together to reassemble cisternae in rat liver cytosol.

4.2.8. The p115 stacking event requires GM130 and giantin.

It has been shown previously that p115 binds to GM130 and giantin on Golgi membranes (Nakamura et al., 1997; Sönnichsen et al., 1998; Seemann et al., 2000; Lesa et al., 2000) and that these interactions are important for COPI vesicle tethering in vitro (Sönnichsen et al., 1998). To test whether these two molecules were required for the p115 stacking function MGF were pretreated with anti-GM130 (NN15) and/or anti-giantin antibodies. The MGF were then resuspended for reassembly via the NSF, p97 (including p115 so stacks will form) or NSF/p97 combined pathway using the purified components.
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When the MGF were resuspended for the p97 or the NSF/p97 combined pathway cisternal regrowth was unaffected by either anti-giantin and/or anti-GM130 (Figure 4.19A-H, 4.20A). In the case of the NSF/p97 complete reaction inclusion of anti-giantin and anti-GM130 did cause a change in the morphology of the cisternae that formed, in that they adopted a wrinkled morphology, characteristic of cisternae generated by the p97 pathway (Figure 4.19B-D, F-H and 4.15C, E). Therefore the presence of these antibodies may be diverting the reaction down the p97 route and away from the NSF route. In contrast to cisternal regrowth the stacking process was severely inhibited in both the p97 and the NSF/p97 catalyzed reactions (Figure 4.19A-H, 4.20B) and the preimmune sera had no effect on this process (Table 4.2). p115 stacking function then requires p115 interactions with GM130 and giantin. Furthermore, this indicates that p115 may be able to tether cisterna to cisterna as well as COPI vesicle to cisterna.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Anti-giantin preimmune</th>
<th>Anti-GM130 preimmune</th>
<th>Anti-giantin preimmune stacks (%)</th>
<th>Anti-GM130 preimmune stacks (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSF/p97</td>
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<td>92±1.1</td>
<td>16±1.2</td>
<td>20±2.0</td>
</tr>
<tr>
<td>p97</td>
<td>95±1.4</td>
<td>97±3.7</td>
<td>21±1.7</td>
<td>18±2.1</td>
</tr>
<tr>
<td>NSF</td>
<td>91±1.9</td>
<td>99±2.2</td>
<td>22±1.7</td>
<td>19±3.4</td>
</tr>
</tbody>
</table>

Table 4.2 Effect of anti-giantin and anti-GM130 preimmune sera on reassembly using pure components. Reaction was carried out as in Figure 4.19, 4.20 except replacing antibodies with the appropriate preimmune serum. Values represent means ±SEM (n=3).

When MGF were pretreated with either anti-giantin or anti-GM130 and resuspended for the NSF pathway, both cisternal regrowth and as a consequence stacking were inhibited (Figure 4.19I-L, 4.20) and the preimmune sera had no effect (Table 4.2). That cisternal regrowth is inhibited strongly suggest that the interaction between GM130, p115 and giantin is essential for NSF mediated Golgi membrane fusion. This may be due to an inhibition of COPI vesicle tethering to Golgi membranes (Sonnichsen et al., 1998).
Figure 4.19 Effect of antibodies against giantin and GM130 on the reassembly process using pure components. MGF isolated through a 0.5M sucrose cushion were either held on ice for 15min (A, E, I), or preincubated on ice for 15min with 1μl anti-GM130 NN15 serum (B, F, J), 1μl anti-giantin serum (C, G, K) or a combination of both (D, H, L). The pretreated MGF were then incubated at 37°C for 60min with; p97 (70ng/μl), p47 (37.5ng/μl) and p115 (30ng/μl; E-H); or NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (30ng/μl; I-L); or all these components combined (A-D). Samples were processed for EM, large arrows denote stacks, small arrows denote single cisternae, and arrowheads denote unfused material. Note the inhibition of stacking but not fusion in B-D and F-H, and the inhibition of fusion in J-L. Bar, 0.5μm.
Figure 4.20 Quantitation of effect of antibodies against giantin and GM130 on the reassembly process using pure components. MGF isolated through a 0.5M sucrose cushion were either fixed and processed for EM, held on ice for 15min, or preincubated on ice for 15min with 1μl anti-GM130 serum, anti-giantin serum, or a combination of both. The pretreated MGF were then incubated at 37°C for 60min with: p97 (70ng/μl), p47 (37.5ng/μl) and p115 (30ng/μl); or NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (30ng/μl); or all these components combined. Samples were fixed and processed for EM, and the percentage cisternal regrowth ±SEM (n=3; A) and the percentage total membrane present as stacks ±SEM (n=3; B) was determined.
4.2.9 Gtn448 inhibits NSF catalyzed cisternal regrowth.

Giantin was first identified using a mAb raised against Golgi membranes (Linstedt and Hauri, 1993). It is predicted to be an extensively coiled-coil, rod like protein, and is a type II Golgi membrane protein with almost all of its mass projecting into the cytoplasm (Linstedt and Hauri, 1993). Deletion analyses of rat giantin have revealed that the N-terminal 15% (i.e. the N-terminal 448 amino acids) of giantin is sufficient to bind p115 both \textit{in vitro} and \textit{in vivo} (Lesa et al., 2000). The N-terminal 448 amino acids of giantin (Gtn448) was able to bind p115 in Golgi detergent extract and cytosol almost as well as full length giantin, and was able to compete with full length giantin for these interactions (Lesa et al., 2000). Overexpression of Gtn448 in NRK cells removed p115 from the Golgi apparatus, in a manner similar to microinjection of N73pep (the minimal p115 binding domain of GM130; Lesa et al., 2000; Nakamura et al., 1997; Seemann et al., 2000). For these reasons it was of interest to determine whether Gtn448 could interfere with NSF catalyzed cisternal regrowth, just as anti-giantin antibodies could.

MGF isolated through a 0.5M sucrose cushion (Figure 4.21A), were preincubated on ice for 15min with either buffer (Figure 4.21B), 3.25\mu M Gtn448 (a 50-fold molar excess over p115; Figure 4.21C), or 3.25\mu M Gtn1967-2541 a giantin fragment which shows no significant p115 binding (Figure 4.21D) and then incubated for 60min at 37°C for the NSF catalyzed reassembly reaction. Gtn448 significantly blocked NSF catalyzed cisternal regrowth (Figure 4.21C, E), and this was dose dependent (Figure 4.21F) and to a similar extent to which anti-giantin antibodies inhibit the reaction (Figure 4.20A). In contrast, stacks reassembled normally when MGF were preincubated with buffer or Gtn1967-2541 (Figure 4.21B, D, E). These results strongly suggest that the N-terminal 448 amino acid domain of giantin is that which participates in p115 function in Golgi reassembly. p115 then binds to the membrane distal portion of giantin. Giantin is anchored to the membrane by its C-terminal 25 amino acids, and is predicted on the basis of glycerol velocity gradients to adopt a 250nm long rod like structure (Linstedt et al., 1995). This is easily sufficient to protrude through the COPI coat (10-15nm) into the cytoplasm, and interaction with
Figure 4.21 Gtn448 inhibits NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion (A) were preincubated for 15min on ice with either: giantin fragment buffer (20mM Hepes-KOH, pH 7.4, 100mM KCl, 1mM magnesium acetate, 0.1mM DTT; B), Gtn448 (C), or Gtn1967-2541 (D) such that the final concentration of giantin fragment in the reassembly reaction was 3.25μM. Pretreated MGF were incubated at 37°C for 60min with NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (7.5ng/μl). Samples were processed for EM and the amount of cisternal regrowth was determined (E). Arrows denote reassembled Golgi stacks and arrowheads denote unfused tubulovesicular material. Bar, 0.5μm. (F) Increasing amounts of Gtn448 were titrated into the above reaction, and the amount of cisternal regrowth determined.
Chapter 4  p115 in Stacking of Reassembling Golgi cisternae

p115 could lead to concatenation to GM130, thus forming a flexible tether which may allow the COPI vesicle to come closer to the Golgi membrane to facilitate v-/t-SNARE pairing, and ultimately lead to membrane fusion.

4.2.10 N73pep inhibits NSF, but not p97 catalyzed cisternal regrowth, and also inhibits p115 dependent stacking of p97 generated cisternae.

GM130 was first identified as a highly immunogenic component of a detergent insoluble Golgi matrix (Nakamura et al., 1995). GM130 is an extensively coiled-coil, rod-like peripheral membrane protein which may have some degree of flexibility and is tightly anchored to the Golgi membrane at its C-terminus by the N-terminally myristoylated GRASP65 (Barr et al., 1998). The N-terminal 73 amino acids of GM130 represent the minimal p115 binding domain (Nakamura et al., 1997). A peptide comprising this domain, N73pep, was titrated into the NSF, p97 and NSF/p97 catalyzed reassembly reactions. The results were very similar to those achieved when anti-GM130 was included in the reaction. N73pep potently inhibited NSF catalyzed cisternal regrowth and as a consequence stacking in a dose dependent manner (Figure 4.22A, B, 4.23A). N73pep very clearly inhibited the rebinding of p115 to reassembling Golgi membranes (Figure 4.24A). As a control reaction the S25D N73pep mutant which binds p115 with a much lower affinity (Lowe et al., 1998b) was added to the reactions at the maximum level and had no effect on either NSF mediated cisternal regrowth/stacking (Figure 4.22A, C, 4.23D) or p115 rebinding to reassembling membranes (4.24B). These data strongly suggest that the effect of N73pep is due to preventing p115 from binding to reassembling membranes. p97 catalyzed cisternal regrowth was unaffected by the presence of N73pep (Figure 4.22D, E, 4.23B) in accordance with previous findings (Nakamura et al., 1997). However, N73pep did inhibit the p115 mediated stacking of p97 generated cisternae (Figure 4.22E, 4.23B), presumably by interfering with p115 binding, as the S25D N73pep mutant was unable to interfere with this process (Figure 4.22D, F, 4.23D). Similar results were obtained with the complete NSF/p97 reaction, in that N73pep inhibited stacking but not cisternal regrowth (Figure 4.22G, H, 4.23C). Those cisternae that did form in the presence of N73pep adopted the wrinkled morphology, indicative of p97 mediated
Figure 4.22 Effect of N73pep on NSF, p97, NSF/p97 catalyzed reassembly reactions. MGF isolated through a 0.5M sucrose cushion were preincubated for 15min on ice with either buffer (A, D, G), N73pep (B, E, H), or N73pep S25D (C, F, I), to achieve a final concentration of 80μM peptide in the reaction. The pretreated MGF were then incubated at 37°C for 60min with: NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl), and p115 (30ng/μl; A-C); or p97 (70ng/μl), p47 (37.5ng/μl) and p115 (30ng/μl; D-F); or all these components combined (G-I). Reactions were processed for EM, large arrows denote stacks, small arrows denote single cisternae, and arrowheads denote unfused material. Note the absence of fusion in B, and the absence of stacks in E and H. Bar, 0.5μm.
Figure 4.23 Quantitation of effect of N73pep on NSF, p97 and NSF/p97 catalyzed reassembly reactions. MGF isolated through a 0.5M sucrose cushion were preincubated for 15min on ice with increasing concentrations of N73pep, and then incubated at 37°C with either: NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (30ng/μl; A); or p97 (70ng/μl), p47 (37.5ng/μl) and p115 (30ng/μl; B); or all these components combined (C). As a control reaction the N73pep S25D mutant was used at the maximum concentration (80μM; D). The percentage total membrane present as cisterneae and stacks was determined. Values represent means ±SEM (n=3).
Figure 4.24 Effect of N73 pep and the mutant N73pep S25D on p115 binding to reassembling Golgi membranes. MGF isolated through a 0.5M sucrose cushion were preincubated for 15min on ice with increasing concentrations of N73pep (A) or the mutant N73pep S25D (B). The pretreated MGF were then incubated at 37°C for 60min with NSF (100ng/μl), α–SNAP (25ng/μl), γ–SNAP (25ng/μl), p115 (30ng/μl), p97 (70ng/μl) and p47 (37.5ng/μl). At the end of the reaction membranes were recovered by centrifugation, washed three times with reassembly buffer, and then fractionated by SDS-PAGE using a 7.5% gel, transferred to nitrocellulose, and probed for GM130 and p115 using specific antibodies.
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4.2.11 Soluble GRASP65 inhibits cisternal stacking without affecting cisternal regrowth.

An NEM sensitive, membrane bound component of MGF is essential for the stacking reaction during reassembly. This factor was identified as GRASP65 a highly conserved, N-myristoylated protein that exists as a tight complex with GM130 on the membrane (Barr et al., 1997). A soluble, nonmyristoylated recombinant form of GRASP65 inhibited stacking, but not cisternal regrowth in the cytosol based reassembly (Barr et al., 1997). This soluble GRASP65 was titrated into the NSF, p97 and NSF/p97 combined pathways of reassembly. In contrast to N73pep, the effect of soluble GRASP65 was very similar on NSF, p97 and NSF/p97 catalyzed reassembly, in that stacking was potently inhibited, but cisternal regrowth proceeded normally (Figure 4.25A,B, D, E, G, H, 4.26A-C). Stacking of cisternae could occur if the soluble GRASP65 was first pretreated with NEM (Figure 4.25C, F, I). Given the absence of cytosol in contrast to previous studies (Barr et al., 1997) it may be that soluble GRASP65 inhibits the stacking process by interacting with a Golgi membrane bound factor. This Golgi membrane bound factor is unlikely to be GM130 as soluble GRASP65 did not act to remove GM130 from the membranes (Figure 4.26D) which is consistent with the fact that the complex between GM130 and GRASP65 is very stable, and can only be reconstituted if both proteins are co-translated (Barr et al., 1998). Similarly, soluble GRASP65 did not appear to interfere with p115 rebinding to reassembling Golgi membranes (Figure 4.26D) nor inhibit NSF catalyzed cisternal regrowth for which p115 is essential (Figure 4.25B, 4.26A), and so presumably does not affect p115 function. The effect of soluble GRASP65 on the stacking reaction would then not appear to be due to disruption of the endogenous GRASP65-GM130
Figure 4.25 Effect of soluble GRASP65 on the NSF, p97, NSF/p97 catalyzed reassembly reactions. MGF isolated through a 0.5M sucrose cushion were preincubated for 15min on ice with either buffer (A, D, G), soluble GRASP65 (B, E, H), or NEM treated soluble GRASP65 (C, F, I) to achieve a maximum concentration of 75ng/µl in the final reaction volume. The pretreated MGF were then incubated at 37°C for 60min with either: NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl) and p115 (30ng/µl; A-C); p97 (70ng/µl), p47 (37.5ng/µl) and p115 (30ng/µl; D-F); or all these components combined (G-I). Reactions were processed for EM, large arrows denote reassembled stacks (which are prevalent in A,C, D, F, G and I), while small arrows denote single cisternae (which are prevalent in B, E and H). Bar, 0.5µm.
Figure 4.26 Quantitation of effect of soluble GRASP65 on NSF, p97 and NSF/p97 catalyzed reassembly reactions. MGF isolated through a 0.5M sucrose cushion were preincubated for 15min on ice with increasing concentrations of soluble GRASP65. Pretreated MGF were then incubated at 37°C for 60min with either: (A) NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl), and p115 (30ng/µl); or (B) p97 (70ng/µl), p47 (37.5ng/µl) and p115 (30ng/µl); or (C) all these components combined. Reactions were processed for EM, and the percentage total membrane present as cisternae (circles) and stacks (triangles) was determined. Values represent means±SEM (n=3). (D) Alternatively, at the end of the combined NSF/p97 catalyzed reaction membranes were recovered by centrifugation, washed three times with reassembly buffer, and then fractionated by SDS-PAGE using a 7.5% gel, transferred to nitrocellulose, and probed for GM130 and p115 using specific antibodies.
interaction and so is not affecting the stacking reaction by preventing p115 function. It may be that the soluble GRASP65 competes with the endogenous GRASP65 for other interactions which help promote stacking by anchoring cisternae together.

4.2.12 p115 is required before GRASP65 at an early stage in stack formation.

To assess the temporal sensitivity of the stacking reaction to N73pep and soluble GRASP65, MGF were incubated in the NSF/p97 purified reaction for increasing time at 37°C. At various time points (time of addition \([t]\) Figures 4.27A-D) the reaction was transferred to ice and either fixed with 2% glutaraldehyde and processed for EM or treated with buffer (KHM, the GRASP65 and N73pep solvent), N73pep, or soluble GRASP65 and then reincubated at 37°C for a total time of 60min.

The time course for the reassembly of stacked regions of cisternae in the NSF/p97 reaction displayed very similar characteristics to the rat liver cytosol catalyzed reaction (compare Figure 4.28A and 4.8). Cisternae first reformed, and then by 15min began to dock and align to form stacks (Figure 4.27A), these stacks then grew laterally, the total percentage membrane present as stacks steadily rising until the end of the incubation (Figure 4.27B-D, 4.28A). In a manner similar to the cytosol catalyzed reaction, the mean number of cisternae per stack increased much more rapidly than did the percentage total membrane present as stacks (Figure 4.29), suggesting stacks first form, and then grow laterally. The percentage total membrane present as cisternae (single and stacked) followed the kinetics in Figure 4.30A. p115 rebound very rapidly to the reassembling Golgi membranes (Figure 4.27A).

Addition of KHM buffer had no effect on the amount of cisternal regrowth nor stacking and had no effect on the amount of p115 bound to the Golgi membranes at the end of the incubation (Figure 4.28B, 4.30B). This suggests that the buffer and transferring the reaction to ice was not detrimental to the process.

The stacking process was sensitive to N73pep for the first 15min of the reaction (Figure 4.27E-H, 4.28C). When added at 15min, the time point when cisternae begin to
Figure 4.27 Temporal sensitivity of the stacking reaction to N73pep and soluble GRASP65. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for the indicated time with NSF (100ng/µl), α–SNAP (25ng/µl), γ–SNAP (25ng/µl), p115 (30ng/µl), p97 (70ng/µl) and p47 (37.5ng/µl). At the indicated time, the reactions were transferred to ice and either: fixed with 2% glutaraldehyde (A-D); or supplemented with N73pep to 80µM (E-H), or soluble GRASP65 to 75ng/µl (I-L). After 15min on ice N73pep and GRASP65 treated samples were transferred to 37°C and incubated for a total time of 60min. Samples were processed for EM, large arrows denote stacked cisternae, while small arrows denote single cisternae (note prevalence in E, I and J). Bar, 0.5µm.
Figure 4.28 Quantitation of the temporal sensitivity of the stacking reaction to N73pep and soluble GRASP65. MGF isolated through a 0.5M sucrose cushion were incubated for the indicated time (time of addition) at 37°C with NSF (100ng/μl), α–SNAP (25ng/μl), γ–SNAP (25ng/μl), p115 (30ng/μl), p97 (70ng/μl) and p47 (37.5ng/μl). At the indicated time, the reactions were transferred to ice and either fixed with 2% glutaraldehyde (A), or supplemented with buffer (B), 80μM N73pep (C), or 75ng/μl soluble GRASP65 (D). After 15min on ice, buffer-, N73pep-, and GRASP65-treated samples were transferred to 37°C and incubated for a total time of 60min. Samples were processed for EM and the percentage total membrane present as stacks at the end of the incubation ±SEM (n=4) was determined. The amount of p115 bound to Golgi membranes at the end of the incubation was also determined (shown below each graph).
Figure 4.29 Changes in the mean number of cisternae per stack during NSF/p97 catalyzed reassembly. Reassembly was conducted as in Figure 4.27A, and the mean number of cisternae per stack was determined. Values represent means±SEM (n=4).
Figure 4.30 Quantitation of the temporal sensitivity of cisternal regrowth to N73pep and soluble GRASP65. MGF isolated through a 0.5M sucrose cushion were incubated for the indicated time (time of addition) at 37°C with NSF (100ng/μl), α–SNAP (25ng/μl), γ–SNAP (25ng/μl), p115 (30ng/μl), p97 (70ng/μl) and p47 (37.5ng/μl). At the indicated time, the reactions were transferred to ice and either fixed with 2% glutaraldehyde (A), or supplemented with buffer (B), 80μM N73pep (C), or 75ng/μl soluble GRASP65 (D). After 15min on ice, buffer-, N73pep-, and GRASP65-treated samples were transferred to 37°C and incubated for a total time of 60min. Samples were processed for EM and the percentage total membrane present as cisternae at the end of the incubation ±SEM (n=4) was determined.
dock and align (Figure 4.27A, 4.9B), the N73pep actually unstacked those stacks that had formed suggesting that p115 was mediating this event (compare Figure 4.28A and 4.28C). At time points later than 15min the reassembled stacks became resistant to added N73pep, and normal stacking was able to proceed (Figure 4.27F-H). Cisternal regrowth was insensitive to N73pep added at any time (Figure 4.30C). RLG stacks are also unaffected by N73pep treatment (Figure 4.31B, D, 4.32) suggesting that this is a shared property of reassembled Golgi stacks and starting RLG stacks. At all time points tested N73pep was able to significantly remove bound p115 from the membranes, such that at the end of the incubation only 15% of the p115 was bound as compared to control reactions (Figure 4.28C). Thus, it was not that N73pep could no longer remove p115 from the membranes at later time points. The requirement for p115 then appears to be a transient event required for the initial docking and alignment of newly formed single cisternae.

The stacking process was sensitive to soluble GRASP65 for the first 30min of the process (Figure 4.27I-L, 4.28D). Soluble GRASP65 acted to unstack Golgi cisternae that had formed prior to this point (compare Figure 4.28A and 4.28D). However, beyond 30min the reassembled stacks became resistant to soluble GRASP65 which is also a property of the starting RLG stacks (Figure 4.31C, E, 4.32). Soluble GRASP65 treatment of starting RLG did not disrupt their stacked structure, neither the percentage total membrane as stacked regions of cisternae nor the mean number of cisternae per stack was affected (Figure 4.32B, C). At no time point did soluble GRASP65 affect p115 binding, the amount bound at the end of the incubation remained constant (Figure 4.28D). That the reassembled stacks remain sensitive to soluble GRASP65 for longer than they do to N73pep suggests that GRASP65 may act downstream of p115 in the stacking pathway. So raising the possibility that the stacking reaction proceeds by an initial p115 dependent tethering step, which is followed by a GRASP65 dependent stacking step.
Figure 4.31 Effect of N73pep and soluble GRASP65 treatment on RLG. RLG (A) at 0.75mg/ml were treated with N73pep (80µM; B and D) or soluble GRASP65 (75ng/µl; C and E) in reassembly buffer and an ATP regeneration system in a final volume of 20µl, and incubated either on ice for 15min (B and C) or at 37°C for 60min (D and E). Reactions were processed for EM, arrows denote the prevalent stacked structures found in all incubations. Bar, 0.5µm.
Figure 4.32 Quantitation of effect of N73pep and soluble GRASP65 on RLG. RLG at 0.75mg/ml were treated with N73pep (80μM) or soluble GRASP65 (75ng/μl) in reassembly buffer and an ATP regeneration system in a final volume of 20μl, and incubated for either 15min on ice or 60min at 37°C. Reactions were processed for EM, and the percentage total membrane present as stacks (A), cisternae (B) and the mean number of cisternae per stack (C) was determined. Values represent means±SEM (n=2).
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4.3 \textit{p}115 maintains normal stacked Golgi architecture under interphase steady state conditions.

4.3.1 Depletion of the interphase system of \textit{p}115.

The above experiments suggest a role for \textit{p}115 in the initial alignment and docking of cisternae as a first step in stack formation. Another question was does \textit{p}115 make a contribution to Golgi organization under steady state conditions? Although prior removal of \textit{p}115 from RLG does not affect the stacked structure \textit{per se}, it does affect the rate at which cisternae unstack during mitotic disassembly (Section 3.3.3), what would be the effect on Golgi structure if incubated in an interphase system depleted of \textit{p}115? An \textit{a priori} prediction would be that if the release of \textit{p}115 from the Golgi membrane at mitosis is the sole determinant of mitotic disassembly, depletion of \textit{p}115 from the interphase situation may compel it to mimic the mitotic situation.

To address these questions RLG were extracted with 1M KCl to remove any \textit{p}115, and then incubated at 37°C for 40min with either sHeLa interphase cytosol (Figure 4.33A), sHeLa interphase cytosol depleted of \textit{p}115 supplemented with \textit{p}115 (to its original level prior to depletion; Figure 4.33B), sHeLa interphase cytosol depleted of \textit{p}115 (Figure 4.33C, D) or sHeLa interphase cytosol supplemented with a 200-fold molar excess (over \textit{p}115) of N73pep (Figure 4.33E). RLG incubated with sHeLa interphase cytosol, or depleted cytosol supplemented with \textit{p}115, maintained stacks, which often adopted a curved morphology (Figure 4.33A, B). The peripheral rims of cisternae (if not circularized) were often blunt ended, or were distended with either a vesicle budding, or having recently fused, there was no accumulation of vesicles at the rims. The effects of removal of \textit{p}115 on Golgi architecture were striking in comparison. For 1M KCl extracted RLG incubated in \textit{p}115 depleted cytosol or cytosol supplemented with N73pep, cisternae and stacks of cisternae persisted although were less frequent (Figure 4.34A, B). Cisternae also tended to be slightly shorter in cross sectional length: 0.44µm and 0.47µm for \textit{p}115 depleted cytosol and N73pep supplemented cytosol, compared to 0.66µm and 0.72µm for cytosol and depleted cytosol with \textit{p}115 added back. This shortening of cisternae in stacks, leaving only the core of the stack, suggested some unstacking had occurred at the peripheral rim. This
Figure 4.33 Effect of incubation of 1M KCl extracted RLG in p115 depleted interphase cytosol. 1M KCl extracted RLG were incubated at 37°C for 40min with either: sHeLa interphase cytosol (A), p115 depleted sHeLa interphase cytosol supplemented with p115 (B), p115 depleted cytosol (C, D), or sHela interphase cytosol supplemented with N73pep (E). Reactions were processed for EM, note the persistence of stacks in all reactions (denoted by small arrowheads), but the accumulation of tubular network (large arrowheads) and small vesicles (arrows) in C-E, and also the reduced cisternal length in C-E. Bar, 0.5μm.
Figure 4.34 Quantitation of effect of incubating KCl extracted RLG with p115 depleted interphase cytosol. 1M KCl extracted RLG was incubated at 37°C for 40min with either sHeLa interphase cytosol, sHeLa interphase cytosol supplemented with N73pep, p115 depleted sHeLa interphase cytosol, or p115 depleted sHeLa cytosol supplemented with p115. Reactions were processed for EM and the percentage total membrane present as cisternae (A), stacks (B), tubular network (C) and vesicles (D) was determined. Values represent means±SEM (n=2).
difference was coupled to a large accumulation of free vesicles (arrows in Figure 4.33C-E) and also tubular network (arrowheads in Figure 4.33C, D) at the peripheral rims of stacks of cisternae. This accumulation was so great that it often extended far away from the peripheral rim (Figure 4.33C, D). The percentage total membrane present as tubular network was increased up to threefold in reactions lacking p115 (Figure 4.34C). The percentage total membrane present as vesicles increased from c. 12% when p115 was present to c. 20% when it was absent, or being competed for by N73pep (Figure 4.34D). These vesicles were the correct size to be COPI vesicles (i.e. c. 60-70nm in diameter).

These observations suggest that the usual events that occur at the peripheral rims of cisternae are perturbed in the absence of p115. Precisely, the accumulation of vesicles suggests that transfer of COPI vesicles between cisternae is affected in the system lacking p115. The presence of tubular network might indicate that a tubular mode of inter-compartmental transport has been implemented as is possible in vitro under some conditions where COPI vesicle budding is blocked (Orci et al., 1991; Elazar et al., 1994b; Happe et al., 1998). These changes may be due to disruption of the giantin-p115-GM130 complex which may crosslink cisterna to cisterna and COPI vesicle to cisterna at the peripheral rims of stacks. These changes in Golgi architecture in the absence of p115 are similar to observations made in vivo, where transport through the Golgi apparatus is inhibited (but not blocked) and COPI vesicles accumulate upon microinjection of N73pep into NRK cells, or upon overexpression of a GM130 mutant lacking the p115 binding site (Seemann et al., 2000).

The fact that cisternae are not completely consumed here as they are in mitosis suggests that the mitotic inhibition of p115 binding to GM130 cannot be the sole determinant responsible for mitotic disassembly. However, the fact that COPI vesicles seem to accumulate suggests that this event may contribute to the COPI dependent pathway of Golgi fragmentation. That the extent of vesicle accumulation is not as great as at mitosis, may indicate that there are other mitotic regulatory events affecting the consumption of COPI vesicles, possibly at the level of the SNAREs. Under these
interphase conditions, vesicles may still be able to fuse as the SNAREs should still be active. However, in the absence of p115 the equilibrium is shifted to the free vesicle, since p115 may facilitate efficient SNARE pairing (Sapperstein et al., 1996; Cao et al., 1998) and so vesicle consumption. As a consequence some cisternal shortening may occur, as is seen. Alternatively, other events which may disrupt Golgi architecture, such as the COPI independent pathway or cisternal unstacking (possibly due to GRASP65 phosphorylation), may be responsible for the greater accumulation of COPI vesicles at mitosis. Since these events will not be occurring under interphase conditions the Golgi remains intact. These observations are consistent with there being several mitotic regulatory events that act in concert to dismantle the Golgi apparatus, and that the inhibition of p115 binding to GM130 is just one of these events.

4.4 A role for a G-protein in stacking of reassembling Golgi cisternae.

4.4.1 GTPγS stimulates cisternal stacking, but not cisternal regrowth during reassembly.

GTPγS stimulates the stacking of Golgi cisternae without affecting the amount of cisternal regrowth in rat liver cytosol catalyzed reassembly (Rabouille et al., 1995c). This discovery was repeated here, MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with rat liver cytosol (10mg/ml) with increasing concentrations of GTPγS (Figure 4.35A-C, 4.36). Inclusion of GTPγS in the reaction stimulated stacking in a dose dependent manner (Figure 4.35A), without stimulating the amount of cisternal regrowth (Figure 4.36B). In fact, in the presence of 0.4mM GTPγS the percentage total membrane present as stacks was c. 36% very close to the level in starting RLG (42%). GTPγS also had the effect of clustering all membranes in the reaction closer together (Figure 4.35B, C and especially F). This suggests a regulatory role for a G-protein in cisternal stacking. The fact that [AlF4]− does not cause this same stimulation of stacking (Rabouille et al., 1995c), suggests that the G-protein involved is more likely to be a small G-protein and not a heterotrimeric G-protein (Chabre, 1990; Kahn, 1991; Wittinghofer, 1997) or a small G-protein: GAP
Figure 4.35 Effect of GTPγS on the reassembly of Golgi stacks. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with rat liver cytosol in the absence of GTPγS (A), or supplemented with 0.1mM (B) or 0.4mM (C) GTPγS. Alternatively, p115 depleted rat liver cytosol supplemented with 0.4mM GTPγS (D) or p115 depleted rat liver cytosol supplemented with p115 and 0.1mM GTPγS (E) or with p115 and 0.4mM GTPγS was used. Reactions were processed for EM, and arrows denote stacks, and arrowheads single cisternae. Note the increasing presence of stacks with increasing GTPγS in A-C and E-F, and the absence of stacks in D. Bar, 0.5μm.
Figure 4.36 Quantitation of effect of GTPγS on reassembly of Golgi stacks. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60 min with either: rat liver cytosol, p115 depleted rat liver cytosol, or p115 depleted rat liver cytosol supplemented with p115 in the presence of increasing concentrations of GTPγS. Reactions were processed for EM and the percentage total membrane present as stacks (A) and cisternae (B) was determined. Values represent means±SEM (n=2).
Cisternae reform but do not stack if reassembled in p115 depleted rat liver cytosol. It was tested whether inclusion of GTPyS could reverse this effect. Supplementing p115 depleted rat liver cytosol with 0.4mM GTPyS did not reverse this effect (Figure 4.35D), single cisternae reformed to the same degree as in the absence of GTPyS (Figure 4.36B), but did not stack (Figure 4.36A). Supplementing the depleted cytosol with purified rat liver p115 to the level it was at prior to depletion reversed these effects. GTPyS was again able to greatly stimulate stacking (Figure 4.35E, F, 4.36A), and the amount of cisternal regrowth was unaffected (Figure 4.36B). This implies that p115 itself was responsible for the effect, and not another factor that had been co-depleted from cytosol. This result suggests that the point of action of the putative G-protein is either coincident with or downstream of p115 in the stacking process.

An interesting possibility as to the identity of the G-protein comes from evidence in yeast that membrane levels of Ypt1p (Rab1) and Uso1p (p115) positively correlate, and are affected similarly by GDI (Cao et al., 1998). A similar situation is found in this system, if RLG is incubated at 37°C for 30min with rat liver cytosol (1mg/ml) in the presence of increasing concentrations of GDI, Rab1 is removed from the membrane and so too is p115 (Figure 4.37). Quantitation reveals that c. 90% of Rab1 is removed, and this compares to the c. 80% removal of p115. Mann I levels are unaffected, suggesting GDI was not causing any differences in membrane recovery. Thus, a possible explanation for the increased stacking in the presence of GTPyS is that it stimulates Rab1 association with the Golgi membrane (Soldati et al., 1994) and in so doing stimulates p115 levels, which in turn stimulates the stacking process. This suggested mechanism will require further work to clarify.
Figure 4.37 Effect of GDI on rab1 and p115 Golgi levels. (A) RLG was incubated at 37°C for 30 min with sHeLa interphase cytosol in the presence of increasing GDI. At the end of the reaction membranes were recovered by centrifugation and washed thrice with buffer, then fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies against p115, mann1, and rab1. (B) Films were scanned by a high resolution scanner at 600 dpi. Pixel densities were determined using NIH Image 1.51. Values represent the mean±SEM (n=2) expressed as a percentage, where 100% corresponds to the amount of marker bound in the absence of GDI.
4.5 Discussion.

4.5.1 A role for p115 in stacking reassembling Golgi cisternae.

A modified cell free assay has been employed to assess more closely p115 function in
cisternal regrowth and cisternal stacking. In this assay the MGF are isolated through a
0.5M sucrose cushion which renders them incompetent for cisternal regrowth in the
absence of added soluble factors due to the virtual absence of the membrane fusion
ATPases NSF and p97. Previously, treatment of the MGF with the cysteine alkylating
reagent NEM inactivated any residual NSF or p97 thus ensuring dependence on added
soluble factors (Rabouille et al., 1995b). However, this treatment inhibited the stacking
of the Golgi cisternae that reformed presumably due to modification of a conserved
cysteine on GRASP65 (Barr et al., 1997). The obviation of MGF NEM treatment
enabled the study of cisternal regrowth and cisternal stacking simultaneously in a
membrane polypeptide environment devoid of alkylated cysteines. Using this system a
novel, essential role for p115 in the stacking of reassembling Golgi cisternae was
elucidated as well as confirmation of the requirement for p115 in NSF-mediated
cisternal regrowth (Rabouille et al., 1995b).

Several lines of evidence strongly suggest a requirement for p115 in the stacking of
reassembling Golgi cisternae. Firstly, p115-depleted cytosol supports full cisternal
regrowth at cytosol concentrations above 1mg/ml, but not cisternal stacking suggesting
these are separable processes. Cisternal stacking is restored by addition of purified
p115 to the depleted cytosol. Reassembly conducted in p115-depleted cytosol at
maximum concentration for periods of well over 1 hour did support some stacking, but
the initial rate and overall extent of stacking were severely retarded. In the reassembly
assay conducted with purified fusion components NSF-dependent reassembly required
p115 for both stacking and cisternal regrowth. While in the p97-dependent reassembly
p115 was required for stacking but not cisternal regrowth. Similarly, when the
NSF/p97 pathways were combined, p115 was only required for cisternal stacking, as
was the case in the reassembly conducted in p115-depleted cytosol. Thus, we conclude
that p115 is able to tether cisterna to cisterna as well as COPI vesicle to cisterna, and in
so doing plays a role in cisternal stacking.
That p115 functions in both the NSF (for membrane fusion and stacking) and p97 (stacking only) pathways suggests that this may be another point where these two pathways intersect and so may be modulated. Syntaxin 5 is also a common component of the two pathways, and may explain why they contribute non-additively to cisternal regrowth (Rabouille et al., 1998). A modulatory role for p115 is perhaps reflected by the different cisternal morphologies the two pathways produce. A clear continuum exists whereby at one extreme the p97 pathway in the absence of p115 only generates long single cisternae whereas the NSF pathway in the presence of p115 generates stacks with three or more short cisternae. When both pathways are combined the result was approximately intermediate with no really long cisternae forming and stacks with only 2-3 cisternae per stack. Why the NSF pathway generates stacks with more cisternae per stack is not yet clear.

The number of cisternae per Golgi stack is a highly polymorphic phenotype both between organisms and even between different cell types in the same organism. For example, mammalian epithelial cells of the seminal vesicle have 2-3 cisternae per stack while the mucous cells of the Brunner's gland have 9-11 cisternae per stack (Rambourg and Clermont; 1997). The functional significance, if any, of these differences remains both unclear and moot (see Section 1.4.4). However, a cell might tailor its Golgi architecture according to its needs by modulation of the p97 and NSF pathways via p115 during Golgi biogenesis or reassembly.

### 4.5.2 The mode of fusion of NSF and p97.

The difference in cisternal length produced by the NSF and p97 pathways was not detected when MGF were pretreated with NEM (Rabouille et al., 1995b). This may reflect an NEM sensitivity of membrane bound components required for the p97 pathway. These may be involved in the tethering step of the reaction since p97 mediated fusion seems to be independent of p115 and has no known tethering molecules. In addition, these cisternal length differences may reflect the mode of fusion p97 and NSF catalyze. That the p97 generated cisternae are longer suggests that it may
be acting to fuse cisterna to cisterna in a homotypic fashion. In contrast, the NSF pathway may be acting to fuse COPI vesicle to its target membrane, a heterotypic fusion event, so generating numerous short cisternae that are less able to fuse homotypically.

The current working hypothesis is that the NSF pathway would reconstitute the Golgi rims while the p97 pathway would reconstitute the cisternal cores as has been suggested before (Rabouille et al., 1995b, 1998). Attempts are being made to verify this model. This model is in contrast to another system in which the drug IQ has been used to disassemble the Golgi apparatus into small fragments. After the removal of IQ, the reassembly pathway has been shown to involve the sequential action of NSF followed by p97 (Acharya et al., 1995a). This sequence does not lend itself to the simple rebuilding of cores by p97 and the rims by NSF. However, this sequence may be dictated by the fact that the fragments were generated by an IQ specific mechanism and not by a mitotic process so that although the end-products (stacked cisternae) are the same, the route of reassembly may well be different.

4.5.3 Wrinkled morphology of p97 generated cisternae.

Another feature of cisternae reassembled in the absence of p115 is their frequent wrinkled, corrugated morphology. This suggests p115 is required for a membrane smoothing event during the reassembly process. Analogy may be drawn to the post-mitotic reassembly of the nuclear envelope. In a cell free system that utilizes *Xenopus* egg extracts and scanning EM to visualize nuclear envelope assembly (Wiese et al., 1997) once membrane fusion has created a fully enclosed nuclear envelope the membrane at first appears wrinkled. The envelope is then ‘smoothed’ by a process that requires active transport by nuclear pore complexes and may be due to the uptake of soluble lamins and reassembly of the nuclear lamina on the nucleoplasmic face. It may be that the 15% of Golgi bound p115 that is resistant to salt extraction is deeply enmeshed in the Golgi matrix (Slusarewicz et al., 1994; Nakamura et al., 1995). The incorporation of this p115 back into this matrix at the end of mitosis may be responsible for the cisternal membrane smoothing event. It is conceivable that the
reformation of the Golgi matrix on the cytoplasmic face of the Golgi membrane causes a concomitant increase in membrane tension and so results in membrane smoothing. Perhaps p115 acts by establishing cis-interactions between GM130 and giantin, or by forming homo-oligomeric structures. In fact, one may compare p115 to the A-type lamins which are also released in a soluble state at mitosis (Gerace and Blobel, 1980), and are also extensively coiled coil dimers. p115 also bears significant resemblance to the nuclear matrix protein NuMA which is capable of self assembling into homo-oligomeric structures (Harborth et al., 1999).

4.5.4 p115 mediated stacking and NSF mediated cisternal regrowth utilize GM130 and giantin.

p115 mediated stacking requires both receptors for p115 on Golgi membranes, giantin and GM130. Pretreatment of MGF with antibodies against GM130 and/or giantin precluded cisternal stacking as well as NSF mediated cisternal regrowth. Interestingly, the minimal p115 binding regions of both giantin (Gtn448; Lesa et al., 2000) and GM130 (N73pep; Nakamura et al., 1997) also inhibited NSF catalyzed cisternal regrowth. Previously, the GM130-p115-giantin complex had been implicated in tethering COPI vesicles that had been isolated in the presence of GTPγS to Golgi membranes (Sönichsen et al., 1998). Since the vesicles could not uncoat it could not be proved that this tethered intermediate reflected a bona fide intermediate in the transport reaction. However, the fact that anti-giantin and anti-GM130 block COPI vesicle tethering and NSF mediated membrane fusion suggests that the GM130-p115-giantin complex does act in COPI vesicle tethering that then leads to NSF mediated membrane fusion.

The fact that GM130 appears to be largely excluded from COPI vesicles, and the relative effects of pre-blocking either COPI vesicles or Golgi membranes with either anti-giantin or anti-GM130 antibodies on subsequent COPI vesicle tethering, suggested that the tether was made up of giantin on the COPI vesicle linked to GM130 on the target membrane via p115 (Sönichsen et al., 1998). The most parsimonious explanation for p115 action in cisternal stacking is that it proceeds through this same
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hetero-ternary complex. This is supported by the fact that preincubation of MGF with either anti-GM130 or anti-giantin alone precludes stacking, suggesting that stacking cannot be operating through just GM130-p115-GM130 or giantin-p115-giantin cross bridges alone.

4.5.5 Implications for the mode of intra-Golgi transport.

Many of the factors required for the reassembly assay have a predominantly cis-Golgi localization. For example, GM130 (Nakamura et al., 1995), GRASP65 (Shorter et al., 1999), and p115 (Nelson et al., 1998). Giantin represents an exception as it is located around the periphery of stacks (Seelig et al., 1994; Shima et al., 1997). One explanation for this apparent cis-bias is that our RLG preparation is enriched for cis-medial markers relative to trans markers (Hui et al., 1998), and so the reassembly assay may be biased towards isolating cis-Golgi acting factors. Alternatively, factors that are necessary to establish the stacked Golgi structure may be concentrated at the cis-face as part of the biogenetic process that is constantly occurring in interphase cells as proposed by the cisternal maturation model of Golgi transport (Pelham, 1998). The mitotic disassembly of the Golgi apparatus may then represent the conversion of Golgi membranes to a form that is essentially the same as VTCs. Indeed, mitotic Golgi clusters and VTCs display very similar morphological characteristics in vivo. Telophase reassembly would then proceed as a process akin to the fusion of these VTCs to form Golgi cisternae.

Whether the cisternal maturation model, the vesicular transport model or a synthetic model is true, all models have a requirement for the transfer of COPI vesicles between successive layers of the stack. The directionality/content of these vesicles varies between models (Pelham, 1998) and awaits in vivo confirmation at least for Golgi enzymes, if not cargo (Orci et al., 1997). That p115 plays a role in the establishment of the Golgi stack indicates that the mechanism of intra-Golgi transport may be ‘hard wired’ in to the structure of the stack. One might envisage the existence of a continuum of the giantin-p115-GM130 heteroternary complex. Whereby, at the cisternal rim this complex links COPI vesicle to cisterna and on moving towards the cisternal core links
cisterna to cisterna. In this way a COPI vesicle may already be linked to its acceptor cisterna before budding is completed. Intra-Golgi transport would then proceed by transfer of COPI vesicles that are pretethered to their acceptor membrane, rather than release of COPI vesicles by the donor membrane followed by capture by the acceptor membrane. This would increase the efficiency of the reaction by eliminating the reliance on a vesicle meeting its target membrane by random collision, and reduce the chance of losing the vesicle in the vast surrounding cytoplasm. Since giantin is most likely to enter the budding COPI vesicle, the orientation of the complex might even help determine the next cisterna with which the COPI vesicle is to fuse. The fact that removal of p115 from the interphase system shortens the length of cisternae in the stack and disrupts the peripheral rim architecture of the stack, in a manner where vesicles (COPI size) and tubular network accumulate provide support for this model.

Such a model would seem to be inconsistent with the findings that budded vesicles, both in vivo and in vitro, will fuse with the correct cisternae even when it is present in a different stack, and do so with the same speed and efficiency (Rothman et al., 1984a, b). However, the in vitro evidence may be explained by mechanical fragmentation breaking tethers and so allowing vesicle transfer between distinct stacks (Dominguez et al., 1999). Alternatively, collisions between stacks may allow the transfer of vesicles between them. It is then interesting to note that when stacks are stably attached to a surface, so collisions between them cannot occur, vesicles no longer transfer between stacks (Weidman et al., 1993). The in vivo evidence comes from the transfer of proteins between distinct Golgi stacks after fusion of cells, one population of which was infected with VSV and deficient in NAGTI activity and the other wild type. Rothman and colleagues now interpret this result differently (Orci et al., 1998). It may be that a combination of the cytopathic effects of viral infection and the pH 5 treatment needed for cell fusion altered the membrane permeability of the cell, and in so doing increased intracellular salt concentrations which would weaken tethers and so COP1 vesicle attachment. Vesicles could than transfer between discrete stacks. Alternatively, as the cytoplasms of the fusing cells forcefully mix, stacks may collide and transiently
Comparison of COPI vesicle production by the Golgi apparatus under interphase and mitotic conditions reveals an apparent capacity to generate twice as many COPI vesicles at mitosis with the same content (Sönnichsen et al., 1996). This suggests the Golgi stack may be seen as a capacitor for COPI vesicle flow. At mitosis the Golgi stack is 'opened up' and eventually disappears and more COPI vesicles form, as compared to the 'closed' stack during interphase. This may be due to more Golgi rim being available for COPI vesicles to bud from, such that as more rim is available COPI vesicle flux increases and vice versa. The amount of rim available for COPI vesicle formation may be determined by how much of the giantin-p115-GM130 complex is sequestered tethering cisterna to cisterna. This complex is abolished during mitosis by Cdc2 kinase mediated phosphorylation of GM130, and may be disrupted by direct phosphorylation of p115 during interphase (Sohda et al., 1998; but see Chapter 6). The ratio of complex tethering cisterna to cisterna and cisterna-COPI vesicle may then be tailored to suit the COPI vesicle flow needs of the cell. That the giantin-p115-GM130 complex is essential for establishing stacked structure after mitosis and acts to stabilize stacked architecture at steady state, suggests that the stacked structure is coupled to processive intra-Golgi COPI vesicle flow.

4.5.6 \textit{p115} acts upstream of GRASP65 in the stacking reaction.

It has been previously shown that GRASP65, which anchors the C-terminus of GM130 to the Golgi membrane, is involved in the stacking process (Barr et al., 1997). How \textit{p115} function and GRASP65 function relate to promote cisternal stacking during reassembly was assessed by determining the temporal sensitivity of the stacking reaction to agents that specifically interfere with \textit{p115} (N73pep) and GRASP65 (soluble, nonmyristoylated GRASP65) function. The soluble GRASP65 neither removed GM130 from the Golgi membrane, prevented \textit{p115} rebinding to reassembling Golgi membranes, nor inhibited NSF mediated cisternal regrowth. Therefore, it seems unlikely that soluble GRASP65 is acting to disrupt the endogenous GRASP65-GM130
interaction and so does not interfere with stacking by preventing p115 function. Rather the soluble GRASP65 is more likely to be competing with the endogenous GRASP65 for other interactions. In the combined NSF/p97 reassembly system both N73pep and soluble GRASP65 potently inhibited stacking without affecting cisternal regrowth. The stacking reaction remained sensitive to soluble GRASP65 for longer than it did to N73pep, suggesting that p115 acts upstream of GRASP65 in cisternal stacking.

The 15min time point of reassembly, where N73pep has its most potent effects, is the stage when single cisternae begin to dock and align to form stacks (Rabouille et al., 1995c). p115 may be required for this initial meeting of the cisternal membranes and then pass on the stacking function proper to another machinery, that likely involves GRASP65. The giantin-p115-GM130 complex would then not be essential for steady state stacking *per se*, and this is consistent with the Golgi stack’s resistance to 1M KCl and N73pep which remove c. 85% of p115. Stacking seems to proceed by the formation of stacks followed by their lateral growth (Souter et al., 1993). Since agents which interfere with p115 and GRASP65 can completely block stacking, this implies that p115 and GRASP65 may affect stacking at all levels of the stack.

Precisely how GRASP65 acts to stack cisternae and precisely how soluble GRASP65 interferes with this reaction remains obscure. One possibility is that the oligomeric state of GRASP65 may be important for anchoring cisternae together. GRASP65 appears to be either a dimer or a trimer (Barr et al., 1998). It may be that GRASP65 monomers insert their myristoyl groups into opposite membranes of adjacent cisternae, so holding them together. Soluble GRASP65 may then prevent the endogenous GRASP65 from interacting with itself and in so doing induce the formation of inactive oligomers. Alternatively, there may be as yet unidentified GRASP65 interacting molecules which are titrated out by the soluble GRASP65 that help to promote stacking. It will be important to determine the precise higher order structure of the GM130-GRASP65 complex, and to elucidate other GRASP65 interacting Golgi molecules before a molecular mechanism of stacking can be established.
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Analogy may be drawn to the proposed mechanism of vesicular transport, where p115 acts at an early stage in tethering the COPI vesicle to its acceptor membrane, and then hands over to the SNAREs to complete the fusion step. Similarly, in cisternal stacking p115 may act at an early stage in tethering cisternal membranes together, and then hand over to another set of molecules that complete the stacking reaction. GRASP65 (and GRASP55; Chapter 5) is an excellent candidate for one of these downstream factors. However, given that p115 acts in conjunction with SNAREs for transport this may also be true for stacking as well.

One of the elaborations of the SNARE hypothesis is that Golgi stacking reflects the v-SNAREs in each cisterna interacting with the cognate t-SNARE in the next (Rothman and Warren, 1994). The intercisternal matrix might be so dense as to act as a fusion clamp so the SNAREs do not promote fusion at the cisternal core. Alternatively, there may be fusion resistant isoforms of the SNAREs specialized for stacking. This extension of the SNARE hypothesis would explain the ordered stacking, close apposition, and constant spacing of stacked cisternae. Furthermore, it is interesting to note that careful localization of the Golgi t-SNARE syntaxin-5 and the v-SNARE membrin by immunoelectron microscopy reveal that these two SNAREs penetrate deeply into the stack (Hay et al., 1998). In PC12 cells syntaxin-5 is found at equal concentrations over the first 4 cisternae of the stack, while membrin is more concentrated in the first two cis-cisternae, but is also present at lower concentrations in later cisternae. Interestingly, these SNAREs do not appear to be restricted to the cisternal rims, the sites of vesicle traffic, but penetrate into the intercisternal matrix between the cisternal cores which would be consistent with a role in cisternal stacking.

GTPγS also stimulates the cytosol driven stacking process, without affecting the amount of cisternae that reform. However, GTPγS is unable to stimulate stacking in p115 depleted cytosol, suggesting the point of action of the GTPγS activated factor is either coincident or downstream of p115 function. The fact that [AlF₄]⁻ is unable to produce the same effect as GTPγS suggests the G-protein of interest may be a small G-protein rather than a heterotrimeric (Kahn, 1991; Wittinghofer, 1997). A possible
Explanation for the GTPγS stimulated stacking would be the enhanced recruitment of Rab1 to the membrane which may in turn stimulate p115 binding to the membrane and so enhance stacking. This model awaits verification, but the involvement of a Rab protein may also indicate that the SNAREs are involved as well, as suggested above. The stacking reaction has also been shown to have a microcystin sensitive component (Rabouille et al., 1995c), the identification and the point of action of which will prove revealing. This component may be a phosphatase which catalyzes the dephosphorylation of GRASP65 and/or GM130 and so facilitates the stacking reaction. The trimeric form of PP2A with the Bα regulatory subunit is the phosphatase responsible for the dephosphorylation of GM130 (Lowe et al., 2000). It may be possible to test this possibility directly by a depletion/add back approach by depleting the system with microcystin coupled to agarose and then adding back purified phosphatases, as has been performed for the homotypic vacuole fusion assay (Peters et al., 1999). The challenge ahead is to further elucidate the pathway that leads to stack formation at the end of mitosis, and to attempt to verify this pathway in vivo.
Chapter 5

GRASP55, a second mammalian GRASP molecule, involved in the stacking of reassembling Golgi cisternae at the end of mitosis
5.1 Introduction

In the previous chapter a cell free approach has clarified a possible molecular sequence of events that may lead to the formation of stacked cisternae at the end of mitosis. The first stage of this sequence entails p115 induced tethering and alignment of nascent single cisternae through simultaneous interaction with its two Golgi receptors GM130 and giantin on adjacent cisternae (Shorter and Warren, 1999). After this stage agents that remove p115 from the membrane (e.g. N73pep) have no effect on the stacked structures that have formed. A putative G-protein may play a role coincident, or downstream of p115 in the stacking reaction. The second stage of the reaction involves GRASP65 action, but the mechanics of how GRASP65 performs this function remain obscure. A soluble form of GRASP65 is able to compete for this stage of the reaction, and may compromise membrane associated GRASP65 activity by competing for as yet unknown binding proteins, or by affecting the oligomeric state of membrane associated GRASP65. An unidentified microcystin sensitive factor is also required for the stacking process (Rabouille et al., 1995c).

In this chapter as part of the search for additional membrane associated factors required for the formation or maintenance of the stacked structure of the Golgi apparatus the identification and characterisation of GRASP55 is reported. GRASP55 is a second mammalian form of the Golgi stacking protein GRASP65. This work was carried out in collaboration with Dr Francis Barr (IBLS, Division of Biochemistry and Molecular Biology, University of Glasgow) who performed the GRASP55 cloning, expression, localization and GM130 binding studies (Figure 5.1-5.9) and whose data is presented here with permission.

5.2 GRASP55, a novel GRASP molecule.

5.2.1 Cloning of a mammalian GRASP65-related protein.

During the course of previous studies on GRASP65 a number of antibodies have been raised against either the full-length protein expressed in bacteria, or synthetic peptides (Barr et al., 1997, 1998). One of these antibodies, FBA19, raised against the sequence YLHRIPTQPSSQYK (underlined in Figure 5.1), which is conserved amongst the
Figure 5.1 Comparison of the GRASP55 and GRASP65 amino acid sequences. Alignment of the GRASP55 (AF110267) and GRASP65 (AF015264) sequences; shading indicates identity and boxed residues are conserved. Residues in GRASP65 important for GMI30 binding are indicated by asterisks. Underlined residues indicate the peptide used to raise FBA19 antiserum. The shared cdc2 kinase site is marked with a triangle. Exclamation mark denotes a highly conserved cysteine residue.
known forms of GRASP65 from various yeasts and C. elegans, recognizes 65kDa and 55kDa proteins in RLG (Figure 5.3A). Other antibodies to GRASP65, which recognize epitopes in the less well conserved C-terminal domain of the protein, recognize only a 65kDa protein in Golgi membranes (Barr et al., 1997). This raised the possibility that there might be a second form of GRASP65, a view supported by the existence of expressed sequence tags (ESTs) with only partial homology to the known rat GRASP65 sequence. A RACE cloning strategy was adopted in order to obtain a clone for the putative GRASP65 homologue, using nested primers designed from a mouse testis EST (Genbank accession number AA061790). To obtain 5' and 3' clones corresponding to this GRASP65 related protein, nested primer pairs TR1 to TR4, and adapters primers AP-1 and AP-2 were used. These clones were sequenced and a new pair of primers designed to amplify the full open reading frame. The putative GRASP65 homologue is encoded by a 2kb cDNA (Genbank accession number AF110267), with a polyadenylation sequence and poly-A tail at the 3' end, and an ATG with a Kozak consensus sequence near the 5' end. Analysis of the predicted open reading frame of this clone revealed that it had a high level of homology, but not identity with GRASP65 in the first 212 amino acids, and after this point became highly divergent (Figure 5.1). This indicated a distinct gene product and not a splice variant of GRASP65.

The cDNA encodes a 454 amino acid protein with a predicted $P_i$ of 4.84 and molecular weight of 55kDa, confirmed by in vitro translation (Figure 5.8A, lane 9) and western blotting with specific antibodies (Figure 5.3A, lane 1), and it was therefore named GRASP55. Like GRASP65, it has a consensus site for myristoylation at the N-terminus (i.e. MGXXXSXX; Johnson et al., 1994) and could be anchored to membranes by means of this modification (Barr et al., 1997). The sequence in this N-terminal first 21 amino acids is slightly divergent in the two proteins, and GRASP55 has an insertion at position 14, the only point in the first 212 amino acids at which the two proteins are not co-linear. This raises the possibility that they are not targeted to the same regions of the Golgi apparatus, or that their interaction with membranes is differentially regulated. Comparison of the first 212 amino acids of GRASP65 and 213
amino acids of GRASP55 reveals that 66% of residues are identical and 14% conserved in this region, with the residues currently known to be important for GM130 binding being identical (asterisks in Figure 5.1; Barr et al., 1998). The region against which the FBA19 antibody was raised is partially conserved between the two proteins, YLHRIPTRPFEEGK in GRASP55 and YLHRIPTQPSSQYK in GRASP65 (underlined in Figure 5.1), thereby explaining why it sees two bands in purified Golgi membranes.

While GRASP65 is the major mitotic phosphoprotein in Golgi membranes, there is no such obvious mitotic phospho-form of GRASP55 (Barr et al., 1997). This could be explained by the fact that GRASP65 has a large number of putative serine phosphorylation sites in its C-terminal domain while GRASP55 does not, especially for glycogen synthase kinase 3 (GSK3) and casein kinase II (CKII), as revealed by running the two sequences through Phosphobase (Kreegipuu et al., 1999). The two proteins do share a conserved potential Cdc2 kinase site at position 219 of GRASP65 (triangle in Figure 5.1; Kreegipuu et al., 1998), raising the possibility that both are in fact regulated by cell cycle dependent phosphorylation, and that this modification may contribute to the unstacking of Golgi cisternae that occurs at mitosis.

To determine the expression patterns of GRASP65 and GRASP55, Northern blots on a panel of rat tissues were performed using a combination of probes specific or common to the two mRNAs (Figure 5.2). A probe specific for GRASP65 recognized a message at the correct size in all tissues, although only very faintly in spleen (Figure 5.2A). As previously reported a second message of approximately 1.5kb was observed in testis (Barr et al., 1997). The GRASP55 specific probe detected two messages of slightly smaller size than seen by the GRASP65 probe, again present in all tissues (Figure 5.2B). The lower band of this doublet was especially noticeable in liver, but was visible in other tissues upon longer exposure of the blot. In testis, the lower band of the doublet was more prominent than the upper band in contrast to other tissues, and a second smaller message this time of approximately 1.4kb was also observed. A probe common to both GRASP65 and GRASP55 gave a complex pattern of bands.
Figure 5.2 GRASP55 and GRASP65 are both ubiquitously expressed. A Northern blot of the rat tissues indicated in the figure was hybridized with probes specific for (A) GRASP65, (B) GRASP55 and (C) a probe common to both GRASP65 and GRASP55. As a control the blot was also hybridized with an actin specific probe (D).
corresponding to the addition of the signals seen with the GRASP65 and GRASP55 specific probes (Figure 5.2C). An actin probe used as a control for the loading of the Northern blot, gave the expected pattern of hybridization (Figure 5.2D). Therefore, messages for GRASP65 and GRASP55 would appear to be present in all tissues tested, with multiple possible splice variants being present in testis.

To confirm that the proteins are actually expressed, western blots were performed using protein extracts from these tissues. These western blots were probed with antibodies specific to GRASP65, GRASP55, and GM130 (Figure 5.3). To demonstrate these antibodies are in fact specific for the GRASP65 and GRASP55, RLG were blotted with these and with an antibody that sees both proteins. In Golgi membranes, the sheep polyclonal FBA34 recognizes a 55kDa protein, the 7E10 monoclonal to GRASP65 sees a 65kDa protein, and the FBA19 antibody recognizes both GRASP65 and GRASP55 (Figure 5.3A). GRASP65 often appears as a doublet, which may reflect either proteolysis or the fact that GRASP65 is extensively phosphorylated during interphase (Barr et al., 1997), and so the two bands may represent distinct phosphorylated forms of GRASP65.

The antibody to GRASP65 detected a protein of 65kDa in all tissues, with a fainter second band in testis at approximately 60kDa possibly corresponding to the second messenger RNA seen in this tissue (Figure 5.3B). Similar results were obtained with an antibody to GRASP55, this detected a 55kDa band in all tissues (Figure 5.3C). Blotting for the GRASP65 partner protein GM130 revealed this protein is also present in all the tissues examined (Figure 5.3D). The ratio of GRASP65 to GM130 does vary between tissues, and this may have implications for tissue specific variations in stacked Golgi structure. Together, these data demonstrate that GRASP55 is ubiquitously expressed in mammalian tissues, and therefore like GRASP65 could act in the stacking of Golgi cisternae.
**Figure 5.3** Tissue Western blots for GRASP55 and GRASP65. (A) Purified rat liver Golgi membranes were fractionated by SDS-PAGE, transferred to nitrocellulose and probed for GRASP55, with polyclonal FBA34, GRASP65, with monoclonal 7E10, GRASP55+GRASP65, with polyclonal FBA19 or GM130, with polyclonal MLO-7. Western blots of the tissues indicated in the figure were probed with these antibodies specific for either GRASP65 (B), GRASP55 (C), or GM130 (D).
5.2.2 Localization of GRASP55 to the Golgi apparatus.

The N-terminal domain of GRASP65 when fused to GFP can target to the Golgi apparatus, something that is abolished by mutations in its GM130 binding site (Barr et al., 1998). Given the similarity between the N-terminal domains of GRASP65 and GRASP55 it was likely that GRASP55 would also target to the Golgi apparatus. To test this, full-length GRASP65 and full-length GRASP55 fused to GFP were transfected into HeLa cells either singly or together (Figure S.4). When HeLa cells were transfected with constructs for either rat GRASP65 (Figure S.4A), or GRASP55-GFP (Figure S.4B) a pattern typical of the perinuclear ribbon-like structure of the Golgi apparatus was observed. To confirm this was indeed the Golgi apparatus, equivalent samples were stained with antibodies to the Golgi marker protein GM130, and GRASP65 (Figure S.4C), or GRASP55-GFP fluorescence visualized (Figure S.4D). Comparison of the individual staining patterns reveals that GRASP55, like GRASP65 and GM130 is localized to the Golgi apparatus. Cells were also transfected with both GRASP65 and GRASP55-GFP to allow a direct comparison of the distributions of these two proteins (Figure S.4E). Comparison of the two images shows that GRASP65 and GRASP55 have similar distributions within the Golgi apparatus.

The targeting of GRASP65 to the Golgi apparatus requires its N-terminal myristoylation site, and a series of residues in the domain which is involved in binding to GM130 (Barr et al., 1998). To find out if the targeting of GRASP55 was similar to that of GRASP65, point mutants were constructed analogous to those known to abolish GRASP65 targeting. When the glycine at position 2 was mutated to alanine, GRASP55 G2A, the protein was found to accumulate in the cytoplasm with only a very weak signal for the Golgi apparatus (Figure 5.5A). Mutations of two residues that cause a complete loss of binding to GM130 and of Golgi targeting in GRASP65, the G196A and H199A mutations (Barr et al., 1998), caused only a partial defect in Golgi targeting when introduced into GRASP55, G197A (Figure 5.5B) and H200A (Figure 5.5C), seen as an increased diffuse cytoplasmic fluorescence relative to the wild-type protein (Figure 5.4B). These data show that GRASP55 localizes to the Golgi apparatus, and that this requires the N-terminal myristoylation site. A recent study
Figure 5.4 Localization of GRASP55 to the Golgi apparatus by immunofluorescence. HeLa cells were transfected with rat GRASP65 (A and C), rat GRASP55-GFP (B and D), rat GRASP65 and GRASP55-GFP (E). Cells were processed for immunofluorescence with antibodies to rat GRASP65 only (A and E), rat GRASP65 and GM130 (C) or GM130 only (D). GFP fluorescence was used to visualize GRASP55 (B and D).
Figure 5.5 Determinants of GRASP55 localization to the Golgi apparatus. HeLa cells were transfected with the GRASP55-GFP mutants G2A (A), G197A (B) and H200A (C). Cells were processed for immunofluorescence with antibodies to GM130 (A-C). GFP fluorescence was used to visualize GRASP55 (A-C).
revealed that myristoylation of GFP chimeric proteins is insufficient to target them to the Golgi, but sufficient for endosome or ER localization (McCabe and Berthiaume, 1999). Targeting to the Golgi apparatus may then require a second lipid modification, a polybasic domain or an interaction with another protein (McCabe and Berthiaume, 1999). Correct targeting of GRASP55 may require an interaction with another protein, as is the case for GRASP65, where the accessory protein is GM130. However, unlike GRASP65, mutations in the putative GM130 binding region have only a small effect on the Golgi localization of GRASP55 implying this does not require interaction with a target protein at this site, or that the mutations have no effect on binding of GRASP55 to its target protein.

To determine where GRASP55 and GRASP65 were localized within the Golgi apparatus, antibody labelling on cryo-sections of HeLa cells transfected with rat GRASP65 and GRASP55-GFP was performed (Figure 5.6). Under the electron microscope it could be seen that the labelling for both proteins was over the Golgi apparatus, consistent with the localization at the light microscope level. GRASP65 labelling was typically seen over the cis-face of the stack (Figure 5.6A). The cis-aspect of the stack was distinguished from the trans-aspect morphologically on the basis that clathrin coated vesicles are only associated with the trans-side of the stack, COP coats are associated predominantly with the cis-side of the stack, and the TGN adopts a more ‘peeling off’ configuration (Rambourg and Clermont, 1997). GM130 has been localized to the cis-Golgi by cryo-EM and this was confirmed by double labelling experiments with the trans-Golgi marker SialylT (Nakamura et al., 1995). Given that GRASP65 interacts with GM130 to form a tight complex, and that the vast majority, if not all, of GM130 is complexed to GRASP65 as determined by immunodepletion of Golgi extracts (Barr et al., 1997, 1998) suggests that GRASP65 would also localize to the cis-Golgi. Furthermore, confocal microscopy and immunofluorescence reveal that GRASP65 and GM130 co-localize very closely at the cis-side of the Golgi and this staining is distinct from that of TGN38 or GalT, bona fide trans-Golgi markers (Barr et al., 1997; Francis Barr and Joachim Seemann, personal communication). Double labelling cryo-EM experiments with a trans-Golgi marker are currently underway to
Figure 5.6 Localization of GRASP55 and GRASP65 within the Golgi apparatus by cryo-electron microscopy. HeLa cells transfected with rat GRASP65 and GRASP55-GFP were processed for cryo-electron microscopy. Cryosections were labelled with a rabbit polyclonal FBA31 or the 7E10 monoclonal antibody to rat GRASP65, or a polyclonal antibody to GFP to detect GRASP55-GFP. (A) Single FBA31 labelling for GRASP65. (B) Single GFP labelling for GRASP55. (C and D) Double labelling for GRASP65 with 7E10, large gold particles marked by arrows, and GRASP55 with anti-GFP, small gold particles. N marks the nucleus in C and the scale bar denotes 0.25 μm.
unequivocally confirm this. In addition, it cannot be ruled out that GRASP65 is present further into the stack but is simply not accessible to antibodies. The observation that GRASP65 only becomes accessible to the small alkylating agent NEM after treatment of stacked Golgi membranes with mitotic cytosol indicates that it is sequestered in some protein complex in these structures, and indicates that it may not be easily accessible to some antibodies under native conditions.

GRASP55 labelling was found to be more over the stack with some labelling over the cis-face of the Golgi (Figure 5.6B), and was at a similar level to GRASP65. Double labelling experiments with a trans-Golgi marker are currently underway to confirm this. Double labelling experiments revealed that GRASP65 labelling (Figure 5.6C, D, large gold), marked by the arrows, is more concentrated at the cis-face of the Golgi stack, while GRASP55 labelling (Figure 5.6C, D, small gold) is seen over the stack and at the cis-Golgi cisternae. The labelling efficiency for GRASP65 in the double-labelling experiments was lower than that seen for GRASP55-GFP, and also lower than for GRASP65 detected by a polyclonal antibody in single labelling experiments. This might be explained by the use of a monoclonal to detect GRASP65, which by definition sees only a single epitope, in the double-labelling experiments, as opposed to the use of a polyclonal antibody, likely to recognize multiple epitopes, to detect GRASP55-GFP.

In order to be able to compare the localizations of GRASP65 and GRASP55 within the Golgi apparatus, the distributions of gold particles corresponding to antibody labelling of the proteins were quantitated. Due to the differences in labelling efficiencies discussed above this was carried out for both the single and double labelling experiments, and the results compared. For the single labellings, the distribution of gold particles over 22, GRASP65 (133 gold particles), and 20, GRASP55 (147 gold particles), Golgi regions with a defined cis to trans polarity were quantitated. Plotting these distributions as a function of cisterna reveals that GRASP65 (Figure 5.7A, shaded bars) is present over the first cisterna, while GRASP55 (figure 5.7A, open bars) is mainly present over the second and third cisternae. A Mann-Whitney test
Figure 5.7 Quantitation of localization of GRASP55 and GRASP65. Distributions of gold particles over Golgi cisternae were quantitated for GRASP65 and GRASP55 in both single (A) and double (B) labelling experiments. The percentage of gold particles is plotted for each Golgi cisterna from cis (1) to trans (5).
revealed that these two distributions were significantly different in location, P=0.000. For the double labellings, the distribution of gold particles for GRASP65 (34 gold particles) and GRASP55 (97 gold particles) over 14 Golgi regions with a defined cis to trans polarity were quantitated. Plotting these distributions as a function of cisterna reveals that GRASP65 (Figure 5.7B, shaded bars) is present over the first cisterna, while GRASP55 (Figure 5.7B, open bars) is mainly present over the second and third cisternae. Again, a Mann-Whitney test revealed these two distributions to be significantly different in location, P=0.0000. From these data it appears that GRASP65 is located at the cis-face of the Golgi, while GRASP55 is found more over the stack. This is consistent for GRASP65 with the localization of its binding partner GM130, to the cis-Golgi at the electron microscope level (Nakamura et al., 1995).

5.2.3 GRASP65 and GRASP55 binding to GM130

Given the high degree of homology between the two proteins in the region previously shown to be important for binding to GM130, it was determined biochemically whether, like GRASP65, GRASP55 interacts with GM130. To test this, the same in vitro transcription-translation system developed to demonstrate and characterize the interaction between GRASP65 and GM130 was used (Barr et al., 1998). In vitro translation reactions were carried out with plasmids encoding wild-type, ΔN441 and ΔC887 deletions of GM130, GRASP65, and GRASP55 as indicated in the figure legend. Aliquots of the total reactions were analysed by SDS-PAGE and autoradiography, the position of GRASP65 is marked by an open triangle and that of GRASP55 by filled triangle (Figure 5.8A, B). To assay for an interaction between GM130 and GRASP65 or GRASP55, immunoprecipitations were performed from these in vitro translation reactions with antibodies to GM130. Analysis of the bound material revealed that antibodies to GM130 precipitated GM130 (Figure 5.8B, lane 1) but not GRASP65 or GRASP55 (Figure 5.8B, lanes 8 and 9). If GRASP65 was translated together with GM130 it was found to be co-precipitated by antibodies to GM130 (Figure 5.8B, lane 2) whereas under the same conditions GRASP55 was not co-precipitated with GM130 (Figure 5.8B, lane 3). Deletion of the N-terminus of GM130, ΔN441, had no effect on the binding of GRASP65 to GM130 (Figure 5.8B,
Figure 5.8 GRASP65 and GRASP55 binding to GM130. Transcription-translation assays were performed using plasmids encoding GM130, GRASP65 or GRASP55 alone, or GRASP65 and GRASP55 with full length GM130 or the N- and C-terminal deletions ΔN441 and ΔC887. Immunoprecipitations were performed using antibodies to GM130 with either 5 or 10μl for single and co-in vitro translations, respectively. Aliquots of the total (A) and immunoprecipitated (B) material were analyzed by SDS-PAGE and autoradiography. The positions of GRASP65 and GRASP55 are marked by an open and closed triangle respectively, in (A) and (B). (C) Golgi membranes (10μg) were extracted in buffer 20mM Hepes-KOH pH 7.3, 200mM KCl, 0.5% (w/v) Triton X-100. This extract was the fractionated by molecular sieving over Superose 6, collecting 1ml fractions. Aliquots of each fraction were analyzed by SDS-PAGE and Western blotting with antibodies to either GM130 MLO-7 (triangles), GRASP65 7E10 (open squares) or GRASP55 FBA32 (closed squares). The distribution of each marker is plotted as a percentage in a given fraction of the total signal for that marker.
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lane 4), while deletion of 100 amino acids from the C-terminus of GM130, AC887, abolished this interaction (Figure 5.8B, lane 6). Again, GRASP55 was unable to bind either of these truncated forms of GM130 (Figure 5.8B, lanes 5 and 7). Therefore, while GRASP65 can specifically bind to the C-terminus of GM130, GRASP55 is unable to under these conditions.

To investigate this further, it was decided to see if GRASP55 exists in a complex with GM130 and GRASP65 when isolated from stacked Golgi membranes. Golgi membranes were extracted in a salt and detergent containing buffer, fractionated by gel filtration, and the distributions of GRASP65, GRASP55, and GM130 determined by western blotting (Figure 5.8C). GM130 (Figure 5.8C, open triangles) and GRASP65 (Figure 5.8C, open squares) were found to co-fractionate by gel filtration, existing as a complex of approximately 1,200 kDa, as reported previously (Barr et al., 1998). In contrast, GRASP55 (Figure 5.8C, closed squares) behaved as a 200kDa protein by gel filtration, clearly resolved from GRASP65 and GM130. These observations are consistent with GRASP55 existing as part of a complex discrete from that containing GM130 and GRASP65. Alternatively, it might be that GRASP55 was dissociated from GM130 under the conditions, 200mM NaCl and 0.5% (w/v) TX-100, used to extract Golgi membranes, and for the co-immunoprecipitation assay.

5.2.4 Soluble GRASP55 and antibodies against GRASP55 block cisternal stacking, but not cisternal regrowth in cytosol driven reassembly.

To provide evidence that GRASP55 functions in the mechanism by which Golgi cisternae come together to form stacks, the effects of soluble recombinant GRASP55 on rat liver cytosol catalyzed reassembly were tested. Similar experiments were used to demonstrate that GRASP65 functions in stacking, and that its point of action is downstream of p115 (Chapter 4; Barr et al., 1997; Shorter and Warren, 1999). MGF isolated through a 0.5M sucrose cushion (Figure 5.9A) were either left untreated or treated for 15min on ice with 1.2μM GRASP55 or GRASP65, followed by incubation at 37°C for 60min with rat liver cytosol (10mg/ml). Reassembly of control MGF (Figure 5.9B) gave stacks with predominantly two to three cisternae per stack, as
Figure 5.9 Stacking of Golgi cisternae is blocked by recombinant forms of GRASP55 and GRASP65. MGF isolated through a 0.5M sucrose cushion (A) were incubated at 37°C for 60min with rat liver cytosol in the absence (B) or presence of 1.2μM soluble GRASP65 (C) or 1.2μM GRASP55 (D). Reactions were processed for EM, note the absence of stacks (arrows) in A, C and D and the prevalence of single cisternae (arrowheads) in C and D. Bar, 0.5μm.
Figure 5.10 Quantitation of effect of soluble GRASPs on rat liver cytosol catalyzed reassembly. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with rat liver cytosol in the presence of 1.2μM soluble GRASP65, or 1.2μM soluble GRASP55, or NEM treated soluble GRASPs. Reactions were processed for EM, and the percentage total membrane present as stacks (A) and cisternae (B) was determined. Bars represent means±SEM (n=3). (C) Increasing amounts of soluble GRASP were titrated into the above reaction, and the percentage total membrane present as stacks and cisternae was determined. Values represent means±SEM (n=2).
expected. When the MGF were pre-incubated with either the recombinant forms of GRASP65 or GRASP55 Golgi stacks were not formed. In these cases a population of single cisternae and some vesicles was observed (Figure 5.9C, D, 5.10A), similar to that seen when MGF are pre-treated with the alkylating agent NEM (Barr et al., 1997). This effect was not due to a decrease in the amount of membrane being incorporated into cisternae, since this was not significantly changed in the presence or absence of soluble recombinant GRASP65 or GRASP55 (Figure 5.10B).

To determine whether GRASP55 is an NEM-sensitive component of the Golgi stacking machinery, like GRASP65 (Barr et al., 1997), reassembly experiments were performed with NEM treated recombinant proteins. When the recombinant GRASP55 and GRASP65 were pretreated with NEM prior to their addition to the reassembly assay, their respective abilities to inhibit stack formation were decreased by approximately 50% (Figure 5.10A). This was in contrast to the effect of NEM treated soluble GRASP65 on the stacking reaction using pure fusion components, where it had little or no effect on the amount of stacking that occurred. The reason for this difference between the cytosol and purified component reassembly systems is unclear. However, again the reduction in membrane incorporated into stacks was not due to any change in the amount of membrane incorporated into cisternae, which was unchanged compared to the incubations where untreated recombinant GRASP55 and GRASP65 were added (Figure 5.10B). These results are consistent with GRASP55 being an NEM-sensitive Golgi stacking protein. This NEM sensitivity could be due to alkylation of cysteine 191 in GRASP65 (192 in GRASP55; ! in Figure 5.1) which is conserved amongst all the known forms of GRASP65 from various yeasts and C. elegans. This cysteine residue is situated just before the conserved GM130 binding domain, and so its alkylation may well disrupt the conformation of the GRASP65-GM130 complex in the context of the membrane.

Titration experiments were performed to ascertain the relative potencies of recombinant GRASP65 and GRASP55 in blocking the formation of Golgi stacks in this cell free system. Since 1.2 µM of each recombinant protein seemed able to block
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Stacking almost completely, progressively lower amounts of the two proteins were titrated into the assay. These experiments revealed the maximal inhibition was similar in both cases, but that GRASP55 is less potent than GRASP65 in blocking the formation of stacks, the IC$_{50}$ is 220nM compared to 80nM (Figure 5.10C, closed squares; closed circles). This effect was not due to an inhibition of membrane fusion, since this was not changed by the addition of either recombinant GRASP65 or GRASP55 (Figure 5.10C, open squares; open circles).

This inhibition of stacking caused by the two soluble forms of GRASP were approximately additive. When low levels of soluble GRASP55 and GRASP65 were added together, the inhibition of stacking was greater than when they were added alone (Figure 5.11, 5.12). Addition of 20nM GRASP65 and 120nM GRASP55 alone to the reaction had a small effect on the amount of stacks that formed, but this was increased when added together at these same levels (Figure 5.11A-C, 5.12A). In fact, when the estimated IC$_{50}$ for GRASP55 and GRASP65 were added together to the reaction, the effect was almost a complete inhibition on stacking (Figure 5.11D-F, 5.12A). Again the amount of membrane incorporated into cisternae was unaffected by these treatments (Figure 5.12D). This additivity may suggest that the soluble GRASPs may be affecting the same target molecule, or different but related target molecules, to inhibit the stacking process. It will be of great interest to identify GRASP interacting Golgi molecules, and whether any of these interact with both GRASP55 and GRASP65.

To provide evidence that the endogenous GRASP55 acts during stacking, the effects of GRASP55 specific antibodies on the formation of Golgi stacks were examined in the cell free system for Golgi reassembly. In these experiments, MGF were either left untreated, treated with preimmune, or immune sera for antibodies specific to GRASP55 (FBA34) or GRASP65 (FBA31), followed by incubation with rat liver cytosol (10mg/ml) for 60min at 37°C to allow the regrowth and stacking of cisternae. Reassembly of control MGF (Figure 5.13A), or MGF treated with the preimmune sera for the GRASP55 (Figure 5.14A, preimmune serum sheep) or GRASP65 (Figure 5.14A, preimmune serum rabbit) antibodies gave stacks with predominantly two to
Figure 5.11 Effect of soluble GRASPs on Golgi stack reassembly is additive. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with rat liver cytosol in the presence of increasing concentrations soluble GRASP65 (20nM in A, 80nM in D) or soluble GRASP55 (120nM in B, 220nM in E) or a combination of both (C and F). Reactions were processed for EM. Arrows denote stacks and arrowheads denote single cisternae. Note that the number of stacks is reduced in C relative to A and B, and in F relative to D and E. Whereas the converse is true for single cisternae, which are more frequent in C relative to A and B, and in F relative to D and E. Bar, 0.5µm.
Figure 5.12 Quantitation of effect of combinations of soluble GRASP55 and GRASP65 on the stacking of reassembling Golgi cisternae. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with rat liver cytosol in the presence of increasing concentrations of GRASP55, or GRASP65, or a combination of both. Reactions were processed for EM and the percentage total membrane present as stacks (A) and cisternae (B) was determined. Bars represent means±SEM (n=2).
Figure 5.13 Antibodies to GRASP55 and GRASP65 block the stacking of Golgi cisternae. MGF isolated through a 0.5M sucrose cushion were either left untreated (A), or treated for 15min on ice with anti-GRASP55 (B), anti-GRASP65 (C) or both antibodies (D), and then incubated at 37°C for 60min with rat liver cytosol. Reactions were stopped and processed for EM. Note the reduction in the number of stacks (arrows) in B and C when compared to A, and their absence in D, where single cisternae (arrowheads) are the major reaction product. Bar, 0.5μm.
Figure 5.14 Quantitation of effect of antibodies to GRASP55 and GRASP65 on the stacking of reassembling Golgi cisternae. MGF isolated through a 0.5M sucrose cushion were left untreated (none), or treated for 15 min on ice with pre-immune sera for GRASP55 (sheep), GRASP65 (rabbit), immune sera to GRASP55 (sheep anti-GRASP55), GRASP65 (rabbit anti-GRASP65), or GRASP55 and GRASP65 (anti-GRASP55+anti-GRASP65) and then incubated at 37°C for 60 min with rat liver cytosol. Reactions were processed for EM and the percentage total membrane present as stacks (A) and cisternae (B) was determined. Values represent means±SEM (n=2).
three cisternae per stack, as expected. When the MGF were pre-incubated with antibodies for either GRASP55 (Figure 5.14A, sheep anti-GRASP55) or GRASP65 (Figure 5.14A, rabbit anti-GRASP65) the number of Golgi stacks formed was decreased by approximately 60%. Treatment of MGF with antibodies to both GRASP55 and GRASP65 (Figure 5.14A, anti-GRASP55+anti-GRASP65) resulted in a greater than 80% inhibition of stack formation, again suggesting that the two GRASPs may contribute additively to the stacking process. These effects were not due to a decrease in the amount of membrane being incorporated into cisternae, since this was not significantly changed from the control value by the presence of any of the immune or preimmune sera tested (Figure S.14B). Representative images (Figure S.13) of these different conditions demonstrate the effects of GRASP55 and GRASP65 in blocking the formation of stacks but not cisternae. The simplest explanation for these results is that endogenous GRASP65 and GRASP55 act together in the pathway by which cisternae come together to form stacks. Alternatively, the high homology between the soluble GRASPs means they may be able to inhibit both GRASP65 and GRASP55 mediated stacking events. However, the antibodies should be more specific for each GRASP, and the partial inhibition with each antibody alone possibly suggests that they may be responsible for the stacking of different cisternae (i.e. cis with medial or medial with trans). This possibility would be consistent with their differential localization within the Golgi stack in vivo. Many further experiments will be required to distinguish between these possibilities.

5.3 Discussion

5.3.1 A role for GRASP55 in stacking Golgi cisternae.

The above data suggest that GRASP55, a GRASP65 related protein from mammals, plays a similar role to GRASP65 in stacking reassembling Golgi cisternae at the end of mitosis. Further experiments will be required to determine whether the stacking reaction displays similar temporal sensitivity to GRASP55 inhibitors as it does to GRASP65 inhibitors, and so whether it also acts downstream of p115. Unlike GRASP65, GRASP55 does not seem to exist in a stable complex with GM130 since an interaction between the two proteins was undetectable by biochemical methods,
although a weak interaction could be detected with the yeast two hybrid system (Shorter et al., 1999). In addition, GRASP65 displayed an apparent cis-Golgi localization similar to GM130 (Nakamura et al., 1995), whereas GRASP55 was found predominantly over the medial-cisternae of the stack. The basis for this differential localization could be explained if GRASP55 is in a complex with a protein other than GM130 which then targets to a different region of the Golgi apparatus. Supporting this idea are experiments showing that GRASP65 and GM130 need to bind to one another to efficiently target to the Golgi apparatus (Barr et al., 1998). Arguing against this are the data showing that mutations in the conserved binding region of GRASP55 have little effect on its Golgi targeting, indicating it might not require interaction with another protein at this site for correct localization. Identification of the proteins binding to GRASP55 should allow this point to be addressed.

This differential localization also suggests that the different GRASPs may be involved in anchoring different cisternae together at different levels of the stack. A curious feature of the GRASP65 labelling is that it is predominantly over the cis-face of the Golgi apparatus, and not in between cisternae, which would be more consistent with a role in stacking cisternae. This may represent an epitope accessibility problem, the epitope being sterically masked due to its presence in a large protein complex, as is indicated by the molecular sieving analysis of Golgi detergent extract. Consistent with this possible accessibility problem is the fact that GRASP65 is only accessible to NEM on MGF and not on RLG. However, it may also be that this is the true distribution of GRASP65, and this concentration on the cis-face of the Golgi would be more consistent with the establishment of Golgi stacks as VTCs mature into nascent cis-Golgi cisternae, as required by the cisternal maturation model of Golgi transport (Pelham, 1998; Glick and Malhotra, 1998). Linstedt has gone even further to suggest that GRASP65 may be required to establish the Golgi stack at the cis-face during the early stages of the maturation process and GRASP55 would act to maintain it as the cisternae mature (i.e. become more medial and trans; Linstedt, 1999). The differential distributions of the GRASPs could be maintained by how efficiently they are incorporated into retrograde COPI vesicles, in a manner similar to Golgi enzymes.
(Glick et al., 1997; Linstedt, 1999). However, that GRASP65 and GM130 are largely excluded from COPI vesicles isolated in the presence of GTPyS, would be inconsistent with such a model (Sonnichsen et al., 1998; Francis Barr, personal communication), provided GTPyS was not affecting the incorporation of GRASP65 and GM130.

That both soluble molecules are able to completely inhibit the formation of stacks in the reassembly assay may be due to them having a common target protein, or related target proteins involved in stacking at all levels of the stack. This would be consistent with the observed additivity of inhibition of stacking by the soluble GRASPs. Identification of GRASP interacting Golgi molecules will provide further insight into how they may be acting. Alternatively, it may be that the in vitro reassembled stacks do not have the same polarity as stacks in vivo, or compared to starting RLG, as preliminary experiments have hinted at (Rabouille et al., 1995c; Catherine Rabouille and Eija Jokitalo, personal communication) and so both GRASPs may contribute to stacking cisternae at all levels of the stack, rather than having overlapping distributions. Another possibility is that the reassembled stacks do have the correct polarity and that GRASP65 and GRASP55 are responsible for stacking different parts of the Golgi stack. This would be consistent with the fact that antibodies against either GRASP are not able to completely inhibit the stacking process when added alone. More work will be required to distinguish between these possibilities.

Precisely how the two GRASPs might act to anchor cisternae together still remains unclear. However, since from molecular sieving analysis reveals that both are in homooligomeric complexes, and that GRASP65 is likely a dimer/trimer (Barr et al., 1998) and GRASP55 a trimer/tetramer (Francis Barr, personal communication), it is possible that the GRASPs anchor cisternae together by inserting their myristoyl groups into adjacent cisternae. The soluble GRASPs may act to break up the endogenous oligomer, so forming oligomers which contain monomers which lack myristoyl groups, and so are inactivated with regard to their stacking function.
5.3.2 Evolutionary conservation of GRASP55 and GRASP65

Why do mammalian cells have two members of the GRASP family of proteins while *S. cerevisiae* only has one? Mammalian cells do have a more complex Golgi structure than this yeast, typically there are between three and five cisternae in a stack with extensive tubular networks at the *cis*- and *trans*-faces, and many small vesicles associated with the edges of the cisternae (Rambourg and Clermont, 1997; Ladinsky et al., 1999). *S. cerevisiae* possess clearly defined Golgi cisternae, but serial sectioning and electron microscopy have shown that only 40% of the cisternae are present in stacks at any one time (see Table 1 in Preuss et al., 1992). However, this is not true of the yeast *S. pombe* which has only one form of GRASP, but c. 80% of identifiable cisternae present in stacks, which contain between 2-9 cisternae (Ayscough et al., 1993). However, the stacking mechanism in *S. pombe* is distinct from that in mammalian cells as it is sensitive to microtubule destabilizing agents such as thiabendazole (Ayscough et al., 1993), whereas in mammalian cells such agents cause disruption of the Golgi ribbon into its component ministacks, but no unstacking *per se* (Cole et al., 1996a; Storrie et al., 1998). A more complex organization of membrane bound compartments in mammalian cells is also reflected in the diversity of other proteins involved in membrane transport, for example there are many more members of the SNARE family of proteins in mammalian cells than in yeast (Advani et al., 1998). The presence of two GRASP molecules may reflect the more complex organization of the Golgi apparatus, or a requirement for additional regulation of the structure of this organelle in mammals. Consistent with this argument are the localizations of GRASP65 and GRASP55 to distinct subcompartments of the Golgi apparatus.

Given this correlation between number of GRASP molecules and complexity of Golgi organization it is also of interest that testis seem to possess multiple splice variants of GRASP molecules (Figure 5.2). Many of the cells of the testis have amongst the highest number of cisternae per stack of all mammalian cells. In fact, the Golgi apparatus of early spermatids has between 8-10 cisternae per stack, more than double that of hepatocytes (Rambourg and Clermont, 1997). However, the Golgi apparatus undergoes a dramatic series of morphological changes during spermiogenesis, and
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culminates in the presence of disorganized Golgi cisternae in the cytoplasmic droplet, a small localized outpouching of cytoplasm of the spermatozoan tail, which is hypothesized to be responsible for glycosylation of plasma membrane substrates (Oko et al., 1993). Such a radically different intracellular morphogenetic programme for the Golgi apparatus may require multiple GRASPs. However, it will still be of interest to determine whether this correlation between multiple forms of GRASPs and the number of cisternae per stack holds for other cells with a high number of cisternae per stack, such as the Mucous cells of Brunner’s Gland which have between 9-11 cisternae per stack (Rambourg and Clermont, 1997), or the nurse cells of \textit{Oniscus} which can have as many as 40 cisternae per stack (Fawcett, 1981).

5.3.3 Mechanism of stack formation during reassembly.

The stacking of nascent Golgi cisternae during reassembly requires a p115 tethering step that may act to align and initially bring cisternae together. It is at this point that the function of p115 in tethering membranes prior to stacking must diverge from that of simply tethering membranes prior to membrane fusion, as is likely to be the case during vesicle transport. If this were not the case, then one would expect Golgi cisternae to dock and fuse with one another, rather than form stacks. There must therefore be additional factors acting to either divert the tethering complexes away from the SNARE-mediated membrane fusion pathway, or prevent SNAREs from completing the fusion reaction, possibly by clamping them in some way (Rothman and Warren, 1994). The clamping of SNAREs, or the diversion of the tethering complex away from the SNAREs may involve the formation of a matrix or lattice-like structure from the many coiled-coil proteins or cytoskeletal elements (esp. ankyrin, spectrin, cytokeratins) associated with the Golgi complex (Chan and Fritzler, 1998; De Mattheis et al., 1998; Hui, 1997). Evidence has been presented for such a structure existing on Golgi membranes, and furthermore that GM130 is a component of this matrix (Slusarewicz et al., 1994; Nakamura et al., 1995). One possibility is that GRASP65 and GRASP55 are key regulators in the nucleation of such a structural matrix. The inhibition of stack formation by recombinant forms of these two proteins could be due to competition for interactions with the endogenous membrane associated GRASP proteins. These
interactions must be with membrane associated components of MGF, since stacks can reassemble from these membranes with the addition of purified membrane fusion components (Shorter and Warren, 1999). Therefore, it will be of great interest to elucidate Golgi membrane associated GRASP interacting molecules, and also to probe for whether SNARE function is required for stacking \textit{per se}.

Whether a tethering complex is destined to be involved in stacking or fusion may be regulated by p115 phosphorylation, which is the focus of the next chapter.
Chapter 6

Casein kinase II-mediated phosphorylation of the vesicle tethering protein p115 is required for NSF catalyzed cisternal regrowth
Chapter 6  
CKII-mediated phosphorylation of p115 in membrane tethering

6.1 Introduction

p115 can act to both tether cisterna to cisterna during the initial phases of post-mitotic stack formation, and also to tether COPI vesicle to acceptor membrane, as a probable prelude to SNARE mediated membrane fusion (Shorter and Warren, 1999; Sönntichsen et al., 1998; Cao et al., 1998). This activity may require the simultaneous interaction between its two Golgi receptors giantin and GM130 in opposite membranes, thus forming a bridge between discrete compartments (Shorter and Warren, 1999; Sönntichsen et al., 1998). To probe further precisely how p115 accomplishes this task the role of p115 phosphorylation in these processes was investigated. p115 is phosphorylated at its extreme acidic C-terminus, the region which is essential for GM130 binding (Nelson et al., 1998), on S942 (in human p115, S941 in bovine p115 and S940 in rat p115) by a Golgi associated kinase in a cell cycle dependent manner (Sohda et al., 1998). At mitosis p115 is predominantly cytosolic (Levine et al., 1996) as binding to the Golgi membrane is inhibited (Levine et al., 1996) due to Cdc2 kinase mediated phosphorylation of GM130 (Nakamura et al., 1997; Lowe et al., 1998b), hence p115 does not come into contact with the Golgi associated kinase and so remains in the dephosphorylated state (Sohda et al., 1998). During interphase p115 is found to be in two forms, a cytosolic phosphorylated form and a dephosphorylated form found associated with the Golgi apparatus. Furthermore, phosphorylation of p115 has been proposed to facilitate its release from the Golgi membrane, and this proposal was reinforced by the fact that the S942A mutation increases p115 membrane binding, while S942D mutation decreases p115 membrane binding (Sohda et al., 1998). This cycle of phosphorylation and dephosphorylation was proposed to regulate p115 membrane association, and possibly act to recycle p115 between soluble and membrane bound states in order to enable multiple rounds of vesicle transport (Sohda et al., 1998).

In this chapter the function of p115 phosphorylation is probed with regard to the ability of p115 to cross-link GM130 to giantin, and with regard to p115 function during reassembly of MGF in the cell free system. The kinase responsible for this phosphorylation is identified as casein kinase II (CKII) or a CKII like molecule. The
results suggest that p115 phosphorylation is required for NSF catalyzed cisternal regrowth, and possibly for the transition from COPI vesicle tethering to COPI vesicle fusion, by increasing the strength of the giantin-p115-GM130 bridge. This work was conducted in collaboration with Barbara Dirac-Svejstrup in the lab, who made all the p115 constructs, and did the biochemistry on the giantin/p115/GM130 interactions and the CKII phosphorylation studies (Figure 6.2, 6.4B, 6.6A, 6.7, 6.11, 6.16, 6.17) and whose data is reproduced here with permission.

The second part of this chapter concerns the coiled-coil domains of p115, and how they may function in the reassembly process. The first coiled-coil domain of p115 bears subsignificant, but notable given the biological context, homology to the membrane proximal H3 coiled-coil domain or SNARE motif of the syntaxin superfamily (Weimbs et al., 1997). This domain contributes one of the four parallel \( \alpha \)-helices to the internal ‘core’ of an assembled SNARE complex (Fiebig et al., 1999), and this ‘core’ complex is proposed to be the minimal machinery required to forcefully drive bilayer mixing (Parlati et al., 1999). A synthetic peptide corresponding to this weakly homologous region of p115 (p115 637-699) is able to inhibit NSF catalyzed cisternal regrowth, while the other coiled-coil domains of p115 have no effect on the reaction. p115 637-699, but not the other coiled-coil domains of p115, is able to retrieve a Golgi v-SNARE: GOS28 (Nagahama et al., 1996), a Golgi t-SNARE protector: Sly1p (Dascher and Balch, 1996) and a Golgi t-SNARE: syntaxin-5 (Dascher et al., 1994) from Golgi detergent extract. Full length p115 is also able to retrieve GOS28 and syntaxin-5 from Golgi detergent extract. These data suggest that the first coiled-coil domain of p115 may interact with the SNARE molecules, and in so doing provide a physical link between the processes of SNARE independent vesicle tethering and SNARE dependent vesicle docking and fusion.

6.2 Role of p115 phosphorylation in post-mitotic Golgi reassembly.

6.2.1 p115 binds to GM130 and giantin via its C-terminal acidic domain.

p115 exists as a myosin II shaped parallel homodimer, with two large N-terminal globular heads, a coiled-coil tail and a short acidic domain at the extreme C-terminus.
Chapter 6  CKII-mediated phosphorylation of p115 in membrane tethering

(Figure 6.1; Sapperstein et al., 1995; Yamakawa et al., 1996). The C-terminal acidic domain of p115 has been shown to be required for binding to GM130 (Nelson et al., 1998), and contains the serine phosphorylated by the putative Golgi associated kinase (asterisk in Figure 6.1B; Sohda et al., 1998). The region of p115 required for giantin binding had not been determined. In order to define the region of p115 required for giantin binding DNA constructs were generated encoding full length p115 (HTA), p115 lacking the acidic domain (HT), p115 lacking the coiled-coil tail and acidic domain (H), or p115 lacking the globular head domain (TA; Figure 6.1). The various DNA constructs were *in vitro* transcribed and translated in the presence of ^35^S-methionine and mixed with Golgi detergent extract. Giantin and GM130 were then immunoprecipitated and co-immunoprecipitation of the different p115 forms was monitored by autoradiography (Figure 6.2A). Very similar patterns of p115 binding were clarified for GM130 and giantin, whereby only those forms of p115 that included the acidic C-terminus (A) co-immunoprecipitated. HTA and TA bound very well in comparison to HT and H which displayed virtually no binding. Interestingly, TA bound twice as efficiently to giantin and GM130 compared to HTA, implying that the globular heads of p115 may exert some negative regulatory effect on p115 binding to GM130 and giantin.

Two synthetic peptides containing S941 were used to provide direct evidence for binding of the acidic domain of p115 to GM130 and giantin. A 75mer comprising a short stretch of the coiled-coil region plus the acidic domain, and a 26mer comprising just the acidic domain, were N-terminally biotinylated, coupled to neutravidin beads, and incubated with Golgi detergent extract. Both peptides selectively retrieved GM130 and giantin, as shown by Coomassie staining and Western blot (Figure 6.2B).
Figure 6.1 Structure of p115 and deletion constructs. (A) Amino acid sequence of bovine p115 (P41541). Analysis of the sequence of p115 reveals a N-terminal globular head domain (1-651), a rod like tail domain (651-934, light grey shading) containing four predicted coiled-coil regions (underlined), and an extreme acidic C-terminus (934-961, dark grey shading). (B) Schematic of constructs used, HTA corresponds to full length p115 (1-961), HT to the head plus the tail domain (1-934), H to the head domain (1-651), TA to the tail plus the acidic domain (651-961), the 75mer to the 75 C-terminal amino acids of p115 (887-961), and the 26mer to the acidic domain of p115 (936-961). The 75mer and 26mer were made as synthetic peptides. Asterisk denotes the serine that is phosphorylated in p115, and that which will be mutated to either alanine or aspartate in the TA mutants (see later).
Figure 6.2 Mapping the binding site in p115 for GM130 and giantin.

(A) Full length p115 (HTA) and truncated forms (HT, H, TA) were in vitro transcribed and translated in rabbit reticulo-lysate in the presence of ^35S-methionine. 1ng of each translate was added separately to Triton X-100 extracts of 100μg RLG (in 100μl) that had been 1M KCl extracted prior to detergent extraction to remove any endogenous p115. Immunoprecipitations were performed with anti-giantin or anti-GM130 antibodies. Co-immunoprecipitation of the various forms of p115 was monitored by SDS-PAGE of the immunoprecipitates followed by phosphor-imager visualization. The amount of p115 in the immunoprecipitates was normalized for the amount of p115 added to each reaction. (B) Biotinylated 75mer or 26mer synthetic peptides were coupled to neutravidin beads at 2mg/ml. 50μl beads were incubated at 4°C for 60min with Triton X-100 extracts of 200μg RLG (in 400μl) that had been 1M KCl extracted prior to detergent extraction to remove any endogenous p115. The bound material was eluted and fractionated by SDS-PAGE followed by Coomassie staining or Western blotting for GM130 and giantin (* giantin proteolytic fragment).
6.2.2 The globular heads of p115 are not required for NSF catalyzed cisternal regrowth nor for stimulating complex formation between GM130 and giantin.

Since TA and the 75mer could bind both giantin and GM130, it was tested whether they could substitute for p115 in NSF catalyzed cisternal regrowth. Therefore, the 75mer synthetic peptide, N-terminally His-tagged recombinant TA, and rat liver p115 were titrated into the NSF catalyzed reassembly reaction (Figure 6.3, 6.4A). Despite its ability to bind GM130 and giantin, the 75mer was unable to substitute for p115 in the NSF catalyzed reaction and as a result very little cisternal regrowth was visualized (Figure 6.3A-C, 6.4A). However, TA was able to substitute for p115 to a certain extent, stacked cisternae were regenerated (Figure 6.3D-F), and in fact TA catalyzed cisternal regrowth approximately 60-70% as well as rat liver p115 (Figure 6.3G-I, 6.4A).

This ability of TA, but not the 75mer, to stimulate cisternal regrowth was coupled to the fact that the TA, but not the 75mer, could stimulate complex formation between GM130 and giantin. Increasing concentrations of 75mer, TA and p115 were added to Golgi detergent extract depleted of p115 and incubated at 4°C for 60min. Giantin was then immunoprecipitated, and co-immunoprecipitation of GM130 was monitored by Western blotting using a specific mAb against GM130. Increasing concentrations of TA and p115, but not 75mer, caused a concomitant linear increase in the amount of GM130 that co-immunoprecipitated with giantin at TA/p115 concentrations below 100nM (Figure 6.4B). TA and p115 stimulated complex formation equally well up to 100nM, after which the process began to saturate, at which point p115 began to stimulate complex formation slightly better (Figure 6.4B). Higher concentrations of p115 could not be tested owing to the difficulty in concentrating purified rat liver p115. The 75mer was completely unable to stimulate complex formation (Figure 6.4B). This complex may represent GM130 linked to giantin via p115 (or permutations of this heteroternary complex, which are possible as all the molecules are in free solution) or GM130 linked directly to giantin, a p115 dependent interaction, but p115 is excluded from the final complex. It is difficult to distinguish between these possibilities as the binary complex of giantin and p115 is also co-immunoprecipitated, so although
Figure 6.3 The globular head domain of p115 is dispensable for Golgi reassembly. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl), and increasing concentrations of either the 75mer (A-C), recombinant TA (D-F), or rat liver p115 (G-I). Reactions were processed for EM. Arrows denote unfused tubulovesicular material (A-D and G) while arrowheads denote reassembled cisternae (E, F, H and I). Bar, 0.5µm.
Figure 6.4 TA can substitute for p115 in NSF mediated cisternal regrowth and in stimulating complex formation between giantin and GM130. (A) MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/μl), α–SNAP (25ng/μl), γ–SNAP (25ng/μl) and increasing concentrations of either rat liver p115 (squares), recombinant TA (circles) or 75mer (triangles). Reactions were processed for EM, and the amount of cisternal regrowth was determined. Values represent means ±SEM (n=3). (B) Increasing concentrations of rat liver p115, recombinant TA or 75mer were added to Triton X-100 extracts of RLG that had been washed with 1M KCl prior to detergent extraction. 100μg of Golgi extract in 100μl was incubated at 4°C for 60min with increasing concentration of p115, or TA, or 75mer. Reactions were then incubated at 4°C for 60min with anti-giantin antibodies, followed by protein A sepharose for 60min at 4°C. Beads were recovered and eluted with SDS-PAGE sample buffer, fractionated by SDS-PAGE, transferred to nitrocellulose, and then probed for GM130 and RGS-His (His- tagged TA) with mAbs. GM130 in complex with giantin is shown as % of input GM130. Quantitations are an average of two separate experiments.
p115 is present, whether it is in a ternary or binary complex remains unclear (Barbara Dirac-Svejstrup, personal communication).

This apparent dispensability of the globular heads for both NSF mediated cisternal regrowth and for stimulating complex formation between GM130 and giantin suggests that they may not be required for p115 function during Golgi reassembly. This is a surprising result given that the globular heads of p115 appear to be the most conserved part of the molecule (Sapperstein et al., 1995).

6.2.3 S941 of p115 is essential for NSF catalyzed cisternal regrowth.

To determine whether phosphorylation of S941 of p115 was required for NSF catalyzed cisternal regrowth TA mutants were generated where the serine was mutated to either an alanine or an aspartate. The alanine mutant (TA [S941A]) should approximate the nonphosphorylated form of TA and the aspartate mutant (TA [S941D]) should approximate the phosphorylated form of TA. TA was chosen in preference to p115, since it is active in the reassembly assay, and thus far it has been impossible to express and purify full length p115 in bacteria. The various forms of TA were titrated into the NSF catalyzed reassembly reaction. TA was able to support cisternal regrowth as previously determined (Figure 6.5A, B, 6.6.A), but neither the TA (S941A) nor the TA (S941D) were able to stimulate cisternal regrowth even at the highest concentrations tested (Figure 6.5C-E, 6.6A). The fact that TA (S941A) cannot support cisternal regrowth suggests that TA must be phosphorylated on S941 for it to accomplish its role in NSF mediated cisternal regrowth. Additionally, the fact that TA (S941D) also cannot stimulate NSF catalyzed cisternal regrowth, indicates that the phosphorylation event per se is intrinsic to the process, or that the aspartate substitution does not mimic the phosphorylated form of TA. Alternatively, the alanine or aspartate substitutions may affect TA conformation, such that it is rendered dysfunctional.

Given this clear difference in the reassembly assay it was tested whether the TA mutants could promote the formation of the tethering complex between GM130 and
Figure 6.5 Effect of TA mutants on NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl) and increasing concentrations of either TA (A and B), TA (S941A; C and D), or TA (S941D; E and F). Reactions were processed for EM, and arrowheads denote reassembled cisternae (prevalent in A and B), and arrows denote unfused tubulovesicular material (prevalent in C-F). Note that the mutant TA proteins do not support any significant cisternal regrowth, Bar, 0.5µm.
Figure 6.6 The TA (S941A) and (S941D) mutants can support complex formation between giantin and GM130, but not NSF catalyzed cisternal regrowth. (A) MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/μl), α–SNAP (25ng/μl), γ-SNAP (25ng/μl) and increasing concentrations of TA (squares), TA (S941A; triangles) or TA (S941D; circles). Reactions were fixed and processed for EM and then quantitated for the amount of cisternal regrowth. Values represent means±SEM (n=2). (B) Increasing concentrations of either TA, TA (S941A) or TA (S941D) were added to Triton X-100 extracts of RLG that had been washed in 1M KCl prior to detergent extraction. 100μg Golgi extract in 100μl was incubated at 4°C for 60min with increasing concentrations of TAs, followed by 60min at 4°C with anti-giantin antibodies, followed by 60min at 4°C with protein A sepharose. The sepharose beads were recovered, washed, and eluted into SDS-PAGE sample buffer, fractionated by SDS-PAGE, transferred to nitrocellulose and probed for GM130 and RGS-His (His tagged TA) using mAbs. GM130 in complex with giantin is shown as % of input GM130. Quantitations reflect the mean of two eperiments.
giantin. Increasing concentrations of the various TA forms were added to Golgi detergent extract depleted of p115 and incubated at 4°C for 60 min. Giantin was then immunoprecipitated, and co-immunoprecipitation of GM130 was monitored by Western blotting using a specific mAb against GM130. All forms of TA were able to stimulate complex formation between GM130 and giantin equally well and in a similar dose-dependent style (Figure 6.6B). If allowed to extrapolate from this detergent biochemistry, this would suggest that the different forms of TA are able to tether membranes together equally well, yet the mutant proteins cannot support the transition from tethering to fusion (as measured by cisternal regrowth).

6.2.4 p115 can be phosphorylated by CKII.

Having determined that the phosphorylation of S941 was essential for NSF catalyzed cisternal regrowth it was then important to determine the kinase responsible for this event. Given that the reassembly was conducted in the absence of cytosol the kinase would have to be associated with the Golgi membrane. This would be consistent with the fact that Golgi purified from HeLa cells is capable of phosphorylating p115 (Sohda et al., 1998). When RLG is incubated with \(^{32}\text{P}\)-ATP or \(-\text{GTP in the presence of purified rat liver p115, p115 became phosphorylated, and either ATP or GTP could serve as the phosphate donor (Figure 6.7A). If TA and TA (S941A) were used as alternative substrates, then TA was phosphorylated in the presence of either ATP or GTP, but TA (S941A) was not, suggesting that S941 is the serine that is phosphorylated by the Golgi associated kinase in full length p115 (Figure 6.7B).}

Since CKII is unusual amongst protein kinases in being able to use GTP as well as ATP as a phosphate source (Gatica et al., 1993; Allende and Allende, 1995), and that the region surrounding S941 is extremely acidic and bears resemblance to a CKII phosphorylation site motif (Figure 6.8; Pinna, 1990; Songyang et al., 1994; Kreegipuu et al., 1998), indicated the kinase may be CKII or a CKII like molecule. Furthermore, although mostly a nuclear or cytosolic molecule (Allende and Allende, 1995; Pinna and Meggio, 1997) subpopulations of CKII have been reported to localize to ER membranes (Ou et al., 1992; Cala et al., 1993) and the Golgi apparatus (Wu et al.,
Figure 6.7 CKII and Rat liver Golgi phosphorylate p115 at the same site.

10μg RLG was incubated at 30°C for 10min with either (A) 0.6μg p115 or (B) 2.5μg TA or TA (S941A) and either 10μM ATP and 5μCi γ-32P-ATP or 10μM GTP and γ-32P-GTP. p115 was released by addition of 1M KCl, and Golgi membranes removed by centrifugation. Samples were fractionated by SDS-PAGE and visualized using the phosphoimager. (C) 10μg RLG was incubated at 37°C for 10min with or without 1μg CKII substrate peptide (RRRDDDSDDDDD) in the presence of 10μM GTP and γ-32P-GTP. Reactions were dotted onto phosphocellulose paper, washed in 100mM phosphoric acid followed by 100% ethanol, dried and the paper counted in the scintillation counter for 1min. (D) 0.2μunits of recombinant human CKII was incubated at 30°C for 10min with either: 5μg TA (lanes 1 and 3), 5μg TA (S941A; lanes 2 and 4) or 0.5μg purified rat liver p115 (lanes 5 and 6) in the presence of 10μM GTP and 5μCi γ-32P-GTP (lanes 1, 2 and 5) or 10μM ATP and 5μCi γ-32P-ATP (lanes 3, 4 and 6). Reactions were fractionated by SDS-PAGE and exposed to X-ray film for visualization. (E) 10μg RLG or MGF were incubated at 30°C for 10min with 5μg TA and 1mM ATP and 20μCi γ-32P-ATP. Samples were processed as above. (F) 30μg RLG was separated by SDS-PAGE, transferred to nitrocellulose and probed for CKII alpha and beta subunits using specific antibodies.
**Optimal motif**  

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p115 (rat)  

|             | E | E | D | E | S | G | D | Q | D |

p115 (human/bovine)  

|             | D | E | L | E | S | G | D | Q | D |

Human a-Hsp90  

|             | E | D | V | G | S | D | E | E | E |

PKA subunit RII  

|             | A | D | S | E | S | E | D | E | E |

Nucleolar protein B23  

|             | E | D | A | E | S | E | D | E | D |

Phosphatase inhibitor 2  

|             | E | Q | E | S | S | G | E | E | D |

Residues in bold face are strongly selected. The underlined serine is the one that gets phosphorylated.

**Figure 6.8 Region surrounding p115 phosphorylation site resembles a consensus casein kinase II phosphorylation motif.** Comparison of the optimal casein kinase II phosphorylation motif determined using a peptide library with the p115 sequence and sequences at the phosphorylation sites of known protein substrates (from Songyang et al., 1996).
1995), and there is even a Golgi-specific CKII like kinase (Lasa-Benito et al., 1996; Lasa et al., 1997a). When RLG were incubated with \([\gamma^{32}P]-GTP\) in the presence or absence of a CKII substrate (RRRDDDSDDDDD), an elevated level of global phosphorylation was observed in reactions containing the CKII substrate, indicating that RLG contains CKII or a CKII like activity (Figure 6.7C).

If the identity of the RLG associated p115 kinase is CKII, pure CKII should also be able to phosphorylate p115. Purified rat liver p115 was incubated with pure recombinant human CKII (Calbiochem), with either ATP or GTP. p115 phosphorylation occurred with either nucleotide (Figure 6.7D). When p115 was substituted with TA or TA (S941A) as substrate, phosphorylation of TA occurred with either nucleotide, but TA (S941A) could not be phosphorylated (Figure 6.7D). This strongly suggests that CKII can phosphorylate p115 at S941. This CKII like activity was also present on MGF, the membrane source in the reassembly reaction, and the ability of MGF to phosphorylate TA was much greater than that of RLG (Figure 6.7E). CKII is heterotetrameric holoenzyme consisting of CKII\(\alpha\) or CKII\(\alpha'\) (an isoform of \(\alpha\)) as the catalytic subunit and CKII\(\beta\) as the noncatalytic subunit. The \(\beta\)-subunits dimerize and link to two \(\alpha\)-subunits together to form a \(\alpha_2\beta_2\) heterodimer (Gietz et al., 1995). Western blotting with specific antibodies revealed that both the \(\alpha\)- (antibody recognizes both forms) and \(\beta\)-subunits of CKII were present on RLG (Figure 6.7F).

6.2.5 Inhibition of CKII inhibits NSF catalyzed cisternal regrowth.

Given this apparent capacity for CKII or a CKII like activity to phosphorylate p115, and the importance of this event for NSF mediated reassembly as revealed by the TA mutant proteins, it was of interest to determine whether CKII activity was also required for this process. Three independent approaches were used to elucidate whether CKII activity was required for NSF catalyzed cisternal regrowth. The effect of either: competitive inhibitors of CKII with respect to ATP, specific antibodies against CKII subunits, or CKII substrate peptides on NSF catalyzed cisternal regrowth was determined.
Chapter 6  CKII-mediated phosphorylation of p115 in membrane tethering

Four different kinase inhibitors were added to the NSF catalyzed reaction at 50μM concentration: staurosporine, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), chrysin and roscovitine. Roscovitine was employed as a control inhibitor as it is specific for Cdc2 and Cdk2 rather than CKII (De Azevedo et al., 1997), and it had little discernible effect on NSF mediated cisternal regrowth (Figure 6.9A, B, 6.11A). Staurosporine is a global inhibitor of protein kinases and acts to compete with ATP for the catalytic domain of the kinase, and does not inhibit CKII unless used in the μM range (Meggio et al., 1995). Staurosporine caused a c. 50% reduction in the amount cisternal regrowth that occurred (Figure 6.9C, 6.11A). When DRB was included in the reaction again a c. 50% reduction in the amount of cisternal regrowth was observed (Figure 6.9D, 6.11A). DRB is a more specific inhibitor of CKII, but may also inhibit other kinases (Shugar, 1994; Yankulov et al., 1995). Another recently defined, but structurally unrelated to DRB, specific CKII inhibitor chrysin (Critchfield et al., 1997) reduced NSF catalyzed cisternal regrowth by c. 70% (Figure 6.9E, 6.11A).

These results correlated well with RLG mediated phosphorylation of purified rat liver p115 (Figure 6.11B). Approximately the same pattern of results is seen: staurosporine and DRB reduce RLG mediated p115 phosphorylation by c. 70%, and chrysin by c. 90%, while roscovitine has very little effect on the amount of p115 phosphorylated (Figure 6.11B).

Next the effects of anti-CKII antibodies and a CKII substrate peptide were determined on NSF catalyzed cisternal regrowth. Treatment of MGF with anti-CKII-α resulted in a 30% inhibition of cisternal regrowth (Figure 6.10A, B, 6.11C), and treatment with anti-CKII-β antibodies resulted in a c. 40% inhibition of cisternal regrowth (Figure 6.10C, 6.11C). This was again paralleled by the in vitro RLG mediated p115 phosphorylation assays, where both antibodies reduced p115 phosphorylation by c. 50% (Figure 6.11D). Addition of the CKII substrate peptide such that it was at a 100-
Figure 6.9 Effect of CKII inhibitors on NSF catalyzed cisternal regrowth.
MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60 min in the presence of NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (30ng/μl) in the presence of either: buffer (A), 50μM roscovitine (B), 50μM staurosporine (C), 50μM DRB (D), or 50μM chrysin (E). Reactions were processed for EM. Arrows denote reassembled cisternae prevalent in A and B, whereas arrowheads denote unfused tubulovesicular material which is more frequent in C-E. Bar, 0.5μm.
Figure 6.10  Effect of anti-CKII antibodies and CKII substrate peptide on NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/µl), α-SNAP (25ng/µl), γ-SNAP (25ng/µl) and p115 (30ng/µl) in the presence of either: buffer (A), 1µl anti-CKII α (B), 1µl anti-CKII β (C), a 100x molar excess (over p115) of CKII substrate peptide (D) or the phosphorylated form of the peptide (E). Reactions were processed for EM. Arrows denote reassembled cisternae which are most prevalent in A and E, whereas unfused tubulovesicular material (arrowheads) is more frequent in B-D. Bar, 0.5µm.
Figure 6.11 CKII inhibitors block NSF catalyzed cisternal regrowth and RLG mediated p115 phosphorylation. (A, C, E) MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/µl), α–SNAP (25ng/µl), γ–SNAP (25ng/µl) and p115 (30ng/µl) in the presence of either CKII inhibitors (A), anti-CKII antibodies (C), or CKII substrate peptide/26mer (E) as described in figures 6.9, 6.10, and 6.14. Reactions were processed for EM, and the amount of cisternal regrowth was determined. Values represent means±SEM (n=2-4). (B, D, F) 10µg RLG was incubated at 30°C for 10min in the presence 0.6µg p115 and 10µM GTP and 5µCi γ-^32P in the presence of either 100µM staurosporine, DRB, chrysin or roscovitine (B), or anti-CKII antibodies (D) or a 100x molar excess (over p115) of CKII substrate peptide/phosphopeptide or a 200x molar excess (over p115) of 26mer:26mer-P (F). p115 was released from the Golgi membranes by addition of 1M KCl and the Golgi membranes removed by centrifugation. Samples were then fractionated by SDS-PAGE and phosphorylation of p115 monitored by SDS-PAGE and phosphoimaging. Values represent means±SEM (n=2-3).
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Fold molar excess over p115 in the reaction inhibited NSF catalyzed cisternal regrowth by c. 70% (Figure 6.10D, 6.11E) and RLG mediated p115 phosphorylation by c. 60% (Figure 6.11F). In contrast, a phosphorylated form of the CKII substrate peptide inhibited NSF catalyzed cisternal regrowth by only 20% (Figure 6.10E, 6.11E), and seemed to slightly stimulate RLG mediated p115 phosphorylation (Figure 6.11F).

Taken together these data strongly suggest that NSF catalyzed cisternal regrowth requires the phosphorylation of p115 at S941 by CKII or a CKII like molecule.

6.2.6 The 75mer and 26mer are able to interfere with the p115 facilitated, NSF catalyzed cisternal regrowth.

Next, it was decided to titrate in the 75mer and 26mer into the NSF catalyzed reassembly reaction in the presence of p115. These peptides should act as a substrate for the kinase, and so competitively inhibit the process. Alternatively, since they can bind to both GM130 and giantin they may compete for p115 binding with the Golgi membrane, and so inhibit the process.

Addition of 75mer at a 50- or 200-fold molar excess over p115 in the NSF reaction caused a drastic reduction in the amount of cisternal regrowth that occurred (Figure 6.12A-C, 6.13). In the presence of the 75mer cisternae failed to reform and instead the predominant reaction product was a series of tethered membrane profiles, both vesicular and tubular, as though the reaction had been halted at this stage of the reaction (asterisks in Figure 6.12B, C). A concern was that the acidic Pi of these peptides (4.05 for the 75mer and 3.47 for the 26mer) may somehow be a non-specific inhibitor of NSF mediated cisternal regrowth. To control for this synthetic peptides of 70 amino acids in length composed of purely aspartate, or purely glutamate, or glutamate and aspartate in a 1:1 ratio were added to the NSF catalyzed reaction at a 200-fold molar excess over p115. These purely acidic peptides had no detectable effect on the reaction, and stacks of cisternae regrew normally (Figure 6.12D-F, 6.13). The effect of the 75mer may then be due to direct interference with p115 function.
Figure 6.12 Effect of 75mer on NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/µl), α–SNAP (25ng/µl), γ–SNAP (25ng/µl) and p115 (10ng/µl) in the presence of either: buffer (A), 50x molar excess (over p115) of 75mer (B), 200x molar excess (over p115) of 75mer (C), poly-D (D), poly-E (E), poly-D/E (F). Reactions were processed for EM. Note the abundance of unfused tubulovesicular material in B and C (arrowheads) and the presence of reassembled cisternae in A, D-F (arrows). Bar, 0.5µm.
Figure 6.13: Quantitation of effect of 75mer on NSF catalyzed cisternal regrowth.

MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60 min with NSF (100 ng/μL), α-SNAP (25 ng/μL), γ-SNAP (25 ng/μL), and β15 (10 ng/μL) in the presence of either: buffer, a 50-fold molar excess of 75mer, a 200-fold molar excess of 75mer, polyglutamate, polyaspartate, or polyaspartate/glutamate (1:1). Reactions were processed for EM and the amount of cisternal regrowth was determined. Values represent means ± SEM (n = 2).
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A similar dose-dependent effect was observed when the 26mer was added at a 50- or 200-fold molar excess over p115 to the NSF catalyzed reassembly reaction. Cisternae failed to reform, and amongst the reaction products were strings of vesicles, and tubules often aligned in parallel to one another (Figure 6.14A, B, 6.15). This indicated that tethering was occurring, but that the fusion process was somehow inhibited. However, if a phosphorylated form of the 26mer were added to the reaction there was little discernible effect on cisternal regrowth (Figure 6.14C, 6.15). This difference in effect on inhibition of NSF catalyzed cisternal regrowth correlates closely with the inhibition of RLG mediated p115 phosphorylation (Figure 6.11E, F). The phosphorylated form of the 26mer inhibits neither NSF catalyzed cisternal regrowth nor RLG mediated p115 phosphorylation. Therefore, the 26mer may be disrupting the reaction by competing with p115 for the Golgi associated CKII like activity. However, the peptide may still be acting to compete for p115 membrane binding sites, although this explanation is unlikely given that neither the 75mer nor the forms of the 26mer are able to compete very efficiently with p115 for binding to Golgi membranes (Barbara Dirac-Svejstrup, personal communication) or to giantin and GM130 in detergent solution (Figure 6.16B). The phenotype of the membranes on inhibition of the reaction with the 26mer is reminiscent of the reaction products when MGF are incubated at 37°C for 60min with just p115 (Figure 6.14D, E; Rabouille et al., 1995b). Under these conditions tubules, and vesicles and any remaining cisternae can be seen to cluster together, as though aligning ready for membrane fusion (or stacking). Membrane fusion presumably cannot occur under these conditions due to the absence of NSF and SNAPs, whose activity in the post-mitotic reassembly of Golgi membranes is independent of NSF ATPase activity, and by extension SNARE complex disassembly (Müller et al., 1999b; Chapter 7). This clustering of membranes is not observed when p115 is omitted, and MGF are just incubated in buffer alone (Figure 6.14F). Thus, inhibition of the NSF mediated cisternal regrowth by the 26mer and 75mer would not appear to be due to any inhibition of membrane tethering, but rather an inhibition of the transition from membrane tethering to membrane fusion. Taken together the data suggest that the phosphorylation of p115 is required for this transition from tethering to fusion.
Figure 6.14 Effect of 26mer on NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/μl), α–SNAP (25ng/μl), γ–SNAP (25ng/μl) and p115 (30ng/μl) in the presence of either: buffer (A), 200x molar excess (over p115) of 26mer (B) or 26mer-P (C). Alternatively, MGF were incubated at 37°C for 60min in the presence of only p115 (30ng/μl; D and E) or buffer alone (F). Reactions were processed for EM. Note the presence of reassembled cisternae (arrows) in A and C, compared to the presence of clustered, unfused tubulovesicular material in B, D, and E (arrowheads), and the well separated tubulovesicular material in F. Bar, 0.5μm.
Incubation

None
Buffer
50x26mer
200x26mer
50x26mer-P
200x26mer-P

Cisternal regrowth (%)

Figure 6.15 Quantitation of effect of 26mer on NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/μl), α–SNAP (25ng/μl), γ–SNAP (25ng/μl) and p115 (30ng/μl) in the presence of either: buffer, a 50x molar excess (over p115) of 26mer or 26mer-P, or a 200x molar excess (over p115) of 26mer or 26mer-P. Reactions were processed for EM, and the amount of cisternal regrowth was determined. Values represent means±SEM (n=4).
6.2.7 Phosphorylation of S941 enhances binding to GM130 and giantin.

Having established a role for S941 in NSF catalyzed cisternal regrowth it was then of interest to determine how phosphorylation of this residue affected p115 function. To this end, it was tested what effect this phosphorylation had on the binding of p115 to its two Golgi receptors GM130 and giantin. Therefore, N-terminally biotinylated 26mer and phosphorylated 26mer were coupled to neutravidin beads and incubated with Golgi detergent extract. Prior to detergent extraction the membranes had been washed with 1M KCl in order to remove any endogenous p115 and CKII (Barbara Dirac-Svejstrup, personal communication). Both forms of the peptide were able to selectively retrieve GM130 and giantin from Golgi detergent extract, however, the phosphorylated form of the 26mer retrieved c. 2-fold more giantin, and c. 5-fold more GM130 (Figure 6.16A).

More stringent binding conditions were tested, by performing the reaction in free solution by mixing biotinylated peptides with Golgi detergent extract (membranes were again washed with 1M KCl prior to detergent extraction), and then retrieving any complexes that had formed by addition of neutravidin beads at the end of the incubation (Figure 6.16B). Under these conditions less GM130 and giantin were retrieved yet once again the phosphorylated form of the 26mer retrieved more GM130 and giantin than the nonphosphorylated form (Figure 6.16B). The 75mer was also tested under these conditions, and was able to retrieve more GM130 and giantin than the 26mer (Figure 6.16B). Furthermore, performing the reaction with the phosphorylated form of the 75mer, caused a c. 10-fold increase in the amount of GM130 and giantin retrieved, as compared to the nonphosphorylated form of the 75mer. These reactions were also performed with Golgi detergent extract where the membranes had not been previously washed with 1M KCl, so endogenous p115 would remain in the reaction (Figure 6.16B). Under these conditions the amount of GM130 and giantin retrieved by the various peptides was much reduced, however, the phosphorylated form of the 75mer was the most effective competitor for GM130 and giantin with the endogenous p115 (Figure 6.16B).
Figure 6.16 GM130 and giantin from Golgi Triton X-100 extract bind the phosphorylated form of the 26mer better than the non-phosphorylated form. (A) N-terminally biotinylated 26mer and 26mer-P were coupled to neutravidin beads at 2mg/ml. RLG was washed with 1M KCl to remove p115, and then extracted in Triton X-100 buffer. To 200µg Golgi extract in 400µl, 50µl beads were added for 60min at 4°C. Beads were washed, and then eluted with SDS-PAGE sample buffer and fractionated by SDS-PAGE, transferred to nitrocellulose, and probed for GM130 and giantin using specific antibodies. (B) Stringent binding conditions allow efficient binding only of the 75mer-P form, and not the 75mer, 26mer, or 26mer-P. RLG were washed with 1M KCl prior to detergent extraction (+p115) or extracted without prior washing (+p115). To 40µg Golgi extract in 40µl, 12µg of either 26mer/26mer-P or 4µg 75mer/75mer-P was added for 60min at 4°C. Neutavidin beads were then added for 60min at 4°C. Beads were then recovered, washed and eluted into SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE followed by Western blot, probing for GM130 and giantin using specific antibodies.
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6.2.8 Phosphorylation of S941 stimulates complex formation between GM130 and giantin.

To clarify the role of p115 phosphorylation in the context of tethering, it was tested whether inclusion of recombinant CKII could promote the p115/TA dependent formation of the putative tethering complex between GM130 and giantin. The TA constructs were utilized in order to exploit mutations at S941. Since the phosphorylation event seemed to be required for the transition from tethering to fusion, it was decided to allow TA dependent GM130/giantin complexes to form and then add the CKII to the reaction. Therefore, increasing concentrations of the various forms of TA were added to Golgi detergent extract devoid of p115 and incubated at 4°C for 60 min. Recombinant CKII was then added and the temperature raised to 30°C for 10 min. Giantin was then immunoprecipitated and co-immunoprecipitation of GM130 was monitored by Western blotting utilizing a mAb specific for GM130. The addition of CKII enhanced the ability of TA to stimulate complex formation between GM130 and giantin, the effect was most potent at low concentrations of TA (Figure 6.17). TA was only phosphorylated when CKII was present in the reaction (Figure 6.17, left panel; Barbara Dirac-Svejstrup, personal communication). The complex between GM130 and giantin was formed in the presence of TA (S941A), but was not improved by the presence of CKII, indicating that phosphorylation of S941 is the sole determinant for the improved efficiency of formation of the complex between GM130 and giantin (Figure 6.17). TA (S941D) did not show elevated complex formation (Barbara Dirac-Svejstrup, personal communication), indicating that either it did not mimic the phosphorylated form of p115 or that the phosphorylation event must occur after complex formation, where it increases the stability of the complex.

Taken together these data suggest that CKII mediated phosphorylation of p115 on S941 increases its ability to stimulate complex formation between GM130 and giantin, or increases the stability of the complex. Furthermore, this effect on the complex must somehow be required for the transition from COPI vesicle tethering to fusion. The precise composition of this complex remains unclear, and may represent either giantin linked to GM130 via p115, or giantin binding directly to GM130, which would be an
Figure 6.17 Addition of CKII enhances the ability of TA to stimulate complex formation between GM130 and giantin. RLG were washed with 1M KCl to remove endogenous p115 and CKII, and then extracted with Triton X-100. Golgi extracts were incubated at 4°C for 60 min in the presence of increasing concentrations of TA or TA (S941A). 0.2 μunits CKII was then added to half of the reactions and the incubation continued at 30°C for 10 min. Subsequently, anti-giantin antibodies were added for 60 min at 4°C, followed by protein A sepharose for 60 min at 4°C. The sepharose beads were then washed and eluted into SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and Western blot, probing with anti-GM130 and anti-RGS-His (TA) antibodies. The amount of GM130 co-immunoprecipitated with giantin is shown as a percentage of input GM130. After blotting, TA phosphorylation was monitored by phosphoimaging the blot. Reactions containing TA and CKII resulted in TA being phosphorylated as shown in right panel.
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event catalyzed by the presence of p115. A GM130 mutant lacking the p115 binding region is not co-immunoprecipitated with giantin in this assay in the presence of p115 (Barbara Dirac-Svejstrup, personal communication). This suggests that it is likely that the complex represents a giantin-p115-GM130 bridge, or that giantin binds to GM130 at the same site as p115, and that p115 somehow facilitates this binding without being a final part of the complex. Purification and precise molecular analysis of the tethering complex will be required to differentiate between these possibilities.

6.2.9 The 26mer and CKII substrate peptide do not interfere with p115 mediated stacking of p97 generated cisternae.

In addition to being essential for the NSF mediated fusion process, p115 also plays a role in stacking p97 generated cisternae, but is not required for the p97 catalyzed fusion process per se. Therefore, it was determined whether CKII phosphorylation of p115 was also required for p115 dependent stacking of p97 generated cisternae, by adding to the p97 catalyzed reaction 26mer or CKII substrate peptide at levels which inhibited NSF catalyzed cisternal regrowth and RLG mediated p115 phosphorylation. Addition of either 26mer or CKII substrate peptide, or their phosphorylated counterparts had very little effect on p97 catalyzed cisternal regrowth or the stacking that was facilitated by p115 (Figure 6.18, 6.19). That stacking was unaffected by these reagents is consistent with the fact that complex formation between GM130 and giantin does not require p115 phosphorylation (Figure 6.17) and that membranes can still be brought into close apposition in a p115 dependent style, even in the presence of agents that prevent p115 phosphorylation (Figure 6.12, 6.14). Therefore, one possibility is that the phosphorylation of p115 at S941 serves as a switch that indicates that this tethering complex is destined to be involved in membrane fusion, and so is passed onto the fusion machinery. Alternatively, the absence of phosphorylation may indicate the tethering complex is to be involved in the stacking process, and so is passed onto the stacking machinery. It may also be that the SNARE molecules are components of both the fusion and the stacking machinery (Rothman and Warren, 1994), and so are downstream in both pathways.
Figure 6.18 Effect of 26mer and CKII substrate on p97 catalyzed Golgi reassembly.

MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with p97 (70ng/μl), p47 (37.5ng/μl) and p115 (15ng/μl) in the presence of either buffer (A), a 200x molar excess (over p115) of 26mer (B) or 26mer-P (C), or a 100x molar excess (over p115) of CKII substrate peptide/phosphopeptide (D and E). Reactions were processed for EM. Note the presence of stacked reassembled cisternae (arrows) in all reactions. Bar, 0.5μm.
Figure 6.19 Quantitation of effect of 26mer and CKII substrate peptide on p97 catalyzed Golgi reassembly. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with p97 (70ng/μl), p47 (37.5ng/μl) and p115 (15ng/μl) in the presence of either: buffer, a 200x molar excess (over p115) of 26mer or 26mer-P, or a 100x molar excess (over p115) of CKII substrate peptide/phosphopeptide. Reactions were processed for EM and the amount of cisternal regrowth and the percentage total membrane present as stacks was determined. Values represent means±SEM (n=2).
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6.3 The first coiled-coil domain of p115 can interact with SNAREs and is required for NSF catalyzed cisternal regrowth.

6.3.1 p115 637-699 and full length p115 are able to retrieve SNARE molecules from Golgi detergent extract.

p115 is a homodimer, with two large N-terminal globular heads, followed by a coiled-coil rich rod like domain, and a short acidic stretch at the C-terminal extremity (Sapperstein et al., 1995; Yamakawa et al., 1996). The coiled-coil rich tail may be responsible for p115 dimerization, and is 45nm in length in mammalian p115 (150nm in Uso1p) and consists of four regions with a high probability of forming coiled-coils (Sapperstein et al., 1995; Barroso et al., 1995). Interspersed between these coiled-coil regions are glycine and proline rich regions, which will disrupt α-helices, and thus are unlikely to enter into coiled-coil interactions. These interspersions may confer some flexibility to the p115 tail, and possibly act as hinges, similar to the tail of myosin I (Lupas et al., 1991; Oas and Endow, 1994).

The first coiled-coil domain of p115 is reported to have weak sequence similarity, but notable given the biological context, to the membrane proximal H3 coiled-coil domain (Kee et al., 1995) or SNARE motif of the syntaxin superfamily (Weimbs et al., 1997). In syntaxin-1, the H3 domain has been implicated in interactions between SNAP-25, VAMP1/synaptobrevin, α-SNAP, and synaptotagmin (Bennett, 1995). While in Sed5p (yeast homologue of syntaxin-5) the H3 domain may be required for homodimerization (Banfield et al., 1994). The H3 domain of syntaxin-1 is hypothesized from nuclear magnetic resonance studies to be the domain contributed by syntaxin-1 to the four helix bundle (SNAP-25 contributes two coiled-coil domains and VAMP1 one) in the ternary complex of syntaxin-1/SNAP-25/VAMP1 that is proposed to drive bilayer mixing (Fiebig et al., 1999; Parlati et al., 1999). The fact that p115 and SNAREs are postulated to act in the tightly coupled, sequential steps of vesicle tethering (p115 dependent), docking and fusion (SNARE dependent; Pfeffer, 1999) suggests that this weak sequence similarity may actually reflect an evolutionary relationship. This would indicate an important role for this domain of p115 in perhaps physically linking the sequential steps of tethering and docking in the transport reaction. Therefore, synthetic
N-terminally biotinylated peptides corresponding to the four coiled-coil domains of p115 were synthesized (Figure 6.20). Structural studies suggest that synthetic peptides of this length should form stable coiled-coils (Hodges, 1988; O'Shea et al., 1989). The relative ability of these peptides to retrieve SNARE molecules from Golgi detergent extract, and their effect of NSF catalyzed cisternal regrowth was then determined.

Biotinylated p115 637-699 was coupled to neutravidin beads and used to probe Golgi detergent extract. Fractionation of the polypeptides retrieved by the p115 637-699 beads by SDS-PAGE followed by silver staining revealed a number of bands that were specific for p115 637-699 as compared to mock beads (asterisks in Figure 6.21A). To attempt to identify these specific bands, the retrieved, fractionated polypeptides were transferred to nitrocellulose and probed using a panel of specific antibodies (Figure 6.21B). p115 637-699 did not retrieve any detectable giantin or GM130 (Figure 6.21B), unlike full length p115 (Figure 6.2; Nakamura et al., 1997; Sönntichsen et al., 1998) and this is consistent with the binding site for both giantin and GM130 being in the acidic tail of p115 (Figure 6.2; Nelson et al., 1998). Neither did p115 637-699 retrieve any p115 (Figure 6.21B) suggesting it was not acting to stably disrupt p115 dimers in detergent solution. The H3 domain of syntaxin-1 has been implicated in binding to α-SNAP (McMahon and Südhof, 1995), thus α-SNAP was probed for, but retrieval could not be detected (Figure 6.21B). Given that specific bands for p115 637-699 compared to mock beads were seen at approximately 22kDa and 24kDa (lower two asterisks in Figure 6.21A), it was tested whether these might correspond to Rab proteins. However, neither Rab6 nor Rab1 retrieval could be detected (Figure 6.21B). However, p115 637-699 was able to selectively retrieve the Golgi localized t-SNARE protector Sly1p (Dascher and Balch, 1996), the short (36kDa) form of the Golgi localized t-SNARE syntaxin-5 (Hui et al., 1997; Hay et al., 1998) and the Golgi localized v-SNARE GOS28 (Nagahama et al., 1996; Hay et al., 1998) from Golgi detergent extract (Figure 6.21B). To estimate the efficiency of the retrieval 10% of the total Golgi detergent extract was also fractionated by SDS-PAGE and transferred to nitrocellulose (Figure 6.21A, B second lane). The most significant retrieval was of
Figure 6.20 p115 coiled-coil domains. (A) Amino acid sequence of rat p115 (P41542), light grey shading denotes the coiled-coil tail, while dark grey denotes the acidic domain. (B) The amino acid sequences of the synthetic peptides: p115 637-699, 728-765, 788-827 and 843-930 used in this study. Each peptide represents a coiled-coil domain of p115.
Figure 6.21 Biotinylated p115 637-699 coupled to neutravidin beads retrieves slyl, syntaxin-5 and Gos-28 from Golgi detergent extract. (A, B) Biotinylated p115 637-699 was coupled to neutravidin beads at 2.8mg/ml. 20μg RLG was extracted with 0.5% Triton X-100, and added to 10μl beads (p115 637-699, or mock) in a final volume of 200μl, and incubated for 60min at 4°C. Beads were recovered, washed in extraction buffer, and eluted into SDS-PAGE sample buffer. Samples were fractionated by SDS-PAGE, and either silver stained (A; asterisks denote bands specific for p115 637-699) or transferred to nitrocellulose (B) and probed for markers with specific antibodies. (C) 10μg of RLG (lanes 1 and 3), or 1M KCl washed RLG (lane 2) were fractionated by SDS-PAGE and transferred to nitrocellulose. The blot was probed without peptide (mock) or with 5μg/ml biotinylated p115 637-699 in 10% milk dissolved in PBS. The blot was then washed for 60min with 10% milk and the probed with avidin-HRP. Bands were visualized by ECL. Arrow indicates specific band for p115 637-699.
GOS28, in that greater than 10% of the total GOS28 was retrieved from Golgi detergent extract. The retrieval of Sly1p was less efficient in that just less than 10% was retrieved. The short form of syntaxin-5 (36kDa, Hui et al., 1997) was also retrieved, but this was much less efficient than GOS28 and Sly1p retrieval. The amounts of GOS28, Sly1p and syntaxin-5 retrieved were not as much as the amounts of GM130 or giantin that can be retrieved from Golgi detergent extract by full length p115, which perhaps suggests the interactions are less stable or more transient in nature.

A Far-Western approach to determine possible p115 637-699 interacting proteins was also taken. This approach that has been successful in identifying p115 interacting proteins previously (Nakamura et al., 1997; Sönnichsen et al., 1998). RLG or 1M KCl washed RLG were fractionated by SDS-PAGE and transferred to nitrocellulose. The blot was then blocked, and probed with the N-terminally biotinylated p115 637-699, and specific bands were revealed by subsequent probing with avidin-HRP and ECL visualization. Omission of the p115 637-699 stage revealed that a doublet above the 66kDa marker, and a band of around 115kDa were interacting with avidin-HRP alone (Figure 6.21C, mock). However, p115 637-699 recognized a band of around 107kDa, the correct size for p115, and this band was removed upon prior 1M KCl washing of RLG, also consistent with it being p115 (Figure 6.21C). This of course cannot be confirmed without sequencing, but possibly suggests that p115 637-699 is required for p115 dimerization as it interacts with the monomeric form of p115 by Far-Western. That bands corresponding to the size of Sly1p, syntaxin-5 or GOS28 were not detected may reflect the different conformation that these proteins will adopt when coupled to a nitrocellulose matrix, as compared to being free in detergent solution. A Far-Western approach may also not be optimal for detecting transient or less stable interactions.

Next it was tested whether full length p115 and the other coiled-coil domains of p115 could retrieve syntaxin-5 and GOS28 from Golgi detergent extract. Owing to the difficulty in concentrating purified rat liver p115 the concentration of the respective ligand was reduced on the beads to 5μM. Full length p115 was also able to retrieve
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GOS28 and syntaxin-5 from Golgi detergent extract (Figure 6.22). Interestingly, full length p115 could retrieve syntaxin-5 more efficiently than p115 637-699, and none of the other coiled-coil domains nor mock beads could retrieve syntaxin-5 (Figure 6.22). Full length p115 could also retrieve GOS28 from Golgi extract, and this retrieval was similar to the amount of GOS28 retrieved by p115 637-699 (Figure 6.22). Once again GOS28 was pulled down more efficiently than syntaxin-5. Neither p115 728-765 nor 788-827 retrieved any detectable GOS28, yet p115 843-930 did, although not as efficiently as either p115 or p115 637-699 (Figure 6.22).

6.3.2 Excess p115 637-699 inhibits NSF catalyzed cisternal regrowth

If p115 637-699 represents a functionally important region of p115, possibly by mediating interactions with GOS28, Sly1p or syntaxin-5, then addition of excess p115 637-699 should compete for such interactions and compromise p115 function. Therefore p115 637-699, and the other coiled-coil domains of p115 were added to the NSF catalyzed reassembly reaction at a 150-fold molar excess over p115. Addition of no peptide, p115 728-765, 788-827, or 843-930 had no effect on the NSF catalyzed reassembly, stacks of cisternae reformed normally (Figure 6.23A-D, 6.24A). That p115 843-930 did not seem to cause any inhibition of cisternal regrowth suggested that the ability of this peptide to retrieve GOS28 from Golgi detergent extract may not be biologically significant. A 150-fold molar excess of p115 637-699 over p115 reduced NSF catalyzed cisternal regrowth by c. 80% (Figure 6.23F, 6.24A), and in fact a 15-fold molar excess of p115 637-699 over p115 in the reaction inhibited cisternal regrowth by c. 40% (Figure 6.23E, 6.24B). The amount of inhibition was strongly dependent on the dose of p115 637-699 (Figure 6.24B). This result strongly suggests that the first coiled-coil domain of p115 participates in NSF catalyzed membrane fusion, and this may possibly be through interactions between GOS28, Sly1p and/or syntaxin-5, thus physically linking COPI vesicle tethering to docking and fusion.
Figure 6.22 Retrieval of syntaxin-5 and Gos-28 from Golgi detergent extract by full length p115 and the other coiled-coil domains of p115. Biotinylated purified rat liver p115, p115 637-699, 728-765, 788-827, and 843-930 were coupled to neutravidin beads at 5μM. 20μg RLG was extracted with 0.5% Triton X-100 and added to 10μl beads in a final volume of 200μl, and incubated for 60min at 4°C. Beads were recovered, washed in extraction buffer and eluted into SDS-PAGE sample buffer. Samples were fractionated by SDS-PAGE and transferred to nitrocellulose, and probed with specific antibodies raised against syntaxin-5 and Gos-28.
Figure 6.23 p115 637-699 interferes with NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (10ng/μl) in the presence of either: buffer (A), 13μM p115 728-765 (B), 788-827 (C), 843-930 (D), 637-699 (F) or 1.3μM p115 637-699 (E). Reactions were processed for EM. Arrows denote reassembled cisternae which are prevalent in A-D, as compared to the more frequent unfused tubulovesicular material (arrowheads) found in E and F. Bar, 0.5μm.
Figure 6.24 Effect of p115 coiled-coil domains on NSF catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with NSF (100ng/μl), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (10ng/μl) in the presence of either: buffer, or a 150x molar excess (over p115) of p115 637-699, 728-765, 788-827, or 843-930 (A) or increasing amounts of p115 637-699 (B). Reactions were processed for EM and the amount of cisternal regrowth was determined. Values represent means±SEM (n=3).
6.4 Discussion

6.4.1 Binding of p115 to GM130 and giantin.

Deletion analysis of p115 and affinity probing of Golgi detergent extract with synthetic peptides revealed that the minimal region of p115 required for binding to GM130 and giantin is the short acidic C-terminus (28 amino acids in *Bos taurus*, 25 amino acids in *Rattus norvegicus*). p115 binds to GM130 at its extreme basic N-terminus (Nakamura et al., 1997), suggesting that the interaction may be electrostatic in nature, and this would explain why p115 can be removed from Golgi membranes by high salt concentrations (Waters et al., 1992b; Levine et al., 1996). In contrast to GM130 where the p115 binding site resides in the first 73 N-terminal amino acids, the N-terminal 448 amino acids of giantin are required for p115 binding (Lesa et al., 2000). This region of giantin is predicted to be mostly coiled-coil and does not seem to have any particular features apart form a stretch of basic amino acids around position 100, that is similar but not identical to that found in the p115 binding site of GM130. However, since the first 186 amino acids of giantin alone, do not associate with p115, this basic stretch is either not required or insufficient for p115 binding. p115 binding to giantin may then require some higher order conformation in giantin.

6.4.2 The globular heads of p115 are dispensable for NSF catalyzed cisternal regrowth.

Even though the acidic C-terminus of p115 is sufficient to bind GM130 and giantin individually it is not sufficient to stimulate complex formation between GM130 and giantin (Barbara Dirac-Svejstrup, personal communication), and neither is the 75mer which includes a stretch of the last coiled-coil domain. This may be due to the fact that the 75mer and 26mer do not form dimers. The TA mutant which does form a dimer (Barbara Dirac-Svejstrup, personal communication) is able to stimulate complex formation, and also stimulate NSF catalyzed cisternal regrowth to approximately 60-70% that of full length p115. This suggests that the globular heads of p115, which comprise c. 70% of the entire molecule are not essential for p115 function during Golgi reassembly, although they possibly play some negative regulatory role, as their presence seems to occlude binding to GM130 and giantin. This lack of function was
surprising given that the head domains appear to be much more conserved than either the tail or the acidic domain (Sapperstein et al., 1995). However, deletion analysis has revealed that the globular head domains are required for proper targeting of p115 to the Golgi membrane in vivo (Nelson et al., 1998). p115 is also localized to VTCs in vivo (Nelson et al., 1998), and binding to these structures does not require interaction with GM130 or giantin (Nakamura et al., 1997; Nelson et al., 1998; Seemann et al., 2000; Lesa et al., 2000), but is affected by deletions in the globular head domain, which causes p115 to localize more with ER like structures (Nelson et al., 1998). The globular heads may then be required for binding to a receptor on VTCs where it may be involved in VTC maturation (Seog et al., 1994). Such interactions may be required to target p115 to the Golgi region, where it may then bind GM130 and giantin (Nakamura et al., 1997; Seemann et al., 2000; Lesa et al., 2000). Another possibility is that the globular heads play a role in the binding of p115 to CTP:phosphocholine cytidylyltransferase (CT) and so be important in the putative alternative function of p115 in phosphatidylcholine biosynthesis (Feldman et al., 1998).

6.4.3 The p115 kinase is CKII or a CKII like molecule.

The acidic C-terminus of p115 contains a serine (S941) that is phosphorylated by a Golgi associated kinase (Sohda et al., 1998). The Golgi associated p115 kinase was identified as CKII or a CKII like molecule on the basis of several observations. First, the amino acid sequence in p115 surrounding S941 resembled a CKII phosphorylation motif. However, the sequence in p115 did lack the canonical acidic residue at position +3, yet some CKII substrates which lack this residue are known (e.g. HMG protein 14, clathrin light chain B [Pinna, 1990], cation-dependent mannose-6-phosphate receptor [Korner et al., 1994]). p115 does have an aspartate at position +2 and +4, and given the acidity of the region it seemed likely that it would serve as a CKII site. S941 is not conserved from p115 in mammals to the p115 homologue Uso1p in S.cerevisiae. However, it is of interest to note that Uso1p has two CKII phosphorylation sites right at the start of the acidic domain at S1770 and S1771. These two sites fit the CKII phosphorylation motif perfectly, in that they both have the canonical acidic residue at position +3 (Pinna, 1990). Second, the Golgi associated kinase could use GTP as a
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phosphate source, a kinase trait peculiar to CKII (Allende and Allende, 1995). Third, recombinant CKII could phosphorylate p115 and TA on S941 in vitro. Fourth, RLG mediated p115 phosphorylation was inhibited by competitive inhibitors of ATP binding to CKII (staurosporine, DRB, chrysin), specific anti-CKII antibodies, and CKII substrates (26mer and CKII substrate peptide). Taken together these data provide evidence that CKII or a CKII like Golgi activity is able to phosphorylate p115 in vitro.

Since the kinase activity has not been purified from our source of RLG it cannot be definitively said that the kinase is actually CKII or an isokinase with similar activity. Therefore, it is of interest that RLG contain a CKII like molecule termed Golgi-enriched-fraction casein kinase (GEF-CK; Lasa-Benito et al., 1996; Lasa et al., 1997a; Lasa et al., 1997b). However, GEF-CK is unlikely to be the p115 kinase activity, since it appears to be insensitive to staurosporine, and cannot use GTP as a phosphate source (Lasa et al., 1997a). In addition, GEF-CK may be an integral membrane protein, with most of its mass projecting into the Golgi lumen, therefore having the wrong orientation to phosphorylate p115 (Pascall et al., 1981; Moore et al., 1985). Specific antibodies against the α- and β-subunits of CKII revealed their presence on RLG, suggesting that active CKII may be present on RLG.

Whether CKII or a CKII like kinase phosphorylate p115 in vivo, or whether the above represents the promiscuous activity of a kinase in vitro will require further experiments. CKII is a heterotetrameric holoenzyme consisting of CKIIα or CKIIα' (an isoform of α) as the catalytic subunit and CKIIβ as the noncatalytic subunit. The β-subunits dimerize via zinc fingers (Chantalat et al., 1999) and link to two α-subunits together to form a α₂β₂ heterodimer (Gietz et al., 1995). CKII is ubiquitously found in the nucleus and cytoplasm (although 3-15 fold enriched in the nucleus relative to cytosol dependent on the cell cycle stage [Lorenz et al., 1993]) of eukaryotes (Allende and Allende, 1995), and is an essential component in living cells as demonstrated by the lethality of its gene disruption in yeast (Padmanabha et al., 1990), and its designation as an oncogene (Xu et al., 1999a). Well over 100 CKII substrates are known (Pinna and
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Meggio, 1997), although understanding the co-ordination of the multitudinous, heterogeneous roles of CKII is in reality only in its infancy. CKII signalling appears to be second messenger independent and seems to play a central role in cell growth and differentiation and may operate at the levels of DNA replication, regulation of basal and inducible transcription, translation and the control of metabolism (for recent reviews see Guerra et al., 1999; Xu et al., 1999a; Pinna and Meggio, 1997; Allende and Allende, 1995). To give just four diverse examples to illustrate the omnipresence of CKII function, the β-subunit of CKII negatively regulates Xenopus oocyte maturation by titrating out the serine/threonine kinase Mos (Chen and Cooper, 1997). This interaction with Mos is independent of CKIIα subunits, suggesting the β-subunit is capable of more promiscuous interactions (Kusk et al., 1999). Mos is rendered inactive by binding to CKIIβ, and expression levels of Mos must rise above that of CKIIβ for there to be free Mos which can then promote oocyte maturation by activating the MAP kinase pathway (Chen and Cooper, 1997). Second, in mammalian fibroblasts, microinjection of anti-CKIIβ can bias CKII distribution and has revealed cell cycle transition from G0 to S requires the presence of a certain functional level of CKII at defined times and at defined cellular locations as follows: for transition of G0/G1 at both the nucleus and the cytoplasm, for transition of early G1 at the nucleus, and for transition of G1/S at the cytoplasm (Lorenz et al., 1993; Pepperkok et al., 1994). Third, mice lacking the CKIIα' subunit exhibit globozoospermia (Xu et al., 1999b) suggesting that different isoforms of CKII may have distinct roles during development. Fourth, CKII is extremely concentrated in the brain, where its function is virtually unknown, but a recent study suggests CKII may regulate NMDA channel gating in hippocampal neurons (Lieberman and Mody, 1999).

In addition to these apparent global regulatory roles for CKII, subpopulations of CKII have also been found associated with intracellular membranes and implicated in diverse secretory processes. ER associated CKII (Ou et al., 1992; Cala et al., 1993) phosphorylates the cytoplasmic tail of calnexin, and in so doing enhances calnexin association with ribosomes thereby leading to increased glycoprotein folding adjacent to the translocon (Chevet et al., 1999). CKII activity is required for transferrin receptor
endocytosis, although the precise targets remain unknown (Cotlin et al., 1999). CKII has also been localized with the Golgi apparatus (Wu et al., 1995) and CKII phosphorylation has been implicated in the correct sorting and Golgi localization of a number of proteins (Alconada et al., 1996; Eng et al., 1999). For example, the retrieval of the endoprotease furin from immature secretory granules (ISG) by clathrin coated vesicles to the TGN requires a CKII mediated phosphorylation of the cytoplasmic tail of furin (Jones et al., 1995; Dittié et al., 1997). This phosphorylation facilitates an interaction between furin and AP1, so enabling sorting of furin into a nascent clathrin coated vesicle (Dittié et al., 1997). Similarly, CKII may modulate clathrin assembly by phosphorylation of the cytoplasmic tails of both cation-dependent and cation-independent mannose-6-phosphate receptors, which enhances AP1 recruitment (Mauxion et al., 1996; Dittié et al., 1999). CKII may also negatively regulate clathrin assembly by phosphorylation of AP180, which weakens its interaction with AP2, an interaction required for clathrin assembly (Hao et al., 1999). CKII has also been found to phosphorylate components of the core membrane fusion machinery: synaptobrevin (Nielander et al., 1995), synaptotagmin, syntaxin-1 and -4 (Bennett et al., 1993b; Foster et al., 1998; Risinger and Bennett, 1999) suggesting CKII mediated phosphorylation may play a modulatory role in SNARE interactions. Therefore, a role for CKII in regulating Golgi membrane fusion processes may not be without precedent. However, the possibility still remains that the responsible kinase is not CKII, but a kinase possessing similar characteristics.

6.4.4 Role of CKII mediated phosphorylation of p115 in post-mitotic Golgi reassembly.

Whatever the precise identity of the p115 kinase, phosphorylation of p115 appears to be crucial for NSF catalyzed cisternal regrowth. Since p115 could not be expressed successfully in bacteria, the functionality of the TA mutant in NSF catalyzed cisternal regrowth was exploited to study the importance of S941 in reassembly. TA mutants where S941 is mutated to alanine or aspartate are not able to support NSF catalyzed cisternal regrowth, but are still able to stimulate complex formation between GM130 and giantin in Golgi detergent extract. This suggested that TA mutants may be deficient
in the NSF mediated cisternal regrowth at the stage of going from COPI vesicle tethering to fusion, and that the p115 phosphorylation event *per se* was required for this event, since the alanine mutant (which will not get phosphorylated) and the aspartate mutant (which may mimic the phosphorylated form of p115) were not functional for cisternal regrowth.

Inhibition of CKII during the NSF catalyzed reassembly reaction using the same range of inhibitors that inhibit RLG mediated p115 phosphorylation caused a parallel inhibition of cisternal regrowth. The most striking phenotype was displayed when the 75mer or 26mer were added at a 200-fold molar excess over p115 to the NSF catalyzed reassembly reaction. Here vesicles and tubules seemed to align in parallel as though in preparation for fusion, in a manner similar to the strings of tethered profiles seen when just p115 was added to the reaction. This effect of the 26mer and 75mer was thought to be more due to inhibition of the kinase, rather than competing for p115 binding to Golgi membranes, since phosphorylated forms of the peptides had no effect on cisternal regrowth yet were better competitors for binding to GM130 and giantin. This vivid phenotype is suggestive of an inhibition at the level of the transition from tethering to fusion. However, why this phenotype was most striking for these peptides, rather than for other methods of preventing p115 phosphorylation (i.e. CKII substrate peptide, CKII inhibitors, anti-CKII antibodies, TA mutants) is unclear.

Phosphorylation of p115 at S941 may enhance p115 binding to GM130 and giantin as revealed by the fact that phosphorylated forms of the 75mer and 26mer are able to retrieve more GM130 and giantin from Golgi detergent extract, and compete better with endogenous p115 for these interactions. This would be consistent with the interaction between GM130 and p115 being electrostatic in nature. In simple terms, the phosphorylation of S941 would make this region of p115 more acidic, and therefore may enhance the strength of an ionic interaction with the basic N-terminus of GM130. Equally the Cdc2 kinase mediated phosphorylation of GM130 at mitosis (Nakamura et al., 1997; Lowe et al., 1998b) may serve to weaken the interaction by making the basic N-terminus of GM130 more acidic.
Recombinant CKII was able to stimulate the p115/TA dependent formation of the putative tethering complex between GM130 and giantin. Addition of recombinant CKII after TA dependent GM130/giantin complexes had formed improved the recovery of this complex, suggesting either more complexes had formed, or that the complex was more stable after CKII action. This effect was specific for phosphorylation of S941, as it was not seen when the nonphosphorylatable TA (S941A) mutant was used. When TA (S941D) was used no elevated complex formation was seen, which suggested that either the aspartate did not mimic the phosphorylated serine very effectively, or that the phosphorylation occurs after GM130-p115-giantin complex formation, and acts to strengthen the interaction.

The precise nature of the tethering complex is still not resolved, in that although p115 is able to stimulate complex formation between GM130 and giantin it is unclear whether p115 participates stoichiometrically forming an integral part of the final complex, or acts catalytically to bind GM130 directly to giantin. p115 (or TA) is also coimmunoprecipitated with giantin, but much more efficiently than GM130, suggesting that this represents the binary complex between giantin and p115. This signal conflates with any signal that may be due to a GM130-p115-giantin (or permutation thereof) heteroternary complex. It would appear the simplest explanation is that p115 crosslinks GM130 to giantin, since a GM130 mutant lacking the p115 binding domain, does not coimmunoprecipitate with giantin in the presence of p115 from Golgi detergent extract, where the Golgi had been washed with carbonate prior to detergent extraction to remove all endogenous p115 and GM130 (Barbara Dirac-Svejstrup, personal communication). However, this does not exclude the possibility that giantin utilizes the same binding site as p115 on GM130. Until analysis of the composition of the tethering complex in isolation is performed, it is not possible to distinguish between these possibilities. This may be resolved when it is possible to generate recombinant molecules which can be added in stoichiometric amounts in solution. However, given the coiled-coil nature of the molecules involved, and their vast sizes, this presents a formidable technical challenge.
How the increased stability of the tethering interaction caused by CKII mediated phosphorylation of p115, may contribute to the transition from tethering to fusion is unclear. It may be that the strengthening of the bridging interaction between COPI vesicle and its target membrane may allow more efficient sampling of the membrane for a cognate SNARE, thus leading to steps downstream of tethering and ultimately membrane fusion. Alternatively, the phosphorylation of p115 may cause an alteration in the conformation of the tethering complex, which enables other protein interactions, such as bringing the cognate SNAREs into contact which would lead to the progression from tethering to fusion. This phosphorylation event did not seem to be required for the stacking of p97 generated cisternae, a process that is also p115 dependent. Thus, phosphorylation may act as a signal marking which tethering complexes are to be involved in fusion and which are to be involved in stacking. At steady state interphase conditions this may imply that if tethering complexes are linking adjacent cisternae in the stack at the peripheral rim, such that a COPI vesicle is pretethered to its acceptor compartment before budding is complete, that the p115 phosphorylation may be dependent on the completion of the budding process. This phosphorylation may then trigger the rearrangements needed to bring the SNAREs into contact and result in bilayer mixing. In this way only tethering complexes linking COPI vesicle to cisternae will be involved in membrane fusion, and not tethering complexes linking cisterna to cisterna, with the result of maintaining the integrity of discrete Golgi compartments.

However, whether this phosphorylation event is required for solely post-mitotic Golgi reassembly or for intra-Golgi transport in addition remains to be determined. Phosphorylation events have been implicated in interphase intra-Golgi transport processes both in vivo and in vitro (Lucocq et al., 1991; Davidson et al., 1992). Cis-medial intra-Golgi transport is insensitive to staurosporine (Davidson et al., 1992; Stuart et al., 1993; Mackay et al., 1993), but transport steps from the medial to later Golgi compartments are staurosporine sensitive (Davidson and Balch, 1993). However, CKII is relatively insensitive to staurosporine (Meggio et al., 1995), and no reports exist in the literature of testing the effects of specific CKII inhibitors on intra-Golgi transport. Phosphorylation would seem to be important at some level since both
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Microcystin and okadaic acid can inhibit intra-Golgi transport, suggesting the involvement of a phosphatase (Lucocq et al., 1991; Davidson et al., 1992; Mackay et al., 1993), which may be PP2A (Davidson et al., 1992). Interestingly, homotypic vacuolar fusion is also microcystin sensitive (Conradt et al., 1994) and the microcystin sensitive factor has recently been identified to be PPI, and is thought to act downstream of SNAREs in the fusion process (Peters at al., 1999). Whether p115 phosphorylation is required for steady state transport reactions will require further experiments.

The yeast homologue of p115, Uso1p, lacks S941 in its acidic C-terminus, but does possess two CKII phosphorylation motifs right at the start of the acidic C-terminus, suggesting Uso1p function may also be modulated by CKII. Precisely how Uso1p functions in vesicle tethering in yeast is less well defined, in that obvious homologues or analogues of GM130 and giantin in yeast have not been identified. Therefore, it is uncertain whether the molecules required and therefore the mechanism of tethering is conserved. Whatever the case, the tethering complex in yeast is likely to be differentially regulated during mitosis as compared to mammals since yeast do not dismantle their Golgi apparatus at mitosis nor stop secretion (Makarow, 1988; Preuss et al., 1992), possibly due to the need to be constantly building a cell wall during cell division (Warren, 1993).

At first glance the results obtained here concerning p115 phosphorylation may seem to contradict those obtained by Ikehara and colleagues (Sohda et al., 1998). These workers found that the majority of phosphorylated p115 resided in the cytosol and displayed a lower affinity for binding to Golgi membranes (Sohda et al., 1998). The phosphorylation of p115 would appear here to increase its affinity for its two Golgi receptors GM130 and giantin. However, this phosphorylation event seems to be required from the transition from COPI vesicle tethering to fusion, which *in vivo* may be a very rapid process. Perhaps phosphorylation of p115 causes conformational changes in the tethering complex which promote membrane fusion and that once fusion is complete p115 is released from the membrane in the phosphorylated state.
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Therefore, p115 phosphorylation would be coupled to completion of the transport reaction and release from the membrane. As a result when the steady state distribution of p115 is studied it would appear that phosphorylated p115 is mostly cytosolic. Dephosphorylation of p115 may then be required to recycle p115 to the correct place, for subsequent rounds of vesicle transport. Further work will be required to establish the cycling properties of p115 in the transport process.

6.4.5 The first coiled-coil domain of p115 participates in NSF catalyzed cisternal regrowth.

A synthetic peptide, p115 637-699, representing the first coiled coil domain of p115 was able to inhibit NSF catalyzed cisternal regrowth in a dose dependent manner, while peptides representing the other coiled-coil domains of p115 had no effect. This strongly suggests that this domain contributes to p115 function in NSF catalyzed cisternal regrowth. This domain of p115 is conserved in Uso1p, and possesses weak sequence similarity to the membrane proximal coiled-coil domain of the syntaxin superfamily, the H3 domain or SNARE motif (Kee et al., 1995; Weimbs et al., 1997). The H3 domain of syntaxin-1 is important for interactions with SNAP-25, synaptobrevin, α-SNAP and synaptotagmin (Bennett, 1995). In fact, the H3 domain represents the minimal SNARE interacting domain of syntaxin-1, and is the coiled-coil contributed by syntaxin-1 to the four parallel α-helices packed into a single bundle at the core of the v-/t-SNARE complex (Hanson et al., 1997; Lin and Scheller, 1997; Hohl et al., 1998; Sutton et al., 1998). This complex forms an extraordinarily thermodynamically stable complex, and is even stable in 0.1% SDS (Hayashi et al., 1994). The energy gained from formation of such a stable complex may be transformed, in concert with close membrane apposition, to drive the membrane fusion reaction. Supportive evidence for this hypothesis comes from the fact that when purified neuronal v- and t-SNAREs are reconstituted into distinct liposome vesicle populations, they are sufficient to drive bilayer fusion and lumenal mixing (Weber et al., 1998; McNew et al., 1999; Nickel et al., 1999; Parlati et al., 1999).
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Given this slight homology suggested an important role for this region of p115, it was determined what, if any, interactions were mediated by p115 637-699. Probing Golgi detergent extract with p115 637-699 coupled to beads revealed a selective retrieval of GOS28, Sly1p and syntaxin-5. The other coiled-coil domains of p115 were unable to retrieve these molecules, with the exception of p115 843-930, the last coiled-coil domain of p115 which was able to retrieve some GOS28. However, this interaction may not be of functional importance since this region of p115 was without effect on NSF catalyzed cisternal regrowth. Full length p115 was also able to retrieve GOS28 and syntaxin-5 from Golgi detergent extract, and an interaction with Sly1p has not yet been looked at. That p115 can interact with SNARE molecules provides direct evidence for a physical link between the processes of COPI vesicle tethering (p115 dependent) and subsequent docking and fusion (SNARE dependent). Therefore, it may be that these interactions are crucial for the transition from tethering to docking and fusion.

At the moment it is not possible to determine which of these interactions are direct, or whether the retrieval of GOS28, Sly1p and syntaxin-5 represents the retrieval of a protein complex. Especially since the v-SNARE GOS28 interacts directly with the short form of the t-SNARE syntaxin-5 (Hay et al., 1997, 1998), and Sly1p interacts directly with syntaxin-5 (Dascher and Balch, 1996). However, GOS28 and syntaxin-5 and Sly1p are unlikely to all be in the same complex. This is because Sly1p is a t-SNARE protector (Pfeffer, 1999), it binds to the N-terminal coiled-coil region of syntaxin-5, and acts to prevent the t-SNARE from binding a cognate v-SNARE unless the appropriate Rab protein is present (Rothman and Söllner, 1997; Lupashin et al., 1997). Therefore, complex formation between GOS28 and syntaxin-5 would require the prior removal of Sly1p from syntaxin-5 (Lupashin and Waters, 1997). It may then be that p115 637-699 is performing at least two discrete interactions. The retrieval of GOS28 by p115 637-699 from Golgi detergent extract was the most efficient, with greater than 10% of the total GOS28 being retrieved, suggesting this may be the primary interaction mediated by p115 637-699. The interaction with Sly1p was more efficient than the interaction with syntaxin-5, suggesting that this may also be a direct
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interaction. These predictions will need to be confirmed by binding experiments using pure recombinant proteins.

p115 637-699 was unable to interact with p115 or α-SNAP in Golgi detergent solution and so was unlikely to be inhibiting NSF mediated reassembly by disrupting p115 dimers, or titrating out the α-SNAP from the reaction. p115 637-699 may then interfere with NSF mediated cisternal regrowth by interacting with either GOS28, Sly1p or syntaxin-5. Since both GOS28 and syntaxin-5 are essential for NSF catalyzed cisternal regrowth (Rabouille et al., 1998) it may be that the peptide binds to these SNAREs and inhibits the reaction in some non-specific steric manner. Alternatively, the peptide may compete with p115 for SNARE interactions, and in so doing inhibit the reaction. It may be that since GOS28 seems to bind preferentially to the p115 637-699, that this domain of p115 may somehow bring the GOS28 into contact with syntaxin-5 and enable *trans*-SNARE pairing. In addition, the first coiled-coil domain of p115 may serve to set up this interaction by ‘deprotecting’ syntaxin-5, by displacing Sly1p (Pfeffer, 1999). That p115 is a dimer may enable it to carry out these interactions between GOS28 and Sly1p simultaneously. The *S. cerevisiae* p115 homologue, Uso1p, has been implicated in ER-Golgi SNARE complex assembly (Sapperstein et al., 1996). A temperature sensitive Uso1 mutant was unable to form Sed5/Sec22/Bet1 (syntaxin-5/rsec22/rbet1; Hay et al., 1997, 1998) complexes at the restrictive temperature. This is most likely due to the absence of vesicle tethering at the restrictive temperature, but may also reflect a more direct, physical role for Uso1p in establishing *trans*-SNARE complexes. Overexpression of v-SNAREs could suppress the temperature sensitive Uso1 mutation, suggesting that Uso1p may not be absolutely required for *trans*-SNARE pairing, but may improve the efficiency of the reaction (Sapperstein et al., 1996). Previously, the displacement of Sly1p has been correlated with the transient association of a Rab protein (Ypt1p) with syntaxin-5 (Sed5p; Lupashin and Waters, 1997). However, this is only a positive correlation and it has not been determined whether Ypt1p and Sly1p share the same binding region on Sed5p. So although transient association of Rab-GTP with the t-SNARE may catalyze *trans*-SNARE pairing, it has not been demonstrated that the binding of the Rab itself actually
displaces Sly1p. The membrane bound levels of Ypt1p and Uso1p correlate very closely (Cao et al., 1998), as do Rab1 and p115 on RLG. Therefore, it may actually be Uso1p which displaces Sly1p by direct interaction, and so allows Ypt1p binding and trans-SNARE pairing. Were this true it would explain why Ypt1p and Uso1p function seem to be so coincident. Since these events are at the actual point of the transition from tethering to docking and fusion it may be that the phosphorylation of p115 that causes a conformational switch in p115 that allows these interactions to occur. This model generates a number of testable predictions, which will enable it to be proven or quickly discarded.

Another direct link between the tethering machinery and the fusion machinery has been elucidated in an endosome fusion assay. The Rab5 interacting protein EEA1 (Simonsen et al., 1998), another extensively coiled-coil molecule (Corvera and Czech, 1998), with a C-terminal ‘FYVE’-finger domain which interacts with phosphatidylinositol-3-phosphate in endosomal membranes (Gaullier et al., 1998; Patki et al., 1998), acts to tether endosomes together and acts upstream of SNAREs to trigger endosome fusion in vivo (Christoforidis et al., 1999a). Furthermore, EEA1 interacts directly with syntaxin-13, and is proposed to incorporate this t-SNARE into large oligomers and somehow in the process activate the t-SNARE and drive the membrane fusion process (McBride et al., 1999). Interestingly, EEA1 has also been shown to interact with the t-SNARE syntaxin-6 (Simonsen et al., 1999). Syntaxin-6 is implicated in TGN to early endosome trafficking (Davanger et al., 1997), and is localized to both these compartments, but tightly co-localizes with EEA1 on endosomal membranes (Simonsen et al., 1999), suggesting that EEA1 may be a more general tethering factor for multiple transport routes. It therefore seems likely that the beginnings of a sophisticated regulatory network is being perceived that may physically couple the sequential phenomena of vesicle tethering, docking and fusion required for constitutive transport/membrane fusion processes.
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7.1 Introduction

Catalysis of cytoplasmic fusion events requires a highly ordered, sequential series of reactions between soluble factors and membrane bound SNAREs. In the simplest scenario a v-SNARE binds to its cognate t-SNARE in the opposite membrane (Rothman, 1994), forming an extremely stable parallel four α-helix bundle (Sutton et al., 1998; Fiebig et al., 1999), the free energy gained from complex formation may then be harnessed to bring membranes into close apposition and forcefully drive bilayer mixing (Weber et al., 1998; Parlati et al., 1999), possibly in concert with downstream factors (Peters and Mayer, 1998; Peters et al., 1999; Ungermann et al., 1998b). At least two soluble AAA proteins (Patel and Latterich, 1998) are strongly implicated in this cytoplasmic fusion process. The first to be characterized was NSF as part of the soluble intra-Golgi transport machinery (Block et al., 1988; Malhotra et al., 1988; Wilson et al., 1989). The second was p97 which catalyzes the fusion of ER membranes (Latterich et al., 1995; Patel et al., 1998; León and McKearin, 1999) and of Golgi membranes fragmented by either IQ (Acharya et al., 1995a) or mitosis (Rabouille et al., 1995b, 1998; Rondo et al., 1997; Shorter and Warren, 1999). Both molecules are highly conserved.

The focus of this chapter is on the function of these two ATPases in the post-mitotic Golgi reassembly process. The first part concerns NSF, and in order to more precisely understand the mechanism of action of NSF, two mutant NSF proteins were tested for their ability to substitute for wild type NSF in supporting cisternal regrowth. The first mutant, NSF (E329Q), harbours a mutation in the D1 ATPase domain (Whiteheart et al., 1994) that debilitates the ATPase and SNARE disassembly activity of the protein (Nagiec et al., 1995), and renders it inactive for intra-Golgi transport and endosome fusion (Nagiec et al., 1995; Colombo et al., 1996). The second mutant, NSF (D604Q) contains the corresponding mutation in the D2 ATPase cassette, and this single amino acid substitution has no effect on SNARE complex disassembly nor intra-Golgi transport (Nagiec et al., 1995). The results obtained complement studies on a temperature sensitive NSF point mutant, NSF (G274E), which was first described in Drosophila melanogaster, as a mutant that induces neuroparalysis at the restrictive
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temperature (Siddiqi and Benzer, 1976; Pallanck et al., 1995), and suggest an NSF function distinct from ATPase-dependent SNARE disassembly is essential for Golgi membrane fusion (Müller et al., 1999b; Schwarz, 1999).

The second part of this chapter concerns p97, and in particular the relevance of a new protein complex that contains p97, but not p47, for post-mitotic cisternal regrowth. p97 has been implicated in a variety of disparate cellular processes other than membrane fusion. The archael homologue of p97, VAT, has chaperone-like activities, where it can either fold or unfold substrates, dependent on the rate of ATP hydrolysis (Golgib et al., 1999). Conditional mutants of p97 (CDC48) in *S. cerevisiae* arrest in mitosis as large budded cells with elongated nuclei spanning the mother-daughter junction (Moir et al., 1982; Fröhlich et al., 1991). A point mutation in the D2 domain of CDC48 (S565G) cause *S. cerevisiae* to exhibit diagnostic markers of early and late apoptosis, such as exposure of phosphatidylserine at the outer leaflet of the plasma membrane, DNA fragmentation and chromatin condensation (Madeo et al., 1997). Similarly, in *C. elegans* p97 may play an anti-apoptotic role (Wu et al., 1999) and in mammalian cells expression of a p97 mutant with mutations in the Walker A cassettes of both the D1 and D2 domains (K251A and K524A) induces apoptosis (Shirogane et al., 2000). Immunohistological analyses of mouse tissues reveal that expression of p97 is tightly controlled in a cell type- and cell differentiation-type manner (Müller et al., 1999a). The localization of p97 to either the cytoplasm or the nucleus, or both varies widely within a given tissue suggesting an elaborate, highly regulated and intermittent function for p97 (Müller et al., 1999a). The intracellular localization of p97 may depend upon tyrosine phosphorylation (Egerton et al., 1992; Zhang et al., 1999), which may cause a conformational change that exposes a nuclear import signal in the molecule (Madeo et al., 1998). Finally, p97 may also play a role in proteolysis since it interacts with proteins of the ubiquitin fusion degradation pathway. CDC48 interacts with Ufd2p, a protein involved in the formation and topology of the multi-ubiquitin chain (Koegl et al., 1999), and Ufd3p, which is involved in the regulation of free cellular ubiquitin in yeast (Ghislain et al., 1996; Koegl et al., 1999). In mammals p97 copurifies in part with the 26S proteasome (Dai et al., 1998). In these same systems
immunodepletion or inactivation of p97 severely compromised ubiquitin-dependent proteolysis of experimental substrates \textit{in vitro} and \textit{in vivo} (Ghislain et al., 1996; Dai et al., 1998; Koegl et al., 1999). It has been proposed that the role of p97 in these processes is in a chaperone capacity, where p97 may catalyze assembly or disassembly of protein complexes involved in ubiquitination or proteasomal targeting (Koegl et al., 1999).

Probing rat liver cytosol with purified p97 attached to beads reveals specific interactions between p97 and two other proteins: Ubiquitin Fusion Degradation protein 1 (Ufd1p) and Nuclear Protein Localization protein 4 (Npl4p; Meyer et al., 2000). p97/Ufd1p/Npl4p exist as a distinct complex to p97/p47 in rat liver cytosol, and Ufd1p binds to the same region on p97 as p47 (Meyer et al., 2000). Ufd1p and Npl4p also exist as a binary complex in rat liver cytosol, and Ufd1p facilitates Npl4p binding to p97, as p97 does not bind to Npl4p alone (Meyer et al., 2000). Yeast lacking Ufd1 exhibit a cell survival defect that is incompletely rescued by one allele of Ufd1, and is thought to act at a post-ubiquitination step in the UFD pathway (Johnson et al., 1995) and to regulate poly(A) polymerase activity (del Olmo et al., 1997). Ufd1 haploinsufficiency seems to contribute to the congenital heart and craniofacial defects seen in humans with microdeletions in chromosome 22q11 (Yamagishi et al., 1999). These congenital heart defects are the most common of all human birth defects and are the leading cause of death in the first year of life (Hoffman, 1995). They are to be due to the defective development of the neural crest derived cells which populate the heart (Van Mierop and Kutsche, 1986). This may be due to an accumulation of certain proteins that would otherwise be degraded and as a result compromises the survival of these neural crest derived cells which are essential for these developmental processes (Yamagishi et al., 1999). Npl4 was originally identified in a screen for temperature sensitive mutants in \textit{S. cerevisiae} which accumulated several nuclear proteins in the cytoplasm (DeHoriatus and Silver, 1996). The NPL4 gene encoded a 64kDa protein that was localized to the nuclear periphery in a manner similar to nuclear pore complex proteins, and was required for nuclear membrane integrity (DeHoriatus and Silver, 1996). Whether the p97/Ufd1p/Npl4p complex had any functional role in Golgi
reassembly was tested. The results obtained suggest that this new complex is not required for Golgi reassembly and that it in fact represents a novel p97 module that directs p97 function elsewhere in the cell.

7.2 Post-mitotic Golgi reassembly in the presence of mutant NSF proteins.

7.2.1 NSF (E329Q) and (D604Q) support cisternal regrowth.

To determine the importance of NSF mediated ATP hydrolysis in NSF catalyzed cisternal regrowth wild type NSF was replaced with either the NSF (E329Q) or NSF (D604Q) mutants, which reflect mutations in the D1 and D2 ATPase cassettes respectively. NEM-treated MGF were incubated at 37°C for 60min with p115, SNAPs and the various NSF proteins, and reactions were then fixed and processed for EM (Figure 7.1). NEM-treated MGF were utilized in order to be consistent with previous experiments conducted with a temperature sensitive NSF mutant, NSF (G274E; Müller et al., 1999b). A lower concentration of NSF (5ng/µl) was used in order to maximize any effects the mutations may cause, and this concentration of wild type protein was sufficient to catalyze significant cisternal regrowth (Müller et al., 1999b). Single cisternae reformed (they did not stack due to the NEM sensitivity of GRASP65) when incubated with wild type NSF at this concentration (Figure 7.1A, 7.2) and this activity was abolished by pretreating NSF with NEM (Figure 7.1B, 7.2). NEM treatment of NSF returned the amount of cisternal regrowth almost to the level when NSF is omitted from the reaction (control in Figure 7.2). NEM treatment of NSF dramatically alters NSF conformation, in that the compact cylindrical hexamer is transformed into ‘splayed’ open structures, and eventually leads to breakdown of NSF into its component monomers (Hanson et al., 1997). These structural changes are very similar to when NSF is deprived of nucleotide (Hanson et al., 1997), suggesting NEM treatment may inhibit nucleotide binding to NSF, which would explain the NEM inactivation of NSF’s ATPase activity (Tagaya et al., 1993). NSF binding to SNAPs and SNAREs is also abolished by NEM treatment (Hanson et al., 1997), and this correlates with the fact that ATP must be bound for these interactions to occur (Söllner et al., 1993a; Nagiec et al., 1995; Hanson et al., 1997). The effect of NEM treatment is
Figure 7.1 NSF ATPase catalytic-site mutants can support cisternal regrowth. NEM-treated MGF were incubated at 37°C for 60min at 37°C with α-SNAP (25ng/μl), γ-SNAP (25ng/μl), p115 (7.5ng/μl) and either NSF wild type (A), NSF (E239Q; C), NSF (D604Q; E) or these same NSF proteins pretreated with NEM (B, D, F). The final concentration of NSF protein in the reactions was 5ng/μl. Reactions were processed for EM. Note the presence of reassembled cisternae (arrows) in A, C and E, and the unfused tubulovesicular material (arrowheads) in B, D and F. Bar, 0.5μm.
Figure 7.2  Cisternal regrowth in the presence of ATPase catalytic site mutants of NSF. NSF proteins (5ng/μl) were mixed with NEM-treated MGF (0.75mg/ml), α-SNAP (25ng/μl), γ-SNAP (25ng/μl) and p115 (7.5ng/μl) in a final volume of 20μl and incubated at 37°C for 60min. Samples were processed for EM and the percentage cisternal regrowth was determined. In some reactions NSF proteins were pretreated with 2.5mM NEM. Values represent means±SEM (n=2).
therefore threefold: it changes the oligomeric state of NSF, inhibits nucleotide binding/hydrolysis and prevents binding to SNAPs and SNAREs.

Interestingly both the NSF mutant proteins NSF (E329Q) and (D604Q) were able to catalyze cisternal regrowth, and prior NEM treatment of the molecules abolished this activity (Figure 7.1C-F, 7.2). The D1 ATPase mutant (E239Q) supported cisternal regrowth to c. 80%, a similar level observed to the temperature sensitive NSF (G274Q) mutant at the permissive temperature (Müller et al., 1999b). This is in contrast with a cell free intra-Golgi transport assay where NSF (E239Q) is completely inactive, and could act to inhibit transport by competing with wild type NSF (Whiteheart et al., 1994). The D1 ATPase mutant possesses c. 25% of ATPase activity of wild type NSF, while the NSF (G274Q) mutant possesses no detectable ATPase activity. Together this suggests that NSF function in post-mitotic cisternal regrowth may have different ATPase requirements as compared to steady state transport reactions. The D2 ATPase mutant (D604Q) could support cisternal regrowth almost as well as wild type NSF, a situation very similar to results obtained in a cell free intra-Golgi transport assay (Whiteheart et al., 1994). The D2 mutant is not so compromised for ATPase activity, as it displays c. 70% the activity of wild type NSF (Whiteheart et al., 1994).

7.3 The p97/Ufd1p/Npl4p protein complex in post-mitotic Golgi reassembly.

7.3.1 p97/Ufd1p/Npl4p complex does not support cisternal regrowth.

p97 exists in at least two complexes in rat liver cytosol, one containing p97 and p47 (Kondo et al., 1997) and another containing p97/Ufd1p/Npl4p (Meyer et al., 2000). It was tested whether this second, novel p97 complex was able to support cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with either p97/p47 (Figure 7.3A) or p97/Ufd1p/Npl4p complex (Figure 7.3B) containing equal concentrations of p97. p97/p47 was able to stimulate the formation of long single cisternae as expected, and this was in sharp contrast to p97/Ufd1p/Npl4p which stimulated almost no cisternal regrowth (Figure 7.3A, B, 7.4). The p97/Ufd1p/Npl4p complex was unable to stimulate any cisternal regrowth even if it
Figure 7.3 The p97/ufd1/npl4 complex does not support cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with either: (A) p97 (70ng/μl) and p47 (35ng/μl); (B) p97 (70ng/μl), ufd1 (35ng/μl) and npl4 (49ng/μl); (C) p97 (70ng/μl) and ufd1 (35ng/μl); (D) ufd1 (35ng/μl) and npl4 (49ng/μl); (E) ufd1 (35ng/μl); or (F) npl4 (49ng/μl). Reactions were processed for EM. Note that cisternae only reassemble in the presence of the p97/p47 complex (arrow in A), while all other combinations generate unfused tubulovesicular material (arrowheads, B-F). Bar, 0.5μm.
Figure 7.4 Quantitation of effect of the p97/ufdl/npl4 complex on cisternal regrowth.
MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with either: p97 (70ng/µl) and p47 (35ng/µl); p97 (70ng/µl), ufdl (35ng/µl) and npl4 (49ng/µl); p97 (70ng/µl) and ufdl (35ng/µl); ufdl (35ng/µl) and npl4 (49ng/µl); ufdl (35ng/µl); or npl4 (49ng/µl). Reactions were processed for EM and the amount of cisternal regrowth determined. Values represent means±SEM (n=3).
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was added at a level five times higher than in Figure 7.3A (cisternal regrowth was 8%±2). Since Ufd1p can bind to p97 independently of Npl4p, a p97/Ufd1p complex was also tested, although this complex is not found in rat liver cytosol (Meyer et al., 2000). p97/Ufd1p was also almost completely unable to stimulate cisternal regrowth (Figure 7.3C, 7.4). Whether the binary complex of Ufd1p/Npl4p and the two proteins in isolation could stimulate cisternal regrowth was also determined (Figure 7.3D-F). Once again these components were unable to promote any cisternal regrowth (Figure 7.4). Taken together these data strongly suggest that the p97/Ufd1p/Npl4p complex does not function in post-mitotic Golgi reassembly, and must serve to redirect p97 to perform other functions *in vivo.*

### 7.4.2 Excess Ufd1p or Ufd1p/Npl4p inhibit p97/p47 catalyzed cisternal regrowth.

Since Ufd1p binds to the same region of p97 as p47 (Meyer et al., 2000) it was determined whether free Ufd1p or Ufd1p complexed to Npl4p could act to disrupt p97 catalyzed cisternal regrowth, possibly by displacing p47 from p97. Npl4p alone was not tested in this manner since it is unable to bind p97 in isolation (Meyer et al., 2000). Therefore, MGF were resuspended for p97 catalyzed cisternal regrowth in the presence of increasing concentrations of Ufd1p or Ufd1p/Npl4p. Both free Ufd1p and Ufd1p/Npl4p antagonized p97 catalyzed cisternal regrowth (Figure 7.5), in a dose dependent manner (Figure 7.6). In fact, both Ufd1p and Ufd1p/Npl4p inhibited cisternal regrowth by over 50% when present at a c. 7-fold molar excess (5μM) over p47 (Figure 7.6). Ufd1p/Npl4p was a more potent inhibitor of the reaction with an IC$_{50}$ of 2.8μM compared to 4.6μM for Ufd1p alone. This correlates with the ability of the Ufd1p and Ufd1p/Npl4p to compete for p47 binding to p97 (Meyer et al., 2000). This effect was specific for p97 as neither free Ufd1p nor Ufd1p/Npl4p had any effect on NSF catalyzed cisternal regrowth (20μM Ufd1p: cisternal regrowth=95.2%±3; 20μM Ufd1p/Npl4p: cisternal regrowth=103.5%±2). Therefore, Ufd1p and Ufd1p/Npl4p represent new specific inhibitors for the p97 catalyzed reassembly reaction.

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Figure 7.5 Effect of ufd1 and ufd1/npl4 on p97/p47 catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with p97 (70ng/μl) and p47 (35ng/μl) in the presence of increasing concentrations of either ufd1 (A-C) or ufd1/npl4 (D-F). Reactions were processed for EM. Note the diminishing amount of reassembled cisternae (arrows) on increasing the amount of ufd1 or ufd1/npl4 in the reaction and the concomitant increase in unfused tubulovesicular material (arrowheads). Bar, 0.5μm.
Figure 7.6 Quantitation of effect of ufd1 and ufd1/npl4 on p97/p47 catalyzed cisternal regrowth. MGF isolated through a 0.5M sucrose cushion were incubated at 37°C for 60min with p97 (70ng/μl) and p47 (35ng/μl) in the presence of increasing concentrations of ufd1 (circles) or ufd1/npl4 (triangles). Reactions were processed for EM and the amount of cisternal regrowth determined. Values represent means±SEM (n=2).
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7.4 Discussion

7.4.1 NSF ATPase activity is not required for NSF catalyzed post-mitotic cisternal regrowth.

The fact that the NSF (E329Q) mutant was able to support cisternal regrowth in place of wild type NSF, suggests a distinct requirement for NSF ATPase activity in post-mitotic cisternal regrowth compared to intra-Golgi transport. NSF (E329Q) is mutated in the D1 ATPase cassette and this reduces the ATPase activity of the molecule by c. 75%, and renders the molecule dysfunctional for intra-Golgi transport, as well as SNARE complex disassembly (Whiteheart et al., 1994; Nagiec et al., 1995). These previous results suggested a positive correlation between NSF catalyzed membrane fusion and NSF ATPase activity. However, another NSF mutant, NSF (G274E) with no detectable ATPase activity or SNARE disassembling ability was also able to catalyze cisternal regrowth (Müller et al., 1999b). Therefore, NSF dependent SNARE disassembly would appear to be uncoupled from membrane fusion of post-mitotic Golgi fragments. Since the disassembly of SNARE complexes is now perceived to be required to recycle SNAREs for further rounds of transport, it may be that this recycling may not be required for post-mitotic cisternal regrowth. Therefore, a prediction would be that MGF contain an abundant source of disassembled SNAREs prior to reassembly. This is possible given that the mitotic cytosol used to disassemble RLG contains NSF, which may act to disassemble SNAREs during the disassembly phase of the reaction. Membrane fusion may be inhibited due to inhibition of other steps in the transport reaction, such as decreased binding of p115 to the Golgi membrane (Levine et al., 1996; Nakamura et al., 1997), but NSF mediated SNARE disassembly may still be occurring. Therefore, selective removal of NSF from mitotic cytosol may make the reassembly reaction dependent on NSF ATPase activity. This prediction is currently under investigation and may equate the discrepancy in the necessity for NSF ATPase activity in other membrane fusion assays (Whiteheart et al., 1994; Colombo et al., 1996), but not for cisternal regrowth (Müller et al., 1999b).

Despite the lack of requirement for ATPase activity, NSF must still be added to the reassembly reaction to obtain any cisternal regrowth in the NSF catalyzed reaction.
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(Rabouille et al., 1995b). NSF must therefore be conducting another reaction that is independent of ATPase activity, and is necessary for Golgi membrane fusion. This may be in stimulating trans-SNARE pair formation in a chaperone capacity or by ‘priming’ the unpaired SNARE molecules, and in so doing keeping them in an active state ready for trans-pairing and fusion. NSF may accomplish priming by facilitating recruitment of accessory factors to the SNARE molecule which keeps it active. In yeast, transfer of LMA1 to a t-SNARE correlates with the presence of NSF and Mg-ATP, but it is unclear whether ATP hydrolysis is essential for this event (Xu et al., 1998; Cao et al., 1998). LMA1 is hypothesized to maintain the t-SNARE in activated state, and is not released until trans-SNARE complex formation, an event which may, in the case of homotypic vacuole fusion, require vacuole acidification mediated by a vacuolar proton ATPase (Ungermann et al., 1999b). Alternatively, NSF may act in a more chaperone like capacity and ensure correct SNARE folding in anticipation of fusion. It is therefore of interest to note that the archael homologue of p97, VAT, displays differential chaperone activity dependent on its rate of ATP hydrolysis. Dependent on the Mg\(^{2+}\) concentration VAT assumes two states with maximum rates of ATP hydrolysis that differ by an order of magnitude (Golbik et al., 1999). In the inactive ATPase state VAT catalyzed refolding of substrates, but in the active ATPase state catalyzed their unfolding. Furthermore, VAT is structurally equivalent to NSF over its entire length, suggesting that the mode of action of NSF may also depend on its rate of ATPase activity (Golbik et al., 1999). The mutant NSF molecules, have reduced ATPase activity so may still be functional for priming or SNARE folding, but not functional for unfolding, or SNARE disassembly processes.

Another alternative is that a checkpoint exists where recruitment of NSF to the site of fusion is essential, in order that NSF can act to disassemble SNARE complexes after fusion has occurred. In this scenario NSF plays no part in the fusion process per se, but must be present in a regulatory capacity, to ensure SNAREs will be unpaired after the fusion event. This may be why NSF can be found associated with synaptic vesicles or clathrin coated vesicles prior to docking and fusion (Hong et al., 1994; Steel et al., 1996). It could also be that NSF participates in the actual fusion process itself, which
may be SNARE independent, as proposed by Nilsson and colleagues. The SNARE molecules would only serve as a specific platform for NSF molecules which drive the actual fusion event (Otter-Nilsson et al., 1999). Therefore, it is of interest that SNAP-NSF mediated SNARE independent liposome fusion occurs best at the point where the SNAP-NSF complex has its lowest ATPase activity, the principal feature of the NSF (E329Q) and (G274E) mutants (Müller et al., 1999b). Whatever the case it seems clear that the reassembly assay may have dissected another role for NSF in membrane fusion that is distinct from NSF ATPase dependent SNARE disassembly. This new, putative role warrants further study.

7.4.2 The p97/Ufd1p/Npl4p complex is not involved in Golgi reassembly.

p97 exists in at least two distinct complexes in rat liver cytosol. The first comprises p97 bound to p47 and this complex is capable of catalyzing post-mitotic cisternal regrowth in vitro (Kondo et al., 1997; Rabouille et al., 1998). The second consists of p97 bound to Npl4p via Ufd1p (Meyer et al., 2000). Ufd1p seems to bind to the same site on p97 as p47 (Meyer et al., 2000). This novel p97 complex seems to have no capacity to reassemble cisternae from MGF, and furthermore excess Ufd1p and Ufd1p/Npl4p are able to inhibit p97, but not NSF catalyzed cisternal regrowth. These molecules then provide another way to distinguish whether the p97 or NSF pathway of reassembly is operative, and may act to inhibit p97 driven reassembly by displacing p47 from p97 (Meyer et al., 2000). Ufd1p/Npl4p is more effective than Ufd1p alone in inhibiting p97 catalyzed cisternal regrowth and in displacing p47 from p97 (Meyer et al., 2000).

The function of the p97/Ufd1p/Npl4p complex then remains to be resolved, but the fact that Npl4p appears to be a resident of the nuclear pore complex in yeast (DeHoriatus and Silver, 1996) suggests that it may be required to translocate p97 into the nucleus where it has also been localized (Peters et al., 1992; Madeo et al., 1998). Similarly, the presence of Ufd1p in the complex implicates p97 further in proteolytic functions as others have found (Ghislain et al., 1996; Dai et al., 1998; Koegl et al., 1999). These apparent alternative functions for p97 suggest that it may be a modular
component associated with many different proteins or complexes, and that it performs a similar function only at different sites within all of these complexes (Patel and Latterich, 1998). This may be related to the chaperone-like activity of p97, which may be important for a general set of folding/unfolding reactions in diverse situations. The p97/Ufd1p/Npl4p complex then represents a Golgi reassembly independent p97 module, which may function at the nuclear pore or in proteolytic situations. The function of this complex is currently under active investigation.
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