Retention and Targeting in the Golgi Apparatus

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

May 1997

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Abstract

A matrix that binds medial Golgi enzymes can be isolated as a detergent insoluble complex. Some components of this complex were identified as cytoskeletal proteins, e.g. cytokeratins and actin. To further analyse this matrix, Golgi membranes were extracted in detergent and salt followed by dialysis and fractionation on a linear sucrose density gradient. An oligomer was identified with an apparent molecular weight of $2 \times 10^6$ D as assessed by size-exclusion chromatography. It contained two medial Golgi enzymes, $\alpha$-1,3-1,6-mannosidase II (Mann II) and $\beta$-1,2-N-acetylglucosaminyltransferase I (NAGT I), along with various other proteins. Some of the components of the oligomer were identified as being proteins homologous to the lectins, ERGIC53/p58 and VIP36, and members of the p24 family of putative cargo receptors. The isolation of the oligomer and the identification of some of its components represent a step closer to the elucidation of the mechanisms of enzyme retention and the structural maintenance of the Golgi apparatus.

The early Golgi t-SNARE, syntaxin 5, is thought to provide targeting specificity for both COPI and COPII vesicles originating from the endoplasmic reticulum (ER) and COPI vesicles on the retrograde pathway. Two forms of syntaxin 5 were shown to be generated from the same mRNA by alternative initiation of translation. The short form (35kD) corresponds to the published sequence (Bennett et al. (1993), Cell, 74, 863-73). The longer form (42kD) is novel, with an N-terminal, cytoplasmic extension, containing a predicted type II ER targeting signal. When grafted onto a reporter molecule, this signal localised the construct to the ER as assessed by immunofluorescence microscopy. This signal could function to retrieve syntaxin 5 from later Golgi compartments but several lines of evidence, including the absence of this longer form from the yeast homologue, Sed5p, point to a function unique to higher organisms.
Table of Contents

Abstract ................................................................................................................................2
List of Figures .....................................................................................................................7
List of Tables ........................................................................................................................8
Abbreviations .......................................................................................................................9
Acknowledgements .............................................................................................................10
Publications ..........................................................................................................................11

Chapter 1 Introduction .................................................................................................12
  1.1 Introduction ......................................................................................................13
  1.2 The Exocytic Pathway .....................................................................................13
    1.2.1 Endoplasmic reticulum ......................................................................13
    1.2.2. Intermediate compartment................................................................16
    1.2.3. Golgi apparatus .................................................................................19
      1.2.3.1 The CGN ............................................................................20
      1.2.3.2 The Golgi stack .................................................................21
      1.2.3.3 The TGN .............................................................................23
  1.3 Vesicular transport ..........................................................................................24
    1.3.1 Coat proteins .......................................................................................24
    1.3.2 Coat assembly .....................................................................................25
    1.3.3 Vesicle budding and uncoating .........................................................26
    1.3.4 Vesicle docking ..................................................................................28
    1.3.5 Vesicle targeting ................................................................................29
      1.3.5.1 SNARE hypothesis .............................................................30
      1.3.5.2 Regulation of targeting .......................................................32
    1.3.6 Vesicle fusion ....................................................................................36
  1.4 Protein Localisation..........................................................................................39
    1.4.1 Retention mechanisms .......................................................................39
    1.4.2 Retrieval mechanisms .......................................................................42
  1.5 Aim of thesis .....................................................................................................44

Chapter 2 Materials and Methods ........................................................................45
  2.1 Biochemistry ....................................................................................................46
    2.1.1 Protein Precipitation .........................................................................46
      2.1.1.1 Chloroform/Methanol Method ..............................................46
      2.1.1.2 TCA Method ......................................................................46
2.1.2 Electrophoresis .................................................................47
  2.1.2.1 1-D SDS-PAGE .....................................................47
  2.1.2.2 2-D IEF-SDS-PAGE ............................................49
2.1.3 Western Blotting .............................................................51
  2.1.3.1 Immobilon-C ...........................................................51
  2.1.3.2 PVDF .................................................................51
  2.1.3.3 Immunodetection ...................................................52
2.1.4 Immunoprecipitation ......................................................52
2.1.5 Isopycnic Centrifugation .................................................53
  2.1.5.1 TMMDS Gradients ...............................................54
  2.1.5.2 KEHM Gradients ...................................................54
  2.1.5.3 Step Gradients .....................................................54
2.1.6 Digestion with Proteases ................................................55
  2.1.6.1 Trypsin ...............................................................55
  2.1.6.2 Chymotrypsin ......................................................55
  2.1.6.3 Proteinase K ..........................................................55
2.1.7 Affinity Purification of Antipeptide Antiserum .......................56
  2.1.7.2 Coupling of peptides ...........................................56
  2.1.7.3 Affinity column .....................................................57
  2.1.7.4 Blot elution method ..............................................57
2.1.8 Assays .............................................................................58
  2.1.8.1 NAGT I .................................................................58
  2.1.8.2 MannII ...............................................................59
  2.1.8.3 GalT .................................................................59
  2.1.8.4 Protein Assays .....................................................60
2.1.9 Rat Liver Golgi Preparation .............................................62
  2.1.9.1 Isolation ...............................................................62
  2.1.9.2 Biochemical Characterisation .................................64
2.1.10 Extraction of Golgi Membranes ........................................65
  2.1.10.1 Using TX-100 alone ............................................65
  2.1.10.2 Using TX-100 and NaCl ......................................66
2.1.11 Gel Filtration ...............................................................66
  2.1.11.1 Choice of media ..................................................66
  2.1.11.2 Calibration of gel filtration columns .......................66
  2.1.11.3 TX-114 extraction ..............................................68
2.1.12 Fixation for Electron Microscopy .....................................68
2.2 Cell Culture .........................................................................69
  2.2.1 Passaging ......................................................................69
  2.2.2 Expansion .....................................................................69
  2.2.3 Double Thymidine Shake-off ......................................69
2.2.4 Long Term Storage ................................................................. 70
2.2.6 Production of Post-Nuclear Supernatants ................................ 70
2.2.6 HeLa Golgi Preparation ............................................................ 71
2.2.7 Immunofluorescence ................................................................. 72
   2.2.7.1 Fixation Methods ............................................................... 72
   2.2.7.2 Antibody staining .............................................................. 73
   2.2.7.3 Mounting ........................................................................... 73
2.3 Molecular biology ................................................................. 75
   2.3.1 Cleaning of oligonucleotide primers ....................................... 75
   2.3.2 Polymerase Chain Reaction (PCR) .......................................... 77
   2.3.3 Gel-extraction of DNA ........................................................... 77
   2.3.4 Ligation and Transformation .................................................. 78
   2.3.5 His-syn5 construction and antibody purification .................... 78
   2.3.6 RACE protocol .................................................................... 79
   2.3.7 Sequencing ........................................................................... 79
   2.3.8 In vitro translation/transcription ............................................. 80
   2.3.9 Transient Transfection .......................................................... 80
      2.3.9.1 p31 constructs ................................................................ 80
      2.3.9.2 Procedures ..................................................................... 80

Chapter 3 Retention in the Golgi apparatus .................................. 82
3.1 Introduction ............................................................................... 83
3.2 Components of the cytoplasmic matrix .................................... 83
   3.2.1 Identification of matrix components ...................................... 84
      3.2.3.1 Actin ................................................................................ 85
      3.2.3.2 Rat uricase ....................................................................... 86
      3.2.3.3 Cytokeratins .................................................................... 86
   3.2.2 Unstacking the Golgi .............................................................. 88
      3.2.2.1 Stereology definitions ....................................................... 88
      3.2.2.2 Effect of salt treatment on Golgi ....................................... 88
      3.2.2.3 Effect of protease treatments on Golgi ............................ 91
      3.2.2.4 Cytokeratins .................................................................... 94
      3.2.2.5 p35 .................................................................................. 94
   3.2.4 Discussion ............................................................................ 96
3.3 Evidence for Kin Oligomers .................................................... 99
   3.3.1 Sucrose gradients ................................................................. 99
   3.3.2 Gel filtration ......................................................................... 103
   3.3.4 Identification of oligomer components .................................. 105
   3.3.5 Discussion ............................................................................ 108
3.4 Summary .................................................................................. 110
Chapter 4  Targeting in the Golgi apparatus ................................................. 111
  4.1 Introduction ............................................................................................ 112
    4.1.1 The Syntaxin family ................................................................. 112
    4.1.2 Sed5p ...................................................................................... 114
    4.1.3 Syntaxin 5 .............................................................................. 115
  4.2 Results .................................................................................................. 116
    4.2.1 Characterisation of syn5 antipeptide antibodies ......................... 116
    4.2.2 42kDa protein ........................................................................ 117
    4.2.3 Expression and tissue distribution ............................................ 119
    4.2.4 N-terminal extension ................................................................ 120
    4.2.5 Alternative initiation ................................................................ 124
    4.2.6 ER targeting signal .................................................................. 125
    4.2.7 Localisation of the 42kD syn5 .................................................. 128
    4.2.8 Syn5-binding proteins .............................................................. 128
  4.3 Discussion ............................................................................................ 130
    4.3.1 Two forms of syn5 .................................................................. 130
    4.3.2 Alternative initiation ................................................................ 130
    4.3.3 ER targeting signal .................................................................. 130
    4.3.4 Possible functions of 42kD syn5 .............................................. 132

Chapter 5  Summary .................................................................................. 134

References ................................................................................................. 137
List of Figures

Figure 1.1 Major compartments in the exocytic pathway ............................................... 14
Figure 1.2 Topology of membrane proteins ................................................................. 15
Figure 1.3 3-D reconstruction of the Golgi apparatus by HVEM ................................. 20
Figure 1.4 Processing of N-linked oligosaccharides .................................................... 22
Figure 1.5 Life cycle of a transport vesicle ................................................................. 27
Figure 1.6 Schematic for vesicle docking ................................................................. 29
Figure 1.7 The SNARE Hypothesis ............................................................................. 30
Figure 1.8 Generation of the Golgi stack from SNARE complexes ......................... 32
Figure 1.9 Hypothetical regulation of vesicle targeting ............................................... 35
Figure 1.10 Golgi enzyme retention by kin recognition model .................................... 41

Figure 2.1 Cross-linking of antibodies to protein-A sepharose beads ....................... 53
Figure 2.2 Affinity purification of antibodies ............................................................ 57
Figure 2.3 Gel filtration calibration ........................................................................... 67
Figure 2.4 Syntaxin 5 primer map ............................................................................. 75
Figure 2.5 Inducible expression of His-syn5 ............................................................... 78
Figure 2.6 Purification of bacterially expressed His-syn5 .......................................... 78
Figure 2.7 5'-RACE reactions .................................................................................. 79

Figure 3.1 Extraction of Golgi stacks with TX-100 and salt ........................................ 84
Figure 3.2 Comparison of human and rat cytokeratins ............................................... 87
Figure 3.3 Morphology of KCl treated Golgi membranes ......................................... 89
Figure 3.4 Effect of KCl treatment on Golgi stacking ............................................... 90
Figure 3.5 Effect of KCl treatment on the average length of Golgi cisternae ............. 90
Figure 3.6 Effect of KCl treatment on Golgi enzyme sedimentation ....................... 90
Figure 3.7 Morphology of chymotrypsin-treated Golgi membranes ...................... 91
Figure 3.8 Effect of chymotrypsin treatment on Golgi stacking ............................. 92
Figure 3.9 Chymotrypsin concentration at which the Golgi unstacks ..................... 92
Figure 3.10 Effect of chymotrypsin treatment on the average length of the Golgi cisternae ................................................................. 92
Figure 3.11 Effect of chymotrypsin digestion on Golgi enzyme sedimentation ........ 93
Figure 3.12 Extraction of chymotrypsin-treated Golgi membranes ....................... 94
Figure 3.13 p35 appears at chymotrypsin concentrations that unstacks the Golgi ........................................................................................................ 95
Figure 3.14 Immunofluorescence of NRK cells using eluted antibodies against p35 ................................................................. 95
Figure 3.15 Isolation of an oligomer in sucrose gradients ......................................... 100
Figure 3.16 Disassembly and reassembly of oligomer formation ............................ 101
Figure 3.17 Membrane proteins are important for oligomer formation ............... 102
Figure 3.18 Gel filtration of the oligomers ................................................................. 103
Figure 3.19 Disassembly and reassembly of the oligomer ........................................ 104
Figure 3.20 TX-114 extraction of oligomers ............................................................... 106
Figure 3.21 2-D gel of the oligomers ....................................................................... 107
Figure 3.22 Western blot of gel filtration fractions with p23/p24 antibodies ........... 107

Figure 4.1 Domains of syntaxin ............................................................................... 113
Figure 4.2 Published amino acid sequence of rat syn5 ........................................... 116
Figure 4.3 Characterisation of antipeptide antibodies against rat syn5 .................. 117
Figure 4.4 Specificity of syn5 antipeptide antibodies .............................................. 117
Figure 4.5 Topology studies of p42 .......................................................................... 117
Figure 4.6 Digestion with chymotrypsin ................................................................. 117
Figure 4.7 Immunofluorescence microscopy of NRK cells ..................................... 118
Figure 4.8 42kD syn5 is not a metabolic intermediate of the 35kD syn5 .................. 118
Figure 4.9 Syn5 is broadly expressed in various rat tissues .................................... 119
Figure 4.10 Syn5 is broadly expressed in different cell lines ................................... 120
Figure 4.11 Expression of 42kD syn5 is not induced by heat shock treatment of NRK cells ............................................................... 120
Figure 4.12 5'-RACE products .............................................................................. 120
Figure 4.13 Sequence of the N-terminal extension of rat syn5 ............................... 121
Figure 4.14 Homologies of syn5 N-terminal extension to human EST entries ....... 122
Figure 4.15 Construction of full-length rat syn5 cDNA ........................................... 123
Figure 4.16 In vitro transcription/translation of various cDNAs of rat syn5 .......... 124
Figure 4.17 Immunoprecipitation of in vitro transcription/translation products ....... 124
Figure 4.18 Immunofluorescence microscopy of p31 constructs ............................. 126
Figure 4.19 Gradient distribution of the syn5 isoforms .......................................... 127
Figure 4.20 Immunoprecipitation of syn5 ............................................................... 129

List of Tables

Table 1.1 Comparison of COPI and COPII proteins ................................................. 25
Table 1.2 Components of SNARE complexes ......................................................... 31
Table 1.3 Regulators of SNARE complexes ............................................................ 33
Table 2.1 Solutions used for SDS-PAGE ............................................................... 48
Table 2.2 Solutions used for IEF ............................................................................ 50
Table 2.3 Sucrose buffers used for purification of rat liver Golgi membranes ....... 63
Table 2.4 Purification of Golgi markers in Golgi preparations ............................... 64
Table 2.5 Oligonucleotide primers sequence and orientation ............................... 75
Table 3.1 Matrix components .............................................................................. 84
Table 3.2 Identification of matrix components by peptide sequence ..................... 86
Table 4.1 The Syntaxin family members ............................................................... 112
Abbreviations

BFA: Brefeldin A
CGN: cis-Golgi network
CK: Cytokeratin
COP: Coat protomer
DMSO: Dimethyl sulfoxide
DTT: Dithiolthreitol
EM: Electron microscopy
ER: Endoplasmic reticulum
Gal T: β-1,4-galactosyltransferase
IF: Immuno-fluorescence
IP: Immuno-precipitation
IPTG: Iso-propyl-thio-galactopyranoside
IQ: Ilimaquinone
MannII: α-1,3,1,6-mannosidase II
NAGT I: β-1,2-N-acetylglucosaminyltransferase I
NAP: NaCl extraction pellet
NEM: N-ethylmaleimide
NSF: NEM sensitive factor
PAGE: Polyacrylamide gel electrophoresis
PMSF: Phenylmethylsulfonyl fluoride
PNM: p-Nitrophenol-α-mannopyranoside
PTA: Phosphotungstic acid
RLG: Rat liver Golgi
RACE: Rapid amplification of cDNA ends
SDS: Sodium dodecyl sulphate
SNAP: soluble NSF attachment protein
SNARE: SNAP receptor
Syn5: Syntaxin 5
TEX: Triton extraction pellet
TGN: trans-Golgi network
TX-100: Triton X-100
TX-114: Triton X-114
Acknowledgements

First, many thanks to Graham, without whom none of the following would have taken place. His passion for science and the pursuit for excellence have been a constant source of inspiration during my time in his lab.

I have benefited enormously from the bunnies in the Warren lab who helped me so generously during the past four and half years. Great many thanks go, in particular, to Tommy "I'll be back" Nilsson for initiating my confidence both in science and in the gym, without which I will surely be mad (as if I am not mad enough already); to Hiro Nakamura for showing me what careful planning and hard work can achieve and for being an excellent teacher in all aspects of molecular biology; to Catherine Rabouille for giving constant encouragement and advice in science and in life; to Paul Slusarewicz for his guidance in biochemical work; to Dave "Couple of hours and you get your sequence" Shima whose enthusiasm started me on the molecular road; to Hisao "What do you think?" Kondo whose dedication to science puts a new meaning into the philosophy of life; to Francis "The tooth is out there" Barr for reading my thesis in its embryonic stages; and to the unique collection of colourful personalities in the lab including Birte "Oh Norm, you can't do that" Sonnichsen, Felicia "But you are cultured!" Hunte, Ginny "Oh my Gaud!" Kicksbush, Rose "Barcardi 151 proof" Watson, Tim "Immaturity award winner" Levine, Vas "Hello, girls!" Ponnambalalalam, and the Chinese gang for keep me company in insane hours on weekends in the empty building: Alan, Julian and Waijing. Special thanks go to Francis, Carlos, Catherine, Nadia, Paul and Sylvie for their most constructive comments in proof-reading my thesis.

Also need to be thanked are the folks at home who first put the idea of a Ph.D in my head: grandparents whose hope for my scientific career made me learn algebra and geometry when other kids were still playing with mud, but alas! no mathematician grandson; parents for not putting or inducing excess pressure, a great achievement for a Chinese family; to sister for her understanding; to my hibou for improving my life by a quantum step in the last year; and to Lewis, whose friendship and generosity provided the vital support in the most critical moments.
Publications

Some of the data described in this thesis have been presented in the following publications:


Chapter 1

Introduction
1.1 Introduction

Two aspects of the exocytic membrane traffic in the cell will be discussed in this thesis: protein retention and vesicle targeting in the Golgi apparatus. Firstly, I am going to give a general overview of the exocytic membrane traffic of the cell. Then I will introduce vesicular transport within the cell, in particular the vesicle targeting step. Lastly, I will discuss the mechanisms that are involved in correct protein localisation in the Golgi apparatus.

1.2 The Exocytic Pathway

The exocytic pathway of eukaryotic cells consists of a number of distinct membrane compartments. The first compartment is the endoplasmic reticulum (ER), where proteins are synthesised and inserted into the ER membrane or translocated into the ER lumen. Once these newly synthesised proteins have been folded and assembled into functional oligomers (Helenius, 1994) they are rapidly transported out of the ER (Pfeffer and Rothman, 1987). In between the ER and the Golgi is the intermediate compartment (IC), an important though controversial compartment involved in the recycling pathway between the Golgi and the ER. The next major compartment is the Golgi apparatus, the focus of this thesis. It receives proteins exiting from the ER, only allowing correctly folded and oligomerised proteins to enter the Golgi stack, where more post-translational modifications occur. After passing through the Golgi apparatus proteins are sorted to their final destinations in the trans-Golgi network, e.g. endosomes/lysosomes, secretory granules and the plasma membrane (Fig. 1.1).

1.2.1 Endoplasmic reticulum

The endoplasmic reticulum (ER) is the largest membrane-bound organelle in a typical eukaryotic cell. It consists of the nuclear envelope and a continuous network of tubules and cisternae extending throughout the cytoplasm. Ribosomes are associated with most of the ER membranes, forming the rough ER. Secretory proteins as well as membrane proteins are synthesised on ribosomes and then enter the ER where folding and oligomerisation occur. The ER also contains a characteristic set of enzymes that are involved in the processing of its resident proteins and those undergoing transport.

The biosynthesis of many proteins involves translocation across the ER membrane (Blobel and Dobberstein, 1975). The vast majority of proteins are translocated across the ER membrane co-translationally in a signal recognition particle (SRP)-dependent manner. The classical signal sequence for directing the proteins to the ER is usually located at the N-terminus of proteins and contains at least six core hydrophobic amino acids (Nothwehr and Gordon, 1990). The signal sequence can be cleaved after translocation or can
Figure 1.1 Major compartments in the exocytic pathway. Newly synthesised proteins are folded and assembled in the endoplasmic reticulum before being transported through the intermediate compartment to the Golgi apparatus, where further modifications occur. When they reach the trans-Golgi network they are sorted to various cellular destinations. Each arrow represents a putative vesicular transport step mediated by COPI and/or COPII coat proteins.
function to anchor the proteins in the membrane, when they are referred to as the signal anchor (Fig. 1.2). The orientation of signal anchor proteins is determined by the properties of the nascent chain and in particular the number of charged residues present in the two hydrophilic regions flanking the hydrophobic core of the signal-anchor sequences (Hartmann et al., 1989).

Of particular interest to this thesis are the type II membrane proteins. All of the resident Golgi enzymes (see next section) adopt this topology and are retained in the membrane using signal anchor sequences, with large lumenal catalytic domains and generally short cytoplasmic sequences. The VAMP and syntaxin family proteins (see section 1.3.5) all have type II topology, but are retained in the membrane by a single hydrophobic region at the C-terminus with most of their mass in the cytoplasm. They fall into a specific group termed tail-anchored proteins (Fig. 1.2).

The precise mechanism of membrane insertion for tail-anchored proteins has remained largely obscure but there appear to be several possibilities. It has been shown to occur post-translationally (Jantti et al., 1994) and is ATP-dependent (Kutay et al., 1995). It may be independent of the classic SRP complex, utilising an as-yet unidentified protein translocation machinery (Whitley et al., 1996). Alternatively the membrane insertion of tail-anchored proteins could be mediated by lipid interactions (Kutay et al., 1995).

After their translocation across the membrane of the rough ER, newly synthesised secretory and membrane proteins undergo folding, post-translational modification and oligomerisation before being transported out of the ER. Whereas most proteins acquire

![Figure 1.2 Topology of membrane proteins. The types of signal sequences of proteins determine their final orientation after membrane insertion. Type I proteins have their C-terminus in the cytoplasm while type II proteins have their N-terminus in the cytoplasm. Tail-anchored proteins have their signal anchors at the C-terminus with short, if any, lumenal domain. Proteins having cleavable signal sequences, which are removed from the nascent protein during insertion by the signal peptidase complex (Evans et al., 1986), and span the membrane once, are type I membrane proteins with their C-terminus in the cytoplasm (e.g. influenza haemagglutinin). In contrast, signal-anchor sequences, which act to target the proteins to the membrane and subsequently function as the membrane anchor of the proteins (High, 1992), and span the membrane once, can be either type I (e.g. glycoporphin C) or type II, with N-terminus in the cytoplasm (e.g. MHC class II invariant chain).](image-url)
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transport competence before leaving the ER, there are examples of proteins that continue
to fold and assemble in later compartments. The intermediate compartment is thought to
be the site of assembly for hepatitis B surface antigen (Huovila et al., 1992) and the site
for trimerisation of some influenza haemagglutinin (Tatu et al., 1995), while connexin 43
has been found to oligomerise in the Golgi apparatus (Musil and Goodenough, 1993).

The ER maintains a high concentration of chaperones and an environment optimal for
protein folding and assembly. It is also the main site of action for a conformation-based
mechanism, termed quality control (Hurtley and Helenius, 1989), that recognises, retains
and selectively degrades, misfolded, incompletely folded or partially assembled copies of
a large number of newly synthesised proteins. By preventing the deployment of
misfolded proteins, quality control ensures the structural integrity of protein products and
also diminishes the risks posed by malfunctioning protein products. It involves a complex
set of phenomena with several underlying molecular mechanisms. Numerous ER
chaperones, such as BiP, calnexin and calreticulin are thought to be involved (Pelham,
1989).

However, the retention and retrieval of misfolded proteins by quality control is not
restricted to the ER. Unassembled MHC class I heavy chains have been shown to cycle
between the ER and cis-Golgi (Hsu et al., 1991). Misfolded vesicular stomatitis virus
glycoprotein (VSV-G) can enter the intermediate compartment, even proceed to cis-Golgi
before being selectively retrieved to the ER by retrograde transport (Hammond and
Helenius, 1994).

1.2.2. Intermediate compartment

There are two distinct membrane bound compartments through which proteins must pass
before entry into the Golgi apparatus. The first is the rough ER itself, the site of protein
synthesis; the second is the organelle that is now referred to as the intermediate
compartment (IC).

It was originally defined in Semliki Forest virus-infected baby hamster kidney (BHK)
cells. Intracellular movement of the viral glycoprotein can be monitored by temperature
changes. At 15°C entry of newly synthesised viral proteins from the ER into the Golgi
apparatus was blocked. They accumulated in a tubulo-vesicular compartment between
the ER and the Golgi, called pre-Golgi vacuoles (Saraste and Kuismanen, 1984). This
effect was reversible: when restored to the permissive temperature the viral proteins
proceeded to the Golgi and the cell surface.

This compartment was also identified from the analysis of intracellular movements of
VSV-G proteins. In Vero cells infected with a temperature sensitive strain of VSV, after
incubation at 15°C the VSV-G proteins accumulated in a tubulo-vesicular compartment
between the ER and the Golgi (Schweizer et al., 1990). This compartment can also be observed at 37°C in vivo in normal rat kidney (NRK) and BHK cells (Saraste and Kuismanen, 1984; Saraste and Svensson, 1991) or in vitro at 32°C using digitonin-permeabilised semi-intact NRK cells (Pind et al., 1994; Plutner et al., 1992). It was called the ER-Golgi intermediate compartment (ERGIC) (Schweizer et al., 1990) or simply, intermediate compartment (Lotti et al., 1992).

Various other names were given to this compartment by different groups stressing one or another aspect of its functional importance: "salvage compartment" (Warren, 1987) where escaped ER proteins were thought to be retrieved; "budding compartment" where coronaviruses were shown to bud (Krijnse Locker et al., 1994; Tooze et al., 1988) and where vaccinia virus acquired its first membrane during assembly (Sodeik et al., 1993); "cis-Golgi network" (Hsu et al., 1991) where MHC class I proteins were sorted; "peripheral elements" (Oprins et al., 1993) based on the localisation of a subunit of the transport vesicle coat proteins (β-COP) and "vesicular tubular clusters" (VTCs) (Balch et al., 1994) consisting of transport intermediates. In this thesis the name intermediate compartment is used.

Biochemically the IC was first characterised by the presence of a trans-membrane protein of 53kDa, ERGIC53 (Schweizer et al., 1988). Antibodies against ERGIC53 stained the same compartment where transport of VSV-G was blocked at 15°C (Lotti et al., 1992; Schweizer et al., 1990). Other markers of the IC include the rat homologue of ERGIC53, p58 (Saraste et al., 1987), rab 1A (Tisdale et al., 1992), rab 2 (Chavrier et al., 1990) as well as the human KDEL receptor (see section 1.4.3) (Griffiths et al., 1994).

However, antibodies to ERGIC53/p58 labelled the IC preferentially but not exclusively. They also labelled the cis-Golgi (Chavrier et al., 1990) and the ER (Saraste and Svensson, 1991), suggesting a recycling pathway. The IC differs in density from both the ER and the Golgi apparatus, and can be separated from them by isopycnic centrifugation (Hammond and Helenius, 1994; Lahtinen et al., 1992; Schweizer et al., 1994; Schweizer et al., 1991). ERGIC53/p58 was subsequently shown to cycle between the ER, the IC and the cis-Golgi. It normally distributed in three peaks on a density gradient: one co-migrating with the ER, one co-migrating with cis-Golgi and a third peak identified as the IC (Itin et al., 1995).

The nature of the connections between the ER and the IC has been controversial. When cells infected with mouse hepatitis virus (MHV) were incubated at low temperature (14°C) or with a non-hydrolysable analogue of GTP, the viral proteins were prevented from acquiring Golgi-specific modifications but transport from the ER to the IC was not affected (Krijnse Locker et al., 1994). This led to the conclusion that the IC is
INTRODUCTION

continuous with the rough ER and that only one vesicular transport step would be required from the ER to the Golgi.

In streptolysin O (SLO) permeabilised cells infected with MHV, direct connections between the IC and the ER were seen by EM (Krijnse Locker et al., 1994). Tubular extensions of ER into the IC have also been observed at 20°C in HEP-2 cells transfected with chimeric proteins containing horse radish peroxidase (Stinchcombe et al., 1995). However, at 37°C these connections are not observed. Golgi morphology was also very different at 20°C when compared to that at 37°C, indicating the tubular extensions, as well as changed Golgi morphology, may be a pathological response to low temperature for this cell type. In Vero cells, morphology of the ER, IC and Golgi remain unchanged at all temperatures tested (10°C, 15°C, 31°C, 37°C and 39°C) and yet no direct, tubular connections were observed between the three compartments (Lotti et al., 1996). Neither were connections to the ER observed in NRK cells at 37°C (Balch et al., 1994; Saraste and Svensson, 1991).

Further evidence against the ER-IC connection was found by morphological analysis of VSV-G transport from the ER to the IC in Vero cells over a wide temperature range (4-32°C) (Lotti et al., 1996). It has been shown that VSV-G could correctly fold and oligomerise, and therefore could be exported from the ER. At 10°C, immunolabelling of VSV-G was clustered on the ER but was scarcely found on the IC despite its close proximity to the ER. When the temperature was shifted to 15°C, transport resumed and the labelling for VSV-G accumulated in the IC. If the ER and the IC were physically connected and there were no vesicular step linking the two, this should not happen. Both immuno-EM and conventional EM showed the morphology of the ER, IC and Golgi remained essentially unchanged except for an increased number of ER protrusions at 10°C. These results demonstrate that VSV-G transport from the ER to the Golgi can be arrested at a step preceding entry into the IC, suggesting that the ER and the IC are separate membrane compartments linked by a vesicular transport step (Lotti et al., 1996).

Biochemically, treatment of cells in a variety of ways, e.g. low temperature, ATP depletion, nocodazole and aluminium fluoride suggest that bi-directional traffic with distinct biochemical requirements occur between the IC and ER (Lippincott Schwartz et al., 1990), and IC and Golgi (Itin et al., 1995; Itin et al., 1995). ERGIC53/p58 accumulated in the IC after treatment with the fungal metabolite brefeldin A (BFA), aluminium fluoride or incubation at 15°C while ATP depletion concentrated the protein in the ER. Synchronised release of ERGIC53/p58 from the IC and from the ER revealed two microtubule-independent recycling pathways: one from the IC to the ER without prior transport to the cis-Golgi, and one from cis-Golgi to the ER that probably involves passage through the IC (Itin et al., 1995). Golgi resident proteins in BFA treated cells appeared to cycle between the ER and the IC in a microtubule-dependent manner.
(Lippincott Schwartz et al., 1990). Addition of nocodazole disrupted this dynamic cycle by preferentially inhibiting retrograde movement, causing Golgi proteins to accumulate in the IC. In the absence of BFA, such an ER recycling pathway was followed by ERGIC53/p58 but not by Golgi proteins, as revealed by temperature shift experiments (Itin et al., 1995).

COPI and COPII are vesicle coat complexes whose assembly is regulated by the ARF1 and Sar1 GTPases, respectively (see next section). They were shown to be recruited separately and independently to ER (COPI, COPII), IC (COPI, COPII), and Golgi (COPI) membranes using stage specific in vitro transport assays to synchronise movement of cargo to and from the IC, and GDP- and GTP-restricted forms of Sar1 and ARF1 proteins to control coat recruitment (Aridor et al., 1995). COPII was responsible for export from the ER, was lost rapidly following vesicle budding and proposed to mediate a vesicular step required between the ER and the IC. COPI was recruited onto the IC where it initiated segregation of the anterograde transported protein VSV-G from the retrograde transported recycling protein ERGIC53/p58. This is supported by the finding that upon release from the 15°C block, the direction of transport for ERGIC53/p58 and the KDEL receptor (see sections 1.4.2 & 4.1.2), both accumulated in the IC at 15°C, diverged. KDEL receptor was found to move along the anterograde pathway to the Golgi apparatus while ERGIC53/p58 was distributed into the ER along the retrograde pathway (Tang et al., 1995). These results suggest that bi-directional vesicular traffic occurs between the ER, IC and the Golgi apparatus (Aridor et al., 1995).

Considering the evidence from biochemical and morphological studies detailed above, the IC is most likely to be a discrete compartment in its own right, physically distinct from both the ER and the cis-Golgi, and that protein transport between the three compartments is mediated by vesicular traffic. The IC thus plays an important role in the ER-Golgi recycling pathway and the segregation of anterograde and retrograde transport.

1.2.3. Golgi apparatus

The Golgi apparatus was first defined at the light microscope level as an intracellular component made visible after application of a specific black reaction devised by Camillo Golgi in the 1870s (Pannese, 1996). This revealed an elaborate reticulum in the cell, which was later given the name of its discoverer. Though the reaction was specific, variations between different cell types were large and the resolution limited. A clearer picture only emerged after the arrival of electron microscope in the 1950s and more reliable stains such as that for thiamine pyrophosphatase (TPPase) (Novikoff, 1967).

Morphologically, the Golgi apparatus is visualised by EM as an extensive reticulum comprising stacks of flattened cisternae connected laterally to each other by tubules and
In introduction

CGN
Golgi
stack
TGN

Figure 1.3 3-D reconstruction of the Golgi apparatus by HVEM (taken from Rambourg 1990)

fenestrated membranes as well as extensive tubular networks on either side of the stack (Mollenhauer and Morre, 1994). It is also characterised by zones of exclusion that surround each stack and by an assortment of vesicles and vesicle buds associated with both the stacks and the tubular networks. A three-dimensional (3-D) reconstruction study using high-voltage electron microscopy (HVEM) shows that the Golgi takes the appearance of a continuous ribbon-like organelle (Fig. 1.3), with a tubular network on either side of the central ribbon that usually display alternating stacked cisternae and highly fenestrated or tubular zones along its length (Rambourg and Clermont, 1990).

Biochemically, the Golgi apparatus can be characterised by its resident enzymes which are abundant and present throughout the Golgi, with their cisternal locations varying from enzyme to enzyme and from cell to cell (Dunphy and Rothman, 1985; Dunphy and Rothman, 1983; Nilsson et al., 1993; Rabouille et al., 1995; Roth, 1987; Velasco et al., 1993). They are responsible for protein processing and glycolipid synthesis. Some of the best characterised functions are the trimming of the oligosaccharide core and subsequent addition of complex structures to the N-linked oligosaccharides on proteins undergoing transport.

The Golgi apparatus is structurally and functionally polarised, reflecting its central role in the secretory pathway between the ER, the plasma membrane and the endosome/lysosome system. A three-compartment functional subdivision of the Golgi is consistent with its structural organisation: The cis-Golgi network (CGN) on the cis, or entry face of the Golgi receives proteins synthesised in the ER via the IC; the Golgi stack where post-translational modifications take place; and the trans-Golgi network (TGN) at the trans, or exit face, on the opposite side of the Golgi stack, mediates sorting and secretion of proteins to their terminal destinations.

1.2.3.1 The CGN

The CGN receives newly synthesised proteins from the ER, and can be thought of as the last quality control step for proteins leaving the ER (Pelham, 1991). Structurally, the CGN consists of the cis-most cisterna associated with an array of tubular networks that
are selectively stained after prolonged osmication (Lucocq et al., 1989; Rambourg and Clermont, 1990). Functionally, the CGN can be defined as the compartment where the Golgi enzyme α-1,2-mannosidase I (MannI) functions (Balch and Keller, 1986), although MannI was also found to be present in other parts of the Golgi (Velasco et al., 1993). Markers for the CGN are found to cycle between the ER, IC and the CGN. These include p28 (Subramaniam et al., 1995; Subramaniam et al., 1996), β-COP (Oprins et al., 1993), syntaxin 5 (see Chapter 4) and its yeast homologue Sed5p (Banfield et al., 1994), gp74 (Alcalde et al., 1994), p210 (Rios et al., 1994) and GM130 (Nakamura et al., 1995).

Some suggest that the CGN and IC are equivalent biochemically. However, by immuno-EM, gp74, p210 and GM130 were all localised to the cis-most Golgi cisterna and the tubular networks associated with it, i.e. the CGN. This localisation pattern is different from the more punctate staining pattern of ERGIC53/p58, the IC marker, under the same conditions, suggesting the CGN is a different compartment from the IC.

1.2.3.2 The Golgi stack

Only properly folded proteins are thought to proceed on to the Golgi stack, consisting of a series of flattened cisternae which are closely apposed to each other (Rambourg and Clermont, 1990). The number of cisternae per stack varies greatly between different cell types but is fairly constant within a particular cell type. In HeLa cells there are typically three cisternae, termed the cis-, medial- and trans-cisternae.

The best characterised function of the Golgi stack is the construction of N-linked, bi-antennary complex oligosaccharides, which involves the sequential action of enzymes located in different parts of the Golgi apparatus (Kornfeld and Kornfeld, 1985). As shown in Fig. 1.4, the first stage continues the trimming of mannose residues started in the ER (step 4), leaving a penta-mannose core (step 5). The second stage involves addition of the first N-acetylgalactosamine (GlcNac) by the enzyme β1,2 N-acetylgalactosaminyltransferase I (NAGT I, step 6), the removal of a further two mannoses by α1,3-1,6 mannosidase II (Mann II, step 7) and the addition of another GlcNac by β1,2 N-acetylgalactosaminyltransferase II (NAGT II, step 8). The last stage involves the addition of galactose by β1,4 galactosyltransferase (GalT, step 10) and sialic acid by α2,6 sialyltransferase (SialylT, step 11). These enzymes are also found in the TGN. Fucose may be added prior to (step 9) or after the addition of sialic acid.

These activities were assigned to the different Golgi cisternae and the associated networks based on the localisation of the enzymes and their products using a variety of techniques. This included subcellular fractionation by isopycnic centrifugation (Dunphy and Rothman, 1983; Goldberg and Kornfeld, 1983), immuno-EM using colloidal gold coated
with protein A (Roth and Berger, 1982; Slot and Geuze, 1983), immunoperoxidase (Dunphy et al., 1985), peroxidase-coupled protein A (Novikoff et al., 1983), peroxidase-conjugated lectins (Tartakoff and Vassalli, 1983) or lectins followed by anti-lectin antibodies and protein A-gold (Griffiths et al., 1982). Initially, most of the enzymes were localised to one or one particular group of cisternae, leading to the view that they were physically separated from each other by the cisternae of the Golgi stack (Dunphy and Rothman, 1985). However, simultaneous localisation of two or more enzymes in one cell type was not achieved due to difficulties in making specific antibodies that worked using immuno-EM techniques (Mellman and Simons, 1992).

This problem was overcome using a combination of confocal microscopy and quantitative immuno-EM by double-labelling of cell lines stably expressing epitope-tagged versions of the Golgi enzymes NAGT I and GalT (Nilsson et al., 1993). These two enzymes

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**Figure 1.4:** Processing of N-linked oligosaccharides. The diagram shows the steps involved in the synthesis of classical N-linked bi-antennary oligosaccharides after initial transfer from dolichol. l=oligosaccharyltransferase, 2=α₁,2-Glucosidase I, 3=α₁,3-Glucosidase II, 4=ER α₁,2-Mannosidase, 5=Mann I, 6=NAGT I, 7=Mann II, 8=NAGT II, 9=FucT, 10=GalT, 11=SialylT. ○=mannose, ■=N-acetylglucosamine, ●=galactose, ▲=glucose, □=fucose, ◆=sialic acid. (Adapted from Kornfeld and Kornfeld, 1985)
were found in two adjacent cisternae instead of a single one, i.e. the enzymes exhibit an overlapping distribution. This observation was later extended to other Golgi enzymes, MannII and SialyIT (Rabouille et al., 1995). Each Golgi cisterna thus contains a unique mixture of enzymes, not a unique set.

This finding indicates that the biochemical compartment may not necessarily be the same as the morphological compartments. It also provides a possible mechanism to generate the Golgi stack, if the overlapping enzymes occupying adjacent cisternae bind to the same cytoplasmic matrix (see also Fig. 1.10 in section 1.4.1 and Chapter 3).

1.2.3.3 The TGN

The TGN does not stain for osmium but can be visualised in the electron microscope after labelling with TPPase (Novikoff, 1967), NBD-ceramide (Pagano et al., 1991; Pagano et al., 1989) or after accumulation of VSV-G proteins at 20°C (Griffiths et al., 1985). It is defined structurally by the tubular-vesicular network located on the trans side of the Golgi stack which, in contrast to the CGN, does not exactly follow the main axis of the Golgi ribbon and thus shows a peeling-off configuration (Rambourg and Clermont, 1990). In one HVEM study of the TGN in NRK cells, most regions analysed contained multiple (2-4) Golgi cisternae that "peel off" from the stack and are continuous at their ends with tubules that contribute to the TGN (Ladinsky et al., 1994). Additional budding vesicular profiles are also visualised along the length of TGN tubules. In other studies, the TGN appears to consist of the trans-most Golgi cisternae continuous with a polygonal network of tubules, as observed both by HVEM (Rambourg and Clermont, 1990) and by confocal microscopy (T. Nilsson, personal communication). It also tends to form a cup shape with the trans-most Golgi cisternae (Mollenhauer and Morre, 1994). The TGN is known to undergo dynamic changes in size, possibly due to membrane removal, and depending on the amount of protein traffic through it (Griffiths et al., 1989). Markers for the TGN include TGN38 (Luzio et al., 1990) and its isoform TGN41 (Reaves et al., 1992).

The TGN sorts proteins and lipids into their various destinations (Fig. 1.1) and receives membrane traffic from the cell surface through the endocytic pathway (Farquhar and Palade, 1981). Certain protein modifications also occur, e.g. galactose α-2,6 sialylation and tyrosine sulfation. The TGN may have lower pH than the preceding Golgi compartments (Anderson and Pathak, 1985). In BFA treated cells, rather than redistributing to the ER, the majority of the TGN collapsed around the microtubule organising centre (Reaves and Banting, 1992) or was found to mix with the recycling endosomal system (Lippincott Schwartz et al., 1991). ATP depletion reduces the ability of BFA to induce a redistribution of Golgi proteins into the ER; however, it has no effect upon the BFA-induced relocalisations of the TGN. These data suggest that the TGN is independent of the Golgi stack.
1.3 Vesicular transport

Transfer of proteins from one compartment to another was originally proposed to be mediated by transport vesicles (Palade, 1975). Subsequent genetic and biochemical analyses have led to the identification of many of the molecular components involved in the budding, transfer, targeting, docking and fusion of such vesicles (Bednarek et al., 1996; Rothman and Wieland, 1996). These steps involve a complex molecular machinery, many components of which are conserved from yeast to mammals (Ferro-Novick and Jahn, 1994).

1.3.1 Coat proteins

Transport of proteins along the exocytic pathway is mediated by vesicles that bud from a donor compartment and fuse with an acceptor compartment. Analysis of the formation of transport vesicles from the ER, the Golgi apparatus and the plasma membrane has demonstrated that, in each case, vesicle budding requires the assembly of a protein coat on the cytosolic side of the donor membrane (Barlowe et al., 1994; Orci et al., 1993).

The first coat proteins to be identified were those present on the clathrin coated vesicles that mediate endocytosis and transport from the TGN to endosomes (Pearse and Robinson, 1990). Cytosolic adaptor proteins specific for either the TGN (AP-1) or the plasma membrane (AP-2) were thought to interact directly with signals present on membrane proteins and form a scaffold onto which the clathrin coats assemble (Robinson, 1994).

The next coat proteins to be identified were on the Golgi apparatus and on vesicles derived from it during an in vitro transport reaction using mammalian cells (Malhotra et al., 1989; Orci et al., 1986). Morphologically, the coat proteins were visualised by EM to have a 10 nm thick electron-dense material surrounding vesicles of 70-75 nm in diameter (Duden et al., 1991; Malhotra et al., 1989; Oprins et al., 1993). This coat was purified biochemically from Chinese hamster ovary (CHO) cells and found to be composed of an oligomeric protein complex (the coatomer) of MW 650-700kD whose subunits are termed COPs (Coat proteins). Also found on the vesicles was the small GTP-binding protein ADP-ribosylation factor (ARF), which is required for coat assembly (Donaldson et al., 1992; Palmer et al., 1993).

More recently the combination of yeast genetics and biochemical analysis of a reconstituted vesicle transport step from the ER to the Golgi apparatus has revealed a new coat consisting of three soluble subunits: the Sec13/31 complex, the Sec23/24 complex and the small GTPase Sar1p. These proteins form an approximately 10 nm thick electron-dense coat on ER-derived vesicles of 60-65 nm in diameter (Barlowe et al.,
Table 1.1. Comparison of COPI and COPII proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>Homologies</th>
<th>Biochemical activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF (Arf1p, Arf2p)</td>
<td>20kD</td>
<td>Sarlp</td>
<td>GTP binding and hydrolysis</td>
</tr>
<tr>
<td>α-COP (Ret1p)</td>
<td>160kD</td>
<td>WD-repeats</td>
<td>KKXX binding</td>
</tr>
<tr>
<td>β-COP (Sec26p)</td>
<td>110kD</td>
<td>β-adaptin</td>
<td>KKXX binding</td>
</tr>
<tr>
<td>β'-COP (Sec27p)</td>
<td>102kD</td>
<td>WD-repeats</td>
<td>KKXX binding</td>
</tr>
<tr>
<td>γ-COP (Sec21p)</td>
<td>98kD</td>
<td>none</td>
<td>KKXX binding</td>
</tr>
<tr>
<td>δ-COP (Ret2p)</td>
<td>61kD</td>
<td>clathrin adaptors</td>
<td></td>
</tr>
<tr>
<td>ε-COP</td>
<td>31kD</td>
<td>none</td>
<td>KKXX binding</td>
</tr>
<tr>
<td>ζ-COP (Ret3p)</td>
<td>20kD</td>
<td>clathrin adaptors</td>
<td></td>
</tr>
<tr>
<td>COPII</td>
<td></td>
<td>ARF-like GTPase</td>
<td>GTP binding and hydrolysis</td>
</tr>
<tr>
<td>Sarlp</td>
<td>24kD</td>
<td>none</td>
<td>GTPase-activating protein for Sarlp</td>
</tr>
<tr>
<td>Sec23p</td>
<td>85kD</td>
<td>none</td>
<td>GTPase-activating protein for Sarlp</td>
</tr>
<tr>
<td>Sec24p</td>
<td>105kD</td>
<td>none</td>
<td>GTPase-activating protein for Sarlp</td>
</tr>
<tr>
<td>Sec13p</td>
<td>33kD</td>
<td>WD-repeats</td>
<td>GTPase-activating protein for Sarlp</td>
</tr>
<tr>
<td>Sec31p</td>
<td>150kD</td>
<td>WD-repeats</td>
<td>GTPase-activating protein for Sarlp</td>
</tr>
</tbody>
</table>

The new vesicle coat has been termed COPII whereas the Golgi derived coat has been renamed COPI (Barlowe et al., 1994). Recent morphological and biochemical studies show that both COPI and COPII vesicles can bud directly from the ER in yeast (Bednarek et al., 1995). The comparison of the various components of the two non-clathrin coats is detailed in Table 1.1.

1.3.2 Coat assembly

The assembly of the COPI coat requires coatomer, ARF and GTP (Ostermann et al., 1993). The first step in the assembly is the recruitment of ARF from the cytosol in its GDP-bound form. Nucleotide exchange catalysed by a GDP-GTP exchange factor is sensitive to BFA. This produces the GTP-bound form of ARF which inserts into the membrane via its N-terminal myristic acid chain. ARF is also a potent activator of phospholipase D (PLD) which catalyses the hydrolysis of phosphatidyl-choline to phosphatidic acid and choline (De Camilli et al., 1996). Production of phosphatidic acid is sufficient for COPI coatomer binding to Golgi membranes and high endogenous or exogenous PLD activity can overcome the ARF requirement. This suggests that another important role for ARF may be to stimulate PLD activity to prime the membrane for coatomer binding (Ktistakis et al., 1996). Interaction of β-COP with bound ARF-GTP is possibly regulated by a trimeric G protein (Donaldson et al., 1992; Donaldson et al., 1991). Coatomer binding to the membrane can be mediated by the Golgi membrane receptor p23 (Sohn et al., 1996). The clustering of ARF and p23 as well as further recruitment of coatomer and associated proteins were suggested to create a curved
membrane and subsequently the formation of COPI coated buds.

The shape of the coated vesicle is thought to be determined by the regular arrangement of coatamer and ARF in their polarised form, as shown to be the case for clathrin coats (Lin et al., 1991). The energy made available when GTP binds to ARF is presumably used to force this protein into a higher energy conformation in which fatty acid is more exposed for membrane insertion (Ostermann et al., 1993). GTP is thought to remain bound to ARF and unhydrolysed. Recycling of ARF would presumably follow GTP hydrolysis when uncoating is triggered later in the pathway (Tanigawa et al., 1993).

Like the assembly pathway for COPI, COPII coat formation starts by binding of Sar1p to the ER membrane. This step requires exchange of bound GDP for GTP, and is catalysed by Sec12p (Barlowe and Schekman, 1993). Sec12p is specific for Sar1p, which is shown by genetic studies to co-operate with two other ER proteins, Sec16p and Sed4p (Gimeno et al., 1995), in the formation of the COPII coat. Sec16p is a large multidomain peripheral protein whose C-terminal domains bind directly to Sec23p and Sed4p, while the N-terminal domain may bind cargo and v-SNAREs (vesicle-associated soluble-NEM-sensitive factor attachment protein receptors, see section 1.3.4) (Espenshade et al., 1995). The complex formed by Sar1p, Sed4p and Sec16p serves as a docking site for Sec23p-Sec24p and Sec13p-Sec31p. Sec23p-Sec24p recognises sorting motifs on cargo and targeting proteins, marking them for inclusion into vesicles. Sec23p is the GTPase-activating protein for Sar1p, and Sec24p is required for binding of Sec13p-Sec31p complex to the membrane. Polymerisation of the Sec13p-Sec31p complex is thought to cluster activated cargo and targeting molecules. Additional binding of these two complexes was suggested to induce COPII coated bud formation. GTP hydrolysis may discharge Sar1p from activated coat-membrane-cargo complexes and promote recycling.

1.3.3 Vesicle budding and uncoating

The budding of the newly-formed transport vesicle occurs by membrane fission within the bud neck. This requires the fusion of adjoining regions of the same lipid bilayer at the base of the bud. In the case of plasma membrane clathrin-coated vesicles, this step requires dynamin, a GTP-binding protein that constricts and pinches the bud neck to release the vesicle (Hinshaw and Schmid, 1995; Takei et al., 1995). Scission of COPI-coated vesicle from the Golgi apparatus requires palmitoyl-CoA (Ostermann et al., 1993; Pfanner et al., 1989). Apparently no additional cytosolic factors are required for the release of COPII vesicles (Bednarek et al., 1995).

GTP hydrolysis is required for uncoating, as mutant ARF or the non-hydrolysable analogue GTPyS inhibit transport with concomitant accumulation of coated vesicles, suggesting that uncoating is a prerequisite for fusion (Ostermann et al., 1993; Tanigawa
Figure 1.5 Life cycle of a transport vesicle. Transport between the donor and acceptor compartment involves coat-assembly on the donor compartment and the incorporation of cargo proteins, vesicle budding and uncoating, and transport to the acceptor compartment where docking and fusion take place. Specificity of vesicle targeting is ensured by SNARE complexes.
et al., 1993). Coat disassembly may be triggered when ARF or Sarlp hydrolyses the bound GTP, and proceeds as a simple reversal of coat assembly, possibly with additional unknown factors, though the mechanism is still unclear.

1.3.4 Vesicle docking

The docking of transport vesicles with their target membrane is thought to be mediated by p115, first identified as a component needed for intra-Golgi transport (Waters et al., 1992). It was found to be identical to TAP, a protein involved in transcytotic membrane traffic (Barroso et al., 1995), and homologous to Uso1p, a yeast protein essential for ER to Golgi transport (Nakajima et al., 1991). Uso1p acts before the hydrolysis of ATP by NSF to initiate vesicle fusion (Lupashin et al., 1996) and even before the formation of the ER-Golgi SNARE (see next section) complexes (Sapperstein et al., 1996).

Three lines of evidence support p115's functions in vesicle docking. Over-expression of certain SNAREs rescues the lethal phenotype of Uso1p-deletion mutants. This is most readily explained as a mass action effect, the decreased efficiency of vesicle docking being compensated by higher levels of the proteins that stabilise the docked state (Sapperstein et al., 1996). Furthermore, TAP/p115 has been shown to bind transcytotic vesicles to apical plasma membranes in the absence of ATP (Barroso et al., 1995). Lastly, the structure of p115/Uso1p is consistent with a docking function. Rotary shadowing reveals a myosin-shaped molecule with two globular heads linked by a long tail, the length of which is comparable to or greater than the width of a transport vesicle (Sapperstein et al., 1996; Yamakawa et al., 1996). Such a large molecule could tether transport vesicles to potential target membranes (Fig. 1.6). Work done in this laboratory shows that GM130, a cis-Golgi matrix protein, interacts specifically with p115 and could provide a membrane docking site (Nakamura et al., 1997).

At the synapse vesicles which store neurotransmitters are docked to the pre-synaptic membrane before Ca^{2+}-activated fusion. Vesicle docking occurs as two synaptic vesicle membrane proteins, synaptobrevin/VAMP (vesicle-associated membrane protein) and synaptotagmin, bind to two proteins on the plasma membrane: syntaxin and SNAP-25 (synaptosome-associated protein of 25kD). These four membrane proteins (also called SNAREs) form a stable docking complex (Fig. 1.6) in a detergent extract of rat brain and has a sedimentation coefficient of 7S on glycerol gradients (Sollner et al., 1993).

Clostridial neurotoxins (tetanus and botulinum toxins) are potent inhibitors of synaptic transmission. Studies with these toxins have shown that VAMP, SNAP-25 and syntaxin are the unique targets for cleavage by one or another species of the toxin, providing compelling evidence that these SNAREs play an essential role in synaptic transmission (Huttner, 1993).
The initial synaptic vesicle docking is likely to be mediated by an interaction between synaptotagmin and SNAP-25 (Schiavo et al., 1997). When syntaxin joins synaptotagmin and SNAP-25 to form a ternary complex, it can then recruit VAMP and initiate the assembly of SNAPs and NSF. Blocking of this step by proteolytic cleavage of VAMP with tetanus or botulinum B neurotoxins (Hunt et al., 1994), deletion of the syntaxin gene (Schulze et al., 1995), or the inactivation of syntaxins by sec1 family proteins (Pevsner et al., 1994) (see next section), would result in non-functional docking of synaptic vesicles. This could mean that the integrity of these SNAREs is necessary for NSF to disrupt the stable docking complex and/or that intact SNAREs are needed for subsequent fusion of lipid bilayers, indicating their role in a post-docking and possibly fusion event (Hunt et al., 1994).

In short, SNAREs are central for vesicle docking but the pairing of SNAREs to confer targeting specificity may occur downstream of a p115/GM130 or synaptotagmin/SNAP-25 mediated docking event.

1.3.5 Vesicle targeting

Given the complexity of the intracellular compartments and the vesicular transport of proteins between them, transport vesicles must fuse with the correct compartment to preserve compartmentation. Targeting is, therefore, essential to intracellular transport.

Targeting operates on two levels: targeting of transport vesicles by proteins on the vesicles, and targeting of proteins themselves. In this section I shall introduce targeting of transport vesicles. Targeting signals within a protein will be discussed under protein localisation.
1.3.5.1 SNARE hypothesis

The SNARE hypothesis (Rothman and Warren, 1994) postulates that on every vesicle undergoing transport there is at least one v-SNARE. It forms a cognate pair with its partner on the target membrane, the t-SNARE. Once the SNARE pair is formed, SNAPs and NSF will bind to assemble the fusion machinery (Fig. 1.7). Targeting specificity is ensured by the SNARE pairs, and various v-SNARE and t-SNAREs have been identified in multiple steps of the secretory pathways from yeast to mammals (Table 1.2). In each of these transport steps, the proteins are localised to either vesicle or target membranes and yeast genetic analyses provided further evidence for the v- and t-SNARE interactions observed in neurons (Ferro Novick and Jahn, 1994).

![Diagram of SNARE Hypothesis](image)

Figure 1.7 The SNARE Hypothesis. The specificity of vesicle targeting is thought to be generated by the complexes that form between vesicle-associated membrane protein (v-SNARE) and its cognate t-SNARE on the target membrane. Subsequent binding of SNAPs and NSF to the SNARE complex leads to the formation of the fusion machinery and upon ATP hydrolysis by NSF membrane fusion is initiated.

The topology and domain organisation of the SNAREs are ideally suited to their function. Most of the syntaxin family of t-SNAREs and the VAMP family of v-SNAREs are type II membrane proteins that are retained in the membrane by a C-terminal signal anchor (section 1.2.1.1 and Fig. 1.2). They are termed tail-anchored proteins with little or no lumenal domain and the majority of the mass is projected into the cytoplasm. The cytoplasmic parts of the SNAREs contain one or more domains predicted to form α-helical coiled-coil structures, allowing pairing between the cognate v- and t-SNAREs (Chapman et al., 1994; Hayashi et al., 1994) and intra-molecular interactions (Hanson et al., 1995). The SNAP-25/Sec9 family t-SNAREs are peripheral membrane proteins. They are inserted into the membrane by palmitoylated side chains attached to the cysteine residues (Oyler et al., 1989). A similar membrane insertion mechanism is used for the ER v-SNARE Ykt6p, which is retained in the membrane by farnesylation at the C-terminal CAAX box (Sogaard et al., 1994).
### Table 1.2: Components of SNARE complexes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Pathway</th>
<th>v-SNARE</th>
<th>t-SNARE</th>
</tr>
</thead>
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<tr>
<td>Mammal</td>
<td>Synapses</td>
<td>VAMP/synaptobrevin synaptotagmin</td>
<td>Syntaxin 1 SNAP-25</td>
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<td></td>
<td>ER-Golgi</td>
<td>rbet1, rat sec22 a/b membrin</td>
<td>Syntaxin 5 Syntaxin 6</td>
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<td></td>
<td>Intra-Golgi</td>
<td>GOS-28</td>
<td>Syntaxin 5</td>
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<td>Golgi-plasma membrane</td>
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<td>Syntaxin 2 Syntaxin 4</td>
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<td>Yeast</td>
<td>ER-Golgi</td>
<td>Boslp, Betlp, Sec22p, Ykt6p/p26</td>
<td>Sed5p Ufe1p</td>
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<td></td>
<td>Intra-Golgi</td>
<td>Sftlp</td>
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<td></td>
<td>Golgi-vacuole</td>
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<td>Pep12p</td>
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<td></td>
<td>Golgi-plasma membrane</td>
<td>Snclp, Snc2p</td>
<td>Sso1p, Sso2p Sec9p</td>
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The SNAREs not only provide the specificity for vesicle docking but also control fusion by acting as a scaffold to recruit the SNAP-NSF complex. In constitutive vesicular transport pathways fusion follows vesicle docking without delay. In highly regulated synaptic vesicle fusion, docked vesicles do not fuse until triggered by the influx of Ca\(^{2+}\) ions. Synaptotagmin, being both a Ca\(^{2+}\) sensor and a v-SNARE, provides the vital link between Ca\(^{2+}\) influx and vesicle fusion (Schiavo et al., 1997). Direct interaction between the t-SNAREs SNAP-25 and syntaxin with N-type Ca\(^{2+}\) channels was also demonstrated (Rettig et al., 1996; Sheng et al., 1996; Wiser et al., 1996), further supporting their role in linking the docking and Ca\(^{2+}\)-triggered fusion of vesicles.

One of the many implications of the SNARE hypothesis is the generation of the Golgi stack, where stacking is simply viewed as an extension of the docking process, with the v-SNAREs in each cisterna interacting with the cognate t-SNAREs in the next (Fig. 1.8). Two possible mechanisms could prevent the fusion of Golgi cisternae that normally follows the formation of the docking complex: the first is to postulate a "fusion clamp" that binds to the SNARE pair, denying access to NSF and or SNAPs; a second possibility is to postulate the existence of fusion-resistant isoforms of the SNAREs that are specialised for stacking. This implication of the SNARE hypothesis would explain three characteristics of the Golgi apparatus: the ordered stacking, close apposition and constant spacing of stacked cisternae (Rothman and Warren, 1994).
Introduction

Figure 1.8 Generation of the Golgi stack from SNARE complexes. The SNARE hypothesis can explain the stacking of Golgi cisternae if the SNAREs can dock with each other but are prevented from subsequent fusion by a fusion clamp, or by the involvement of isoforms of the SNARE pairs that are unable to trigger fusion. SNARE pairs at different locations are shown in different shades of grey.

1.3.5.2 Regulation of targeting

The effectiveness of SNAREs in conferring specificity of vesicle targeting is dependent on their restricted intracellular localisation. v-SNAREs are membrane associated both prior to vesicle formation and following vesicle fusion. Thus, they will not only be present in transport vesicles but also be found on both the donor and the target membranes unless immediately captured by coat proteins to be incorporated into recycling vesicles that bring the v-SNAREs back to the donor compartment. In addition, both v- and t-SNAREs are shown to be synthesised and then inserted into the ER membrane before they are delivered to their destinations by vesicle transport (see section 1.3.1). Therefore, in the context of a living cell, there will be mixed populations of v- and t-SNAREs on vesicles and membrane compartments alike. If SNAREs alone determined the specificity of vesicle traffic and compartment identity, the cell would be plagued with constant mis-targeting of vesicles as a consequence of these SNAREs being present on inappropriate membranes in their active form. This implies that the SNAREs must be maintained in an inactive state after biosynthesis and until their final destination is reached. Additional levels of regulation on the formation of SNARE pairs must exist to ensure the fidelity of vesicle transport.

The coat proteins on the transport vesicle may, to some extent, help to restrict SNARE accessibility by virtue of their thickness (Schekman and Orci, 1996), but tighter regulation is more likely to be mediated by proteins. A family of proteins related to the yeast SEC1 gene product is most likely to be involved in this regulation. The gene...
product Sec1p participates in the yeast constitutive secretory pathway from the Golgi apparatus to the plasma membrane and is required for exocytosis (Novick et al., 1981; Novick et al., 1980). In both yeast and animal cells, members of the Sec1 family are shown to interact with members of the syntaxin family of t-SNAREs.

Over-expression of yeast t-SNARE Sso1/Sso2 (Table 1.2) suppresses mutations in the SEC1 gene, suggesting that they interact with the Sec1 protein (Aalto et al., 1993). Sly1, a yeast Sec1 homologue, first identified as a suppressor of loss of Ypt1 (Dascher et al., 1991), is required for ER-Golgi transport (Ossig et al., 1991) and is shown to interact with the ER-Golgi t-SNARE Sed5p (Sogaard et al., 1994). The mammalian homologue, rSly1, forms a native complex with syntaxin 5 in vitro (Dascher and Balch, 1996). Another yeast Sec1 homologue, Slp1, is required for transport between the Golgi and the vacuole (Aalto et al., 1992).

n-Sec1/rb-Sec1 is the mammalian homologue of yeast Sec1p. Biochemical studies have shown that n-Sec1/rb-Sec1 binds syntaxin 1, forming a complex that is distinct from the previously identified 7S and 20S docking and fusion complexes (Garcia et al., 1994). Recombinant n-Sec1 inhibits VAMP or SNAP-25 binding to syntaxin, suggesting the n-Sec1 binding to syntaxin precedes and/or regulates formation of the SNARE complexes (Pevsner et al., 1994).

<table>
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<th>Table 1.3. Regulators of SNARE complexes</th>
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<tr>
<td>Organism</td>
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Further evidence for the regulatory role of the Sec1 family comes from studies in neurobiology. The Sec1 homologue in the nervous systems of Caenorhabditis elegans is unc-18. It belongs to a group of genes defined by mutations with a paralytic phenotype and accumulations of acetylcholine, suggesting a defect in neurotransmitter release (Hosono et al., 1992). The mammalian homologue of unc-18 is munc-18, which was shown to bind syntaxin 1 and prevent in vitro interaction with SNAP-25 and VAMP.
INTRODUCTION

(Hata et al., 1993). Over-expression of Rop (Ras opposite), the Sec1 homologue in Drosophila melanogaster, led to a dramatic reduction of the number of spontaneous vesicle fusion and defective neurotransmitter secretion, suggesting Rop may restrict the ability of vesicles to dock or of docked vesicles to fuse (Salzberg et al., 1993). However, Rop over-expression does not alter significantly the Ca^{2+}-dependence of neurotransmitter release. This indicates that Rop plays a negative role in neurotransmitter release in vivo, possibly by modulation of docking of synaptic vesicles or activation of a pre-fusion complex (Schulze et al., 1994).

Though the mechanism by which the Sec1 family act is still unclear, these observations strongly suggest that Sec1 family functions primarily as a negative regulator of the SNARE complex formation by interacting with the syntaxin family t-SNAREs. The observation that n-Secl is not in a stable complex with syntaxin 1 also suggests that the Sec1 family may have additional functions (Garcia et al., 1995).

Another key regulator in vesicle targeting is the Rab family proteins (in yeast they are members of the Ypt1/Sec4 family), which are proposed to modulate v-SNARE functions. The Rab family (Table 1.3) represent over 30 small GTPases that are localised to various intracellular membranes and are required in multiple steps of the secretory, endocytic and recycling pathways (Novick and Brennwald, 1993; Pfeffer, 1994). They are cytosolic proteins attached to membranes via two geranyl-geranyl side chains added to their C-terminal cysteines (Magee and Newman, 1992). They are also present in the cytosol as a stoichiometric complex with guanine-nucleotide dissociation inhibitor (GDI), which can apparently interact with multiple Rab proteins. A current model (Fig. 1.9) suggests Rab proteins regulate SNARE complex formation by a cycle of activating and inactivating v-SNAREs (Pfeffer, 1996).

GDI has been shown to deliver cytosolic GDP bound Rab proteins to specific membrane compartments (step 1). The removal of GDI and Rab bound to GDI by immunodepletion led to a complete loss of the ability of cytosol to stimulate vesicular transport between late endosomes and the TGN in vitro (Dirac-Svejstrup et al., 1994). Membrane recruitment of Rab was also accompanied by the displacement of GDI (step 2) in a reaction catalysed by a proposed GDI-displacement factor (GDF) (Dirac-Svejstrup et al., 1997). Nucleotide exchange catalysed by a GTP-exchange factor (GEF) followed (step 3) and the GTP bound Rab is associated with a nascent transport vesicle on the membrane of the donor compartment (step 4). Recent findings indicate Rab proteins are required for transport vesicle formation (Nuoffer et al., 1994; Riederer et al., 1994) and that v-SNAREs on the nascent vesicle would be inactive unless complexed with an appropriate Rab protein in its GTP bound form (Lian et al., 1994). Rab proteins were also found to be in a complex with VAMP, SNAP-25 and syntaxins in a detergent extract of bovine brain (Horikawa et al., 1993). In yeast, numerous genetic interactions between the
In introduction ônor Membrane GDP-Rab GTP-Rab Rab-GDI
GDP-Rab GTP-Rab v-SNARE t-SNARE Rab-GDI GDP-Rab GTP-Rab
Figure 1.9 Hypothetical regulation of vesicle targeting. Rab family proteins regulate SNARE complex
formation by interacting with v-SNAREs: (1). GDI presents the Rab-GDI complex to specific donor
membrane during vesicle formation. (2). Dissociation of GDI from Rab may be catalysed by GDF,
followed closely by (3). a nucleotide exchange catalysed by GEF. (4). GTP-Rab is recruited onto
nascent transport vesicles containing inactivated v-SNAREs. In an unidentified step v-SNAREs are
activated in association with appropriate GTP-Rab. Uncoating of vesicles also takes place. (5).
SNARE complex formation takes place to accomplish vesicle docking, leading to fusion. (6). After
vesicle fusion a GAP increases the GTPase activity of Rab and converts it into GDP-Rab. (7).
Unoccupied cytosolic GDI then retrieves GDP-Rab for another round of action. Sec1 family proteins
exhibit negative regulation of t-SNAREs though the mechanism is still unclear: (i). Sec1 bound to t-
SNARE on the target membrane and renders it inactive. (ii). Dissociation of Sec1 protein activates t-
SNARE, though it is unclear whether this is a result of or precedes v-SNARE binding.

genes encoding Rab proteins, v-SNAREs and the Sec1 family have been documented
(Lian and Ferro-Novick, 1993; Lian et al., 1994). Mutant but not the wild type Sly1p, a
Sec1 family member (Table 1.2) could compensate for the loss of Ypt1p, an essential
yeast ER-Golgi Rab protein, suggesting Sly1p acts downstream of Ypt1p (Dascher et al.,
1991). Sec9p, the yeast homologue of SNAP-25 (Table 1.2), is a potent suppressor of a
mutation in Sec4p, a yeast Rab protein, indicating Sec4p acts upstream of this t-SNARE
(Brennwald et al., 1994). Although direct interactions between Rab proteins and v-
INTRODUCTION

SNAREs/Sec1 family have yet to be shown, these findings indicated a possible role of Rab proteins in displacing Sec1 family proteins to activate t-SNAREs (Fig. 1.9, step i) as well as activating v-SNAREs to form the SNARE complex on the target membrane (Fig. 1.9, step 5). This is likely as v- and t-SNARE interaction was not detected in temperature-sensitive ypt1 mutant yeast strains at non-permissive temperatures (Sogaard et al., 1994). After vesicle fusion, Rab-specific GTPase activating proteins (GAPs) are thought to facilitate GTP hydrolysis, converting Rabs to the GDP bound form and inactivating v-SNAREs again on the target membrane (Fig. 1.9, step 6). Cytosolic GDI then retrieves GDP bound Rab proteins from the target membranes and recycles them to their membranes of origin (Fig. 1.9, step 7).

1.3.6 Vesicle fusion

Study of intracellular transport led to the identification of a protein complex in detergent extract of brain that migrates on glycerol gradients with a sedimentation coefficient of 20S (Wilson et al., 1992). This complex consists of NSF (N-ethylmaleimide (NEM) sensitive factor), a cytosolic ATPase required for the vesicular transport between Golgi cisternae (Block et al., 1988), SNAPs (soluble NSF-attachment proteins) (Clary et al., 1990) and their membrane-bound SNAP-receptors (SNAREs) in the presence of a non-hydrolysable ATP analogue or ATP without magnesium (Sollner et al., 1993). Disassembly of this complex by NSF is thought to be a critical step in the molecular events which lead to vesicle fusion with the target membrane. Inactivation of NSF led to the accumulation of uncoated transport vesicles (Malhotra et al., 1988). Binding of NSF to membranes requires α-SNAP and γ-SNAP (Sollner et al., 1993), which in turn require their receptor SNAREs for membrane association.

The SNAREs isolated from the 20S complex are all proteins forming the docking complex at the synapse: the v-SNAREs synaptobrevin/VAMP, and the t-SNAREs syntaxin and SNAP-25 (Sollner et al., 1993). Later findings showed that synaptotagmin is also an essential part of the SNARE complex and it binds SNAP-25 to form a high-affinity complex to which syntaxin binds, both in the presence or absence of calcium (Schiavo et al., 1997). This constitutes a scaffold for VAMP binding, thus illustrating the synergistic nature of interactions between the two t-SNAREs and the two v-SNAREs.

Each SNARE complex binds between three and six SNAPs (Hayashi et al., 1995). Syntaxin has been identified as the primary substrate for α-SNAP, which also binds weakly to SNAP-25. Synaptobrevin/VAMP, although incapable of binding α-SNAP individually, induced a third α-SNAP binding site when associated with syntaxin and SNAP-25 into hetero-trimers (Hayashi et al., 1995). NSF binds to syntaxin through α-SNAP, and with higher affinity in the presence of γ-SNAP (Wilson et al., 1992). In the presence of ATP and magnesium, NSF catalyses a conformational change in itself which
abolishes binding to SNAPs, disrupts the coiled-coil pairing between SNAREs, thus dissociating the SNARE complex (Sollner et al., 1993). In vitro studies of yeast S. cerevisiae vacuole inheritance showed the fusion of vacuole-derived membrane vesicles requires Sec17p (α-SNAP), Sec18p (NSF), and ATP for an early stage of the reaction that results in Sec17p release, which can even precede vesicle docking (Mayer et al., 1996). NSF thus may function to activate membranes for fusion rather than in bilayer fusion per se. The disassembly of the SNARE complex is an essential step in the pathway leading to membrane fusion, although the nature of events between ATP hydrolysis by NSF and lipid bilayer fusion remains to be understood.

At the synapse, Ca\(^{2+}\)-regulated fusion of synaptic vesicles to the pre-synaptic membranes also requires NSF and SNAPs (Pallanck et al., 1995). The brain-specific β-SNAP has been shown to bind synaptotagmin, linking this Ca\(^{2+}\) receptor to the fusion machinery (Schiavo et al., 1995). Direct interactions between SNAP-25 and synaptotagmin was also shown. Further evidence indicates that SNAP-25 and syntaxin directly interact with the N-type Ca\(^{2+}\) channel and that this interaction modifies the gating properties of the channel (Rettig et al., 1996; Sheng et al., 1996; Wiser et al., 1996). Together these support the idea of a link among the SNARE complex, the Ca\(^{2+}\) channel and the likely role synaptotagmin plays as a Ca\(^{2+}\) sensor in synaptic vesicle fusion events.

However, the proposed sequence of fusion events at the intra-Golgi transport step could not explain the rapid release of neurotransmitters upon Ca\(^{2+}\) influx with a time delay of only 60\(\mu\)s (Sabatini and Regehr, 1996). Neither were there data supporting the requirement of ATP hydrolysis for exocytosis from neurones or endocrine cells (Hess et al., 1993; Thomas et al., 1993). These apparent discrepancies can be reconciled by an alternative model for vesicle fusion at the nerve terminal. Here, NSF and SNAPs are required to induce a fusion-competent state for the docked vesicles upon ATP hydrolysis, possibly involving the intra-molecular interaction of syntaxin via its N- and C-terminal coiled-coil domains (Hanson et al., 1995). After NSF and SNAPs dissociate the synaptotagmin-containing SNARE complex is left in a metastable conformation that can be rapidly regulated by Ca\(^{2+}\) to trigger membrane fusion (O'Connor et al., 1994).

On the basis of their roles in both intra-Golgi transport and in vitro studies of synaptic vesicle docking and fusion, NSF and SNAPs are believed to promote vesicle fusion to target membranes throughout the secretory pathway (Rothman and Warren, 1994). This is consistent with the essential roles of their yeast homologues Sec17p (α-SNAP) and Sec18p (NSF) play at multiple stages of the secretory pathway (Kaiser and Schekman, 1990; Wilson et al., 1989).

One notable exception to the general fusion machinery detailed above is the fusion of Golgi-derived vesicles with the apical plasma membrane in polarised Madin-Darby canine
Introduction

Kidney (MDCK) cells. In streptolysin-O (SLO)-permeabilised MDCK cells, transport from the TGN to the basolateral plasma membrane is inhibited by anti-NSF antibodies and stimulated by \( \alpha \)-SNAP but not those from the TGN to the apical cell surface. Furthermore, apical transport is insensitive to Rab-GDI and clostridial neurotoxins (see next section), which inhibit basolateral transport (Ikonen et al., 1995). Another example can be found in the transport from endosomes to the TGN, which has been shown to be independent of NSF (Goda and Pfeffer, 1991). These results provide evidence that mechanisms other than the NSF-SNAP-SNARE-mediated fusion operate in the exocytic pathway.

It is now apparent that NSF belongs to a family of related and conserved ATPases involved in membrane fusion events (Erdmann et al., 1991; Frohlich et al., 1991; Thorsness et al., 1993). Functions of the members of this ATPase family may overlap but each may employ a distinct mechanism to mediate membrane fusion. One such NSF-like family member, p97, is an abundant hexameric ATPase present in all eukaryotic cells and is localised to both the nucleus and cytoplasm (Peters et al., 1990). It has been shown to facilitate the reassembly of mitotic Golgi fragments into longer cisternae (Rabouille et al., 1995). Reassembly can also be mediated by a mixture of NSF, SNAPs and p115, but the reassembled cisternae had different morphologies, indicating that p97 and NSF are not redundant ATPases but play distinct roles in membrane fusion (Rabouille et al., 1995). Moreover, reassembly of functional Golgi stacks in permeabilised cells after their vesiculation using the sponge metabolite illimaquinone (IQ) requires an NSF-dependent stage and a p97-dependent stage, confirming the distinct roles NSF and p97 play in Golgi reassembly (Acharya et al., 1995; Acharya et al., 1995). p97 is also shown to be involved in yeast karyogamy, the fusion of the nuclear envelopes of haploid nuclei, whereas NSF or \( \alpha \)-SNAP are not required (Latterich et al., 1995; Latterich and Schekman, 1994).
1.4 Protein Localisation

Newly synthesised proteins are transported from the ER to and through the Golgi apparatus to their various destinations (Fig. 1.1). Recent observations indicate that signals for transport exist and that secretory proteins are selectively exported from the ER and concentrated into the transport vesicles (Balch et al., 1994; Mizuno and Singer, 1993). However, proteins involved in post-translational modifications such as protein folding and glycosylation are not transported to cellular destinations other than the compartments where they normally reside. How these proteins are delivered to and retained in the appropriate compartments along the secretory pathway are subjects of intense investigation.

Special signals contained within proteins have been shown to be important in their maintenance in specific compartments. There are at least two types of such signals to ensure correct protein localisation: retention and retrieval.

A retention signal acts only in but not before or after the correct compartment. It would permit anterograde movement along the secretory pathway until the correct compartment had been reached. It would then prevent any further movement by denying proteins access to transport vesicles. So far, signals of this type have only been found in the membrane-spanning domain and flanking sequences.

A retrieval signal, on the other hand, would only act once the protein had left the compartment in which it normally resides. This signal would depend on specific binding to components involved in retrograde transport. This type of signal is often composed of a short discrete amino acid motif located at the termini of the proteins.

Since retention can never be 100% efficient, any leakage will have to be salvaged by the retrieval system. At the same time, it is difficult to imagine a retention mechanism that operates entirely by salvaging lost proteins. Indeed, the presence of both types of signals in the ER resident protein, calreticulin (Sonnichsen et al., 1994), and a TGN resident protein, TGN38 (Ponnambalam et al., 1994), strongly suggests that more than one mechanism operates to ensure correct protein localisation along the secretory pathway (Nilsson and Warren, 1994).

1.4.1 Retention mechanisms

Retention of Golgi resident enzymes has been studied in great detail in the past few years. All characterised Golgi enzymes are type II signal anchor proteins anchored in the membrane by their uncleaved signal peptide (Fig. 1.2). This sequence further serves as a retention signal as the membrane-spanning domain (the signal anchor) and part of its
flanking regions have been shown to be sufficient for correct Golgi localisation (Machamer, 1993; Nilsson et al., 1993) Munro, 1991 #152; Nilsson, 1991 #159; Swift, 1991 #158; Machamer, 1993 #272; Machamer, 1993 #150; Nilsson, 1993 #149; Smith, 1993 #156. Replacing the membrane-spanning domains and part of the flanking regions of various reporter molecules with corresponding regions of different Golgi enzymes localise the reporter molecules to the parts of the Golgi apparatus where these enzymes normally reside (Nilsson et al., 1993). This effect cannot be saturated by overexpression, suggesting it is not a receptor-mediated mechanism and that the membrane-spanning domain functions as a retention signal. Two models have been put forward to explain how this might happen.

The first model is based on retention through interaction with other residents to form large oligomers unable to enter the transport vesicle. The first membrane-spanning domain of the M-protein of avian coronavirus mediates the formation of large oligomers of a reporter molecule, the VSV-G protein, in the cis-Golgi (Weisz et al., 1993). Extensive mutagenesis of this domain showed that polar residues, lining one face of a predicted α-helix, are important both for the retention and oligomerisation of the M-protein (Machamer et al., 1993). This type of sidedness of polar residues is also found in the membrane-spanning domains of other resident Golgi enzymes, suggesting a role for these in Golgi retention.

Another, perhaps better characterised example, comes from works on purified Golgi membranes in our laboratory. Large structures can be isolated after detergent extraction of Golgi membranes. These structures consist mainly of medial-Golgi enzymes and can be reversibly disassembled by the addition of salt (Slusarewicz et al., 1994). We have also shown that medial-Golgi enzymes interact with each other via the membrane-spanning domains and part of the flanking regions (Nilsson et al., 1996). Artificial retention in the ER of the medial-Golgi enzyme NAGT I by replacing the cytoplasmic domain with that of an ER resident protein, p33 (see Chapter 3.1), results in the retention of another medial-Golgi enzyme, MannII, and vice versa (Nilsson et al., 1994). Other medial-Golgi enzymes may also be involved in this interaction, as increasing levels of retention of NAGT I in the ER resulted in a complete disappearance of the Golgi stack. This not only argues for the formation of large oligomers between medial-Golgi enzymes but also suggests that they play an important role in maintaining Golgi structure (Nilsson et al., 1994). Further evidence was obtained by altering the membrane-spanning domain of NAGT I, which does not affect its Golgi localisation but has a dramatic effect on Golgi cisternal morphology (Nilsson et al., 1996).

The ability to form large oligomers is not restricted to the medial-Golgi enzymes. Overexpression of GalT and SialylT, two trans-Golgi enzymes, lead them to back up into the ER (Munro, 1991; Nilsson et al., 1991), an observation consistent with premature
oligomerisation caused by high levels of expression. Because the membrane-spanning domains were necessary to mediate this effect, it was postulated that these domains interact with each other to form oligomers too large to be incorporated into transport vesicles, by a mechanism termed kin recognition (Fig. 1.10) (Nilsson et al., 1994; Nilsson et al., 1993).

![Diagram of Golgi enzyme retention by kin recognition model.](image)

Figure 1.10 Golgi enzyme retention by kin recognition model. Each Golgi enzyme is assumed to be a homodimer (Fleischer et al., 1993) in which the lumenal domains are bound by interactions between the catalytic domains and/or the stalk region. The membrane spanning domains and the stalk region are free to bind to the corresponding regions of their kin. After synthesis in the ER, the homodimers interact with the kin oligomers existing in the Golgi cisternae. Their size prevents them entering the transport vesicles budding from the cisternal rim (thick lines) thus achieving retention. Here two kinds of kin oligomers are depicted: hetero-oligomer between MannII and NAGT I, and homo-oligomer of GalT. Interactions between the cytoplasmic domains of Golgi enzymes and the intercisternal matrix may also contribute to Golgi protein retention and could provide a mechanism for stacking the Golgi (see 1.2.3.2).
Similarly, retention of ER proteins is shown to maintain the composition of the ER. Cross-linking studies show many ER resident proteins have affinity for each other, particularly when Ca\(^{2+}\) and ATP are present (Nakai et al., 1992). All the major lumenal proteins have negatively charged domains that bind Ca\(^{2+}\) and ATP (Nigam et al., 1994; Sonnichsen et al., 1994) and Ca\(^{2+}\) depletion results in secretion of normally retained proteins (Booth and Koch, 1989; Suzuki et al., 1991). The ER resident membrane proteins might also interact with lumenal proteins to form an extensive matrix (Booth and Koch, 1989; Nakai et al., 1992) similar to the one proposed for the Golgi (kin recognition) (Nilsson et al., 1994; Nilsson et al., 1993).

The second model postulates that membrane thickness could contribute to the retention of Golgi proteins. It has been shown the ER has a lower cholesterol content than the plasma membrane and that a cholesterol gradient exists across the Golgi stack (Orci et al., 1981). Consequently membrane thickness and rigidity increases from the ER through the Golgi to the plasma membrane. The observation that the membrane-spanning domain of SialyIT could be altered or replaced with polyleucines without loss of Golgi localisation suggests that the primary sequence of this domain is not important in specifying Golgi localisation (Dahdal and Colley, 1993; Munro, 1991). Increasing the length of the membrane-spanning domain overrides the Golgi retention signal and directs several Golgi resident enzymes to the plasma membrane (Munro, 1991); Masibay, 1993 #154. Taken together, this suggests that the thickness of the membrane determines the Golgi enzyme retention and the distribution of Golgi enzymes across the stack would reflect the different lengths of their membrane-spanning domains (Bretscher and Munro, 1993). However, the length of the membrane-spanning domain, although an important parameter, is not sufficient by itself for Golgi retention (Masibay et al., 1993). Furthermore, several cis-Golgi enzymes possess longer membrane-spanning domains than trans-Golgi enzymes, indicating that membrane thickness and the length of transmembrane domain cannot be the sole determinant in protein localisation but it might operate in parallel with other mechanisms.

### 1.4.2 Retrieval mechanisms

Special signals on the molecule have been identified and shown to be necessary and sufficient for retrieval, the best studied case being the KDEL motif present on secretory proteins and its retrieval back to the ER.

For soluble ER resident proteins as well as some type II membrane proteins that have their C-terminus within the luminal side of the ER, the C-terminal tetrapeptide has been shown to function as a retrieval signal at some post-ER compartments. The optimal signal in mammalian cells is the tetrapeptide Lys-Asp-Glu-Leu, or KDEL (Munro and
Pelham, 1987) and HNEL (Bu et al., 1995; Bu et al., 1997); whereas in yeast S. cerevisiae it is HDEL (Pelham et al., 1988) or DDEL in Kluyveromyces lactis (Lewis et al., 1990). Eliminating the KDEL signal from these proteins results in their slow secretion, confirming that they also have effective, but imperfect, retention signals. When transplanted onto various reporter molecules, the KDEL (and related) signal localises them to the ER but at the same time these proteins display post-translational modifications that only occur in the Golgi, indicating they have left the ER at least once before being brought back, confirming it is indeed a retrieval signal (Pelham, 1989).

This retrieval process is receptor-mediated and the KDEL receptor (see section 4.1.2) has been subsequently identified and cloned both in yeast (Lewis et al., 1990) and in mammalian cells (Lewis and Pelham, 1990). Binding to the KDEL-containing ligand occurs in a pH-dependent manner (Wilson et al., 1993) and would induce receptor oligomerisation and recycling to the ER, where the ligand will be discharged and the monomeric receptor would return to the Golgi (Townsley et al., 1993). This retrieval system could function from the IC to as far downstream from the ER as the TGN (Griffiths et al., 1994), where proteins are terminally glycosylated. However, retrieval from the TGN was inefficient and some KDEL-containing proteins reached the cell surface (Martire et al., 1996).

Type I membrane proteins can be efficiently retained in the ER by the C-terminal di-lysine motif, KKXX or KKKXX, in their cytoplasmic domain. When transplanted onto reporter molecules, they acquire Golgi-specific post-translational modifications (Jackson et al., 1993). This suggests that the KKXX motif functions as a retrieval signal via a retrograde transport route in a manner analogous to the retrieval of KDEL proteins from post-ER compartments by the KDEL receptor. The KKXX motif can also function in yeast as proteins bearing this signal can be transported to the Golgi, modified by Golgi enzymes and then retrieved to the ER (Gaynor et al., 1994; Townsley and Pelham, 1994).

Two lines of evidence show that the COPI coat proteins interact with the KKXX motif and are responsible for retrieving escaped ER residents from the Golgi. First, coatomer binds KKXX and related peptides in vitro, both in yeast and mammalian cytosol, but not mutated sequences that are inactive as retrieval signals (Cosson and Letoumeur, 1994). Second, a yeast mutant that failed to retain a reporter molecule with the KKXX motif is shown to have mutations in genes coding for α-COP (ret1) and γ-COP (sec21) (Letoumeur et al., 1994). Moreover, an existing β'-COP mutant (sec27) displays similar phenotype and coatomers from this mutant, as well as from the α-COP mutant, failed to bind KKXX in vitro. These results argue strongly that coatomer binds directly to proteins having the KKXX motif and this interaction is required for their retrieval.
INTRODUCTION

Whereas the ER retention motif for type I membrane protein is based on double lysine motifs, that for type II membrane proteins is based on double arginines. The minimal requirements of this motif were found to be two arginines (RR), spaced by no more than one amino acid, located within the first five amino acids at the N-terminus of type II membrane proteins (Schutze et al., 1994). Depending on the exact sequence context in which the basic residues are placed, in some cases the arginine were found to be replaced by lysine without impairing the function. Proteins with such ER retrieval motifs are TRAM (Gorlich et al., 1992) and p63 (Schweizer et al., 1993; Schweizer et al., 1995), both ER residents. The similarities to the KKXX motif suggest that both signals probably function via a common retrograde transport pathway.

1.5 Aim of thesis

I started the project to identify components responsible for stacking the Golgi cisternae and provide biochemical evidence for the existence of the kin oligomers (Fig. 1.10). One implication of the SNARE hypothesis is the generation of the Golgi stack (Fig. 1.8) from closely apposed cisternae with the cognate v- and t-SNARE pairs docked but prevented from fusion by an additional fusion clamp (Rothman and Warren, 1994). I therefore studied the Golgi t-SNARE, syntaxin 5, hoping to identify its fusion clamp. Whilst pursuing this I noticed an additional, longer form of syntaxin 5, and this was investigated in greater detail.
Chapter 2

Materials and Methods
All reagents were of analytical grade or better and were purchased from either Sigma or BDH, unless indicated otherwise. Radiochemicals were all purchased from NEN. All water was filtered by the Whatman Milli-Q system. Stock solutions used frequently and those requiring special attention were listed at the end of each section.

2.1 Biochemistry

2.1.1 Protein Precipitation

If necessary, protein was precipitated prior to electrophoresis to either reduce the sample volume or remove reagents which would interfere with the running of the gels. Two methods were employed. For samples of up to 500μl, the methanol/chloroform method (Wessel and Flugge, 1984) was used. Samples of up to 1 ml volume were precipitated using trichloroacetic acid (TCA).

2.1.1.1 Chloroform/Methanol Method

Samples were made up to 0.1 ml with H₂O. 0.5 ml of methanol was added, and then 0.2 ml of chloroform was added. Next, 0.3 ml of water was added. If the initial volume of sample was larger than 0.1 ml, correspondingly smaller amounts of water was added to keep the total volume below 1.2 ml. This was followed by vortexing and spinning at 12,000 rpm for 1 min. The upper phase was discarded to leave the interface and the lower phase. Finally, 0.3 ml of methanol were added and the precipitate was spun down at 12,000 rpm for 2 min to pellet the precipitate. The supernatant was removed and samples were incubated at 37°C for 10 min to evaporate excess solvent. Pellets were then dissolved in SDS-PAGE loading buffer and processed for electrophoresis.

2.1.1.2 TCA Method

Samples were made up to 1 ml with H₂O and 0.1 ml of 0.15% sodium deoxycholate was added. After vortexing and a 5 min incubation at 4°C, 0.2 ml of 72% (w/v) TCA was added and the sample left on ice for 1 hr. Samples were spun at 14,000 rpm for 5 min in a bench top microfuge and the supernatants discarded. The pellets were washed and resuspended in 1 ml of acetone that had been pre-cooled on dry-ice. Following a second spin, samples were washed once more in acetone and spun followed by evaporation of excess solvent for 10 min at RT. The resulting pellets were dissolved in SDS-PAGE loading buffer and processed for electrophoresis.
2.1.2 Electrophoresis

2.1.2.1 1-D SDS-PAGE

One dimensional electrophoresis separates proteins by their relative molecular weight (MW) and the different mobility in polyacrylamide gel. This was carried out according to Blobel and Dobberstein, 1975. The methods used are described below.

Gel Casting

Gels consisted of either a single percentage or a linear gradient of, e.g. 7.5-15% or 10-15%, acrylamide. The separating gel and the stacking gel solutions were made as described in Table 2.1. Polymerisation was initiated by the addition of 100 µl 10% (w/v) ammonium persulphate to each solution just before use.

The gels were poured at a thickness of 0.75 mm or 1 mm using a simple two-chambered gradient maker into the Bio-Rad 24x16 cm gel casting assembly or the Mini-gel assembly. The separating gels were overlaid with distilled water to ensure a smooth interface and were allowed to set. Once set, the water was removed and the gel washed once with stacking solution, to which 100 µl of ammonium persulphate solution had been added, before pouring the stacking gel.

Sample Preparation

300 µl of concentrated (3x) SDS-PAGE loading buffer was diluted with 0.65 ml of water and 50 µl of 1 M DTT/β-mercaptoethanol. Samples were dissolved in 40 µl of this solution by shaking for 30 min. Samples were then heated at 95°C for 5 min and cooled to RT. The samples were then spun for 14,000 rpm for 5 min in a bench top micro-centrifuge before being loaded onto the gel with a Hamilton syringe. In addition, 0.75 µl of Bio-Rad high and/or low molecular weight markers were processed as above and loaded in one lane.

Electrophoresis

Gels were placed into the apparatus and the upper and lower tanks filled with SDS-PAGE running buffer. Gels were electrophoresed at a constant current of 35-60 mA per gel for 2-4 hr with the water cooling system operating or left to run overnight at 4 mA per gel. For mini-gel system, gels were run at constant voltage of 120 V for 30-60 min.

Fixing and Staining

Gels were stained by a 10 min incubation in a solution containing 0.25% (w/v)
CoMmAssie Brilliant Blue R. This solution also contained 7.5% (v/v) acetic acid and 50% (v/v) methanol which served to fix the gel by protein precipitation. Gels were then destained by numerous washes in 7% (v/v) acetic acid/40% (v/v) methanol. Silver staining protocol was carried out as described by the manufacturer (Daiichi Chemicals). Gels were then photographed or dried under vacuum using a Model 453 gel drier (Bio-Rad) or dried at RT sandwiched between two sheets of transparent membranes (BioSciences).

Stock Solutions

Acrylamide/bis-Acrylamide (40%; 29:1): Dissolve 6.6g of N,N'-methylene bis-acrylamide in 100 ml of H2O, add 193.4g of acrylamide and make up to a final volume of 500 ml. Add 20g of deionising AG 501-X8 beads (Bio-Rad) to remove any acrylic acid and stir for at least 1 hr. Filter solution through Whatman No.1 paper and store at 4°C.

Ammonium persulphate (10% (w/v)): Just before use dissolve 100 mg in 1 ml of H2O. Can be stored at 4°C for up to one week.

Coomassie Blue Stain: Dissolve 5.0g in a solution composed of 100 ml of methanol, 150 ml of acetic acid and 850 ml of H2O. Filter through Whatman Number 1 paper and store at RT.

DTT (1M): Dissolve 15.4 mg in 1 ml of H2O and use immediately. If stored in -20°C do not freeze-thaw more than 3 times.

Iodoacetamide (0.5M): Dissolve 9.25mg in 100µl H2O and use immediately.

SDS (20% (w/v)): Dissolve 20g of SDS in a final volume of 100 ml of H2O and filter before storage at RT.

SDS-PAGE Running Buffer (5x Stock): Dissolve 150g of Tris, 720g of glycine and 25g SDS in a final volume of 5l H2O. Dilute 5-fold with water before use.

SDS-PAGE Sample Buffer: Dissolve 34.2g of sucrose and 4 mg of bromophenol blue in 10 ml of 2M Tris pH8.8 and 2.5 ml of 200 mM EDTA and make up to 100 ml with

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<tr>
<td>40% Acrylamide/bis (29:1)</td>
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H₂O. Pass through a 0.45μm nitrocellulose filter and store at room temperature.

**Tris pH6.8 (0.5M):** Dissolve 15.1g of Tris in 200 ml H₂O, adjust the pH to 6.8 with HCl and make up to a final volume of 250 ml. Store at 4°C.

**Tris pH8.8 (2M):** Dissolve 121.1g of Tris in 400 ml H₂O, adjust the pH to 8.8 with HCl and make up to a final volume of 500 ml. Store at 4°C.

### 2.1.2.2 2-D IEF-SDS-PAGE

Two dimensional electrophoresis is considered the method with the highest resolution for the separation of complex protein mixtures. This technique separates proteins in terms of their isoelectric points in addition to their molecular weights, and is used to identify new proteins as well as to detect alterations to known proteins, e.g. phosphorylation of a protein can be easily detected. The protocol is adapted from the Cell Biology Handbook and described briefly below.

**Gel Casting**

The first dimension is a tube gel run either in capillary tubes (Bio-Rad) compatible with Mini-gel (Bio-Rad) apparatus, or in large tubes compatible with Millipore 2-D system. The gel solutions (Table 2.2) were poured into the tubes either by capillary action (Bio-Rad) or directly with a syringe (Millipore) taking care that no air bubbles were trapped.

**Sample preparation**

Protein samples were solubilised in lysis buffer directly and shaken at 30°C to prevent the 8 M urea from crystallising at low temperature. Any aggregates still present were centrifuged to clear before loading onto the tube gels.

**1st Dimension: Isoelectric focusing**

The tube gels contain electrolytes and need to be pre-focused to set up a pH gradient. This varies in time depending on the length and capacity of the tube gels. For the Bio-Rad tube gels this takes 10 min while for Millipore system this takes about 3 hr. The end point is reached when there is no more electric current under high voltage.

Samples solubilised in lysis buffer were then applied onto the tube gels and overlaid with buffer to prevent spilling during isoelectric focusing. To make sure all proteins reach their respective isoelectric point, several pH gradients were tested to achieve the desired separation for particular proteins.

**2nd Dimension: SDS-PAGE**

The tube gels were extruded from the 1st dimension tube by using a syringe filled with
**MATERIALS AND METHODS**

SDS-PAGE running buffer sealed to one end of the tube. The tube gels were subsequently equilibrated in SDS-PAGE loading buffer and laid flat on top of the normal SDS-PAGE gels. Care was taken to orientate the extruded tube gels so that the acidic end is always to the same side.

**Stock solutions**

**H$_3$PO$_4$ running buffer (10 mM):** 1.146 ml concentrated H$_3$PO$_4$ (85%) was diluted into 21 H$_2$O.

**IEF acrylamide (acrylamide 28.38% : bis-acrylamide 1.62% w/v):** Dissolve 5.676 g acrylamide and 0.362 g bis-acrylamide to 20 ml final volume in H$_2$O. Filter through 0.45 μm filter and store at 4°C no more than two weeks.

**Lysis buffer (9.8 M urea, 2% ampholine, 4% NP-40, 0.1 M DTT):** Dissolve 29.43 g of ultra-pure urea (BRL), 2.5 ml 40% ampholines pH 7-9 (LKB), 20 ml 10% (w/v) NP-40 and 771.5 mg DTT to 50 ml final volume with H$_2$O at 30°C waterbath. 0.5 ml aliquots were stored at -80°C until use.

**NaOH running buffer (20 mM):** 0.8 g NaOH pellets were solubilised in 1 l H$_2$O and degassed extensively before use.

**Overlay buffer (8M urea, 1% ampholine, 5% NP-40, 10 mM DTT):** Dissolve 9.6 g urea, 0.5 ml 40% ampholine pH 7-9 (LKB), 10 ml 10% (w/v) NP-40 and 30 mg DTT to 20 ml final volume with H$_2$O. Store at -80°C in 0.5 ml aliquots until use.

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<tr>
<td>Urea</td>
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2.1.3 Western Blotting

Western blotting was performed onto two types of membrane matrix. Immobilon-C (Amersham) was used for immuno-blotting and PVDF (polyvinilydene difluoride; Immobilon-P, Millipore) for solid-phase protein sequencing. Transfer was carried out in various Semi-Phor semi-dry blotters (Stainless steel electrode: Hoeffer Scientific Instruments; Carbon electrodes, Carbon and fibre glass electrodes). Unfixed gels were soaked in 100 ml of the appropriate transfer buffer as was the membrane, and six pieces of 3MM paper (Whatman) which were cut to the same size as the gel slice to be blotted. The filter and gel were sandwiched between two sets of three sheets of 3MM paper and placed into the apparatus with the membrane facing the anode. Care was taken to avoid the inclusion of air bubbles in the sandwich and between the electrodes.

2.1.3.1 Immobilon-C

Immobilon-C is a nitrocellulose membrane with a nylon backing which confers mechanical strength. Nitrocellulose is a matrix which binds proteins by hydrophobic interaction. Transfer buffer was made up with 20 ml of TG Buffer (0.2 M Tris/1.5 M glycine), 40 ml of methanol and 1.0 ml of 20% (w/v) SDS and H$_2$O to 200 ml final volume. Transfer was carried out at a constant current of 100 mA for 1 hr. Blots were stained with Ponceau S solution to determine the position of molecular weight markers which were marked with a pencil and then probed with antibodies as described below.

2.1.3.2 PVDF

PVDF is a strong matrix which can withstand the conditions encountered in a solid-phase peptide sequencing (Matsudaira, 1987). The matrix binds proteins by ionic interaction and, therefore, blotting in SDS should be avoided since this anionic detergent will block the binding sites on the membrane. Blotting was, therefore, carried out in a highly alkaline pH in order to ensure that all acidic residues on the proteins being blotted were ionised. Transfer buffer was made up using 25 ml of 100 mM 3-Cyclohexylamino-1-propanesulfonic acid (CAPS) pH11, 5 ml of methanol and H$_2$O to a final volume of 250 ml and transferred as above but at 250 mA for 2 hr in the cold room. Note that these conditions were determined specifically for the blotting of the protein of interest. Methanol is required to strip of any SDS remaining in the gel from the membrane. However, small amounts of SDS will help the transfer of proteins onto the membrane without blocking the filter. The SDS and methanol concentrations, current and transfer-time required for the transfer of any given protein must be determined for each individual polypeptide.

Proteins were visualised on the membranes using sulpho-rhodamine B as described by
(Pappin et al., 1990). This dye binds water and after the membranes have been thoroughly dried under vacuum for 30 min the only water available is the molecular water trapped by the proteins on the membrane. Sequencing was carried out on a MilliGen 6000 solid phase protein sequencer in the ICRF Protein Sequencing Laboratory by Dr. Darryl Pappin and Dinah Rahman.

2.1.3.3 Immunodetection

Western blots were probed using the ECL (Enhanced Chemi-Luminescence) kit supplied by Amersham. Blots were blocked by overnight incubation in blocking buffer (5% low fat milk powder, 0.2% (w/v) Tween-20/PBS) at 4°C. Milk powder was purchased from the Iceland supermarket chain. Blots were then sealed into plastic bags containing 5 ml/100 cm² of blocking buffer to membrane containing the appropriate antibody (spun at 14,000 rpm for 5 min in a bench top microfuge to sediment any aggregates) with care being taken to exclude any air bubbles. Blots were then placed on a rocker and incubated at RT for 1 hr under a bag of water. The blot was then washed twice briefly with blocking buffer followed by 1 hr wash. The blot was then sealed into a fresh bag with blocking buffer containing horse-radish peroxidase-conjugated anti-rabbit IgG (TAGO) at 1/1000 dilution and incubated for 1 hr as before. The blot was washed again as above. The blot was then washed briefly in 0.5% (w/v) Tween-20/PBS and once with PBS alone. The blot was then placed onto a plastic dish and 6 ml of a 50:50 mixture of the ECL reagents poured over it. After 1 min, the excess was drained off and the blot placed onto the shiny side of a piece of benchcote. The blot was covered with cling-film and exposed to X-ray film (X-OMAT AR, Kodak) for periods of 1 sec-5 min.

Stock solutions

**Blocking Buffer:** Dissolve 50 g of low-fat milk powder in 900 ml of PBS. Add 10 ml of 20% Tween-20 and make up to 1 litre with PBS.

**CAPS pH 11.0 (100 mM):** Dissolve 11.1 g of CAPS in 450 ml of ddH₂O. Adjust the pH to 11.0 with NaOH and make up to 500 ml. Store at RT.

**TG Buffer (0.2 M Tris/1.5 M glycine):** Dissolve 12.1 g of Tris and 56.3 g glycine in a total of 500 ml of H₂O. Store at RT.

**Tween-20 (20% (w/v)):** Dissolve 100 g in a total of 500 ml of H₂O. Store at 4°C.

2.1.4 Immunoprecipitation

Immunoprecipitation (IP) was carried out using either Protein A-Sepharose (PAS) or antibodies cross-linked to PAS.

The samples were made up to 0.4 ml with H₂O and 0.4 ml of 2x IP lysis buffer (40 mM...
Tris pH 8.0, 300 mM NaCl, 1% (w/v) TX-100) was added. 80 μl of 10% (w/v) PAS was added along with 4 μl of polyclonal serum which had been pre-spun at 14,000 rpm for 5 min. The samples were then rotated at 4°C for 2 hr and spun at 14,000 rpm for 5 sec on a bench top centrifuge. The supernatant was removed and the beads resuspended in 1 ml of IP wash buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.1% TX-100). Samples were then vortexed briefly and spun again to recover the beads. The above process was carried out two more times to yield the final pellet of beads.

In addition to the normal lysis buffer detailed above, lysis buffer containing NP-40 or TX-114 instead of TX-100 and RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC (deoxycholate), 0.1% SDS and 50 mM Tris pH 8.8 in the presence of protease inhibitor cocktail (PIC) was also used for immunoprecipitation experiments.

Cross-linking of antibodies to the PAS was performed using dimethylpilimidate (DMP) (Pearce) as described (Harlow and Lane, 1988) and the efficiency of cross-linking was assessed by SDS-PAGE (Fig 2.1).

**Stock solution**

*Borate buffer (0.2 M):* Dissolve 1.9 g of Borax (Sodium Tetraborate) in 100 ml of H<sub>2</sub>O and heat to aid solublisation. Take care not to boil. pH to 9.0 with NaOH.

*IP lysis buffer (5x):* Dissolve 484 mg of Tris, 1.75g of NaCl and 1g of TX-100 in 80 ml of H<sub>2</sub>O and adjust pH to 8.0 with HCl. Make up to 100 ml and store at 4°C.

*IP wash buffer:* Dissolve 1.2g of Tris, 4.4g of NaCl and 0.5g of TX-100 in 450 ml of H<sub>2</sub>O and adjust pH to 8.0 with HCl. Make up to 500 ml and store at 4°C.

*Protein-A sepharose (PAS, 10% (w/v)):* Suspend 5g of PAS in a final volume of 50 ml of 2x IP lysis buffer. Store at 4°C with 0.02% sodium azide and stir before use.

*TX-100 (10% (w/v)):* Dissolve 10g in a final volume of 100 ml of H<sub>2</sub>O and store at 4°C.

### 2.1.5 Isopycnic Centrifugation

Linear isopycnic sucrose gradients were poured into Beckman Ultraclear SW-40 rotor tubes using an Auto Densi-Flow automated fractionator (Buchler Instruments) at a flow rate of 1.5 ml/min coupled to a gradient maker (Hoefer Scientific Instruments). The chambers of the device were filled with 5.5 ml of the high- and 6 ml low-concentration sucrose buffer respectively.
After pouring, 0.5 ml aliquots of sample were loaded onto each gradient and the tubes balanced with sucrose-free buffer. Samples were spun at 40,000 rpm at 4°C for 16 hr in an L8-70M ultracentrifuge and the fractions were collected with the same apparatus in 1 ml aliquots from top to bottom and at the same flow rate.

2.1.5.1 TMMDS Gradients

These gradients contained TX-100 and were used in studies on detergent-extracted Golgi membranes. The ranges of the gradients used were either 0.25-2 M or 10-40% (w/v) of sucrose. The gradient buffers were made up as TMMS buffer containing higher than normal sucrose concentrations, i.e. 15 g/100 ml for 15%, 40 g/100 ml for 40%. 1 M DTT was added to each gradient buffer to a final concentration of 1 mM just before use.

2.1.5.2 KEHM Gradients

These gradients contained no detergent and were used for the subcellular fractionation of post-nuclear supernatants from cells grown in culture. Because of the presence of cytosolic proteases in these samples, the gradients also contained a cocktail of protease inhibitors (PIC). Cycloheximide was also added to prevent detachment of ribosomes from the rER in order to maximise the density of this compartment. The range of the gradients was 20.5-63% (w/v) sucrose (0.6-1.84 M) and the gradient solutions were made up as follows using stocks of 5xKEHM buffer and 2.3 M sucrose. The 20.5% buffer contained 4 ml of 5xKEHM, 5.1 ml of 2.3 M sucrose, 20 μl of PIC, 60 μl of 3 mg/ml cycloheximide and 10.8 ml of H₂O. The 63% buffer was made up in the same way except that 16 ml of 2.3 M sucrose and no H₂O were used.

2.1.5.3 Step Gradients

These gradients were used to purify rat liver Golgi (see section 2.1.9) and to study the subcellular localisation of rat syntaxin 5. Rat liver post-nuclear supernatant (PNS) was produced by centrifuging rat liver homogenate at 1000 g for 30 min at 4°C. 1 ml of this PNS was then layered on top of a 12 ml step sucrose gradient between 2 M and 0.5 M at 0.25 M steps. After centrifuging at 30,000 rpm for 18 hr to equilibrium, 1 ml fractions were taken and assayed for enzyme activities. The fractions were then diluted 1/3 with buffer, and the membranes pelleted at 100,000 rpm for 30 min at 4°C. Sample buffer was then added to the pellet to solubilise and boiled at 95°C for 5 min before loading onto SDS-PAGE.

Stock Solutions

**EDTA (200 mM):** Dissolve 7.4 g in 70 ml of H₂O and adjust pH to 7.2 with KOH. Make up to 100 ml and store at RT.
**MATERIALS AND METHODS**

**HEPES pH 7.0 (50 mM):** Dissolve 1.2 g in H$_2$O, adjust pH to 7.0 with KOH and make up to a final volume of 100 ml. Store at RT.

**KEHM Buffer (5x):** Dissolve 9.2 g of KCl and 29.8 g of HEPES in 400 ml of ice-cold H$_2$O. Add 2.4 ml of 2M MgCl$_2$ and 50 ml of 0.5 M EGTA. Adjust pH to 7.4 with KOH and make up to 500 ml with H$_2$O. Store at 4°C and dilute 5 times before use.

**Protease Inhibitor Cocktail (PIC):** This 1000x stock solution contains 1 mg/ml of antipain, aprotinin, chymostatin, leupeptin and pepstatin, 1 M benzamidine and 40 mg/ml PMSF (phenyl-methane-sulphonyl-fluoride). Dissolve 5 mg of the first four, 783 mg of benzamidine and 40 mg of PMSF in 5 ml of DMSO (dimethylsulphoxide). Store as 200 µl aliquots at -20°C. Dilute 1000 times into the sample being used.

**TMMS Buffer:** Dissolve 1.05 g of MOPS, 2 g of TX-100, 10 g of sucrose and 5 µl of 2 M MgCl$_2$ in 80 ml of ice-cold H$_2$O. Adjust the pH to 7.0 with NaOH and make up to 100 ml. Filter and store at 4°C. Just before use, make up a 1 M solution of DTT and dilute 1000-fold into an aliquot of buffer to create TMMDS buffer.

### 2.1.6 Digestion with Proteases

#### 2.1.6.1 Trypsin

Trypsin type XIII from bovine pancreas was made up to 10 mg/ml in 10 mM HCl and incubated at RT for 3 hr before an overnight incubation at 4°C. The protease was divided into aliquots and stored at -20°C. This treatment inactivated any trace amounts contaminating chymotrypsin (Fleischer, 1981).

Increasing amounts of trypsin were added to constant amounts of Golgi membranes, which were then diluted to a protein concentration of 1 mg/ml with MMS buffer (optionally, 10% (w/v) TX-100 was also added to a final concentration of 0.25%). Samples were then incubated at 25°C for 30 min. Reactions were stopped by the addition of a 1000th volume of 40 mg/ml PMSF.

#### 2.1.6.2 Chymotrypsin

Chymotrypsin type VII from bovine pancreas was made up and used exactly as trypsin. Proteolysis was also carried out exactly as with trypsin.

#### 2.1.6.3 Proteinase K

Proteinase K (Boehringer Manheim) was made to a final concentration 10 mg/ml in 10 mM Tris pH7.0. This was divided into aliquots and stored at -20°C. Varying amounts were added to Golgi membranes which were then diluted to the appropriate concentration of protein with MMDS buffer. Samples were incubated at 4°C for 30 min. Reactions were stopped by the addition of a 1000th volume of 40 mg/ml PMSF.
2.1.7 Affinity Purification of Antipeptide Antiserum

The central idea of this protocol was originally conceived by Dr. Tommy Nilsson. The details of the procedures were adapted from the product information sheets for Thiopropyl Sepharose 6B (Pharmacia) and Antibodies (Harlow and Lane, 1988).

The published peptide sequence of rat syn5 (Bennett et al., 1993) was analysed using the pepstructure program in GCG according to Kyle-Doolittle and searched for stretches of peptides suitable for antibody production. The two criteria for a site to be chosen are:

1. the antigenic index is greater than 1.1;
2. the surface prediction probability is greater than 0.9.

The peptides were synthesised with a cysteine residue added to the N-terminus for subsequent affinity purification of the antisera. Peptides were conjugated to KLH using glutaldehyde and injected into rabbits with complete Freund's adjuvant, all according to standard protocol (Harlow and Lane, 1988). Test bleeds were first screened on Western blots then affinity purified.

2.1.7.1 Removing Protecting Groups of Thiopropyl Sepharose

1g thiopropyl sepharose 6B were taken and swollen in 10-15 ml water for 30 min. Water was then changed to buffer containing 0.3 M NaHCO₃, 1 mM EDTA, pH 8.4 by spinning down beads and decanting the liquid phase twice. 1% w/v DTT was added to 10 ml beads/buffer suspension and left at RT for 40 min. This removes protecting groups to leave the beads in a free-thiol form.

The reaction was stopped by washing with buffer containing 0.1 M acetic acid, 0.5 M NaCl and 1 mM EDTA. At least 400 ml were used for 1 g dry beads. This was done by splitting the bead suspension into two 50 ml Falcon tubes and washing as before. Finally the buffer was changed to 50 mM Tris-HCl, pH 7.5.

2.1.7.2 Coupling of peptides

10-20 mg of synthetic peptides were dissolved in 10 ml 50 mM Tris-HCl pH 7.5 containing 0.5 M β-mercaptoethanol or 1% (w/v) DTT. The dissolved peptides were mixed with activated beads and left at RT for 1 hr and then dialysed overnight in 50 mM Tris-HCl pH 5. This low pH is to ensure efficient binding of peptides to beads via the formation of disulphide bridges when the reducing agent is dialysed away.

Also make sure the molecular weight cut-off (MWCO) of the dialysis membrane used retains the peptide but let out the reducing agent. The supernatant from the beads after
dialysis was discarded and the buffer was changed to 50 mM Tris-HCl pH 8. Then 25 mM iodoacetamide was added to the beads and left at RT for 15 min to alkylate any remaining free thiol groups.

2.1.7.3 Affinity column

This is essentially the same as the protocol in Antibody (Harlow and Lane, 1988) pp314-315 except one modification: the serum was diluted 1:5 in 50 mM Tris-HCl, pH 7.5 and filtered if sediments were visible.

The normal column volume is about 1.5 ml beads (1g dry beads swells to 3 ml or so, the amount used was adjusted accordingly). When eluting the antibodies off the column, the protein concentration of the fractions was checked at OD 280 nm after neutralisation with 2 M Tris.

Antibody concentration was easily performed by ultra filtration using an Amicon 8010 pressure concentrator fitted with a 30kD MWCO membrane (YM30; Amicon) at a nitrogen pressure of 45 p.s.i. This procedure uses pressurised nitrogen gas to push solutions through the filter while retaining antibodies. Buffer was changed to PBS or whatever was required. The volume of the final product can be easily controlled to yield antibody concentrations of 1 mg/ml or higher. Between uses, the membrane was stored in 10% (v/v) ethanol. Alternatively, buffer exchange and antibody concentration can be achieved by using Centricon 30 (Amicon) spin columns incorporating a membrane filter with MWCO of 30kD and a volume of up to 15 ml.

Addition of 1-2 mg/ml BSA to the affinity purified antibodies as carrier protein was optional but recommended as proteins degrade if present at concentrations lower than 1 mg/ml. However, if added before concentrating BSA may block the filter. A typical example of affinity purification of antipeptide antibody is illustrated in Fig 2.2. The dark material above the 42kD band and the spots below are most likely keratin contamination, which was completely eliminated after affinity purification.

2.1.7.4 Blot elution method

Affinity purification can also be achieved by binding the antibodies to proteins immobilised on nitrocellulose blot as for Western blotting and then elute with 0.2 M glycine/HCl pH 2.8 for 2 min followed by neutralisation with 0.5 M Tris (Burke et al., 1982). However, this method only gives very small amount of antibodies.
MATERIALS AND METHODS

Stock Solutions

Glycine (1 M): Dissolve 7.4 g of glycine to a final volume of 100 ml of H₂O and pH with HCl as required. Store at RT.

Triethylamine (100 mM): Weigh out 1.012 g triethylamine in 90 ml H₂O. pH to 11.5 with HCl and adjust final volume to 100 ml. Use immediately.

Tris pH 7.4 (10 mM)/150 mM NaCl: Dissolve 1.2 g of Tris and 8.8 g of NaCl in 900 ml H₂O, adjust the pH to 7.4 with HCl and make up to a final volume of 1 litre.

2.1.8 Assays

Several enzymes were assayed during the course of this study as markers of various intracellular compartments. All samples were assayed in duplicate and the results averaged.

2.1.8.1 NAGT I

This medial-Golgi enzyme catalyses the addition of N-acetylglucosamine onto Man₅-GlcNac₂ N-glycans (Fig 1.4, step 6). This assay measures the transfer of tritiated GlcNac from a UDP-[³H]GlcNAc donor onto ovalbumin, which contains incompletely processed N-linked oligosaccharides that can act as a substrate for NAGT I (Vischer and Hughes, 1981).

The assay mixture was made up as follows: 500 μl of 0.5M Tris/maleate pH 6.9, 500 μl of 200 mg/ml ovalbumin, 50 μl of 1 M KCl, 100 μl of 50 mM UDP-GlcNac, 50 μl of 10% (w/v) TX-100, 12.5 μl of 2 M MnCl₂, 12.5 μl of 2M MgCl₂, 50 μl of 100 μCi/ml UDP-[³H]GlcNAc and 2.72 ml of H₂O. 80 μl of this assay mix was added to 20 μl of sample (or 20 μl of H₂O as a blank) and incubated for 2.5 hr at 37°C. The reaction was stopped by the addition of 1 ml of ice cold 1% phosphotungstic acid/0.5 M HCl (PTA/HCl) to precipitate the proteins. The samples were centrifuged at 14,000 rpm for 7 sec on a bench top centrifuge and the supernatants discarded. The pellets were resuspended in a fresh 1 ml of PTA/HCl and spun as before. The pellets were then resuspended in 1 ml of ice cold 95% ethanol (Hayman Ltd.) and subjected to a final centrifugation step. After removal of the ethanol the pellet was dissolved in 50 μl of 2 M unbuffered Tris and 200 μl of 5% (w/v) SDS by shaking at RT for 1 hr. 1 ml 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.

This assay was shown to be linear in the 5-100-fold dilution range for rat liver homogenate, intermediate fraction and Golgi membranes, requiring approximately 0-65 μg of protein for the homogenate and intermediate fraction and 0-12 μg for Golgi membranes (Slusarewicz et al., 1994).
2.1.8.2. MannII

Mannosidase II acts directly after NAGT I by catalysing the conversion of GlcNAc-Man$_5$-GlcNAc$_2$ to GlcNAc-Man$_3$-GlcNAc$_2$. (Fig. 1.4, step 7)

MannII assay is spectrophotometric and utilises this enzyme's ability to cleave the artificial substrate p-nitrophenol-α-D-mannopyranoside (PNM) to release p-nitrophenol whose absorbance can be monitored at a wavelength of 400 nm.

The MannII assay was based on the method of (Bischoff and Kornfeld, 1984). An assay mix was made up containing: 2.9 ml of sodium phosphate pH 6.0, 100 µl of 10% (w/v) TX-100 and 1 ml of 20 mM PNM. 80 µl of this mixture was added to each 20 µl sample (and 20 µl of water to act as a blank) and the samples incubated at 37°C for 1 hr. Reactions were stopped by the addition of 1 ml of 0.25 M Na$_2$CO$_3$. The blank was used to zero the spectrophotometer, and the absorbance of the samples was measured at a wavelength of 400 nm. The assay was shown to be linear for rat liver Golgi membranes which have been diluted 2-100 times, or containing up to 36 µg of protein (Slusarewicz et al., 1994).

The problem inherent in the MannII assay is its lack of specificity. The substrate is cleaved not only by MannII, but also by the mannosidases of the ER and by lysosomal mannosidase (Bischoff and Kornfeld, 1984). However, a mannosidase inhibitor swainsonine, which inhibits all mannosidases except Mann I and ER mannosidases (Dorling et al., 1980; Tulsiani et al., 1982), abolished all of the MannII activity in this assay. This indicated that there are no MannI or ER mannosidases activities in this assay. A contamination from the lysosomal mannosidases is unlikely because lysosomes and microsomal membranes are not large contaminants of purified rat liver Golgi membranes (Slusarewicz et al., 1994).

2.1.8.3 GalT

The activity of this trans-Golgi enzyme was measured by a technique developed by (Bretz and Staubli, 1977). This enzyme catalyses the addition of galactose onto N-linked oligosaccharides bearing the GlcNAc$_2$-Man$_3$-GlcNAc$_2$ structure (Fig. 1.4, step 10). Such an incompletely processed structure is carried by the glycoprotein ovomucoid. This assay is very similar to that for NAGT I except that it measures the incorporation of tritiated galactose, from a UDP-[3H]Galactose donor, into a different acceptor, ovomucoid.

An assay mixture was made up containing: 500 µl of 0.4 M sodium cacodylate pH 6.6, 500 µl of 175 mg/ml ovomucoid, 15 µl of β-mercaptoethanol, 100 µl of 10 mM UDP-
galactose, 100 µl of 10% (w/v) TX-100, 50 µl of 0.2 M ATP, 100 µl of 2 M MnCl₂, 25 µl of 100 mCi/ml UDP-[³H]galactose and 2.6 ml of H₂O. 80 µl of assay mix was added to each 20 µl sample and to 20 µ of H₂O to serve as a blank and the samples incubated at 37°C for 30 min. The reactions were stopped, processed and counted exactly as for the NAGT I assay (see section 2.3.4, above). The only difference occurred in the SDS-solubilisation of the ethanol-washed pellet which occurred much more readily, in the space of a few minutes.

The linearity of this assay for rat liver homogenate, intermediate fraction and Golgi membranes was tested in much the same way as that for NAGT I. Although the GalT assay is significantly more sensitive than that for NAGT I, its linearity in response to increasing sample concentrations is very similar (Slusarewicz et al., 1994).

2.1.8.4 Protein Assays

Sample protein concentrations were determined using the BCA Protein Assay Reagent (Pierce Chemicals) as described in the handbook. Standard curves were constructed using samples containing 0, 10, 20, 30 and 40 µg of protein (using 0, 5, 10, 15 and 20 µl of 2 mg/ml Bovine Serum Albumin diluted to 50 µl with water), and sample volumes were 50 µl at the appropriate dilution. Normal incubation time is 30 min at 37°C although 1 hr at RT was used sometimes. The spectrophotometer was set to zero by a blank sample containing 50 µl of water and subsequent absorbance were measured at 562 nm. The BCA assay was used to test samples containing TX-100 but this assay is very sensitive to the presence of reducing agent, e.g. DTT.

Bradford protein assay (Bio-Rad) was used to test samples containing reducing agents such as DTT or β-mercaptoethanol but it is very sensitive to the presence of detergent, e.g. TX-100. 200 µl reagent was mixed with 600 µl water and 200 µl sample was added to complete a 1 ml final volume. The reaction is instantaneous and the absorbance measure at 595 nm after 5 min as further delay will result in the formation of precipitates. Standard curves were constructed as for BCA assay, except the sample volume is 200 µl.

Assay mixtures and stock solutions

GalT: 50 mM sodium cacodylate pH 6.6, 21.9 mg/ml ovomucoid, 50 mM β-mercaptoethanol, 0.25 mM UDP-galactose, 0.25% (w/v) TX-100, 2.5 mM ATP, 50 mM MnCl₂, 0.5 µCi/ml UDP-[³H]galactose.

MannII: 145 mM sodium phosphate pH 6.0, 0.25% (w/v) TX-100, 5 mM para-nitrophenol-α-mannoside (PNM).
MATERIALS AND METHODS

NAGT I: 62.5 mM Tris/maleate pH 6.9, 25 mg/ml ovalbumin, 12.5 mM KCl, 1.25 mM UDP-GlcNAc, 0.13% (w/v) TX-100, 6.25 mM MnCl₂, 6.25 mM MgCl₂, 1.25 μCi/ml UDP-[³H]GlcNAc.

ATP pH 7.0 (0.2 M): Dissolve 605 mg in 3 ml of H₂O. Adjust to pH 6.5-7.0 with 1 M NaOH and make up to a final volume of 5.0 ml. Store at -20°C in 200 μl aliquots. Do not freeze-thaw more than 5 times. Stable for months.

GlcNAc (1 M): Dissolve 221 mg in 1 ml of H₂O and store at -20°C.

MgCl₂ (2 M): Dissolve 40.7 g of MgCl₂.6 H₂O in a final volume of 100 ml of H₂O and store at RT. Lasts forever.

MnCl₂ (2 M): Dissolve 9.9 g of MnCl₂.4 H₂O in a final volume of 25 ml of H₂O and store at RT.

Ovalbumin (200 mg/ml): Dissolve 1 g ovalbumin in H₂O to a final of volume of 5 ml. Pass through a 0.45 μm nitrocellulose filter and store at -20°C in 500 μl aliquots.

Ovomucoid (175 mg/ml): Dissolve 1 g ovomucoid in H₂O to a final of volume of 5.7 ml. Pass through a 0.45μm nitrocellulose filter and store at -20°C in 500 μl aliquots.

PNM (20 mM): Dissolve 60.3 mg of para-nitro-phenol-α-mannoside (PNM) in 10 ml of DMSO. Store at RT.

PTA (1% (w/v))/0.5 M HCl: Dissolve 5 g PTA in 478 ml of H₂O final volume. Add 22 ml of 11.4 M HCl and store at 4°C.

Sodium cacodylate pH 6.6 (0.4 M): Dissolve 17.12 g sodium cacodylate in 180 ml of H₂O, adjust to pH 6.6 with HCl and make up to a final volume of 200 ml. Store at 4°C.

Sodium phosphate pH 6.0 (0.2 M): Dissolve 2.84 g of Na₂HPO₄ in a final volume of 100 ml of H₂O, and 2.72 g of NaH₂PO₄ in a further 100 ml of H₂O. Mix 87.7 ml of NaH₂PO₄ and 12.3 ml of Na₂HPO₄ and store at RT.

Tris/maleate pH 6.9 (0.5 M): Dissolve 6.06 g Tris and 5.81 g maleic acid in H₂O, adjust to pH 6.9 with NaOH and make up to a final volume of 100 ml. Store at RT.

UDP-galactose (10 mM): Dissolve 25 mg in H₂O to a final volume of 4.42 ml and store at -20°C in 200 μl aliquots.

UDP-GlcNAc (50 mM): Dissolve 60 mg in a final volume of 1.84 ml of H₂O store in 200 μl aliquots at -20°C.
2.1.9 Rat Liver Golgi Preparation

Golgi membranes were routinely prepared and characterised as described (Cell Biology Handbook). The protocol that has been used most frequently is detailed below.

2.1.9.1 Isolation

Rat liver Golgi were prepared using extensive modifications of the procedure of (Leelavathi et al., 1970; Slusarewicz et al., 1994). A discontinuous sucrose-gradient using the buffers A-E was prepared as outlined in Table 2.3. The refractive index of the various solutions was measured using a 0-50% Delta refractometer (Bellingham and Stanley Ltd., Tunbridge Wells, UK.). The most crucial is perhaps buffer C, which should read 9% and buffer D, which should read 26.5%. Six discontinuous sucrose-gradients, consisting of 15 ml of buffer D were poured into Beckman SW-28 Ultraclear rotor tubes and kept on ice.

Four to six rats were starved for 24 hr prior to sacrifice. Livers were quickly immersed in 200 ml of ice-cold buffer C and swirled and squeezed occasionally to expel blood and to speed cooling. 36-45 g of liver was placed into fresh buffer C and cut into several pieces to release as much blood as possible. Excess buffer was decanted to leave a volume of less than 80 ml, and the livers were minced into small pieces with a pair of fine scissors.

This material was homogenised by gently pressing through a 150 μm-mesh steel laboratory test sieve (Endecotts Ltd.) with the bottom of a conical flask and the homogenate was collected in a plastic tray. This is a relatively gentle method of homogenisation and reduces the possibility of cisternal unstacking by mechanical shear (Hino et al., 1978). The homogenate was poured into a 100 ml measuring cylinder and buffer C was added to a final volume of about 100 ml, followed by thorough mixing.

16 ml of this homogenate was overlaid onto each of the gradients, and was topped up with buffer B. These gradients were centrifuged in a L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) using a SW-28 rotor at 28,000 rpm for 1 hr at 4°C. A 100 μl aliquot of the homogenate was kept on ice for enzyme assays.

After centrifugation, the lipid layer on the surface of the gradient was removed by aspiration. The membrane fraction from the interface between buffers C and D was collected using a Pasteur pipette (approximately 3-5 ml from each gradient). These membranes were pooled and diluted to 26.5% (w/w) sucrose using buffer A. A 100 μl aliquot was kept for enzyme assay.
This intermediate fraction was poured into two to four SW-40 centrifuge tubes (10 ml per tube) and underlaid with 3-4 ml buffer D. The samples were then centrifuged at 29,000 rpm for 45 min at 4°C. This step solves the problem of overloading the gradients in the first spin, and it further separates possible contaminants, e.g. ER and lysosomes, from the final Golgi fractions.

Table 2.3 Sucrose buffers used for purification of rat liver Golgi membranes.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose concentration</td>
<td>0 M</td>
<td>0.25 M</td>
<td>0.5 M</td>
<td>0.86 M</td>
<td>1.3 M</td>
</tr>
<tr>
<td>0.5 M potassium phosphate pH 6.7</td>
<td>20 ml</td>
<td>40 ml</td>
<td>80 ml</td>
<td>20 ml</td>
<td>12 ml</td>
</tr>
<tr>
<td>2 M sucrose</td>
<td>-</td>
<td>25 ml</td>
<td>100 ml</td>
<td>43 ml</td>
<td>39 ml</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.25 ml</td>
<td>0.5 ml</td>
<td>1 ml</td>
<td>0.25 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>Water</td>
<td>79.8 ml</td>
<td>134.5 ml</td>
<td>219 ml</td>
<td>36.8 ml</td>
<td>8.9 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>100 ml</td>
<td>200 ml</td>
<td>400 ml</td>
<td>100 ml</td>
<td>60 ml</td>
</tr>
<tr>
<td>Refractive index</td>
<td>9%</td>
<td>16%</td>
<td>26.5%</td>
<td>38%</td>
<td></td>
</tr>
</tbody>
</table>

The supernatant was removed by aspiration, and the membrane felt at the interface of buffer C and D was collected as before. This suspension was diluted to 9% sucrose concentration with buffer A, placed into one to two fresh SW-40 centrifuge tube, and underlaid with 1-2 ml of buffer E to form a sucrose cushion. This sample was centrifuged at 7000 rpm for 30 min at 4°C.

The supernatant was discarded and the final membrane felt collected and resuspended in 4.5 ml of buffer B. The final volume was measured and the membranes divided into 500 μl aliquots. These were snap-frozen in liquid nitrogen and stored at -80°C. Such membranes could be thawed and re-frozen at least twice without significant loss of enzymatic activity or change in Golgi stacking and cisternae morphology.

Stock Solutions

**Phosphate Buffer (0.5 M potassium phosphate pH 6.7):** Make up 500 ml solutions of 0.5 M anhydrous K₂HPO₄ (43.6 g) and 0.5 M anhydrous KH₂PO₄ (34 g). To 400 ml of the latter, gradually add the former until the pH reaches 6.7. Store at 4°C.

**Sucrose (2 M):** Dissolve 342.3 g in H₂O by stirring at 50°C. Make up to a final volume of 500 ml and store at 4°C.
2.1.9.2 Biochemical Characterisation

Golgi membranes were shown to be biochemically pure in two ways. Firstly, it was shown that preparations were highly enriched in the Golgi enzymes GalT and NAGT I. Secondly, a concomitant decrease in the specific activities of the lysosomal marker β-hexoseaminidase and the ER marker NADH-cytochrome c reductase was demonstrated.

Golgi preparations were assayed routinely for GalT activity. The preparations were typically purified 80-fold over the homogenate for this enzyme (Table 2.4 A). Occasionally, the preparations were assayed for NAGT I activity (Table 2.4 B). In this case, the purification was in excess of twice that for GalT. This difference is probably due to some loss of the *trans*-Golgi network during purification since this organelle contains a significant amount of GalT but no NAGT I (Nilsson *et al.*, 1993). Coupled to this large enrichment of Golgi markers in these Golgi preparations, there was also a depletion of enzymatic markers of the lysosomes and ER (Slusarewicz *et al.*, 1994).

The GalT, NAGT I and protein assays were carried out as detailed in section 2.8. Homogenates, intermediate-fractions and Golgi membranes were diluted 200, 20 and 5 times respectively before assaying for protein and all samples were diluted 20 times for the GalT and NAGT I assay. These dilutions ensured that sample concentrations were in the linear range for these assays (see section 2.8).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mg/ml)</th>
<th>[Protein] (mg/ml)</th>
<th>[GalT] (nmol/h/ml)</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>78.0</td>
<td>82.8±3.6</td>
<td>760.0±57.4</td>
<td>9.4±0.6</td>
<td>100.0</td>
<td>1.0x</td>
</tr>
<tr>
<td>Intermediate</td>
<td>65.7±4.0</td>
<td>5.7±0.4</td>
<td>236.8±16.3</td>
<td>45.0±3.2</td>
<td>25.6±1.4</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>Golgi</td>
<td>4.5±0.1</td>
<td>2.8±0.2</td>
<td>2060.0±228.7</td>
<td>749.0±70.0</td>
<td>15.5±1.5</td>
<td>81.8±6.9</td>
</tr>
</tbody>
</table>

Table 2.4 Purification of Golgi markers in Golgi preparations

(A) shows the enrichment of a *trans*-Golgi marker, GalT; and (B) a *medial*-Golgi marker, NAGT I, over the homogenate in both the intermediate fraction and the final Golgi preparation. The GalT table was compiled using the results from 24 separate fractionations and are presented as the mean ±SEM. for each parameter. Note that the specific activity, yield and purification-fold are not, therefore, arithmetically related to the GalT and protein concentrations. The NAGT I table shows the average results from two fractionations.
To determine the concentration of GalT in any given sample, a 10μl aliquot of the assay mixture, mixed with 40μl of 2M unbuffered Tris and 200μl of 5% SDS, was counted with the samples to allow calculation of the specific activity of the UDP-Galactose in the reaction. The specific activity of the UDP-Galactose in DPM/nmole was calculated as shown below:

\[
S. A. \text{ UDP-Galactose} = \frac{(\text{DPM of standard} - \text{DPM of blank})}{1.5} \tag{i}
\]

The protein concentration, in mg/ml, of each sample was calculated based on the slope (m) and y-intercept (c) of the standard curve obtained, as detailed below:

\[
[\text{Protein}] = \frac{((\text{Absorbance of sample} - c) \times \text{Dilution Factor} \times 0.02)}{m} \tag{ii}
\]

The concentration of GalT activity in nmoles/hr/ml was next calculated as follows:

\[
[\text{GalT}] = \frac{((\text{DPM of sample} - \text{DPM of blank}) \times 2000)}{\text{S. A. UDP-Galactose}} \tag{iii}
\]

The specific activity GalT, in nmoles/hr/mg, in any given sample was calculated by dividing the enzyme activity by the protein concentration. GalT yields were calculated from the ratio of the total enzyme in the intermediate and Golgi fractions and the homogenate. Finally, the purification-fold was calculated by dividing the specific activities of GalT in the intermediate and Golgi fractions by the homogenate.

Determination of NAGT I concentrations was very similar. Again a 10μl standard of assay mixture was counted. The calculation of the specific activity of UDP-GlcNAc was exactly as in equation (i) above, except that 2000 in equation (iii) was replaced by 400 to also yield a value in nmoles/hr/ml.

2.1.10 Extraction of Golgi Membranes

Golgi membranes were extracted in either TX-100 alone or TX-100 and NaCl. These procedures are detailed below.

2.1.10.1 Using TX-100 alone

Rat liver Golgi membranes were spun at 50,000 rpm for 5 min at 4°C in a TL-100 ultracentrifuge (Beckmann) using either TLA-100.1, TLA-100.2 or TLA-100.3 rotors. The membranes were then resuspended in TMMDS buffer (2% (w/v) TX-100, 50 mM MOPS pH 7.0, 0.1 mM MgCl₂, 1 mM DTT, 10% (w/v) sucrose), using a P200 Gilson pipette until the suspension was homogenous (10-40 passages) and to a final protein concentration of 1 mg/ml. The samples were incubated on ice for 30 min and spun at 20,000 rpm at 4°C for a further 30 min. The supernatant was removed and the tube containing the pellet rinsed gently in two volumes of TMMDS before resuspension in one volume of TMMDS until the suspension was homogenous.
2.1.10.2 Using TX-100 and NaCl

Extraction in the presence of salt was either carried out on intact Golgi membranes using 150 mM NaCl/TMMDS buffer or on the Triton extracted pellet using TMMDS supplemented with various NaCl concentrations as described in the text.

In the former case, Golgi membranes were extracted directly with 150 mM NaCl/TMMDS at 1 mg/ml of protein using the procedure described above for TX-100 alone. In the latter case, Triton pellets were resuspended in NaCl/TMMDS in the same volume as the initial Triton extraction and incubated and spun as before. The pellets were rinsed in two volumes of salt/TMMDS and resuspended in one volume of the same buffer. Extraction of Triton-pellets using 150 mM NaCl/TMMDS produced an insoluble pellet termed "the matrix" (see chapter 4).

2.1.11. Gel Filtration

2.1.11.1 Choice of media

Three kinds of gel filtration media were used to separate mixtures of protein molecules at different molecular size ranges. Superose-6, Sephacryl 400 and Sephacryl 300.

Superose 6 is a cross-linked agarose-based matrix that has an exclusion limit of $4 \times 10^7$ D and a wide separation range between $5 \times 10^3$ - $5 \times 10^6$ D. It was used initially to separate the large oligomers isolated from rat liver Golgi extracts in the presence of TX-100 and salt.

Sephacryl is a hydrophilic, rigid allyl dextran cross-linked to a bis-acrylamide matrix that offers high recovery and minimum non-specific binding of proteins. It has a higher resolution at the higher molecular weight range. Sephacryl 300 HR was used to separate proteins within the range of $10^4$ - $1.5 \times 10^6$ D, while Sephacryl 400 HR was used to separate proteins within $2 \times 10^4$ - $8 \times 10^6$ D.

2.1.11.2 Calibration of gel filtration columns

Calibration was carried out by running combinations of the standard molecular weight markers on the column in appropriate buffers so that the separation can be clearly charted and a calibration curve can be drawn to determine the approximate molecular weight of the sample. It is important to bear in mind that gel filtration measures the Stokes radius, and the relative molecular weight is an estimate assuming proteins adopt a globular conformation. Protein that adopts a more linear conformation will elute later than globular proteins of the same MW and the estimation of its MW will be less than the real value.
Markers used were: Blue Dextran (2x10^6 D), Thyroglobulin (669kD), Apoferritin (440kD), Catalase (232kD), Bovine serum albumin (66kD), Ovalbumin (43kD) and cytochrome C (17.5kD). An example of the calibration using different gel filtration media is illustrated in Figure 2.3.

Figure 2.3 Gel filtration calibration. Superose 6 (Top panel), Sephacryl 300 HR (Middle panel) and Sephacryl 400 HR (Bottom panel) were calibrated using markers solubilised in TMMS buffer. Only three markers were shown to illustrate the difference in resolution between the three types of media.
2.1.11.3 TX-114 extraction

Non-ionic detergents exist as micelles in solutions at concentrations exceeding their critical micelle concentration (Helenius and Simons, 1975). As the temperature is raised, the micelle molecular weight rises until, at a temperature known as the cloud point, the solution becomes turbid. This turbidity is due to the phase separation of the detergent from the solvent (possibly due to micelle aggregation). The cloud point temperature is a function of the hydrophilicity of the detergent. Hydrophobic detergents have low cloud points and vice versa.

Triton X-114 is the second most hydrophobic of the Triton X series of non-ionic detergents, containing on average only 7 or 8 hydrophilic oxyethylene residues. It therefore has a low cloud point of about 20°C compared with, for example, 64°C for the more hydrophilic TX-100.

This low cloud point has been exploited as a method for isolation of membrane spanning proteins (Moremen et al., 1991; Pryde and Phillips, 1986). During phase separation, amphipathic proteins (such as those that span the lipid bilayer) preferentially enter the detergent phase while hydrophilic proteins remain in the aqueous phase. Although the behaviour of individual proteins varies with regards to their phase partitioning, this provides a qualitative method of isolating membrane spanning proteins and of determining whether a novel protein spans the lipid bilayer.

Unless specified, TX-114 extractions were carried out as follows. The sample was taken up in 200 µl of TX-114 Extraction Buffer (200 µl of Phosphate buffer, 2.5 µl of 2 M MgCl₂, pre-condensed TX-114 to give a final concentration of 1% (w/v) and H₂O to a final volume of 1 ml). This was incubated on ice for 10 min followed by a 3 min incubation at 37°C and a further 10 min on ice. The sample was then spun at 14,000 rpm at 4°C for 5 min in a benchtop microfuge to remove unsolubilised material. The supernatant was then incubated at 37°C for 3 min and spun at RT for 5 min at 5000 rpm to yield a detergent pellet and an aqueous supernatant.

2.1.12 Fixation for Electron Microscopy

Golgi membranes were fixed for electron microscopy in a solution containing 60 µl of 50% (w/v) glutaraldehyde (Fluka), 125 µl of 2M sucrose, 2.5 µl of 2M MgCl₂, 200 µl of Phosphate buffer pH 6.7 and 612.5 µl of H₂O. 100 µg of Golgi membranes were fixed in suspension overnight and dehydrated and embedded as described by (Pypaert et al., 1991). Samples were examined on a Phillips CM10 transmission electron microscope.
2.2 Cell Culture

All parental cells were grown in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) containing 4.5 g/l glucose and supplemented with 10% (v/v) foetal calf serum (FCS; GIBCO), 2 mM glutamine, 1% (v/v) non-essential amino acids (GIBCO) and 100 U/ml each of penicillin and streptomycin in an atmosphere of 5% CO₂/95% air at 37°C. For transformed cells the medium was supplemented with 0.5 mg/ml Geneticin 418 (G-418 sulphate; GIBCO). Trypsin solutions were supplied by the ICRF Central Services and consisted of 0.25% (w/v) trypsin in versene solution.

In addition to parental HeLa and NRK cells, various stably-transfected Hela cell-lines produced by Dr. T. Nilsson in this laboratory were also grown in culture. The cell-line 4:12 (Nilsson et al., 1993) expresses NAGT I which is tagged with the myc-epitope (Evan et al., 1985), while 4:48 (Nilsson et al., 1994) expresses myc-tagged NAGT I carrying the cytoplasmic domain of the ER resident form of human invariant chain, p33 (Lotteau et al., 1990). Syn5myc cell line expresses a myc-tagged rat syntaxin 5 (Banfield et al., 1994) in HeLa cells, and NRK9:3 cell line expresses MannII tagged with VSV-G epitope.

2.2.1 Passaging

Cells were grown on 150 cm² flasks (Falcon) and passaged every two to three days, by which time they had reached 80-90% confluence. The medium was removed and replaced with 5 ml of trypsin/versene solution which was itself removed after 10 sec. Cells were left at RT until they had been dislodged and were resuspended in 10 ml of fresh DMEM/G-418. After 10 passages in a glass pipette, 1 ml of the cell suspension was added to new flasks containing 12 ml each of fresh medium. In general, HeLa cells were split 1/5 and NRK cells 1/10.

2.2.2 Expansion

For the production of post-nuclear supernatant (PNS), each cell line was grown in five 850 cm² roller-bottles (Falcon). Firstly, the cells were expanded into five 150 cm² flasks by the method described above. Once they had reached 60-70% confluence, the cells were trypsinised and each aliquot added to a roller-bottle containing 100 ml of DMEM without G-418. These cells were grown until 70-80% confluent (approximately 3 days) whilst rotating at 0.25 rpm.

2.2.3 Double Thymidine Shake-off

This protocol was adapted from (Bootsma et al., 1964). Essentially, it utilises the fact that incorporation of thymidine into DNA is inhibited when thymidine is present at
large molar excess of other bases. When cells are released from the first thymidine block, they are allowed to go through the cell cycle once, and blocked again when they enter G phase. After two blocks, more than 90% of the cells exit the G phase at the same time. This level of synchrony is difficult to achieve using nocodazole block. As cells enter mitosis, they become less adherent to the culture flask surface and can be easily shaken off into the medium for collection.

Five roller bottles of HeLa cells were grown to sub-confluency. Normal growth medium were replaced with medium containing 2 mM thymidine. After 24 hr cells were washed three times in normal medium and grown for a further 15 hr. Normal growth medium were replaced with 2 mM thymidine medium for another 24 hr. Cells were washed three times in normal medium, and grown as usual. 6 hr after the second thymidine block, roller bottle were spun at full speed for 5 min (Wheton Roller Culture Apparatus) to shake off dead cells. The medium was collected and dead cells spun down before replacing the clear medium in the roller bottles. The shakeoff of mitotic HeLa cells was started 12 hr after the release from the second thymidine block and mitotic cells were collected at 1 hr intervals for 4-5 hr. Half of the medium was taken out and kept at 37°C while the roller bottles was spun at full speed for 5 min with the other half of the medium. After decanting the medium containing the mitotic cell suspension, the other half of the medium kept at 37°C was replaced into the roller bottles to prevent cells drying. Mitotic cells were spun down at 2000 rpm for 2 min and were snap-frozen in liquid nitrogen and stored in -80°C.

2.2.4 Long Term Storage

Cells were grown to confluence in 150 cm$^2$ flasks and harvested. The cells were spun at 1400 rpm for 3 min and resuspended in 3 ml of 90% (v/v) FCS/10% (v/v) DMSO, and divided into three 1 ml aliquots. Cells were slowly frozen in an insulated styrofoam box stuffed with cotton wool at -80°C for 48 hr and then stored in liquid nitrogen.

Cells were thawed by warming at 37°C, resuspended in 10 ml of growth medium and spun at 1400 rpm for 3 min. The pellet were resuspended in a further 12 ml of growth medium and grown in a 150 cm$^2$ flask as before.

2.2.6 Production of Post-Nuclear Supernatants

Once cells had reached 70-80% confluency, 1 ml of 3 mg/ml cycloheximide (Boehringer Manheim) was added and the cells incubated for a further 10 min. At this point, sterile conditions were no longer maintained. The medium was poured off and kept and the cells were then trypsinised with 20 ml of trypsin per bottle and rotated.
until the cells had been dislodged. Trypsin was then quenched by the addition of 30 ml of the used medium and the cells collected into 50 ml centrifuge tubes (Falcon). Cells were recovered by centrifugation at 1400 rpm for 3 min in a 500E bench-top centrifuge (Jouan). The cells were resuspended and pooled in 50 ml of used medium containing 50 µl of 20 mg/ml cytochalasin B and 1 ml of 1 M HEPES pH 7.4. The cells were then rotated at 4°C for 45 min to depolymerise intracellular actin. and recovered as before.

The cells were subjected to a swelling step by resuspending in 25 ml of 10 mM TEA pH 7.4/150 mM KCl buffer containing 0.25 ml 3 mg/ml cycloheximide and incubated at 4°C for 10 min. Cells were then recovered, resuspended in 25 ml of KEHM buffer (50 mM HEPES pH 7.4, 50 mM KCl, 10 mM EGTA, 1.92 mM MgCl₂) containing 25 µl of 1 M DTT and 0.25 ml of 3 mg/ml cycloheximide and recovered again. This final cell pellet was taken up in an equal volume of KEHM containing 1 mM DTT, 30 mg/ml cycloheximide and 1x Protease Inhibitor Cocktail (PIC).

The cell suspension was homogenised with 10-15 passes in a ball-bearing homogeniser with a 10µm clearance (Balch et al., 1984). Cell breakage was checked by staining with Trypan Blue and examined under inverted microscope. Nuclei were removed by a 5 min spin at 3000 rpm at 4°C in a microfuge. The PNS was removed with care being taken not to disturb the nuclear pellet. The PNS was divided into aliquots of 0.5 ml, frozen in liquid nitrogen and stored at -70°C.

2.2.6 HeLa Golgi Preparation

HeLa cells were grown in 8 trays (24x24 cm²) to confluency. Cells were incubated in 10 µg/ml nocodazole to depolymerise the microtubules and 20 µg/ml cytochalasin B in 10 mM HEPES pH 7.4 to disrupt actin polymerisation. 1 ml 200 µg/ml stock nocodazole, 20 µl 20 mg/ml stock cytochalasin B and 200 µl 1M HEPES pH 7.4 were added to 20 ml medium directly and incubated for 45 min at 37°C.

Cells were scraped with rubber policeman and collected in 50 ml tubes (Falcon). Pooled cells were spun down at 500 g for 2 min in Jouan centrifuge and the pellet pre-swollen with 150 mM KCl, 10 mM TEA pH 7.4 for 10 min on ice.

Cells were then washed twice in 50 ml 150 mM KCl, 10 mM EGTA, 50 mM HEPES-KOH pH 7.4, 2 mM MgCl₂ and PIC (KEHM). The final pellet was resuspended in equal volume of KEHM and homogenised in 30" ball for 15-20 passes. Cell breakage was checked by Trypan Blue staining. The homogenate was spun for 10 min at 2000 g and the PNS was recovered.

The PNS was top-loaded in SW40 tubes onto discontinuous gradients containing 3 ml 1.6 M Sucrose, 4 ml 1.2 M Sucrose, 4 ml 0.8 M Sucrose in KEHM and PIC. The
gradients were balanced and spun at 25,000 rpm for 2.5 hr at 4°C. Golgi fractions were collected from the 0.8/1.2 M sucrose interface as the density of the Golgi is 1.13 g/ml, corresponding to 0.96 M sucrose. Bottom loaded PNS was adjusted to 1.3 M sucrose by adding 1:5 1.6M sucrose and topped up with 3 ml each of 1.2 M and 0.8 M sucrose in KHEM/PIC.

### 2.2.7 Immunofluorescence

This is routinely carried out to test antisera and to study the subcellular localisation of proteins of interest.

#### 2.2.7.1 Fixation Methods

**Para-Formaldehyde (PFA) Fixation (For staining surface and internal antigens)**

PFA cross-links arginine and lysine residues and achieves the fixation of proteins. It may alter some epitopes against which the antibodies were raised, and if this is the case other fixation methods should be used to uncover the epitope.

Cells were plated onto sterilised glass coverslips (dunked in ethanol and flamed) in 6-well Greiner trays at least two days before the experiment. The cells should not be over-confluent for best results. The growth medium was rapidly replaced with 2 ml of 3% PFA in PBS containing Ca$^{2+}$ + Mg$^{2+}$ (PBSA+B+C). [Holding the Pasteur pipette in one hand and Gilson pipette in the other, sucking off the old solution and quickly adding the fresh solution.] The coverslips were never allowed to dry out.

Cells were fixed for 20 min at RT (could be stored for up to 1 week at 4°C) and washed 3 times with 2 ml CMF-PBS (PBSA). The aldehyde groups were quenched with 2 ml of 50 mM NH$_4$Cl in CMF-PBS and left for 10 min then washed 3 times with 2 ml of CMF-PBS. Next, 0.1% TX-100 in CMF-PBS was added and left for 4 min to permeabilise the cells and expose intracellular antigenic sites. The cells were washed 3 times with CMF-PBS and a further 3 times with CMF-PBS + 0.2% Gelatin over a 5 min period (This blocks non-specific binding sites).

**Formaldehyde Fixation (For staining surface antigens)**

For staining surface antigens TX-100 treatment was omitted from the protocol outlined above.

**Methanol/Acetone Fixation**

This fixation method works on the principle of dehydration and precipitation of proteins rather than cross-linking basic residues.
MATERIALS AND METHODS

Methanol and acetone were stored at -20°C in glass petri dishes (plastic will be dissolved by acetone) before transferring the coverslips directly from the medium to the cold methanol for 4 min and then to the acetone for a further 4 min. The coverslips were air dried and washed in PBS and PBS+ 0.2% gelatin as above.

Methanol Fixation

Methanol was stored at -20°C before transferring the coverslips directly from the medium to the cold methanol (cell side up) for 6 min and to CMF.PBS at RT for 30 sec. The coverslips were then washed as before in PBS and PBS+0.2% gelatin.

2.2.7.2 Antibody staining

The first antibody was diluted in CMF-PBS + 0.2% gelatin (usually 1:100) and centrifuged at 14,000 rpm for 5 min at 4°C to remove aggregates. The coverslips were incubated with the antibody in a "wet box" (24" x 24" plastic tray with 3MM Whatman filter paper soaked in water and a small length of parafilm placed on top). 100μl of the diluted and centrifuged antibody were placed onto the parafilm. The cover slip from the well were taken and excess liquid removed by touching a small piece of filter paper to the edge of the coverslips. The coverslips were placed cell-side down onto the drop of antibody solution and left for 20 min to 1 hr, depending on the antibody titre, at RT. The coverslips were returned to the wells cells-side up after the first antibody incubation and washed 3 times with CMF-PBS + 0.2% gelatin. The second antibody was incubated as above.

2.2.7.3 Mounting

The coverslips were wash 3 times with CMF-PBS + 0.2% Gelatin followed by 3 times with CMF-PBS before mounting onto a slide. 15 μl of Citifluor solution was put onto a slide. The coverslips were washed thoroughly, by successively immersing it into three beakers each containing distilled water and excess water removed by touching the edge to a piece of filter paper. The coverslips were gently lowered (cell side down) onto the drop of Citifluor, taking care not to trap any air bubbles. Excess Citifluor was removed with pieces of filter paper and the edges of the coverslips were sealed with nail varnish and left at RT to set before viewing under a fluorescent microscope.

The mounted slides can be stored at 4°C in the dark for months.

Stock Solutions

0.1% Triton X-100 in CMF-PBS: Add 1/100th volume 10% TX-100 stock to CMF-PBS.
**MATERIALS AND METHODS**

**3% (w/v) Formaldehyde in PBS containing Ca\(^{2+}\) and Mg\(^{2+}\):** To 400 ml of PBS A, heated to 80°C in the fume hood and stirring add 12.6 g para-formaldehyde. Hold the temperature at 80°C, using a hot plate stirrer. The solid dissolves, generating formaldehyde. If necessary add ~10 drops of 2 M NaOH to dissolve any precipitate. Cool the solution to RT and add 10 ml each of PBS B and PBS C. Filter through Whatman No.1 filter paper and store in 15 ml aliquots at -20°C for long term or at 4°C for a few weeks.

**50 mM-NH\(_4\)Cl in CMF-PBS:** Dissolve 267 mg NH\(_4\)Cl in 100 ml CMF-PBS and make up fresh just before using.

**CMF-PBS + 0.2% gelatin (w/v):** Make up a stock solution containing 2% (w/v) fish skin gelatin by weighing out 1 g gelatin into a 500 ml beaker and add 400 ml PBS A then add 100 ml water to make the volume to 500 ml. Filter through a 0.45 μm nitrocellulose filter and store for 1-2 weeks only at 4°C.

**Nocodazole (664 μM):** Dissolve 5 mg in 25 ml of DMSO and store at -20°C in aliquots of 1 ml. Use only once.

**PBS (Phosphate Buffered Saline):** Made up by the ICRF Central Services containing 10 mM sodium phosphate pH 7.2, 150 mM NaCl and 3 mM KCl.
2.3 Molecular biology

Most working solutions, techniques and experimental procedures were followed or adapted from (Sambrook et al., 1989). All water used in RNA work was treated with diethylpirocarbonate (DEPC).

Primers used for the cloning and RACE of rat syntaxin 5 are detailed in Table 2.5. Their positions in relation to the syn5 cDNA are indicated in Fig 2.4.

Figure 2.4 Syntaxin 5 primer map. Relative positions of various primers with the complete syntaxin 5 cDNA were shown with sense (5'-3') primers indicated on top of the syn5 sequence and antisense (3'-5') primers indicated under the syn5 sequence.

2.3.1 Cleaning of oligonucleotide primers

Primers were cleaned by adding 200 µl TE and leaving to dissolve for 5 min at RT. They were then vortexed and 200 µl buffer-saturated phenol was added. This will get rid of any residual salt from synthesis as DNA partition preferentially into the organic phase of the phenol/buffer mixture. They were vortexed again before spinning for 5 min at 14,000 rpm in a benchtop centrifuge. The supernatant was transferred to new tubes to which 200 µl CIAA was added before vortexing and spun as before.
### Materials and Methods

Table 2.5 Oligonucleotide primers sequence and orientation

<table>
<thead>
<tr>
<th>5'-3'</th>
<th>Sequence</th>
<th>3'-5'</th>
<th>Sequence</th>
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<tr>
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<td>CGGGATCGGACCCAGGAGTTCC</td>
<td>Syn5.02</td>
<td>GGCAAGGAAGACCACAAAGATG</td>
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<td>Syn5.07</td>
<td>AGCAGAGGCAATCCAGAACC</td>
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<td>Syn5.08</td>
<td>AG</td>
</tr>
<tr>
<td>Syn5.04</td>
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<td>Syn5.09</td>
<td>CTCGGGACTCTCCCTCCAGAACC</td>
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<td>GGGATCGGAC</td>
<td>Syn5.10+</td>
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<td>TM-BamHI</td>
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<td>TTTT</td>
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<tr>
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<td>N04</td>
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<td>CACCCTGATC</td>
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<td>GCTCATTGGGAGGAT</td>
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<td>N08-SSS</td>
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<tr>
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<td>SP2</td>
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<tr>
<td></td>
<td>CCTGTACACAGGGGTGTTT</td>
<td></td>
<td>GCCACCGATTGGAGG</td>
</tr>
</tbody>
</table>
The supernatant was again transferred to new tubes to which 550 µl 95% ethanol and 20 µl 3 M NaAc was already added. This step precipitates DNA in high salt. After vortexing the solutions were stored on dry ice for 5 min to facilitate precipitation of short DNA strands. Afterwards they were spun for 5 min, 14,000 rpm at 4°C and the supernatant discarded. The pellets were washed carefully with 200 µl 80% EtOH and spun again at 5 min, 14,000 rpm, discarding the resultant supernatants. The final pellets were dried under vacuum and re-solubilised in 200 µl (double distilled water (ddw).

The OD was measured at 260 nm and 280 nm to calculate primer concentrations. The ratio of OD$_{260}$/OD$_{280}$ should be about 1.5. OD$_{260}$ 1.00 = 20 µg/ml = 20 ng/µl assuming the molecular weight is 650 D/b.p.

2.3.2 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a Perkin-Elmer 9600 machine. Reaction volume was normally 100 µl consisting of the following reagents carefully added under a tissue culture hood using dedicated PCR pipetteman and sterilised tips to minimise contamination: 10 µl Perkin-Elmer PCR buffer (10x), 2 µl 10 mM dNTP mix, 1 µl each of the sense and anti-sense primers at 10 pmole/µl, 83 µl ddw, 2 µl target cDNA at 1 ng/µl, and 1 µl Taq polymerase (Perkin-Elmer) or 50:50 Taq:Pfu (Clontech) mixture. The reactions were then gently mixed and liquid spun down. After the temperature reaches 94°C, 30 cycles of 1 min at 94°C (denature), 1 min at 53°C (anneal) and 2 min at 72°C (extension) were carried out followed by a further 7 min at 72°C at the end and the tubes equilibrated to 4°C for subsequent analysis and storage. Most of PCR products in this thesis were obtained by this cycle unless otherwise indicated.

2.3.3 Gel-extraction of DNA

After visualisation of the DNA in ethidium bromide-containing agarose gels, the DNA band was exercised with a razor blade and DNA extracted directly from the gel slice using either Geneclean II (NEB) or QIAquick (Qiagen) following the manufacturer's protocol. Essentially, the agarose gel slice was solubilised in NaI buffer at 45°C. Geneclean II kit utilises glass beads to bind DNA while QIAquick kit uses a filter to bind DNA. After several washes with buffer the bound DNA was eluted with TE or H$_2$O. QIAquick kit can be spun in the centrifuge, so the recovery is much higher than Geneclean, which has a dead volume around the glass beads. Therefore most work was done with the QIAquick kit.
2.3.4 Ligation and Transformation

Ligation at 16°C overnight using conventional ligase (NEB) was first used but was replaced with the Express Ligation Kit (Boehringer Mannheim) which only took 5 min at RT for efficient ligation following the manufacturer's protocol at vector:insert molar ratio of 1:3.

Two methods were used to transform competent *Escherichia coli* and both gave good transformation efficiency: Electroporation was carried out at 200 Ω, 25 μFD, 1.8 kV with time constant about 4.6; and heat shock was carried out at 42°C for 90 s.

2.3.5 His-syn5 construction and antibody purification

Syn5 cDNA was cloned into vector pQE9 (QIAexpressionist, QIAGEN) using the BamHI site, creating an in-frame His-tag fusion protein. Induction of expression was by adding IPTG and the N-terminal His6 tag was used for subsequent affinity purification of the expressed protein all according to manufacturer's protocol. His-syn5 seemed to have a high level of expression (Fig 2.5) but the protein was insoluble and had to be purified under denatured condition using 6M urea and ProBond resin (Invitrogen) (Fig 2.6). Essentially, the His tag binds nickel ion with high affinity, immobilising the His-syn5 proteins on the resin while other cellular proteins were washed away. Elution of His-syn5 from the resin was achieved by using 0.5 M EDTA to chelate the nickel ions and releasing bound His-syn5 (Fig. 2.6) or using 0.5 M imidazole to compete with the His-tag for nickel binding. Elution of bound His-syn5 can also be carried out using a pH gradient (pH 6.3-4.5) but this was not used. Purified His-syn5 was subsequently precipitated and used to generate antisera that react with multiple epitopes on the molecule, instead of the single epitope as in the case of the peptide antibodies. Test bleeds were first screened and then affinity purified on Nickel column with His-syn5 bound.

![Figure 2.5](image1.png) Inducible expression of His-syn5. Whole cell extracts of E. coli strain JM101 were separated by SDS-PAGE. In the absence(lanes 1&6) or presence (lanes 2, 3, 4&5) of pQE9 plasmids containing His-syn5 before (lanes 1, 2&4) or after (lanes 3, 5&6) induction with IPTG.

![Figure 2.6](image2.png) Purification of bacterially expressed His-syn5. Whole cell extracts of bacteria expressing His-syn5 (lane 2) were bound to nickel columns and washed extensively with extraction buffer containing 6M urea. Bound His-syn5 could be eluted by adding 0.5 M imidazole (lane 1) or 0.5 M EDTA (lane 3).
2.3.6 RACE protocol

Poly A mRNA was isolated from rat (Sprague-Dawley) liver and used for 5' RACE using the Marathon cDNA Amplification Kit (Clontech). Briefly, using poly dT primer the isolated poly A+ mRNA was reverse transcribed into single strand cDNA using reverse transcriptase. Then the double strand cDNA was synthesised, essentially creating a library of cDNA from rat liver. Special adaptor sequences were ligated to either end of the cDNA for subsequent RACE reaction. Using a gene specific primer and a adaptor primer (AP1 or AP2) unknown sequence further upstream from the gene specific primer can be amplified by PCR and their sequences determined. Higher specificity and lower background can be achieve using nested primers for a second round of RACE (Fig. 2.7).

The AP2 primer has been modified to contain a restriction site for subsequent cloning. AP2-BamH: CGCGGATCCACTCACTATAGGGCTCGAGCGGC.

![Diagram of RACE protocol](image)

Figure 2.7 5'-RACE reactions. Lines represent DNA with 5' to the left-hand side.

2.3.7 Sequencing

DNA sequence was determined in both directions by appropriate oligonucleotide primers. Manual sequencing and automatic sequencing using the ABI PRISM Dye Terminator Cycle Sequencing System (Perkin Elmer) gave identical results of the RACE product. In essence, the manual sequencing reaction incorporates radio-labelled nucleotide homologues that arrest DNA elongation. Consequently, a population of
DNA, all of different lengths, was generated terminating at the radio-labelled nucleotide homologue. By using all four nucleotide homologues the sequence can be deciphered from the four DNA ladders starting with the same primer sequence. The automatic sequencing uses the same random-termination principle but uses fluorescent dyes that are machine-readable instead of radio-labelled nucleotide homologues. Ambiguity in sequence was reduced by sequencing in both directions.

2.3.8 In vitro translation/transcription

This was done according to the TNT coupled reticulocyte lysate system (Promega). Basically, 1 μg of DNA template was added using the TNT lysate in the presence of appropriate buffers, RNA polymerase, amino acid mixture, [35S]-labelled methionine and RNase inhibitor for 1 hr at 30°C. The translation products were separated directly by SDS-PAGE or a 5-fold amount was incubated with 15 μg affinity-purified, anti-NHU4 or NHU5 antibodies for 1 hour at room temperature. The complexes were recovered using Protein A-Sepharose (Pharmacia).

Translation exclusively from the first, newly identified, ATG is ensured by mutating the second ATG to CTG. This was done by PCR using primers syn5.15 & 10 and syn5.16 &19 followed by ligation of the two fragments carrying the mutation. Translation from the second ATG is ensured by using the original cDNA [Banfield, 1994 #1740] and from both ATG by using the full-length cDNA.

2.3.9 Transient Transfection

2.3.9.1 p31 constructs

The first 16 amino acid of syn5 N-terminal extension was grafted onto the N-terminus of p31. This was done by designing pairs of oligonucleotides with sticky ends that covers the first 16 amino acids (48 b.p.) of syn5 and the first 19 amino acids (57 b.p.) of p31 at the SalI restriction site. The p31 start codon ATG was mutated into CTG to ensuring translation only of the chimeric sequence. The RKR motif (primers N01-06) was changed into SSS motif (primers N07 &08) to show the functional difference. This 2 sets of oligonucleotides were synthesised and cleaned, annealed and then ligated to the SalI-cut p31 cDNA in an eukaryotic expression vector pSRα. Transformed bacteria were grown and the plasmid DNA was cleaned by caecium chloride density gradient before transfection.

2.3.9.2 Procedures

All solutions were pre-warmed to 38°C before transfection.
Day 0  Cells were split onto 10 cm Petri dishes (30-40% confluency). 10 ml of medium and 10% FCS in the medium were added per dish.

Day 1  20 μg of plasmid DNA were added into a final volume of 160 μl of 1/10 TE and put in water bath for 5 min. at 38°C. 160 μl Ca-mix was carefully added and left in water bath for 10 minutes. 320 μl 2xHBS was then added, with the tube on the vortex at full speed and the HBS added drop by drop. The mixture was vortexed 4-5 times to ensure optimal mixing and left for 15 minutes in water bath. Turning the water bath off before this incubation is crucial to keep the tubes still. The final precipitate was added to cells and mixed carefully with the medium.

Day 2  Cells were checked after 16-20 hours for the presence of the fine precipitate which should be moving by Brownian motions. The cells were then washed with 1x TBS 2-3 times until precipitate was gone. New medium was added and cells left for 8 hours before splitting onto coverslips.

Day 4  Process cells for immunofluorescence

Stock solutions

2xHBS 1000 ml: 50 ml of 1M HEPES stock, 7.5 ml of 100 mM Na₂HPO₄ stock and 7.5 ml of 100 mM NaH₂PO₄ stock were mixed, pH adjusted to pH 6.74 carefully and filtered to sterilise.

Ca-mix 500 ml: 36.75 g of 500 mM CaCl₂, 8.175g of 280 mM NaCl and 50 ml of 1M HEPES stock were mixed, pH adjusted to pH 6.74 carefully and filtered to sterilise.

TBS: 25 mM Tris pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7mM CaCl₂ and 0.5 mM MgCl₂ were mixed as a 10x stock of TBS but with a separate stock of 15 mM Na₂HPO₄ (25x). Just before use dilute TBS and add 25x Na₂HPO₄ to a final concentration of 0.6 mM.
Chapter 3

Retention in the Golgi apparatus
3.1 Introduction

As discussed earlier one model for Golgi protein retention, the kin recognition model, is based on the formation of oligomeric complexes (section 1.4.1). Proteins sharing the same cisterna form complexes too large to enter transport vesicles. These complexes could be immobilised by binding to an intercisternal matrix. Given the overlapping distribution of Golgi enzymes (section 1.2.3.2), binding to such a matrix could generate an ordered stack of cisternae (Fig. 1.10).

This model proposes two types of oligomeric complexes: the intercisternal matrix and the kin oligomer. The first part of this chapter deals with the matrix and attempts to identify some of its protein components. The second part is an attempt to identify a kin oligomer complex containing medial Golgi enzymes and perhaps other proteins.

3.2 Components of the cytoplasmic matrix

It has long been known that intercisternal material exists between the cisternae of the Golgi stack. This material has been visualised, using tannic acid or negative staining, as highly structured, intercisternal bridges (Cluett and Brown, 1992). These are sensitive to protease digestion under conditions that unstack the Golgi but preserve the integrity of the individual cisterna, indicating they may be responsible for holding Golgi cisternae together into a stack.

Extracting purified Golgi stacks with the non-ionic detergent TX-100 and low salt, a readily sedimentable structure, termed the matrix, enriched in the medial Golgi enzymes, MannII and NAGT I, was isolated (Slusarewicz et al., 1994). These enzymes were found to reversibly bind to the matrix, which is shown to have a cytoplasmic location (Slusarewicz et al., 1994). MannII and NAGT I have been shown to be present in two or more cisternae (Nilsson et al., 1993; Rabouille et al., 1995), so it is likely that the matrix which they bind is intercisternal, and such a binding may be involved in stacking.

The approach taken here was to prepare this intercisternal matrix and analyse the protein components that are candidates for stacking and retention of proteins. The candidate proteins in the matrix might be expected to be insoluble after treatment with detergent and salt as are the constituents of other structural cellular components, e.g. the nuclear lamina (Aebi et al., 1986; Dwyer and Blobel, 1976); an as-yet uncharacterised ER matrix (Hortsch et al., 1987) and many intermediate-type filaments (Tezuka and Freedberg, 1972).
3.2.1 Identification of matrix components

The Golgi matrix to which medial Golgi enzymes bind was isolated from Golgi membranes by sequential extraction using detergent and salt. Extractions were performed on Golgi membranes that were biochemically pure (section 2.1.8) and contained large numbers of stacked cisternae (Fig. 3.3 & 3.7, top panels). Stereological analysis showed that 69% ± 8.9 (n=5) of Golgi stacks in these preparations contained two or more cisternae. These membranes were extracted at a protein concentration of 1 mg/ml in TMMDS buffer containing 2% (w/v) TX-100, 50 mM MOPS, pH 7.0, 0.1 mM MgCl₂, 1 mM DTT and 10% (w/v) sucrose (section 2.1.10). After incubation on ice for 30 min and centrifugation at 20,000 rpm for 30 min at 4°C, the supernatants and pellets were separated. The pellet, termed TEX (Triton extraction pellet), was washed once with TMMDS buffer and re-extracted in 150 mM NaCl/TMMDS. After incubation on ice for 30 min and centrifugation at 20,000 rpm for 30 min at 4°C, the supernatant and pellet were again separated. The pellet was termed NAP (NaCl extraction pellet). These samples and an aliquot of an equivalent amount of Golgi membranes were precipitated and analysed by SDS-PAGE. The gel was stained using Coomassie Brilliant Blue R and examined to determine whether any abundant proteins were found preferentially in the TEX and NAP (Fig. 3.1).

There are 10 major proteins that are consistently observed in the NAP (Fig. 3.1). To identify these proteins by peptide sequencing would be both time-consuming and
require large amount of material, therefore mass spectrometry was used as a preliminary identification method before committing to conventional protein sequencing.

NAP proteins were blotted onto PVDF membranes and visualised by staining in sulphorhodamine B according to protocol (section 2.1.3.2). Blotted protein bands were excised and digested with specific proteases. The peptide fragments generated from the digestion were separated in the mass spectrometer and their molecular weight (MW) determined to the nearest Dalton. Accurate peptide MW "fingerprint" maps following the digestion of sample proteins can be obtained by this method. This is then compared to a MOWSE (molecular weight search) peptide mass database generated from calculated peptide MW of theoretical complete digestions using a range of specific proteases.

For each protein, digestion with one specific protease will generate a distinct set of peptides. As mass spectrometry provides an accurate MW of the peptides, sample proteins can be uniquely identified using as few as 3-4 experimentally determined peptide masses when screened against a fragment database derived from theoretical digestions of over 50,000 proteins (Pappin et al., 1993). Accuracy in identifying the sample protein can be greatly improved by using several proteases and then compare the peptide MW maps to the database. If the sample consists of a known protein, mass spectrometry provides a rapid identification in a fraction of the time using less material than conventional protein peptide sequencing. If, however, no matches have been found by mass spectrometry, then peptide sequencing of the unknown protein can be considered.

3.2.3.1 Actin

The prominent band at 45kD (Fig. 3.1 d) was identified as actin. The proteolytic fragments generated from trypsin digestion of the blotted protein matched the pattern generated from a theoretical trypsin digestion of actin peptide sequence in the MOWSE database. The top 50 matches out of a total of 51093 proteins searched were all actin.

The actins are from human, rat and other species. This is consistent with the high homology of actin peptide sequences between species as it is a major component of the cytoskeleton of the cell. Its presence in the NAP proteins reflects the known capacity of actin to polymerise into filaments and bundles that may resist extraction with detergent and salt. However, it is unlikely that actin is a component of the matrix because it is resistant to treatment with cytochalasin, and it thus probably a contaminant (P. Slusarewicz, thesis).
3.2.3.2 Rat uricase

Analysis by mass spectrometry indicated the protein of 37.5kD (Fig. 3.1 e) could be rat uricase, an enzyme involved in purine metabolism, with 11 out of 20 peptide fragments matching the MOWSE database entry in terms of fragment's MW. To confirm the amino acid composition of these fragments, they were esterified and the change in MW was compared to that of the theoretically esterified rat uricase fragments. Of the 20 amino acids, only aspartic acid (D) and glutamic acid (E) can be esterified, increasing the MW by 14 (addition of one ester bond and $\text{CH}_2$). If the peptide fragments have the same MW but different amino acid composition, after esterification the resultant MW should be different. The increase in MW after esterification for seven peptides showed the same acidic peptide sequence composition (Table 3.2, underlined amino acids) as those generated from the published rat uricase sequence (Accession no. P09118).

Uricase (EC 1.7.3.3) is a copper-containing oxidase responsible for the hydrolysis of uric acid (Christen et al., 1970). It is known to be localised in peroxisomes where it forms large paracrystalline cores (Tsukada et al., 1966). It is not present in human and some primates but other mammals have this enzyme. Tissue distribution of uricase was studied in rat and was shown by immunoblotting and RNA slot blot analysis to be preferentially in the liver. The contents of uricase in other rat tissues were estimated to be less than 2% of that in the liver (Motojima and Goto, 1990).

Peroxisomes are known contaminants of the rat liver Golgi membrane preparations (Leelavathi et al., 1970). Considering the high level of distribution of uricase in the liver, and its paracrystalline core structure, it is feasible that this enzyme resists extraction by detergent and salt and is concentrated in NAP proteins. It was a probably a contaminant and therefore not investigated further.

### Table 3.2 Identification of matrix components by peptide sequence

<table>
<thead>
<tr>
<th>Rat uricase</th>
<th>Rat cytokeratin 18</th>
<th>Rat cytokeratin 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEVLLPLDNYPYK</td>
<td>TRDGKLVSE</td>
<td>AQUIASSGLTVE</td>
</tr>
<tr>
<td>DVDFEAYWGAVR</td>
<td>SSDIMSK</td>
<td>GQRQAQE</td>
</tr>
<tr>
<td>EVATSVQLTLR</td>
<td>SRLE</td>
<td>LAMRQSVE</td>
</tr>
<tr>
<td>DQFTTLPVEK</td>
<td>LGNMQGLVE</td>
<td>IDLDMSKMNQINLE</td>
</tr>
<tr>
<td>NDEVEFVR</td>
<td>KLKLE</td>
<td></td>
</tr>
<tr>
<td>KFAGPYDR</td>
<td>SRLE</td>
<td></td>
</tr>
<tr>
<td>FAGPYDR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.3.3 Cytokeratins

Analysis by mass spectrometry indicated that the two proteins of 60kD and 52kD (Fig. 3.1 b & c) were cytokeratins, intermediate filament-like proteins consisting of an
酸性（类型I）和碱性（类型II）亚单位（Coulombe, 1993; Stewart, 1993）。为了确认这一点，两个蛋白质的肽段测序被进行（表3.2）。六个内部肽段来自60kD蛋白质匹配了鼠类型II角蛋白8（CK8, Accession no. M63482）的肽段序列，四个内部肽段来自52kD蛋白质匹配了小鼠和人类型I角蛋白18（CK18, Accession nos. X12881& M26326）的肽段序列。完整的鼠角蛋白18序列不在数据库中，但是部分序列与小鼠和人类序列的高度同源。因此，似乎不太可能是从人角蛋白18中测序的，而是来自鼠肝脏高尔基体膜。

为了进一步调查，鼠角蛋白8（CK8）和人角蛋白8的等电点相同，但CK18的等电点存在微小差异。这可能归因于物种间CK18组成差异。角蛋白是敏感的蛋白酶降解，降解产物是一个特征三联体低分子量蛋白质，大约14kD和14kD以下，与NAP蛋白质一致（如图3.1和3.2）。鼠角蛋白的弱染色（图3.2条带1）可能反映其在制备过程中对降解的敏感性，导致其降解产物的高染色强度。这强烈表明NAP的60kD, 52kD和14kD以下（图3.1 b, c, g & h）是鼠角蛋白及其降解产物。

角蛋白是一个大型多基因家族，包含两种多肽类型，即酸性（类型I）和碱性（类型II）的。它们在免疫学、肽段测绘、mRNA杂交，以及氨基酸序列数据（Parry and Steinert, 1992）的基础上被区分。酸性（类型I）角蛋白可以被进一步细分到至少两个不同的亚型，基于它们羧基末端序列，这可以解释人和鼠CK18（图3.2）的等电点差异。序列差异的类型I角蛋白含有功能域，可能解释了观察到的偏好共表达类型II角蛋白。在物种间角蛋白序列存在显著保守性，甚至扩展到3’-非编码mRNA中。

Fig. 3.2 Comparison of human and rat cytokeratins. Equivalent amount of rat cytokeratins prepared from rat liver (lane 1) and human cytokeratins from MCF-7 cell line (lane 2) were analysed by SDS-PAGE and stained with Coomassie Blue.
regions (Quinlan et al., 1985). The building block of cytokeratin intermediate filaments is a heterotypic tetramer, consisting of two type I and two type II polypeptides arranged in pairs of laterally aligned coiled coils (Stewart, 1993). The resultant filaments are resistant to extraction by salt and only become soluble in 4 M urea, explaining its appearance in NAP proteins (Franke et al., 1981; Franke et al., 1983).

3.2.2 Unstacking the Golgi

If the matrix observed between Golgi cisternae were responsible for Golgi stacking, it follows that after treatment with specific proteases, key components of this matrix would be degraded, leaving the cisternae unstacked.

It has been demonstrated that more than 95% of the Golgi stacks remain intact at 4°C for 1 hr (Cluett and Brown, 1992), therefore all incubations with salt or proteases were carried out under these conditions. After such treatment the Golgi membranes were fixed in glutaldehyde (section 2.1.11), embedded in Epon resin, and impregnated with osmium tetroxide and stained with uranyl acetate/lead citrate before sectioning and examination by transmission-electron microscopy.

3.2.2.1 Stereology definitions

At least seven randomly selected electron-micrographs (Magnification: 15,500x) were taken for quantitation. A Golgi cisterna was defined as being a membrane profile whose length was at least four times its width. Stacked cisternae overlapped each other by at least half their length, with a gap between them that was less than half their width.

3.2.2.2 Effect of salt treatment on Golgi

Purified Golgi membranes were incubated with increasing amounts of KCl before fixing for EM studies. Morphology of the Golgi stacks did not change with KCl concentration up to 1 M (Fig. 3.3).

Quantitation of the electron micrographs showed that neither the stacking (Fig. 3.4) nor the average length of Golgi cisternae (Fig. 3.5) was affected after incubation with KCl at concentrations up to 1 M. The percentage of cisternae in stacks was 69% ± 8.9 (n=5) and this is consistent with published data (Cluett and Brown, 1992) though the authors observed extensive membrane damage at high salt concentrations, which was not observed here. The average length of cisternae was 0.5 μm ± 0.08 (n=5).
Figure 3.3 Morphology of KCl treated Golgi membranes. Golgi membranes were incubated in the absence (top panel) or presence (bottom panel) of KCl (final conc. 1 M) at 4°C for 1 hr before preparation for EM. Both panels consisted of numerous intact Golgi stacks (G), each containing more than two closely apposed cisternae. Other membrane profiles were also observed, most likely swollen cisternae or contaminants. Bar = 0.5 μm
The salt treatment of the Golgi membranes did not change the sedimentability of *medial* Golgi enzymes (Fig. 3.6). After incubation with KCl the Golgi membranes were washed and re-isolated before extracting in TMMDS buffer at 4°C for 30 min. The activities of three Golgi enzymes were assayed in the supernatant and the pellet after sedimentation at 20,000 rpm for 30 min. More than 65% of the activities for the *medial* Golgi enzymes MannII and NAGT I were present in the pellet (TEX) at KCl concentration of up to 0.5 M and dropped to 55-60% at 1 M KCl. Less than 16% of the activity for the *trans* Golgi/TGN enzyme GalT was in the pellet, indicating the TGN might be fragmented under the extraction conditions (Slusarewicz et al., 1994). The recovery for all three enzymes were between 80-100%, indicating there was minimal amount of inactivation during the incubation and sedimentation procedures.
3.2.2.3 Effect of protease treatments on Golgi

Eleven proteases were tested for their abilities to unstack the Golgi but leave the cisternae intact (Cluett and Brown, 1992). Chymotrypsin, elastase, proteinsase K and subtilisin were found to unstack the Golgi in vitro. A broad spectrum protease like proteinase K would be expected to cleave a large number of proteins, making identification of potential candidates for Golgi stacking very unlikely. Therefore chymotrypsin was chosen for this study because it was shown to unstack the Golgi at 0.1 mg/ml and it is the most specific of the proteases tested.

Figure 3.7 Morphology of chymotrypsin treated Golgi membranes. Golgi membranes were incubated in the absence (top panel) or presence (bottom panel) of chymotrypsin (final conc. 1 mg/ml) at 4°C for 1 hr and quenched with PMSF. The membranes were then fixed and prepared for EM as before. The top panel contained numerous intact Golgi stacks (G) while the bottom panel contained very few stacks. Instead, numerous single cisternae were observed. Bar = 0.5 μm
Golgi membranes were digested with chymotrypsin at various concentrations by incubating on ice for 1 hr. All reactions were adjusted to a final protein concentration of 1 mg/ml with 10 mM Tris-Cl pH 7.8, the pH optimum for chymotrypsin. After quenching with 10 mM PMSF/DMSO on ice for 5 min, the membranes were spun down at 50,000 rpm for 5 min at 4°C before processing for EM.

As shown in Fig. 3.7, stacked Golgi cisternae were no longer observed after prior treatment with chymotrypsin at a final concentration of 1 mg/ml. Instead, numerous single cisternae were observed. These single cisternae were not the result of selective degradation of all but one Golgi cisterna within a stack. If this were the case, then there should be a selective loss of enzymes in those cisternae that were digested. This was shown by assaying the enzyme content of single cisterna and no selective loss of enzyme markers were observed (Cluett and Brown, 1992).

Unstacking occurred between chymotrypsin concentrations of 0.1-1 mg/ml (Fig. 3.8), where less than 5% of intact cisternae remained in Golgi stacks, in agreement with published data (Cluett and Brown, 1992). Intermediate concentrations were also tested to define a more precise point at which unstacking occurs. Less than 20% cisternae were found in Golgi stacks at chymotrypsin concentration of 0.25 mg/ml while less than 5% were in stacks at 1 mg/ml (Fig. 3.9). Quantitation of the electron micrographs showed that the average length of Golgi

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**Figure 3.8** Effect of chymotrypsin treatment on Golgi stacking. Increasing amounts of chymotrypsin (CT) were added to 100 µg of Golgi membranes and incubated at 4°C for 1 hr before quenching and fixing for EM studies. Amount of Golgi cisternae bound is expressed as percentage of the total cisternae counted ± SEM.

**Figure 3.9** Chymotrypsin concentration at which the Golgi unstacks. Increasing amounts of chymotrypsin (CT) were added to 100 µg Golgi membranes and incubated at 4°C for 1 hr before quenching and fixing for EM studies. Amount of Golgi cisternae bound is expressed as percentage of the total cisternae counted ± SEM.

**Figure 3.10** Effect of chymotrypsin treatment on the average length of the Golgi cisternae. Golgi membranes were incubated in various chymotrypsin (CT) concentrations before quenching and fixing for EM. Average length of all cisternae counted was presented ± SEM.
Cisternae did not decrease with increasing chymotrypsin concentrations during incubation and remained at around 0.48 μm ± 0.06 (n=8) with even a small increase at high levels of chymotrypsin (Fig. 3.10). This indicated that the single cisternae were intact and not digested by chymotrypsin, consistent with the biochemical findings of (Cluett and Brown, 1992).

Chymotrypsin treatment of Golgi membranes did not change the sedimentation of Golgi enzymes (Fig. 3.11). The membranes were treated with chymotrypsin, quenched as before and re-isolated, then extracted in TMMDS buffer for 30 min on ice, and spun down at 20,000 rpm for 30 min at 4°C. Enzyme activities for MannII, NAGT I and GalT in the pellet and supernatant were assayed.

For the medial Golgi enzymes MannII and NAGT I, the percentage activity in the pellet remained constant at all chymotrypsin concentrations, much the same as in KCl treated Golgi membranes (cf. Fig. 3.6). The unusually low activity of NAGT I in the control sample (Fig. 3.11, asterisk) may be due to experimental error as enzyme recovery in this sample was also much lower (66%) than for others, which is 80-100%. The trans Golgi/TGN enzyme GalT, on the other hand, was detected by activity mainly in the supernatant with less than 15% in the pellet with recovery at around 80%. This again indicated the TGN might be fragmented during the extraction procedure (Fig. 3.6 and (Slusarewicz et al., 1994)). These results confirmed that only unstacking of Golgi cisternae has taken place with cisternae remaining intact both morphologically and biochemically.

Figure 3.11 Effect of chymotrypsin digestion on Golgi enzyme sedimentation. 100 μg of chymotrypsin treated Golgi membranes were extracted in TMMDS buffer for 30 min. After a 30 min spin at 20,000 rpm at 4°C, the supernatant and pellet were assayed for enzyme activities. The data are plotted as the percentage of the total activity found in the pellet. Total activity recovery are plotted above the histograms.
3.2.2.4 Cytokeratins

If the matrix contains candidate proteins responsible for stacking, after protease treatment these proteins will be selectively degraded. To test this, NAP proteins derived from Golgi stacks pre-treated with chymotrypsin were analysed by SDS-PAGE (Fig. 3.12). The prominent protein bands at 45kD (actin) and 37.5kD (uricase) were resistant to prior treatment of chymotrypsin. Some higher molecular weight proteins showed decreased staining intensity with increasing chymotrypsin concentration. The most striking of this was the two cytokeratins at 60kD and 52kD (Fig. 3.12), which were clearly visible in the control (lane 1) and the mock digested sample (lane 2). The staining became much reduced at chymotrypsin concentration of 0.01 mg/ml (lane 3) and was completely invisible at 0.1 mg/ml (lane 4), at which point the Golgi was shown to start unstacking (Fig. 3.8).

3.2.2.5 p35

Cytokeratins were selectively degraded under conditions preced^1 which the Golgi unstacks, suggesting a likely involvement in Golgi stacking. An alternative approach could be taken: under such conditions, key stacking components of the matrix might be degraded and the fragments solubilised. By observing what has been released into the supernatant after proteolysis, using specific antibodies, and tracing back to the protein from which such fragments were derived, identification of stacking candidates might also be achieved.

Golgi membranes were treated with increasing concentrations of chymotrypsin and separated by SDS-PAGE followed by transfer to nitrocellulose membrane. Western blot using antibodies generated against TEX (from Dr. T. Kreis) showed that a 35kD protein (p35) started appearing in the TEX supernatant at chymotrypsin concentration of 0.01 mg/ml (Fig. 3.13), precedes that of Golgi unstacking.

![Figure 3.12](image-url) - Extraction of chymotrypsin-treated Golgi membranes. 500 μg of Golgi membranes were incubated in the absence (lane 1), buffer alone (lane 2) or presence of chymotrypsin (lane 3: 0.01 mg/ml; lane 4: 0.1 mg/ml; lane 5: 1 mg/ml) for 1 hr at 4°C before quenching with PMSF and sequential extraction in TMMDS and 150 mM NaCl. Fractionation by SDS-PAGE (10% gel) was followed by staining with Coomassie Blue.
This protein is potentially a degradation fragment from a matrix component that is specifically digested by chymotrypsin at a concentration that unstacks the Golgi but leaves the cisterna intact. To identify the original protein from which the fragment was derived, anti-TEX antibodies specific for the p35 band blotted on nitrocellulose membrane were eluted directly from the membrane (section 2.1.7.4). Using this antibody to stain NRK cells, a tight peri-nuclear staining pattern was obtained (Fig. 3.14). This staining pattern is identical to MannII staining using a monoclonal antibody 53FC3 (Burke et al., 1982) in a double-label experiment, indicating that the antibody specific for p35 recognised a protein localised to the Golgi apparatus.

Unfortunately, further efforts to pinpoint the candidate protein by Western blotting did not reveal any higher molecular weight proteins from which p35 could derive. The
epitopes which the p35 antibodies recognise could be masked in the original protein on nitrocellulose membranes after blotting but exposed in PFA-fixed NRK cells. The amounts of p35 was also too small to allow further analysis of its protein sequence, it was thus not pursued further.

### 3.2.4 Discussion

The Golgi apparatus is unique among the numerous intra-cellular organelles in that it consists of a stack of flattened cisternae which are held together as a cohesive unit. The rat liver Golgi preparation protocol (section 2.1.8) is sufficiently effective in preserving the stacked morphology of the Golgi, with 69% cisternae present in the stack (Fig. 3.3).

In an investigation of structural components that could account for the proteinase sensitivity of Golgi stacks, uniform rectangular elements have been visualised between stacked Golgi cisternae (Amos and Grimstone, 1968; Franke et al., 1972; Mollenhauer and Morre, 1975) and on disrupted, intact Golgi membranes using negative stain or tannic acid enhancement of positive stain (Cluett and Brown, 1992). These bridge-like structures were not artefacts because they were found both in vivo and in vitro, their dimensions between intact Golgi membranes were identical to those seen on single, unstacked Golgi membranes; they were removed following protease treatment under conditions that resulted in Golgi unstacking, and the heights of these elements were exactly the same as inter-cisternal space, i.e. ~11 nm. They could also be readily distinguished from clathrin coats and COP coats as they were much larger and wider than the bristles seen on clathrin-coated membranes (Pearse and Robinson, 1990) and were found on the planar faces of the cisternae, not on dilated rims or bud profiles where COP coats appear to form.

Biochemical investigations have led to the identification of a cytoplasmic matrix to which medial Golgi enzymes specifically bind (Slusarewicz et al., 1994). This could represent the intercisternal material seen by EM (Cluett and Brown, 1992) and enhance the retention of Golgi enzymes and play a role in Golgi stacking since proteolysis of this matrix unstacks the cisternae. After sequential extraction of purified Golgi membranes with TX-100 and salt, there are 10 major insoluble components in the matrix (Fig. 3.1). Using a combination of mass spectrometry and protein sequencing, some of these components were found to be cytoskeletal proteins (actin and cytokeratins) while others turned out to be contaminants present in the original Golgi preparation (rat uricase, Table 3.1). Work done in this laboratory has led to the identification of another component, GM130, a cis-Golgi matrix protein (Nakamura et al., 1995). Other unidentified components might arise from matrices from different parts of the Golgi stack that are responsible for binding cis- and/or trans-Golgi proteins.
Various aspects of the involvement of the cytoskeleton with the Golgi apparatus have been studied in different organisms. Microtubules are the major constituents of the cytoskeleton. They are involved both in these intracellular transport processes and in the spatial organisation of cytoplasmic organelles. The positioning of the Golgi apparatus has been shown to depend on an intact interphase microtubule network and perturbation of microtubules with exogenous agents such as nocodazole affected the integrity of the Golgi apparatus (Duden et al., 1990; Kreis, 1990). The importance of microtubules in Golgi stacking has been studied in the fission yeast Schizosaccharomyces pombe and it was shown that the disruption of the microtubule network can cause unstacking of Golgi cisternae (Ayscough et al., 1993). Involvement of the actin filaments and intermediate filaments in Golgi structure and stacking is less well characterised.

One aspect of the involvement of actin and the Golgi was studied in the budding yeast S. cerevisiae. Mutations in SAC1, a gene identified by virtue of its allele-specific genetic interactions with yeast actin defects, were also capable of suppressing lethalities associated with yeast Golgi defects and sec9 (yeast SNAP-25 homologue, see section 1.3.5.1 and Table 1.2) secretory vesicle defects (Cleves et al., 1989). These genetic data are consistent with the notion that the secretory pathway and actin cytoskeleton function was co-ordinated in the cell and both could be modulated by the same proteins. Secondly, Golgi-derived vesicles were shown to bundle actin filaments in an ATP-dependent manner via the mechanoenzyme myosin-I in the intestinal brush border (Fath and Burgess, 1993). Lastly, in the slime mould Dictyostelium discoideum alteration of the actin network led to the dispersal of the Golgi apparatus into vesicles distributed throughout the cell. A 24kD protein, comitin, that specifically binds F actin, was localised to the Golgi and proposed to link the Golgi apparatus to the actin network (Weiner et al., 1993). However, actin's role in providing structural framework to the Golgi stack is less obvious. This is reflected in the finding that actin is not sensitive to chymotrypsin digestion at concentrations that unstacks the Golgi (Fig. 3.12). These evidence shows that actin is involved in vesicular transport and maintaining Golgi morphology, but does not necessarily show it is a component of the matrix.

The function of the cytokeratins in the Golgi matrix is currently uncharacterised. Their sensitivity to chymotrypsin at concentrations that precede Golgi unstacking indicates a possible role in providing a cytoplasmic link between Golgi proteins and the cisternal membrane, a function analogous to that of lamins.

Both actin and cytokeratins are involved in cytoskeleton anchoring to plasma membrane and cell adhesion. If they were involved in Golgi stacking, their importance will be difficult to assess beyond the current level using the protease digestion
approach. As cytoskeletal proteins are also involved in providing structural support to many other aspects of cellular function, genetic manipulations, e.g. mutation or knockout, will affect the cell as a whole and their specific contribution to Golgi stacking will thus be difficult to dissect from their general functions in other parts of the cell. An obvious way to test this is to do immuno-EM to see if they are present between stacked Golgi cisternae.

Following the proteolytic release from the stack, the individual cisternae maintained their flattened morphology and remained so for over an hour at 4°C. A 35kD protein was observed to be released into the supernatant under conditions that precede Golgi unstacking. There are some indications that a protein of higher MW may be responsible for the release of p35 (Fig. 3.13), but due to technical difficulties in carrying out further identification using the eluted antibodies specific for p35, the identification of this higher MW protein was not completed. Immunofluorescence data suggest that p35 and/or the higher MW protein is in the Golgi apparatus (Fig. 3.14). Two possibilities exist: (i). p35 could be a degradation fragment derived from a higher MW component of the matrix that is sensitive to chymotrypsin digestion; or (ii). p35 could be an intact, chymotrypsin-insensitive protein itself but was released by the selective degradation of other anchor proteins. From the data available, these two possibilities could not be distinguished.
3.3 Evidence for Kin Oligomers

Molecular biology work showed that MannII and NAGT I form at least a heterotetramer, and that over-expression of either of these medial enzymes destroyed Golgi morphology, suggesting that the kin oligomers could be involved in maintaining cisternal structure (Nilsson et al., 1994). These enzymes were also shown to bind to the intercisternal matrix and form a large, readily sedimentable structure (Slusarewicz et al., 1994). To further investigate this, and to provide evidence for the kin-recognition model of Golgi protein retention, I started characterising a large oligomer isolated from the Golgi membranes.

3.3.1 Sucrose gradients

To provide biochemical evidence for the existence of large kin oligomers, two properties of such proposed structures were used to isolate them from the Golgi membranes. Firstly, these structures must be large enough to be excluded from transport vesicles and, therefore, should be able to sediment on a density gradient away from monomers or other components of the Golgi membranes. Secondly, these oligomers must be able to disassemble and reassemble under suitable conditions, as during mitosis the Golgi fragments into clusters of vesicles and reassembles as the cell exits mitosis (Lucocq et al., 1989; Souter et al., 1993); the oligomers must also undergo the same round of mitotic disassembly and reassembly if they are involved in maintaining Golgi cisternal structure.

Golgi membranes were first treated with KCl or Na$_2$CO$_3$ to strip away cytosolic components that are not tightly bound to the membrane. The membranes were then extracted in the non-ionic detergent TX-100 under conditions that medial Golgi enzymes were shown to be present in an insoluble complex (Slusarewicz et al., 1994). After centrifugation to equilibrium, the fractions were assayed for activity of the medial Golgi enzymes, MannII and NAGT I.

Fig. 3.15 shows a typical sedimentation profile for TEX with some solubilised MannII at fraction 3 (sucrose density 25%) while most of MannII is present in the insoluble material at the bottom of the gradient (A). In KCl (B) treated Golgi membranes, MannII activity in fraction 3 constitutes about 30% of the total enzyme activity found in the gradient. In addition to the insoluble material at the bottom of the gradient, there is also a peak in fraction 10 (sucrose density 58%). This peak could contain oligomers that are large enough to be sedimented away from the solubilised MannII in fraction 3 and yet allow separation from aggregates of membranes at the bottom of the gradient. The MannII activity of carbonate treated Golgi (C) was very low (less than 10%) compared to that of the untreated or KCl washed Golgi (B), indicating that the enzyme
Figure 3.15 Isolation of an oligomer in sucrose gradients. TEX from 5 mg of (A) untreated Golgi membranes; (B) Golgi membranes treated with 1 M KCl and (C) Golgi membranes treated with 0.11 M Na₂CO₃, pH 11.5 were fractionated on linear sucrose gradients. 1 ml fractions were taken and the activity of the medial Golgi enzyme, MannII, was assayed and plotted with sucrose density assessed by reading the refractive index.

may have been inactivated by the high pH treatment of the carbonate wash. Although the distribution of MannII activity across the gradient gave the same pattern as for KCl-treated Golgi membranes, carbonate washing was not used for subsequent experiments.

To test whether the MannII-containing material in fraction 10 could be reassembled after treatment with salt, KCl washed membranes were extracted in TMMD buffer containing 150 mM NaCl, a condition known to solubilise MannII (Slusarewicz et al., 1994) and should lead to the dissociation of the oligomer (Fig. 3.16A). The disassembled oligomer components were then dialysed against TMMS. Only specific
molecules should reassemble into the oligomer structure with irrelevant molecules excluded. A similar peak at sucrose density 58% was observed after dialysis (Fig. 3.16B). This indicated that the dense MannII containing material was not the result of a non-specific aggregation of proteins as it can be disassembled (Fig. 3.16A) and reassembled (Fig. 3.16B).

To investigate if membrane proteins play any role in the oligomer formation, KCl washed Golgi membranes were extracted in 150 mM NaCl/TMMD buffer containing 1% TX-114 and phase separated at 37°C (section 2.1.11.3). The detergent phase containing membrane proteins and the detergent insoluble pellet were dialysed separately against (TX-114)TMMD or after mixing. After centrifugation on linear sucrose density gradient, no oligomers reassembled in the detergent supernatant or pellet dialysed alone (Fig. 3.17 A&B) while a peak at sucrose density 58% was observed in the dialysed mixture (Fig. 3.17 C) and shown by enzyme activities to contain two medial Golgi enzymes MannII and NAGT I. This peak is identical (fraction 10, sucrose density 55%) to the one observed in KCl washed Golgi membranes extracted in 150 mM NaCl/TMMD (containing TX-100) and dialysed against TMMD (Fig. 3.17 D), and similar in enzyme activity for the medial Golgi enzymes MannII and NAGT I.
Figure 3.17 Membrane proteins are important for oligomer formation. 3x2 mg of Golgi membranes were washed in 1 M KCl and extracted in 150 mM NaCl/(TX-114)MMDS buffer for 30 min at 4°C before phase separation at 37°C for 5 min. Detergent phase supernatant (A) and detergent phase pellet (B) was separated and dialysed against (TX-114)MMDS overnight. (C): the aqueous phase supernatant and pellet were mixed before dialysis. (D) 2 mg of Golgi membranes were washed in 1 M KCl and extracted in 150 mM NaCl/TMMDS before dialysing against TMMDS alone. The dialysed material was loaded onto continuous sucrose gradients (0.5-2 M) and spun to equilibrium. 1 ml fractions were taken. Enzyme activities for MannII and NAGT I were assayed.
3.3.2 Gel filtration

The material in sucrose gradients that was sedimented away from soluble monomers and contained medial Golgi enzymes was likely to form the proposed kin oligomer. To further dissect the components of this complex structure, size-exclusion gel filtration chromatography was used to separate the various components.

A Superose 6 column was initially used to obtain an estimate for the size of the oligomer. The medial Golgi enzymes eluted at the fraction immediately after the exclusion volume of the Superose 6 column (fraction 10), indicating an estimated MW of 2x10^6 Dalton (2MD), much in excess of the MW of enzymes themselves (MannII: 125kD, NAGT I: 50kD). Fig. 3.18 shows an experiment where both NAGT I and MannII activities were assayed from gel filtration fractions and that the two peaks coincided.

NAGT I was shown to be more soluble than MannII during TX-100 extraction (Slusarewicz et al., 1994). It is also a less abundant protein than MannII in the Golgi (Moremen et al., 1991): Rough estimates suggest that NAGT I constitutes less than 0.5% of total Golgi protein (Oppenheimer and Hill, 1981) while Mann II constitutes approximately 1% of the total Golgi proteins (Moremen et al., 1991; Tulsiani et al., 1977). The protein concentrations from fractions obtained from the gel filtration column are very low in comparison to those from sucrose density gradients. The low abundance of NAGT I in the dilute gel filtration fractions could lead to a higher degree of inaccuracy in the measurements of its distribution. MannII assay is also simpler and more sensitive than NAGT I assay, and NAGT I always behaves similarly to MannII.
Figure 3.19 Disassembly and reassembly of the oligomer. A. After gel filtration on Sephacryl 400 XK 8/40 column, the oligomer elutes at fraction 13 (2MD) with an intermediate peak at fraction 21 (630kD). B: In the presence of 500 mM NaCl, the oligomer can be disassembled into MannII dimers, eluting at fraction 23 (230kD). C: After dialysis of the pooled fractions in B, the 2MD oligomer reassembles and elutes at fraction 13 with an intermediate peak again at fraction 21.

(Slusarewicz et al., 1994). For these reasons, MannII was used as the standard assay for characterising oligomer assembly and disassembly in subsequent gel filtration studies.

To test the specificity of this 2MD complex obtained by gel filtration, Sephacryl 400 HR column was used for its higher resolution at a high MW range than Superose 6 (section 2.1.11.1). As shown in Fig. 3.19A, the 2MD peak was again obtained with
MannII-containing structures eluting as a smear of intermediate MW and a broad peak at fraction 21 (630kD). After equilibrating the column in 500 mM NaCl/TMMS and reapplying the samples in the same buffer, all the MannII activity eluted in a position corresponding to 230kD (Fig. 3.19B), consistent with the MW of MannII dimers. Fractions containing the peak MannII dimer activities (fractions 20-26) were collected and the NaCl dialysed away before reapplying to the column. The 2MD complex was again obtained (Fig. 3.19C), indicating the oligomer can be repeatedly disassembled and reassembled.

3.3.4 Identification of oligomer components

To identify the protein components of the 2MD oligomer, TX-114 extraction (section 2.1.11.4) of the fractions collected from Superose 6 columns (Fig. 3.18) was carried out. Both the detergent phase containing hydrophobic proteins and the aqueous phase containing hydrophilic proteins were separated on SDS-PAGE and stained with Coomassie Blue (Fig. 3.20). The protein profiles shown in Fig. 3.20 did not cofractionate in fraction 10, thus they could represent a mixed population of oligomeric complexes of similar size.

To identify the proteins in the oligomer complexes, 2-D gels were used to construct a clearer picture of the various components. Proteins from fraction 10 were methanol-chloroform precipitated and solubilised in 2-D lysis buffer before IEF and SDS-PAGE. A reproducible pattern was obtained from several experiments and a typical large scale gel is shown in Fig. 3.21. Direct peptide sequencing of some of the components in fraction 10 from well-separated spots in the 2-D gels (carried out by Dr. T. Nilsson in EMBL) indicated the two proteins of 65kD were novel; below this were three proteins of 50kD, which turned out to be lectin-like molecules that display sequence homology to ERGIC53/p58; actin was also identified; the distinct ladders in the middle of the gel were members of the VIP36 recycling proteins; and the low MW proteins around 20kD were Rab6 and members of the p24 family of putative cargo receptor proteins.
Figure 3.20 TX-114 extraction of oligomers. Gel filtration fractions from Superose 6 columns were subjected to TX-114 extraction and the detergent phase (A) separated from the aqueous phase (B). Each sample was then precipitated using methanol-chloroform and the precipitates solubilised in SDS-PAGE sample buffer, separated on a 10% gel, and stained with Coomassie blue. The 2MD complex was present in fraction 10. Arrows indicate proteins of interest.
Figure 3.21 2-D gel of the oligomers. Large scale 2-D gel was performed by pooling fraction 10 from several gel filtration runs and precipitate the material by methanol-chloroform method. The precipitates were solubilised in 2-D lysis buffer and separated by IEF and then SDS-PAGE, followed by staining with Coomassie blue. Proteins of interest are indicated.

Western blotting of Superose 6 fractions using anti-CHO p24 and anti-rat p24 antibodies confirmed the identity of the p24 proteins (Fig. 3.22).

Figure 3.22 Western blot of gel filtration fractions with p23/p24 antibodies. Fractions from Superose 6 column were precipitated, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Antibodies specific for CHO-p24 and rat-p24 were used to probe the membranes followed by secondary antibodies conjugated to peroxidase and visualised using the ECL system.
3.3.5 Discussion

It was recently shown that medial enzymes can be isolated, in vitro, as a detergent insoluble complex of a supra-molecular nature (Slusarewicz et al., 1994). This complex was not the consequence of non-specific aggregation since similar interactions could be demonstrated in vivo. Expressing the medial enzyme, NAGT I, in the ER resulted in the accumulation of another medial enzyme, MannII, in this compartment (Nilsson et al., 1994). The resulting depletion of medial enzymes caused the Golgi apparatus to lose its stacked morphology. This shows that Golgi enzymes and/or proteins binding these enzymes have a structural role and also argues that medial enzymes form large oligomers.

To further analyse the composition of the oligomers, Golgi membranes were extracted in detergent and salt followed by dialysis and isopycnic centrifugation on a linear sucrose density gradient (Fig. 3.16). An oligomer was identified with an apparent MW of 2MD as assessed by size-exclusion chromatography (Fig. 3.17). It can be disassembled again by addition of 500 mM NaCl and reassembled after dialysis, further indicating its affinity for constituent molecules (Fig. 3.18). It contained the two medial Golgi enzymes, Mann II and NAGT I, along with various other proteins (Fig. 3.21).

Some of them are identified as novel proteins (p65), some are cytoskeletal proteins (actin), others are proteins implicated in different stages of vesicular transport (Rab6, VIP36 and p24).

The presence of actin in the oligomer was not surprising, as the extraction protocol is based on the isolation of the matrix and the possible role of actin in the Golgi was discussed previously (section 3.2.3.1).

Rab proteins are ras-like GTP-binding proteins believed to regulate specific transport steps of vesicular transport by regulating v-SNARE functions (section 1.3.5.2). Rab6 is associated with medial and trans Golgi cisternae and membranes of the TGN as assessed by confocal microscopy (Antony et al., 1992; Goud et al., 1990). Mutational analysis of Rab6 indicates it is involved in intra-Golgi transport (Martinez et al., 1994). As Rab proteins spend a significant portion of their lifetime in the cytosol bound to GDI (Fig. 1.9), identification of Rab6 in the oligomer suggests the conditions used to analyse the composition of the oligomer may lock Rab6 in the membrane-bound state.

VIP36 is a type I membrane protein isolated from MDCK cells as a component of the glycolipid-enriched detergent-insoluble complex (Fiedler et al., 1994). It is localised to the Golgi apparatus and the cell surface, and belongs to a family of lectins in the secretory pathway that might be involved in the trafficking of glycoproteins and/or glycolipids. Mutational studies imply that VIP36 functions as a lectin in trafficking
between the plasma membrane and the Golgi (Fiedler and Simons, 1996).

ERGIC53/p58 was found to be a mannose-binding lectin, sharing sequence homology with VIP36 (Arar et al., 1995). It is known to cycle between the ER, the IC and the cis-Golgi (section 1.2.2) and could facilitate the transport of glycoproteins between the ER and the Golgi (Arar et al., 1995). ERGIC53/p58 contains a cytoplasmic ER-retrieval signal, KKXX (section 1.4.2). Over-expressed ERGIC53/p58 is transported to the cell surface and rapidly endocytosed. Surprisingly, the endocytosis signal was identified as being KKFF and, like the ER-retrieval signal, situated at the C-terminus of proteins (Itin et al., 1995). The minimal consensus sequence determined by substitution mutagenesis (K-K/R-F/Y-F/Y) was related to the ER-retrieval consensus (K-K-X-X). Internalisation of VIP36, which cycles between the plasma membrane and the Golgi, is also mediated by a signal at its C-terminus that matches the endocytosis consensus sequence (Itin et al., 1995).

The identification of proteins homologous to ERGIC53/p58 and VIP36 in the oligomer indicates that it contains not only medial Golgi enzymes and associating proteins but also proteins from both the cis- and trans- side of the Golgi stack. The identification of short, cytoplasmic ER retrieval signals that resemble endocytosis signals on these proteins suggests that the same mechanism for protein retention and retrieval may be used in multiple steps of the secretory pathway and that the structural maintenance of the Golgi apparatus is intimately linked to protein retention.

Further indication of the multiple roles the oligomer constituent proteins play in protein retention and Golgi structure come from the identification of members of the p24 family proteins in the oligomer (Fig. 3.21). p24 family proteins are proposed to operate as cargo receptors, selecting proteins for inclusion into budding vesicles (Fiedler et al., 1996; Schimmoller et al., 1995; Stamnes et al., 1995). All p24 family members are type I membrane proteins with a single membrane spanning domain and a signal sequence (Fig 1.2, Chapter 1.2.1). The bulk of the p24 family proteins are present in the lumen of the secretory compartments. The C-terminal portion display the highest homology between family members, suggesting it is important for the function of these proteins (Fiedler et al., 1996). Many of the p24 family members have a dibasic ER targeting signal, KKXX or RRXX, at their cytoplasmic, C-terminus. The KKXX motif has been shown to bind specifically to coatomer (Cosson et al., 1996; Cosson and Letourneur, 1994; Letourneur et al., 1994) (section 1.4.2). Although the RRXX motif did not bind to coatomer under high-salt conditions and that it was originally found at the cytoplasmic N-terminus for type II membrane proteins (Schutze et al., 1994), it is possible that under physiological conditions coatomer can interact with this motif, perhaps in combination with other binding sites (Stamnes et al., 1995).
The lumenal domains of the p24 family share less similarity among various family members, but they do contain heptad repeats predicted to form α-helical coiled-coils. These motifs are involved in protein-protein interactions, raising the possibility that p24 family members may form homo- or hetero-oligomers. The identification of p24 family members in the Golgi oligomer lends some support to the idea of the formation of hetero-oligomers and illustrates the close link between protein retention/retrieval and the structure of the Golgi apparatus. Further analysis of p24 and other membrane components of the oligomer should lead to additional insights into the mechanism of Golgi protein retention and structural maintenance, as well as transport vesicle formation and cargo selection.

In addition to the biochemical separation of oligomer components, it was also noticed during the preparation of pure MannII (P. Slusarewicz, personal communication), after passing through hydroxylapatite (HA) column MannII no longer exists in a 2MD complex but elute as MannII dimers with an estimated MW of 200kD. This indicates that the HA column separated some factors responsible for holding MannII dimers into a higher order oligomer. Investigation into proteins bound to the HA column could lead to the identification of such factors.

### 3.4 Summary

The cytoplasmic matrix might provide the link between cisternae, possibly by interacting with the cytoplasmic tails of resident Golgi enzymes. The identification of cytoskeletal components points to a structural role of the matrix in maintaining Golgi stacking.

The characterisation of the kin oligomer is still in a very preliminary stage. Some of its sub-units were identified, e.g. proteins homologous to ERGIC53/p58 and VIP36, and the p24 family proteins. Functional importance of these will become more apparent after further identification and characterisation of other components.
Chapter 4

Targeting in the Golgi Apparatus
4.1 Introduction

The Golgi apparatus receives the entire output of newly-synthesized proteins from the ER (section 1.2.3). Resident ER proteins that enter transport vesicles are retrieved back to the ER (section 1.4.3). Targeting of the vesicles in the ER-Golgi transport step becomes an important issue if the anterograde and the retrograde transport vesicles are to fuse with their correct destination compartments.

The syntaxin family of proteins (Bennett et al., 1993, section 1.3.5) are localised to various membrane compartments in the cell, and are t-SNAREs according to the SNARE hypothesis. Syntaxin 5 is one of the molecules shown to be involved in the ER-Golgi trafficking step. In this chapter I shall describe in greater detail syntaxin 5 and its possible functions.

4.1.1 The Syntaxin family

Syntaxins are a family of type II tail-anchored proteins (section 1.2.1 and Fig. 1.2) that are broadly expressed in different tissues (Bennett et al., 1993; Bock et al., 1996). The ubiquitous expression of syntaxin 2, syntaxin 4, syntaxin 5 and syntaxin 6 suggests an involvement in pathways common to all cell types, whereas the more restricted pattern of expression for syntaxin 1 and syntaxin 3 in neural tissues suggests an involvement in more specialized pathways, e.g. neurotransmitter release for syntaxins 1A/1B (Table 4.1). Syntaxins destined for different compartments of the secretory pathway are thought to be first inserted into the ER and subsequently transported to their final locations using vesicular traffic (Jantti et al., 1994; Kutay et al., 1995).

<table>
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<th>Table 4.1 The Syntaxin family members</th>
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<td><strong>Family</strong></td>
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<td>Syntaxin 1A</td>
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In addition to sharing various levels of sequence homology to each other (21-84% identity), the syntaxin family members also share several common structural features (Fig. 4.1). All are about 300 amino acids in length and end with a region of highly hydrophobic residues at the C-terminus. This domain is 17-25 amino acid in length and is of sufficient hydrophobicity to serve as a membrane anchor. Each member of the syntaxin family also contains several domains predicted to form α-helical coiled-coil structures, thought to be involved in protein-protein interactions (Chapman et al., 1994; Hayashi et al., 1994). A 70 a.a. coiled-coil region near the C-terminus displays the highest level of homology within family members and was shown to mediate interaction between the neurospecific isoform syntaxin 1A and N-type calcium channel (Sheng et al., 1994), while a more N-terminal coiled-coil was shown to mediate α-SNAP binding to syntaxin 6 (Bock et al., 1996).

Figure 4.1 Domains of syntaxin. Syntaxins are type II membrane proteins with a short C-terminal membrane anchor and the major part of the proteins projecting into the cytoplasm. There are several predicted coiled-coil domains that are thought to interact with other proteins. A particular 70 amino acid domain near the C-terminus shows the highest homology between family members and have been shown to be the binding site for several proteins in the synapse.

When expressed in COS cells, syntaxins 1, 2 and 4 were shown to localise to the plasma membrane (Bennett et al., 1993). Syntaxin 5 and syntaxin 6 have been localised to the Golgi (Banfield et al., 1994; Bennett et al., 1993; Hardwick and Pelham, 1992; Hardwick and Pelham, 1994). In polarised MDCK cells, syntaxins 1A/IB were present only in intracellular structures, syntaxin 2 on both the apical and basolateral surface whereas syntaxin 3 and syntaxin 4 were restricted to the apical and basolateral surface respectively (Gaisano et al., 1996; Low et al., 1996).

While syntaxin family members form a docking/fusion complex (20S complex) in vitro (Sollner et al., 1993) with other proteins in the presence of detergent, its exact role in vesicle docking/ fusion is still unclear. It has been shown that NSF binds syntaxin through α-SNAP and upon ATP hydrolysis catalyses a conformational change that dissociates the 20S complex (Hanson et al., 1995). Studies by (Sheng et al., 1994) showed a direct interaction between the cytoplasmic domains of syntaxins and N-type calcium channels. This could have an important role in the targeting and docking of synaptic vesicles near N-type calcium channels, enabling tight structural and functional association of calcium entry sites and neurotransmitter release sites.
4.1.2 Sed5p

As introduced in section 1.4.2, HDEL (His-Aps-Glu-Leu) is the C-terminal sorting signal in *S. cerevisiae* that has been shown to be necessary and sufficient for soluble ER proteins that enter transport vesicles to be retrieved from later compartments back into the ER (Pelham *et al.*, 1988). The mammalian equivalent is the C-terminal KDEL sequence for soluble ER proteins (Munro and Pelham, 1987). Retrieval to the ER is thought to be receptor mediated.

The *ERD2* gene codes for the HDEL receptor that retrieves ER proteins from the secretory pathway and returns them to the ER (Lewis *et al.*, 1990). The gene product Erd2p is a 26kD integral membrane protein whose abundance determines the efficiency and capacity of the retrieval. It is essential for the maintenance of Golgi structure and normal protein transport, and is required for growth (Semenza *et al.*, 1990). In the absence of Erd2p the Golgi apparatus is both functionally and morphologically perturbed.

A family of multicopy suppressor *SED* genes (suppressors of *ERD2*-deletion) that allow *S. cerevisiae* to grow in the absence of Erd2p have been isolated (Hardwick *et al.*, 1992). Sequence analysis indicates that all these *SED* genes code for membrane proteins: *SED1* encodes a probable cell surface glycoprotein; *SED2* is identical to *SEC12*, a gene required for the formation of ER-derived COPII vesicles (section 1.3.2); *SED3* is the structural gene for dolichol-P-mannose synthase DPM1; *SED4* encodes a protein whose cytoplasmic domain is 45% identical to that of Sec12p, and is thought to be a GEF that facilitates nucleotide exchange by ARF-1 prior to COPII coat assembly (section 1.3.2); *SED5* encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex (Hardwick and Pelham, 1992); and *SED6* is identical to *ERG6*, and encodes a putative methyltransferase required for ergosterol synthesis (Hardwick and Pelham, 1994).

The absence of *ERD2* causes an imbalance between membrane flow into and out of the Golgi apparatus, and the *SED* gene products are supposed to compensate for this either by slowing transport from the ER or by stimulating vesicle budding from Golgi membranes. However, the *SED* genes encode very different proteins with clearly different functions. It is not obvious how and why over-expression of the *SED* genes should rescue the *erd2* mutant. The exact mechanism by which *SED* genes suppress *ERD2* deletion is, therefore, not clear due to limitations in the genetic approach.

4.1.3 Syntaxin 5

The mammalian homologue of Sed5p is syntaxin 5 (syn5). Rat syn5 cDNA sequence
Targeting was obtained by screening a rat macrophage cDNA library with a probe derived from a *Drosophila* homologue of yeast Sed5p (Bennett *et al.*, 1993). Sequence homology between yeast Sed5p and rat syn5 is not particularly high: 35% identity and 57% similarity. On the other hand, human syn5 and rat syn5 are 97% identical.

In yeast, Sed5p is present in structures that have no obvious connections to the ER (Hardwick and Pelham, 1992) and forms complexes with known v-SNAREs (section 1.4.2) found on ER-derived transport vesicles (Sogaard *et al.*, 1994). By analogy, the syn5-containing structures in mammalian cells should be the destination for vesicles budding from the ER. Indeed, syn5 has been localised to the cis-side of the Golgi (Banfield *et al.*, 1994) and is shown to play an analogous function of Sed5p in ER to Golgi transport in mammalian cells (Dascher *et al.*, 1994); Hay, 1997. Sed5p/syn5 are believed to specify target membrane identity, therefore are Golgi t-SNAREs according to the SNARE hypothesis (section 1.3.5).

A crucial feature of the SNARE hypothesis is that the t-SNARE must be a resident of the target membrane. This is in marked contrast to the cognate v-SNARE which must cycle between the two compartments linked by vesicles. The v-SNARE will share the same compartment as the t-SNARE immediately after membrane fusion but is presumably recycled back to the compartment from which it originated so that it can be re-incorporated into vesicles for another round of vesicle transport. The presence of an active t-SNARE in another compartment would compromise compartmental identity. Signals must, therefore, exist to localise t-SNAREs to particular compartments.

Targeting signals are studied in detail for Sed5p (Banfield, 1994 #1740), which normally resides in the Golgi of yeast. The mammalian homologue, syn5 (Bennett, 1993 #4492), is located in a tubulo- reticular network on the cis-side of the Golgi stack, as is the transiently expressed *Drosophila* homologue, dSed5p. Chimeras between dSed5p and other mammalian syntaxins (from other locations) showed that more than one signal was present [Banfield, 1994 #1740]. The membrane-spanning domain was sufficient but not necessary to localise the chimeras to the cis-Golgi. Additional signals were found in the cytoplasmic domain though their precise nature was not determined.

In carrying out further studies on syn5, a number of anti-peptide antibodies were found to recognise an additional, longer form of syn5 in a wide variety of rat tissues and cell lines. We also noticed that the untranslated region upstream from the putative start codon in the published sequence [Bennett, 1993 #4492] encodes amino acids with no in-frame stop codon or nonsense codon. This led to the cloning of further upstream sequence and an N-terminal extension containing a functional ER targeting signal was found.
4.2 Results

4.2.1 Characterisation of syn5 antipeptide antibodies

Using affinity purified antipeptide antibodies (Fig. 4.2) to probe purified rat liver Golgi membranes, one major and two minor proteins were revealed (Fig. 4.3). The major protein (35kD) had a molecular weight consistent with that of the published rat syn5 sequence ((Bennett et al., 1993); 35kD vs. 34.1kD). The staining pattern was resistant to carbonate washing (Fig. 4.3, lane 5) or pre-treatment with 1M KCl (Fig. 4.3, lane 3). These results are consistent with the syn5 being a type II integral membrane protein.

The minor protein of lower molecular weight (33kD) was likely a degradation product, cleaved near the membrane anchor, since it was largely removed by carbonate washing (Fig. 4.3, lane 5). It was also not found in whole liver extracts (Fig. 4.8, bottom panel) or NRK cells (Fig 4.9).

Prior treatment of the antibodies with the specific peptides abolished staining of all three proteins showing that the reaction was specific (Fig. 4.4, middle panel). Treatment with the irrelevant peptides did not change the antibody staining pattern (Fig. 4.4, bottom panel).

Clearcut Golgi staining in indirect immunofluorescence was not obtained using affinity purified antipeptide antibodies against syn5 (data not shown). Several different protocols for fixing cells have been tried but they did not make any marked improvements in the quality of the immunofluorescence data.
4.2.2 42kD protein

Another minor protein (42kD) was also observed using these antipeptide antibodies raised against different parts of the published sequence (NHU1, 3 and 4; Fig. 4.3) and

Figure 4.3 Characterisation of antipeptide antibodies against rat syn5. Golgi membranes from rat liver (25 µg, lane 1) were separated by SDS-PAGE and Western blotted using affinity-purified antipeptide antibodies as indicated. Antibody binding was not affected when Golgi membranes were pre-treated with 1 M KCl (lane 2: supernatant; lane 3: pellet) or 0.1 M Na_{2}CO_{3}, pH 11.5 (lane 4: supernatant; lane 5: pellet).

Figure 4.4 Specificity of syn5 antipeptide antibodies. Golgi membranes from rat liver (25 µg/lane) were separated by SDS-PAGE and Western blotted using affinity-purified antipeptide antibodies as indicated (top panel). Antibody binding was blocked by prior treatment with the specific peptides (middle panel) but was not affected with irrelevant peptides from MannII sequence (bottom panel).

Figure 4.5 Topology studies of p42. Golgi membranes from rat liver (25 µg/lane) were fractionated by SDS-PAGE (15% gel) and Western blotted using affinity-purified, anti-peptide antibodies which recognised both forms of syn5 (NHU4) or only the longer form (NHU5). Antibody binding was blocked by prior treatment with the specific peptides (lanes 1 and 5). Binding was not affected when Golgi membranes were pre-treated with 0.1 M Na_{2}CO_{3}, pH 11.5 (lanes 3 & 7) but was abolished by pre-treatment with 0.05 mg/ml trypsin (lanes 4 & 8). The asterisk indicates a likely proteolytic fragment of the 35kDa syn5.

Figure 4.6 Digestion with chymotrypsin; Golgi membranes from rat liver (25 µg/lane) was treated with increasing amount of chymotrypsin (lane 1: control; lane 2: 0.001 mg/ml; lane 3: 0.01 mg/ml; lane 4: 0.1 mg/ml) and fractionated by SDS-PAGE (15% gel) and transferred to a nitrocellulose membrane. The blot was cut in two and Western blotted using affinity-purified His-syn5 antibodies (bottom half) or polyclonal antibodies against MannII (top half).
in all cases staining was blocked by pre-treatment of the antibodies with the peptide to which it was raised but not by any of the other peptides (Fig. 4.4). This suggests that the 42kD protein is not a non-specific cross-reaction of the antibodies used.

This was confirmed by raising an antipeptide antibody to part of the sequence upstream of the predicted start site (NHU5; Fig. 4.2). I focused on the N-terminus because there were no predicted stop codons in the published upstream sequence which meant that translation from an upstream start codon might be possible, explaining the longer form.

The NHU5 antipeptide antibody only recognised the 42kD protein, not the 35kD syn5 (Fig. 4.5, lane 6). Labelling was specifically blocked by the NHU5 peptide (Fig. 4.5, lane 5) and was resistant to carbonate washing (Fig. 4.5, lane 7) but sensitive to protease (Fig. 4.5, lane 8) under conditions where lumenally-oriented proteins such as Mannosidase II were insensitive (Fig. 4.6). These results are consistent with the 42kDa protein being an integral membrane protein, with most of its mass projecting into the cytoplasm, exactly the same topology as the 35kD syn5 (Fig. 4.5, lanes 1-4).

NHU5 gave some Golgi-like staining pattern, which seemed to co-localise with GM130 staining (Fig. 4.7), but the background was very high and the nucleus was also stained, indicating specificity was very low.

The 42kDa protein could represent an intermediate in the biosynthetic pathway of syn5 that is cleaved to yield the mature 35kDa product. This was tested by treating NRK cells with cycloheximide for increasing times up to 8 hours. If it were a metabolic intermediate of the 35kD syn5, NRK cells were incubated in the presence (lane 1) or absence (lane 2) of 10 μg/ml cyclo-heximide for 8 hr then fractionated by SDS-PAGE and Western blotted using affinity-purified antibodies to recombinant His-syn5.
precursor, the levels should drop with time as it is converted to the mature form. As shown in Fig. 4.8, this did not happen, the ratio of the 35kDa to the 42kDa forms remaining constant over time. Post-translational modifications such as phosphorylation (T. Levine, personal communication) could also not explain the difference in molecular weight suggesting that the 42kDa protein is an isoform of syn5.

4.2.3 Expression and tissue distribution

Syn5 is broadly expressed as shown both by Northern blot (Bennett et al., 1993 and Fig. 4.9, top panel) and Western blots. A variety of rat tissues were probed with an affinity-purified antibody raised to recombinant syn5 (His-syn5). All tissues contained the 42kDa protein in addition to the 35kDa syn5 (Fig. 4.9, bottom panel). Labelling was blocked by pre-treatment of the antibody with the recombinant protein (data not shown). The absolute amounts varied from tissue to tissue as did the relative amounts within a tissue. There was very little of either form in heart or muscle tissue. Additional forms were observed in liver, lung, spleen and testis but their significance is presently unclear. Both forms were also observed in various cultured cell lines (Fig. 4.10). The lower apparent molecular weight for both forms in 3T3 cells may represent a cell-type specific modification that affects the migration on SDS-PAGE though the nature of this was not investigated.

The 42kD form of syn5 is not predicted in yeast syn5 sequence. The presence of this new, longer form in mammalian cells might point to functions specific in mammals. One possibility is that the expression of the 42kD form of syn5 could be a response to some sort of stress. This was tested by treating NRK cells at 42°C for increasing amount of time and then let the cells recover at normal temperature (Fig. 4.11). The ratio of the 42kD to 35kD forms of syn5 did not change with the duration of heat-shock, indicating the 42kD form was normally expressed in cells under physiological conditions, not induced by the stress of heat-shock.
4.2.4 N-terminal extension

By using specific antibodies a 42kD protein with the same biochemical properties as the 35kD syn5 was identified. Its resistance to extraction with sodium carbonate showed that it is a membrane protein. Its complete sensitivity to proteases showed that it had most of its mass on the cytoplasmic side of the membrane. It reacted with three antipeptide antibodies raised to rat syn5 as well as uniquely with an anti-peptide antibody raised to the predicted N-terminal extension. Treatment for extended times with cycloheximide showed that the 42kD protein was not a precursor of the 35kD syn5 since no change in the relative amounts was observed. Both these two proteins were broadly expressed in all rat tissues and cultured cell lines tested and the expression was not stress-induced. All these evidence suggests that there is an isoform of syn5 present and its sequence contains the previously considered untranslated regions at the 5' end in the published DNA sequence.

To find the upstream start codon, further sequence was obtained using 5' RACE on a poly A+ RNA library isolated from rat liver (section 2.3.7). Using synthesised oligonucleotides syn5.11 and syn5.10 as the gene-specific primers, DNA fragments of 800 b.p. and 1000 b.p. were obtained (Fig. 4.12). This is 200 b.p. longer than what the
Targeting

Published syn5 sequence would give using these primers, (Fig 2.3). The length of DNA obtained should be 600 b.p. and 800 b.p. respectively. These fragments were extracted from the agarose gel and cloned into pBSII KS+ plasmid and transformed into E.coli strain JM101 for sequence analysis.

Six independent clones of different lengths were sequenced. The overlapping sequences were all identical. A predicted, in-frame, start codon (ATG) was found 162 bases (54 amino acids) upstream of the original start site. An in-frame stop codon (TGA) was located 120 bases further upstream of this new ATG (Fig. 4.13). The predicted N-terminal extension in rat syn5 showed very high homology (87-90% similarity; 84-89% identity) to human EST database entries H21702, H83290, T09399, T30862, T31170 and W96260 (Fig. 4.14). The predicted molecular weight of the novel syn5 isoform, starting at the upstream start codon, is 39.8kDa, similar to the 42kDa observed by Western blotting (Fig. 4.3, 4.4, 4.5).

![Genbank sequence alignment](image)

Figure 4.13 Sequence of the N-terminal extension of rat syn5. Extra nucleotide sequence (small letters) and the predicted amino acid sequence (italics) were obtained through 5'RACE upstream from the published sequence. The first predicted start codon (atg), the second start (ATG) and the stop codon (tga) in the extra sequence are boxed. Two longest RACE clones are indicated (arrowheads).
Figure 4.14. Homologies of syn5 N-terminal extension to human EST entries. Nucleotide sequence of the rat syn5 N-terminal extension (finalsynS) was aligned to human EST database entries using the gel assemble program in GCG and the consensus is shown. For syn5 sequence the ORF is shown in capital letters with the second start codon shown as atg. For EST entries unknown nucleotides are shown as N. Reverse background highlights differences in sequences. The consensus is shown in capital letters if all sequences are identical.
To check if the sequence obtained by 5'-RACE gives rise to proteins of the same MW as observed by Western blotting, coupled in vitro transcription/translation was carried out using the rabbit reticulocyte system. Briefly, cDNA coding for the whole syn5 was constructed from the RACE products and the published cDNA (Fig. 4.15) and inserted into pBSII/KS+ vector with T3 and T7 primer sites flanking the insert. Using T3 or T7 polymerase inserts in either directions can be translated into proteins. Translation was done in the presence of 35S-methionine for subsequent detection of the translated products using fluorography.
As seen in Fig. 4.16 (lane 1), two distinct bands were obtained after the full length cDNA was translated in vitro. The apparent MW of these two bands was identical to that obtained by Western blot. The ratio between the p35 and p42 (44%) also reflects the in vivo ratio (40-45%).

**4.2.5 Alternative initiation**

Northern blot analysis of rat tissues suggested that there was only one mRNA for syn5 (Fig. 4.9 and Bennett et al., 1993) raising the possibility that alternative start sites determine the translation of the two syntaxin isoforms.

Evidence in favour of this possibility was obtained by in vitro transcription/translation using cDNAs engineered in such a way that translation could only occur from the first or second ATG (section 2.3.8). Translation from the second ATG was ensured using the cDNA encoding the original published sequence. Translation from the first ATG involved using the full-length cDNA and changing the second ATG to CTG (encoding isoleucine).

As shown in Figure 4.16, transcription/translation of the full-length cDNA yielded both the 35 and 42kD forms of syn5 (lane 1). Translation from the first ATG yielded the higher, 42kD (lane 3 &4), form, whereas translation from the second ATG yielded the 35kD form (lane 2).

To relate these proteins to those identified by Western blotting (Figure 1A), they were immuno-precipitated using antibodies to the common part of the syntaxin (NHU4) and to the new N-terminus (NHU5). As shown in Figure 4.17, the NHU4 antibodies precipitated both the 35 and 42kDa forms (lanes 1-3) whereas the NHU5 antibodies only recognised the 42kDa form (lanes 4-6). Interestingly, a small amount of the 35kDa form was also precipitated when the full-length cDNA was transcribed and translated in vitro (lanes 5 & 6).
translated suggesting the formation of hetero-dimers (lane 7). This presumably reflects
the known capacity of syn5 to form dimers [Banfield, 1995 #2704].

4.2.6 ER targeting signal

A possible role for the N-terminal extension in the 42kDa form of rat syn5 was revealed
by examination of the sequence. As shown in Fig. 4.13, there was a double-arginine
motif (RKR) at positions 4 and 6 from the start methionine. Exactly the same motif
was found in the human sequence (EST entries T09399, T31170 and T30862).

This motif was first identified in human MHC Class II invariant chain. Two major
forms are synthesised differing by an N-terminal extension of 16 amino acids (Strubin
et al., 1984). The form lacking this extension (p31) moves to endosomes whereas the
form containing this extension (p33) is localised to the ER (Lotteau et al., 1990). A
double-arginine near the N-terminus of the extension was shown to be both necessary
and sufficient for localising reporter molecules to the ER. Extensive mutagenesis
studies defined a consensus sequence for this motif (Schutze et al., 1994). The double-
arginine in the N-terminal extension of syn5 matches this consensus.

To confirm this experimentally, the first 16 amino acids of the N-terminal extension
were grafted onto the p31 invariant chain (section 2.3.9). In another construct, the
double-arginine motif (RKR) was replaced by SSS. The original start methionine in p31
was changed to an isoleucine so that only the full-length fusion protein was synthesised.

HeLa cells were transiently transfected with these constructs. After 48 hr, the cells
were fixed, permeabilised and labelled with antibodies to the lumenal domain of
invariant chain followed by secondary antibodies coupled to FITC. As shown in Figure
4.18, p31 was localised to large vacuolar structures previously identified as endosomes
(Romagnoli et al., 1993; top panel). In marked contrast, the p31 with the N-terminal,
double-arginine motif was localised to the endoplasmic reticulum, comprising the
nuclear envelope and an extensive reticular network throughout the cytoplasm (middle
panel). This location was determined by the double-arginine motif because the
chimeric p31 in which this motif was replaced by SSS was again localised to
endosomes (bottom panel).
Figure 4.18 Immunofluorescence microscopy of p31 constructs. HeLa cells were transfected with cDNAs encoding the constructs shown above each panel. Top: p31; Middle: The first 16 N-terminal amino acids of the 42kDa syn5 were grafted onto the N-terminus of p31; Bottom: The RKR motif was changed to SSS. Note that the p31 start methionine was changed to isoleucine in the chimeras to ensure translation only of the full-length constructs. 48 hours after transient transfection the cells were fixed, permeabilised and labelled with a monoclonal antibody to p31 followed by secondary antibodies coupled to FITC. (Bar=10 μm)
Figure 4.19 Gradient distribution of the syn5 isoforms. Post-nuclear supernatants from rat liver homogenates were sedimented to equilibrium on a 0.5-2 M sucrose gradient. The re-isolated membranes were fractionated by SDS-PAGE and Western blotted using antibodies to a recombinant syn5, an ER marker (calnexin) and a marker for the intermediate compartment (ERGIC53/p58). Results were visualised using ECL and quantitated. The membranes were assayed directly for the Golgi marker enzyme, β 1,4-galactosyltransferase.
4.2.7 Localisation of the 42kD syn5

Though the transient transfection studies clearly showed that the double-arginine motif in the 42kD syn5 was both necessary and sufficient to localise a reporter molecule to the ER, this does not mean that the motif is functional in syn5 in vivo. If it is, then the distribution of the two syn5 forms should differ. This was assessed by fractionation of rat liver.

Post-nuclear supernatants were prepared from rat liver homogenates and sedimented to equilibrium on sucrose gradients. Membranes were recovered from each fraction, and proteins separated by SDS-PAGE then Western blotted using antibodies to markers of the Golgi, intermediate compartment and ER as well as the two forms of syn5. The results were quantitated using ECL and appropriate internal controls. A typical experiment is shown in Figure 4.19 (A-C). The results from three experiments were averaged and are presented in Figure 4.19 (D).

Both forms of syn5 were found in two peaks though in different amounts (Fig. 3.19 A). More than 60% of the short syn5 was present in the lighter peak which co-fractionated with the Golgi marker, GalT (Roth and Berger, 1982) (Fig. 4.21 B and D). The longer syn5 was mostly present in the denser peak which overlapped partially with the bulk ER marker, calnexin (Wada et al., 1991) (Fig. 4.19 B). In fact, both peaks of the longer syn5 matched almost exactly the two peaks of the IC marker, ERGIC53/p58 (Lippincott Schwartz et al., 1990; Saraste et al., 1987; Schweizer et al., 1988; Schweizer et al., 1990) (Fig. 4.19 C).

The precise localisation of the 42kD form has so far been precluded by the fact that the antibodies to the N-terminal extension (NHU5) or to both forms of syn5 (NHU1-4) do not work for either immunofluorescence or immuno-EM, possibly because the highest affinity population of antibodies were retained on the peptide column and did not elute, or that in cells fixed for immunofluorescence or immuno-EM the epitopes for antibody binding are somehow masked.

4.2.8 Syn5-binding proteins

Using antipeptide antibody (NHU4) against both forms of syn5 cross-linked to PAS beads to immunoprecipitate a detergent extract of purified rat liver Golgi, a protein of apparent MW of 64kD (p64) was specifically co-immunoprecipitated with syn5 as observed on silver stained gel (Fig. 4.20). It was not associated with syn5 if the
antibody was pre-treated with NHU4 peptide, indicating its association with syn5 in detergent extract was specific.

The apparent MW of p64 matches that of the Sec1 family protein (section 1.3.7) that regulates t-SNARE functions and were shown to associate with known t-SNAREs. Therefore I decided to scale up the immunoprecipitation to identify p64 as antibodies against the Sec1 family proteins were not available.

The immunoprecipitates were blotted onto PVDF membranes for sequence determination by mass-spectrometry. However, p64 did not transfer very well either by semi-dry blotting or using wet-blotting method. In addition, it does not react with the sulpho-rhodamine dye used to visualise the position of blotted proteins. Furthermore, p64 has the same molecular weight as human keratins, a common source of contamination that swamps the signal in mass spectrometry.

To get around these technical difficulties, numerous Coomassie stained p64 bands were excised from the gels and sent to Dr. J. Vandekerckhove's lab in Belgium for direct sequencing. Regrettably nothing came out of this collaboration. This protein was later identified as being rSly1 (Dascher and Balch, 1996) using the same approach. Over-expression of rSly1 was shown to neutralize the dominant negative effect of excess syn5 on ER-Golgi transport, suggesting that rSly1 positively regulate syn5 function (Dascher and Balch, 1996).

Figure 4.20 Immunoprecipitation of syn5. Antipeptide antibody NHU4 was cross-linked to PAS beads and used to immunoprecipitate a detergent extract of Golgi membranes. The beads were boiled directly in SDS-PAGE sample buffer and fractionated (10% gel.) and silver stained. PAS-antibody was added (lane 1) or treated with specific peptides (lane 2). Supernatant after immunoprecipitation (lane 3) was also loaded on the gel.
4.3 Discussion

4.3.1 Two forms of syn5

The two forms appear to be generated from a single mRNA by alternative initiation of translation. Northern blotting suggested a single species of mRNA (Bennett et al., 1993) and Fig. 4.9) and in vitro transcription/translation of the full-length cDNA generated both forms. Translation exclusively from the first ATG yielded the 42kD form whereas translation from the second yielded the 35kD form. These were shown by immuno-precipitation to be the same proteins as those observed by Western blotting. Both were precipitated by antibodies to the common part of the sequence but only the 42kD form was precipitated by antibodies to the predicted N-terminal extension.

The 42kD protein was the minor form. Quantitation of rat tissue blots (Fig. 4.9) showed that the longer form constituted 25 to 40% of the total syn5. In NRK cells it comprised 40-45% (Fig. 4.8) and after in vitro transcription/translation in a reticulocyte lysate, 40% (Fig. 4.16 & 17). There was about 40% in rat liver (Fig. 4.9) but only about 10 to 20% in the Golgi membranes purified from rat liver (Fig. 4.3-6). This is because less of the 42kD form co-fractionated with Golgi membranes when compared with the 35kD form (Fig. 4.19 A & B). The minority amounts of the 42kD form can best be explained by the alternative initiation sites on the syn5 mRNA.

4.3.2 Alternative initiation

The bases flanking the initiation site determine the efficiency with which it is recognised by the scanning ribosome. The optimal sequence is: gcc gcc A/Gcc (AUG) GA/Cu, the relative importance of each residue being indicated by UPPER CASE BOLD>UPPER CASE>lower case (Grünert and Jackson, 1994). When applied to the syn5 mRNA, the first start site has only 4 matches to the flanking bases (tgc gac teg (ATG) ate) whereas the second start site has 7 (gcc gac Act (ATG) tCc) including an important A at position -3. These start sites sequences would predict that less of the longer form is translated, in accordance with the experimental results.

4.3.3 ER targeting signal

Examination of the N-terminal sequence revealed a double-arginine motif which was first identified as an ER localisation signal in invariant chain and other type II membrane proteins (Schutze et al., 1994). Invariant chain exists predominantly in two forms which differ by an N-terminal extension of 16 amino acids containing the motif (Strubin et al., 1986). The motif in the 42kD syn5 was shown to operate as an ER
targeting signal by grafting it onto the end of the p31 invariant chain lacking the motif. Instead of moving to endosomes as p31 normally does, the chimera was localised to the ER. Targeting to the ER was dependent on the double-arginine motif since conversion of RKR to SSS restored transport to the endosomes. It is still not clear whether the double-arginine motif acts as a retention or a retrieval signal (Nilsson et al., 1994) but the latter seems more likely given the functioning of the closely related double-lysine motif found in type I membrane proteins (Jackson et al., 1990; Nilsson et al., 1989). This motif functions as a retrieval signal (Jackson et al., 1993) by interacting with the coatamer of retrograde COPI vesicles (Cosson and Letourneur, 1994; Letourneur et al., 1994).

Retrieval could provide a functional explanation for the N-terminal extension in syn5. Multiple signals operate to locate the short form of syn5 in the early Golgi (Banfield et al., 1994) and similar signals are likely present in other t-SNAREs. In one well-documented case, the t-SNARE complex, syntaxin 1 and SNAP-25, was found in synaptic vesicles as well as the plasma membrane (Walch Solimena et al., 1995) suggesting that retention signals might not be sufficient to localise t-SNAREs completely to the target compartment. If this were to apply to syn5 then any of the protein that strays beyond the early Golgi compartment could be returned to the ER using the double-arginine motif on the long form. It is not clear why this motif would then permit subsequent transport from the ER to the early Golgi.

There is, however, precedence in those type I proteins that carry a double-lysine motif (section 1.4.2) yet are not located in the ER. The mannose-binding lectin, ERGIC53/p58, carries such a motif yet is located in the intermediate compartment. The motif appears to be weakened by the presence of an adjacent double-phenylalanine permitting exit from the ER (Itin et al., 1995). The same may be true for syn5 so further work will be needed to determine whether other residues in the extension are responsible for weakening the ER targeting signal.

Of course, in order for this salvage mechanism to work on the entire population of syn5 molecules, the long form, being in the minority, would have to form hetero-dimers or probably higher order oligomers with the short form. Homo-oligomers have been reported for the Drosophila Sed5p (Banfield et al., 1994) and the possibility of hetero-oligomers is suggested by the in vitro transcription/translation experiment presented in Figure 4.17 (lane 4). In addition, the long form would either have to serve as a functional t-SNARE or at least not inhibit the function of its short partner. Preliminary experiments have shown that both forms of syn5 are present in the 20S fusion complex that also contains NSF and SNAPs. Furthermore, hydrolysis of ATP breaks up this
complex releasing both forms (T. Levine, personal communication).

### 4.3.4 Possible functions of 42kD syn5

Salvage of lost syn5 molecules may not, however, be the only function because syn5 behaves, not as one, but as two populations on sucrose gradients (Fig. 4.19). One population is enriched in the short form and fractionates with the Golgi apparatus. The other population is enriched in the longer form and fractionates with the ER/intermediate compartment. Two possible functions suggest themselves.

The first is that the long form is involved in the biosynthesis of the syn5 complex in the ER. This possibility is based on the precedent set by the biosynthesis of the MHC Class II complex. The two forms of invariant chain (p31 and p33) are generated by differential initiation of translation which then form mixed trimers. This is followed by the stepwise addition of three Class II a/b dimers. This is an unusually long process and the ER targeting signal in p33 is thought to delay exit until assembly of the nonamer is complete (Lamb and Cresswell, 1992).

t-SNAREs could be at least as complex. Each probably contains one or more copies of a syntaxin family member, a SNAP-25 family member and/or a Sec1/Sly1 family member (Bennett, 1995; Pevsner, 1996; Pevsner et al., 1994). The assembly of such a complex might be sufficiently time-consuming to merit the presence of an ER targeting signal to delay exit. However, t-SNAREs are tail-anchored proteins and probably are post-translationally inserted into the ER membrane independent of the SRP (section 1.2.1). The ER targeting signal would only be required if the assembly takes place after membrane insertion of the various components.

After movement to the Golgi, the short form of syn5 would take up residence leaving the longer form to recycle back to the ER for further rounds of assembly. Such a catalytic role could explain the lower levels of the longer form.

If localisation to the ER is essential for the assembly of t-SNARE complexes, it should not be limited to syn5. Interestingly, many of the published sequences of syntaxin family members have no upstream, in-frame, stop codons raising the possibility that longer forms exist. Those that do have up-stream stop codons (e.g. syntaxins 1 and 3) might exist as alternative transcripts. More work is clearly needed to examine upstream sequences and the early stages of t-SNARE assembly.

The second possible function is based on the fact that the longer form is not predicted from the budding yeast sequence (Hardwick and Pelham, 1992). There are four in-
frame stop codons in the 30 to 101 amino acids upstream of the putative start methionine. The yeast sequence, and most others, has what at first sight appears to be an ER targeting signal but the double-arginine motif is split by an aspartic acid which presumably inactivates its function. If the yeast lacks the longer form with an ER targeting signal, what differences are there between the yeast and mammalian exocytic pathways that might explain the need for the longer form in mammals?

The most striking difference is the physical separation of the Golgi apparatus and the ER exit sites in mammals. In yeast the distances are very small because of the small size of the cell and the fact that there are multiple dispersed Golgi throughout the cell cytoplasm (Preuss et al., 1992). Vesicles budding from the ER are never far from a Golgi. In mammals, the distances are much larger. Most of the ER exit sites are in the cell periphery (Bannykh et al., 1996) and cargo must be transported ten or more microns to the Golgi ribbon in the juxta-nuclear region (Griffiths et al., 1995; Saraste and Svensson, 1991). Vesicles budding from the ER might need a representative of the Golgi apparatus nearby and such an outpost could be provided by the longer syn5. This outpost might be needed to form the tubulo-vesicular intermediates that are transported along microtubules to the juxta-nuclear ribbon (Balch et al., 1994; Saraste and Svensson, 1991). The short form of syn5 would remain in the Golgi ribbon receiving cargo from local exit sites. Such a function would explain the enrichment of the short form in Golgi fractions and the co-fractionation of the longer form with ERGIC53/p58, which is known to cycle through this peripheral route (Itin et al., 1995; Lippincott Schwartz et al., 1990).

A proper test of this function will, however, only be possible once antibodies are available that can localise the longer form using microscopic techniques and can be used to block the function after micro-injection.
Chapter 5

Summary
I started the project to identify the components responsible for stacking the Golgi cisternae. A detergent insoluble cytoplasmic matrix was isolated and this could be responsible for Golgi stacking as proteolytic digestion of the matrix resulted in unstacking (Slusarewicz et al., 1994). By using a combination of mass spectrometry and protein sequencing, some of the components of the matrix were identified as being cytoskeletal proteins, e.g. actin and cytokeratins, while others turned out to be contaminants from the rat liver, e.g. rat uricase. The involvement of cytoskeletal proteins in the structure of the Golgi apparatus has been studied for mitotic assembly and disassembly of the Golgi and in drug-induced disassembly (Ayscough et al., 1993; Cleves et al., 1989; Duden et al., 1990; Fath and Burgess, 1993; Kreis, 1990; Lippincott Schwartz et al., 1990; Weiner et al., 1993). However, their importance in the stacking of the Golgi cisternae was not well characterised. This was not pursued further due to limitations in the protease digestion approach and difficulties in dissecting their specific contribution to Golgi stacking from their general functional importance in other parts of the cell.

Another objective in my project was to provide biochemical evidence for the existence of the kin oligomers. By adapting the protocol that isolated the cytoplasmic matrix to which medial Golgi enzymes bind (Slusarewicz et al., 1994), I extracted salt-washed Golgi membranes in detergent and salt followed by dialysis and sucrose density fractionation. An oligomer of estimated molecular weight of 2mD was isolated by size-exclusion chromatography. This oligomer was not the result of a non-specific aggregation of Golgi proteins as it can be repeatedly disassembled by addition of salt and reassembled after dialysis. It contained the medial Golgi enzymes MannII and NAGT I among numerous other proteins. Using 2-D gel to further separate the oligomer sub-units, some of the components were identified as being proteins homologous to the lectins ERGIC53/p58, which cycles between the ER, the IC and the Golgi (Itin et al., 1995; Lippincott Schwartz et al., 1990), and VIP36, which cycles between the Golgi and the plasma membrane (Fiedler et al., 1994; Fiedler and Simons, 1996; Itin et al., 1995). Other components were proteins involved in the regulation of vesicular transport (Rab6) and vesicle cargo receptors (p24 family proteins). Yet other components prove to be novel proteins (p65). These data suggest that the kin oligomers do exist in the Golgi and they not only contain medial Golgi proteins but also proteins in the cis- and trans- side of the Golgi apparatus. Further characterisation of the oligomer components are required before their functional importance in protein retention and structural maintenance of the Golgi apparatus can be investigated.

One of the implications of the SNARE hypothesis is the generation of the Golgi stack from closely apposed cisternae with the cognate v- and t-SNARE pairs docked but prevented from fusion by an additional fusion clamp (Rothman and Warren, 1994).
therefore studied the Golgi t-SNARE, syntaxin 5, as an alternative approach to the problem of Golgi stacking and was hoping to identify its fusion clamp. Using anti-peptide antibodies specific for syn5 to immunoprecipitate a detergent extract of Golgi membranes, I have found a protein of 64kD, similar in molecular weight to the putative fusion clamp identified in neuronal tissues (n-sec1, 64kD) (Pevsner et al., 1994), that specifically co-immunoprecipitated with syn5. Unfortunately, direct peptide sequencing did not provide any information on the identity of this protein and it was later identified to be rSly1 (Dascher and Balch, 1996).

Whilst pursuing this I found a longer form of syn5 (42kD) that contains an N-terminal extension in addition to the published syn5 (35kD) sequence. This isoform was generated from the same piece of syn5 mRNA by alternative initiation of translation. Further analysis of the new syn5 isoform revealed it contained a predicted type II ER targeting signal (MIPRKKR). When grafted onto a reporter molecule, the human MHC class II invariant chain p31, this signal localised the construct to the ER as assessed by immuno-fluorescence microscopy. This signal could function to retrieve syntaxin 5 from later Golgi compartments or participate in the biosynthesis of t-SNARE complexes.

It was also found that the 35kD syn5 was enriched in the Golgi (55-65%) and the 42kD syn5 co-fractionated with ERGIC53/p58, which is known to cycle between the ER, the IC and the Golgi (Itin et al., 1995; Lippincott Schwartz et al., 1990). In addition, the longer form is absent from the yeast homologue, Sed5p (Hardwick and Pelham, 1992). The functional test of these is currently being carried out in the lab using a new anti-peptide antibody against the first 14 amino acid of the N-terminal extension by immuno-EM and micro-injection techniques.


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