Protein transport and the reassembly of the Golgi apparatus during telophase in HeLa cells

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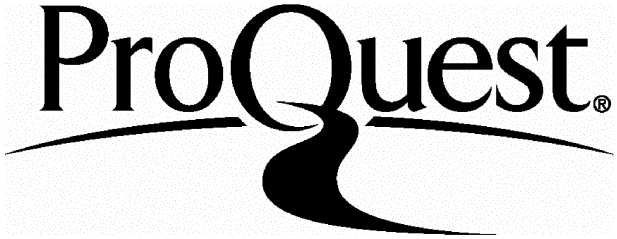

Abstract

At the onset of M phase in animal cells, the Golgi apparatus undergoes disassembly. The pathway of disassembly involves the generation of several hundred discrete Golgi stacks which break down further to yield vesicles. These vesicles are dispersed throughout the mitotic cytoplasm which subsequently provide the building blocks for the reassembly of two daughter organelles during telophase. Mitosis is also characterised by the arrest of a number of membrane-mediated transport processes including transfer of newly synthesised protein from the endoplasmic reticulum to the Golgi apparatus. The kinetic relationship between the resumption of this protein transport with the reassembly of the Golgi stack during telophase has been investigated in HeLa cells. Prometaphase-arrested cells were pulse-labelled with ^{35}S-methionine and chased in the absence of nocodazole to allow passage through mitosis and into G1. Resumption of transport of histocompatibility antigen (HLA) molecules to the *medial* and *trans* Golgi cisternae was measured by monitoring the resistance to endoglycosidase H and the acquisition of sialic acid residues respectively. Transport to the plasma membrane was measured using neuraminidase to remove sialic acid residues on surface HLA molecules. The half-time for transport to each of these compartments was about 70 min longer in cells progressing out of mitosis than in G1 cells. The half-time for reassembly of the Golgi stack, quantified using stereological procedures, was also 70 min, suggesting that both transport and reassembly are triggered at the same time. However, since reassembly is a more rapid process than the delivery of protein from the ER to the Golgi apparatus, the Golgi stack has reassembled by the time newly synthesised protein reaches it.

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CHAPTER 1: INTRODUCTION

1.1 Overview

Mitosis in animal cells is characterised by extensive reorganisation of the cytoarchitecture. One of the most striking of these mitotic transformations is the vesiculation of the Golgi apparatus. At the onset of mitosis, the apparatus undergoes disassembly, followed by dispersal and partitioning during mitosis, and finally reassembly of two daughter organelles at the end of mitosis.

Mitosis in mammalian cells is also characterised by the inhibition of a number of membrane-mediated transport processes. These include transport of newly synthesised plasma membrane proteins through the secretory pathway from the endoplasmic reticulum to the *trans* Golgi network, stimulated secretion of histamine by mast cells, receptor-mediated endocytosis and recycling of the transferrin receptor.

A simple hypothesis has been suggested which links the arrest of protein traffic through the Golgi apparatus with the fragmentation of the organelle during mitosis: if transport were to be arrested by the inhibition of membrane fusion while vesicle budding was maintained, the necessary consequence for the Golgi apparatus would be its disassembly (Warren, 1985). Conversely, at the end of mitosis, restoration of membrane fusion would allow both the resumption of vesicle traffic and the reassembly of the Golgi apparatus.

It is this latter aspect of Golgi reassembly which I have pursued during my thesis work. In particular, I have investigated the relative kinetics of protein transport recommencement and the morphological reassembly of the organelle to address the possibility that restoration of membrane fusion might be the trigger which allows both these processes to proceed.

In the course of this introductory chapter, I propose to review the current state of our understanding of a number of areas of cell biology relevant to the experimental work undertaken. Since the aim of the project was the relationship between protein traffic and the reassembly of the Golgi apparatus during M phase, there are three primary topics which immediately suggest themselves as important areas for review, namely intracellular transport, mitosis and Golgi morphology. I shall begin with a review of the progress that has been made in unravelling the complexity of the secretory pathway including the emerging picture of the molecular machinery underlying vesicular traffic. Mitotic control will be the second major topic to be discussed, including mitotic arrest of membrane traffic and the

morphological changes occurring during mitosis. This will lead conveniently to a review of the structure of the Golgi apparatus, both during interphase and mitosis.

1.2 Intracellular transport

In order to simplify the present discussion of intracellular transport, I shall focus on the exocytic pathway rather than endocytosis. Since the work undertaken has been confined to the default or constitutive pathway, the discussion of regulated secretion will be brief.

1.2.1 Compartmentalisation

The diversity of function within the cell requires an exquisite degree of organisation of structure at the subcellular level. Moreover, the necessity for efficiency in regulating the enzymatic changes that occur second by second also means that these reactions must be regulated both temporally and spatially. By doing so, the cell maximises the efficiency of its resources. The outcome of these selective pressures is the extensive series of membrane-bound compartments within the cell each one responsible for a defined set of reactions, carried out by a defined array of enzymes upon a defined series of substrates.

The biosynthetic secretory pathway is no exception to this requirement for efficiency in regulation. The pathway is responsible for the biosynthesis of proteins and lipids, their modification and processing, and the delivery of these proteins and lipids to the appropriate destination, whether an existing compartment within the cell, the plasma membrane or a destination outside the cell, in the case of multicellular organisms. There is also a need for degradation of unwanted material and partial digestion of molecules to be used in another form, reactions which clearly must be carefully enclosed and regulated. The efficient function of this pathway is essential then for the generation and maintenance of different compartments, for cell growth, division and differentiation.

The essential function of the secretory pathway in general combined with the diversity of specific modifications undertaken as molecules pass along the pathway has led to the generation of a series of compartments. The pioneering work of Palade and co-workers (Palade, 1975) in the 1960's and early 1970's identified the most crucial compartments of the regulated secretory pathway. Subsequent research has led to the further description of many other compartments and subcompartments that all play a role in the secretion of proteins and lipids. The process of identifying these structures and elucidating their function has been an exciting one, and it is clear that there remains a great deal to explore both in terms of other potentially important organelles as yet

obscure, and in the precise nature of the function of those organelles already identified.

1.2.2 The anatomy of the secretory pathway

Figure 1.1 represents a schematic of the biosynthetic secretory pathway in animal cells as we currently understand it. The essential features of this pathway are the endoplasmic reticulum (ER), the cis-Golgi network (CGN), the Golgi apparatus, the trans-Golgi network (TGN), the lysosomes and the secretory granules (SG).

The most extensive compartment of the endomembrane system is the ER, comprising a series of interconnected membrane sacs enclosing a central lumen. The ER can further be subdivided into regions lacking ribosomes, the so-called smooth ER, where fatty acids and lipids are synthesised, and ER with attached ribosomes (rough ER) which forms the site of protein synthesis and is connected to the nuclear envelope. Proteins are co-translationally translocated across the membrane of the rough ER, and are then transported via the transitional elements of the ER and the CGN to the Golgi apparatus.

The structure of the Golgi apparatus will be discussed in the final section of the chapter. Briefly, the organelle consists of a series of flat membrane sacs or "cisternae" with dilated rims, analogous to a stack of pitta bread. The stack of cisternae may be further subdivided into three subcompartments, namely *cis*, *medial* and *trans* both by structural and functional criteria. The orientation of these subcompartments shows a distinct polarity with respect to the ER, proteins arriving at the *cis* face of the organelle and then proceeding in a vectorial manner through the stack to the *trans* face at the other side. The CGN may be thought of as the entry control point of the Golgi apparatus ensuring that only legitimate cargo proteins continue to the Golgi while the TGN may be described as the sorting depot where proteins and lipids are allocated to appropriate destinations. The distinct nature of the CGN and TGN is most readily verified by subjecting the cell to temperature blocks. A shift to 20°C induces an enlargement of the TGN (Saraste and Kuismanen, 1984; Lippincott-Schwartz, *et al.*, 1990), while shifting the temperature to 15°C causes an accumulation of protein in the CGN (Griffiths and Simons, 1986).

Having successfully passed along this secretory conveyor belt, proteins and lipids can then be transported by default to the cell surface, or be diverted to lysosomes or secretory granules. Lysosomes provide the cell with the controlled environment required for the degradation of particular molecules. The membrane-limited interior of the lysosome is maintained at pH 5, the optimal pH for the degradative enzymes localised there. This ensures that the effect of a lysosomal enzyme

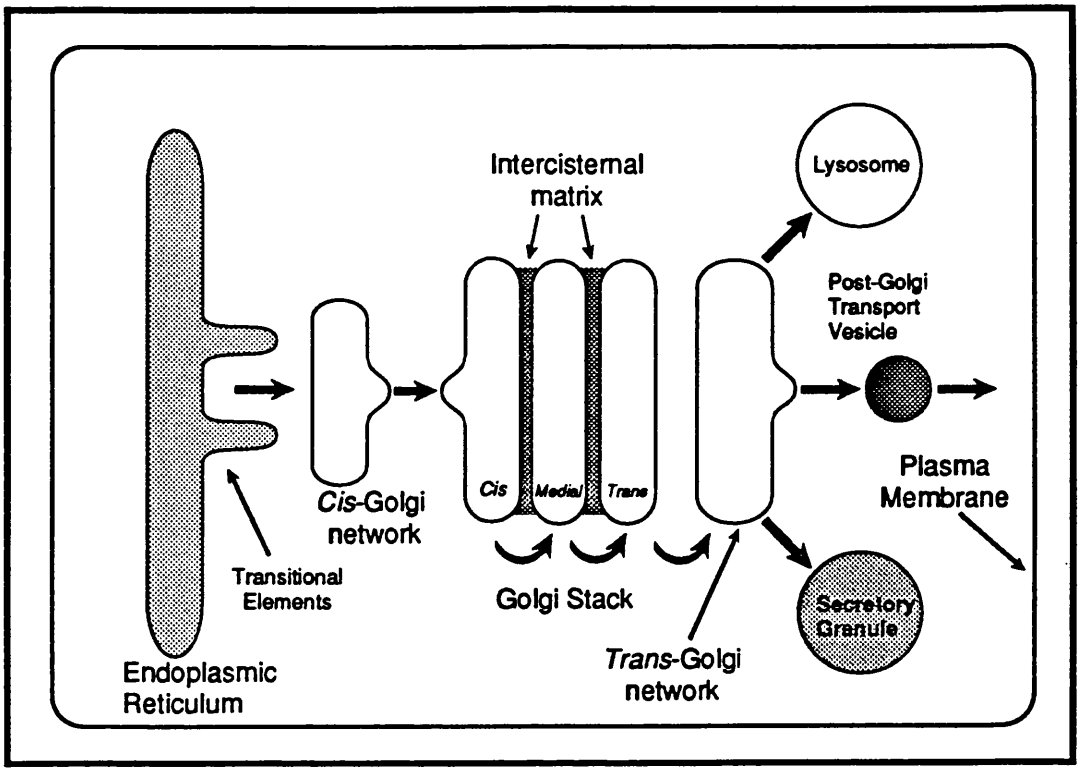

Figure 1.1 The secretory pathway in animal cells.

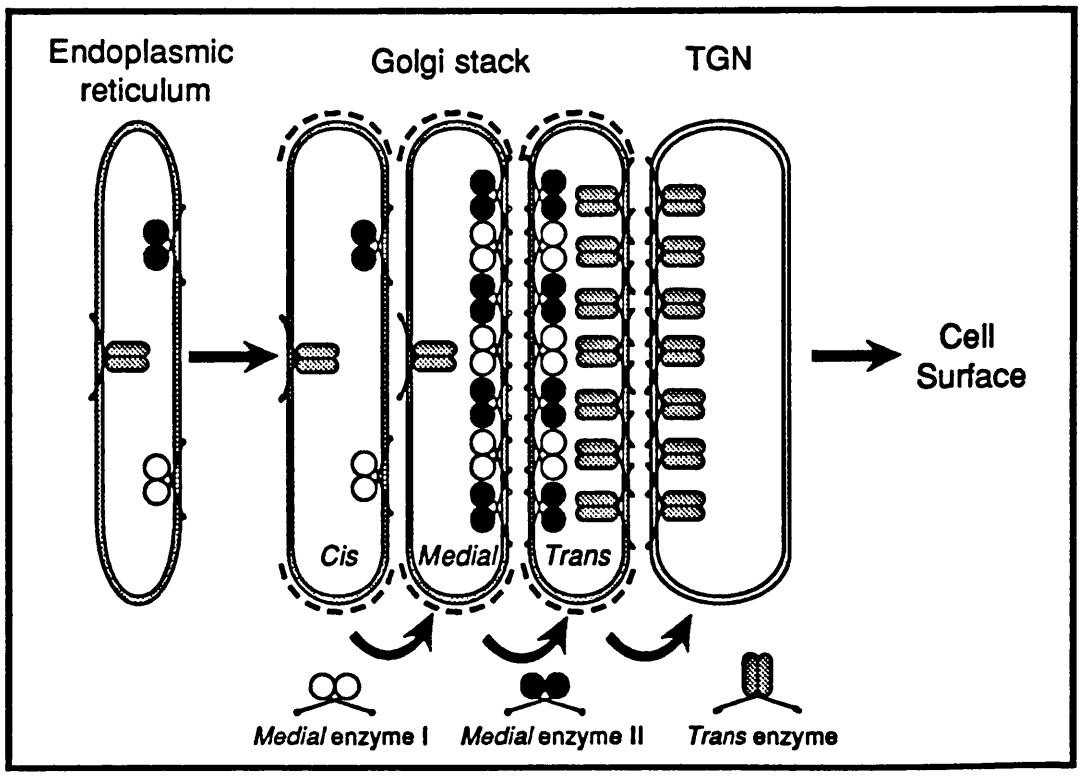

Figure 1.2 Retention of Golgi enzymes by "kin recognition".

leaking into the cytoplasm will be minimal since the pH level outside the lysosome is around 7, emphasising the importance of compartmentalisation within the cell.

1.2.3 Maintaining structure within the secretory pathway

The preceding description of the compartments along the secretory pathway has highlighted the high degree of organisation of the pathway and its polarity, both with respect to structure and function. It is clear then that the underlying mechanism of vesicle traffic between these compartments must also be controlled with respect to the content of carrier vesicles and the sequence of transport steps. The cell must both ensure that proteins that are to be secreted reach their appropriate destination and that proteins which are to be maintained as resident components of a particular compartment do not join the flow of transported molecules through the compartment and consequently arrive at a totally inappropriate location. In the following section, I shall outline what is known of the mechanisms whereby the cell achieves selectivity of transfer through the secretory pathway.

1.2.4 The nature of transport signals

Investigation into the nature of transport signals has led to a current model of regulation of protein sorting involving four distinct mechanisms which ensure the correct distribution of proteins through the secretory pathway (for reviews, see (Pfeffer and Rothman, 1987; Huttner and Tooze, 1989). The first mechanism is that of default whereby secretory proteins pass along the secretory pathway as part of the bulk flow; there is no specificity in this case, and no requirement for the protein to arrive at any particular destination other than the plasma membrane. The second level of sorting is that of diversion. Proteins that have a diverting signal pass by default along the secretory pathway until they reach the appropriate sorting depot (now recognised as the TGN) and are then diverted from the default pathway to a particular destination. A third level of sorting is that of retention which applies to functional molecules of the secretory pathway as opposed to secretory "passengers". Clearly, these proteins must be maintained in their appropriate compartment since the basic premise of compartmentalisation is that the resident molecules of a compartment define its particular function; loss of resident proteins implies loss of function. One mechanism of ensuring residency is to attach a signal to appropriate proteins which anchors them to the correct compartment. Finally, there is the solution of retrieval, ensuring that any functional residents of a compartment that escape and pass downstream of their appropriate compartment can be retrieved and returned to the right location. This solution obviously requires an efficient means of recognising escaped residents and

relocating them. Since retention of resident Golgi proteins is of particular relevance to my thesis work, I shall discuss these signals in more detail.

1.2.5 Golgi retention signals

Recent work from various laboratories has shown that the transmembrane region of resident Golgi molecules provides the signal required to specify retention within this organelle.

Investigation of the E1 glycoprotein of avian coronavirus (Swift and Machamer, 1991) involving the construction of chimeric proteins demonstrated the necessity and sufficiency of the first of the three transmembrane spanning domains of E1 for efficient Golgi retention. A number of subsequent studies of the retention requirements of endogenous Golgi proteins have similarly demonstrated the necessity of retention signals residing within the transmembrane spanning domain (Munro *et al.*, 1991; Nilsson, *et al.*, 1991; Aoki, *et al.*, 1992; Colley, *et al.*, 1992; Russo, *et al.*, 1992; Teasdale, *et al.*, 1992; Wong, *et al.*, 1992). In particular, Nilsson and co-workers (1991), analysing galactosyltransferase (GT), demonstrated that the crucial region of the enzyme was the lumenal half of the transmembrane domain together with a small number of residues from the proximal region of the lumenal domain. A cytoplasmic domain was also required but since the cytoplasmic region of the reporter protein served as well as that of GT, it seems that this region is required for stability rather than a specific interaction. Munro and colleagues (1991) investigated the retention requirement of sialyltransferase (ST), another type II protein resident within the *trans* Golgi. Chimeric protein constructions similarly demonstrated that the transmembrane domain conferred Golgi localisation, with additional efficiency of retention requiring the proximal sections of the molecule on either side of the membrane.

In spatial terms, the requirement of a retention mechanism within the Golgi may be viewed as a mechanism for preventing the lateral movement of resident enzymes into the dilated rims of each cisternae where vesicle budding occurs. Several mechanisms suggest themselves and may not necessarily be exclusive; a combination of a number of different methods may be utilised to ensure highly efficient retention of Golgi resident proteins. One mechanism might rely on a receptor protein being responsible for binding to and retaining functional proteins. However, such a protein would still have to have the capacity for selective retention in different subcompartments of the stack. Such specificity might be encoded in specialised membrane domains through the stack. An alternative hypothesis is that of protein oligomerisation caused by interactions of the transmembrane domains of Golgi proteins resident within the same cisterna (see Figure 1.2). Such aggregates would be prevented from entering dilated rims and

hence would not enter transport vesicles. Again, lipid gradients and/or domains might confer subcompartment specificity to such a retention mechanism. A prediction of such a "kin recognition" hypothesis would be that the alteration of residency of one Golgi enzyme to the ER would therefore cause the alteration of residency of other Golgi enzymes usually retained by oligomerisation with the first enzyme. By fusing an ER retention signal to a *medial* Golgi enzyme, Nilsson and co-workers have been able to induce ER localisation of another *medial* enzyme thereby confirming this prediction (Nilsson, *et al.*, 1993b). A recycling salvage receptor within the Golgi is yet a further refinement of Golgi retention which awaits demonstration.

With regard to the TGN, investigation of retention signals have focused on one particular protein, TGN38 which resides predominantly within the TGN but also recycles between the plasma membrane and TGN (Reaves, *et al.*, 1993). Cleavage of the cytoplasmic domain from TGN38, a resident of this compartment caused the protein to be secreted to the cell surface (Luzio, *et al.*, 1990) and chimeric experiments identified an 11 amino acid segment of the cytoplasmic domain responsible for TGN-localisation (Humphrey, *et al.*, 1993). More recent studies indicate that the signal within the cytoplasmic domain confers retrieval to the TGN, while a second signal within the transmembrane region confers TGN retention (Sreenivasan Ponnambalam, personal communication).

Although much progress has been made in the understanding of protein sorting along the secretory pathway, it is clear that there remains an enormous amount that is still obscure. That this should be so is a measure of the complexity of the protein sorting task faced by the components of the secretory pathway.

1.2.6 Lipid traffic

Before proceeding to a discussion of the molecular mechanisms of vesicle-mediated transfer of proteins along the secretory pathway, it is worth commenting on the nature of lipid traffic in eukaryotic cells. The considerable technical challenges of lipid biochemistry have meant that lipid research has in many ways lagged behind that of protein studies. However, the recognition of the crucial nature of lipid modifications of proteins together with the importance of lipid metabolism in cell physiology including cell signalling pathways has given rise to exciting developments in this area of exploration (reviewed by Pagano, 1990).

Since lipids form the basic building bricks of membrane bilayers, it is clear then that the biogenesis and distribution of lipids are of essential importance to the generation of the endomembrane system within the cell overall. Moreover, analysis of the structure of membrane compartments reveals the fact that lipid

species are not distributed randomly through the cell, but that particular species are enriched in particular locations. This localised enrichment of certain types of lipid may even apply within the same bilayer; there are many instances of an asymmetry between the cytoplasmic and lumenal leaflets of a bilayer. Since no individual compartment is capable of synthesising all of its component lipids, there is a basic requirement for efficient transport of lipids to appropriate compartments. Such transport may take the form of vesicle-mediated transfer, lateral diffusion along membrane bridges that may form transiently between compartments or by direct cytosolic transfer, either by a protein-mediated or protein-independent pathway. Once positioned in the correct compartment, lipid composition may be further altered by lipid degradation, modification or sorting within the leaflet or by a flip-flop mechanism between leaflets.

The technical challenge of dissecting these steps of transport of lipids has been greatly facilitated by the use of fluorescent lipid analogues containing N-(4-nitrobenzo-2-oxa-1,3-diazole) aminocaproic acid (C_6-NBD-fatty acid). The pathway of transport of lipids can then be monitored by carefully assessing the rate and sequence of labelling of different compartments within the cell after labelling with a specific lipid analogue (Koval and Pagano, 1989). Another major breakthrough is the *in vitro* reconstitution of lipid transport. Of particular interest for the study of the relationship between lipid and protein transport through the Golgi apparatus is a system that Wattenberg has reconstituted which demonstrated that glycoprotein and glycolipid transport through this organelle proceed with the same kinetics, suggesting that transport of these molecules occur by the same vesicle-mediated mechanism (Wattenberg, 1990). Research into membrane biology has shown that the energy requirement for the fusion of two lipid bilayers is enormous. Since vesicle-mediated traffic requires such events to occur at every round of transport, the mechanism of fusion must entail a highly refined enzymatic component. Such a prediction has been shown to be the case in studies to dissect transport at the molecular level.

1.2.7 Molecular mechanisms of vesicle-mediated transport

Having discussed some of the mechanisms the cell uses to regulate the contents of the carrier vesicles which ship secretory cargoes from one compartment to the next along the secretory pathway, and particularly how the functional components of each compartment are maintained within their appropriate compartments, it is of interest to review what is currently known of the molecular mechanisms underlying this vesicular traffic. The molecular components of the transport machinery form an entirely distinct subset of molecules along the secretory

pathway and have been found to be common to many different compartments of the pathway.

For the sake of simplicity, I propose to discuss the elucidation of the transport machinery within the Golgi apparatus first through *in vitro* reconstitution of these transport events, and then extend this to transport at other stages of the secretory pathway, illustrating the ways in which yeast genetics have confirmed the cell-free results and in many cases, uncovered new ground in the pursuit of a molecular understanding of intracellular transport.

1.2.8 Molecular dissection of intra-Golgi transport

The initial steps towards the reconstitution of transport through the Golgi apparatus in a cell-free system were made by Rothman and co-workers in the early 1980's. Early cell fusion experiments demonstrated protein transport between Golgi stacks of fused cells and that this transport was targeted and unidirectional. No lateral or retrograde transport could be detected (Rothman, *et al.*, 1984a,b).

Reconstitution of cell-free transport was made easier by the fact that the Golgi apparatus may be fractionated from other organelles relatively simply yielding Golgi stacks that display remarkably undisturbed morphology; recognisable stacks of cisternae are readily seen under the EM. If these Golgi stacks are isolated from cells that have been previously infected with Vesicular Stomatitus Virus (VSV) and are then incubated with ATP and cytosol (100,000g supernatant) from uninfected cells, 75nm vesicles containing VSV G protein are seen to form from each cisterna. Moreover, the concentration of G protein within the vesicles is equivalent to that of the cisternae and no "empty" vesicles may be seen (Balch, *et al.*, 1984; Orci, *et al.*, 1986). These observations indicated that the vesicles which form are *bona fide* transport vesicles identical to those which mediate bulk flow protein transfer between Golgi cisternae *in vivo.*

The development of a quantifiable cell-free assay paved the way for the subsequent molecular dissection of intra-Golgi transport (Balch, *et al.*, 1984). This assay relies on the absence of N-acetylglucosaminyltransferase I from the *medial* Golgi of clone 15B Chinese hamster ovary (CHO) cells. By mixing "donor" Golgi membranes from these 15B cells that have been infected with VSV and uninfected "acceptor" Golgi membranes from wild-type CHO cells, transport of VSV G protein from the *cis* cisterna of the donor Golgi to the *medial* cisterna of the WT acceptor membranes may be monitored; only the WT Golgi can catalyse the addition of tritiated N-acetylglucosamine to the G protein backbone. This assay provides a convenient means of monitoring transport-coupled glycosylation.

Using this assay Rothman and colleagues have gained considerable insight into the steps required during a single round of intercisternal transport. These steps may be broadly defined as coated vesicle budding at the donor compartment, vesicle scission, vesicle targeting, vesicle uncoating, and vesicle fusion at the acceptor compartment. A number of selective inhibitors of these successive steps were of crucial importance in the characterisation of the process. The use of GTPγS for example caused the accumulation of coated vesicles (Melançon, *et al.*, 1987). Use of this inhibitor therefore allowed the purification of Golgi coated vesicles (GCV) (Malhotra, *et al.*, 1989) and allowed the biochemical analysis of the proteins of the coat (Serafini, *et al.*, 1991). These proteins could be separated as a family of so-called "coat" proteins or "COPs", designated α,ß,γ,δ and a small GTP-binding protein previously described as the ADP-ribosylation protein or "ARF". These proteins were found to be present in stoichiometric amounts on the outside of transport vesicles and that they were peripherally associated; these observations were all consistent with their role in the formation of vesicle coats.

Subsequent cloning and sequencing of the ß-COP gene (Duden, *et al.*, 1991) revealed homology to a structural component of clathrin-coated vesicles of the endocytic pathway designated ß-adaptin, responsible for linking the clathrin lattice to the underlying membrane. Although GCV lack clathrin, the discovery of this homology between structural components of these two sorts of coated vesicles at least raises the possibility that there may be other levels of structural similarity between the coats of vesicles isolated from different sections of the secretory pathway. However, there remains a basic difference in the fact that CCV are selective carriers while GCV are bulk carriers, seemingly without the capacity to concentrate proteins within their contents.

Immuno-electron microscopy (immuno-EM) reveals the fact that the coat proteins are absent from the surface of Golgi membranes prior to transport *in vitro* and that the bulk of ß-COP is present in the cytosol in a complex with other coat proteins of 650-700K (Duden, *et al.*, 1991; Waters, *et al.*, 1991); this complex has been termed the "coatomer". ARF is also freely soluble in the cytosol as a single protein independent of the coatomer. In the light of the myristylation of the amino terminus of the ARF protein (Kahn, *et al.*, 1987), the fact that only ARF in the GTP-bound but not the GDP-bound state will spontaneously insert into lipid bilayers (Kahn *et al.*, 1991) and the necessity of ARF for ß-COP binding to Golgi membranes (Donaldson, *et al.*, 1992), Rothman and co-workers have suggested a model which might explain the formation of coated vesicles from the donor Golgi membrane. This model is summarised in Figure 1.3 and suggests that ARF is inserted into the donor membrane in its active GTP-bound state in which the myristate moiety is exposed, allowing interaction with lipids. This membrane

attachment causes the binding of the other coat proteins. After scission of the coated bud and targeting to the acceptor membrane, the GTP is hydrolysed, leading to uncoating of the vesicle and recycling of ARF to the inactive cytoplasmic pool. Such a model would explain the sensitivity of uncoating to GTPγS since without GTP hydrolysis there could be no ARF release, and therefore no uncoating. Another possible feature of such a mechanism is that the specificity of targeting to the correct acceptor membrane might be generated by particular ARF proteins since there is known to be a family of these proteins.

1.2.9 Proteins required for fusion of transport vesicles

The first protein required for vesicle fusion at the acceptor membrane was identified as a result of its sensitivity to N-ethylmaleimide (NEM). NEM causes an accumulation of uncoated vesicles and inhibition of transport-coupled glycosylation. By incubating NEM-pretreated membranes with cytosolic extracts, it is possible to restore transport. The factor responsible for transport restoration has been purified to homogeneity and is termed the NEM-sensitive fusion protein (NSF). NSF is present in cytosol as a 76K homotetramer (Block, *et al.*, 1988) which is stabilised by ATP and is required for membrane fusion but not for budding (Malhotra, *et al.*, 1988). Sequence analysis shows that NSF has three domains (Tagaya *et al.*, 1993). The two domains nearest to the C-terminus consist of two direct repeats which each contain an ATP-binding site. Mutation of these sites inhibits NSF function indicating that ATP hydrolysis is essential for fusion.

NSF is unable to bind to Golgi membranes on its own; a series of other factors mediate fusion. Of specific interest, three soluble NSF attachment proteins (SNAPs) have been purified: α-SNAP (35K), ß-SNAP(36K) and γ-SNAP(39K) (Weidman, *et al.*, 1989; Clary, *et al.*, 1990). The first two of these are very similar structurally while the third is very different, suggesting that either α-SNAP or β-SNAP may act in concert with γ-SNAP. SNAPs cannot bind NSF in solution but require the activity of an integral membrane receptor in the acceptor Golgi membrane to mediate NSF membrane binding. NSF, α-SNAP, γ-SNAP and the receptor assemble together in a 20S particle which is likely to be the core of the fusogenic particle at the acceptor membrane (Wilson, *et al.*, 1992). The precise mechanism of fusion remains obscure for the moment, though Rothman and colleagues have postulated a working model for the interaction of NSF, SNAPs and the SNAP receptor (see Figure 1.4). Recent work from the Rothman laboratory has shed considerable light on the docking mechanism by identifying two proteins, syntaxin and synaptobrevin, which suggest themselves as neuronal docking receptors situated on the vesicle membrane and the target presynaptic membrane (Whiteheart, *et al.*, 1993). It is tempting to speculate that this discovery

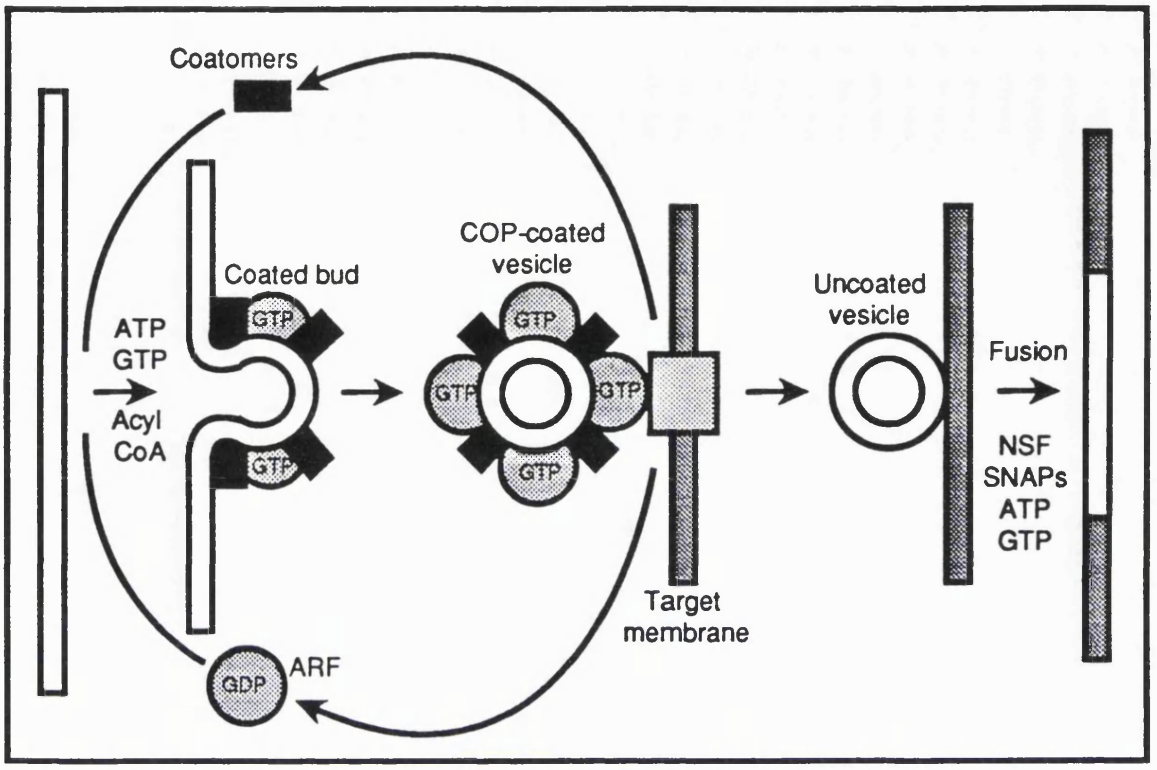

Figure 1.3 The ARF cycle during a single round of intra-Golgi transport.
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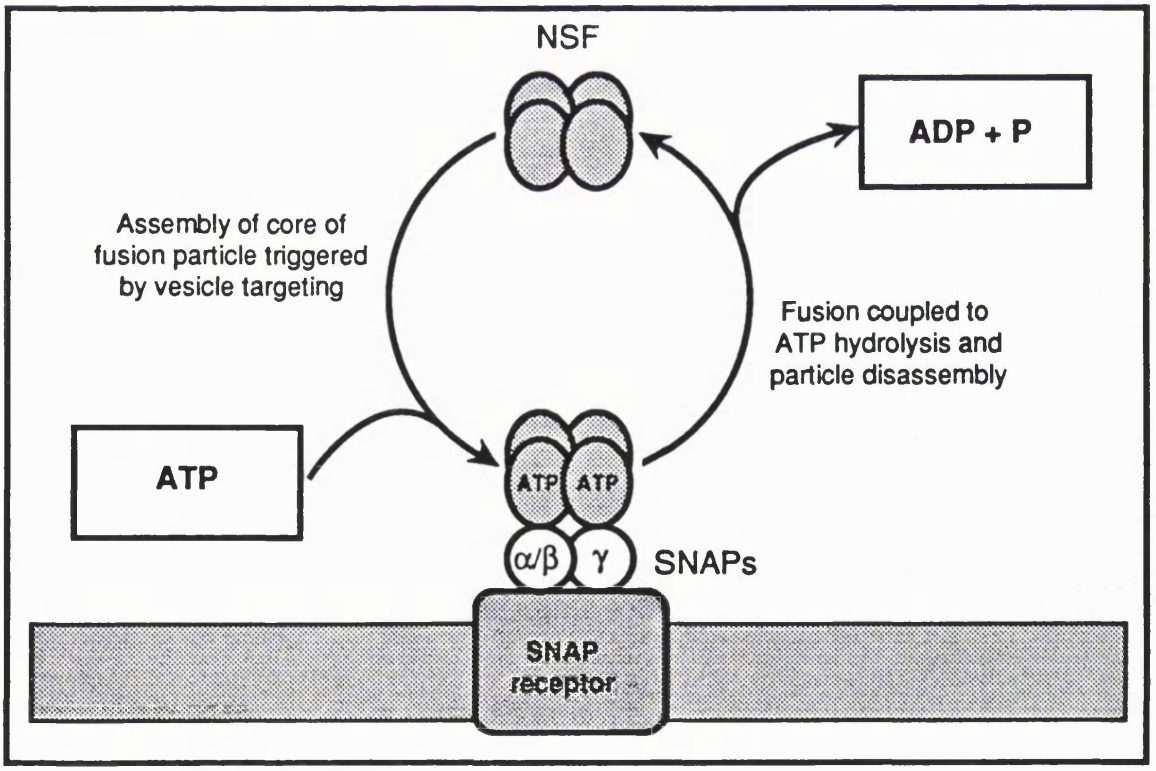

Figure 1.4 Model illustrating SNAP function during intra-Golgi transport.
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may be the key to unlocking the question of targeting specificity required in vesicular transport. The compartmental restriction of such vesicle SNAP receptors ("v-SNAREs") to appropriate donor membranes and target SNAP receptors ("t-SNAREs") to corresponding acceptor membranes would provide an ideal mechanism for specifying targeting information within both the exocytic and endocytic pathways (Warren, 1993a). Other cytosolic factors shown to be required for vesicle fusion after uncoating include acyl-CoA (Pfanner, *et al.*, 1989) and a novel peripheral membrane protein termed p115 (Waters, *et al.*, 1992).

1.2.10 Molecular dissection of ER to Golgi transport

The study of ER to Golgi transport *in vitro* required a different strategy to that of the Golgi system described above since homogenisation causes extensive fragmention of the ER. Much milder conditions were therefore required and these were achieved by generating semi-intact cell populations in which perforations were made in the plasma membrane allowing the passage of small soluble molecules into the cytoplasm of cells but maintaining the complex intracellular architecture, particularly that of the secretory pathway.

Reconstitution of ER to Golgi transport in such a perforated system has shown a requirement for both ATP and cytosol (Beckers, *et al.*, 1987). More specifically, this transport has been also shown to be dependent on NSF (Beckers, *et al.*, 1989; Wilson, *et al.*, 1989) and ARF (Balch, *et al.*, 1992), suggesting the universality of the emerging picture of transport mechanisms through the secretory pathway.

Further weight to confirm this conclusion has come from the extensive investigation of factors responsible for transport in the budding yeast, *Saccharomyces cerevisiae*. This research has revealed the existence of over 40 essential components of the secretory pathway in yeast. Just as the use of selective inhibitors led to the accumulation of transport intermediates in the case of *in vitro* biochemical work, so mutagenesis of genes encoding essential components of the secretory pathway led to a similar proliferation of such intermediates. These genes have been designated "*sec*" (short for secretory) genes and by isolating temperature-sensitive mutants defective in these genes, secretory transport can be shown to occur only at the permissive temperature.

Mutation of the *sec17*, *18* or *22* genes for example blocks transport from the ER to the Golgi in yeast (Kaiser and Schekman, 1990), and causes the accumulation of 50nm vesicles which mediate this step of transport. The sec18p has been shown to be the yeast homologue of mammalian NSF, since it can replace NSF in the mammalian transport assay system (Wilson, *et al.*, 1989). Sec18p is also required

for intra-Golgi transport in yeast and also for Golgi to cell surface transport (Graham and Emr, 1991). The sec17p has been confirmed as the yeast homologue of α-SNAP (Clary, *et al.*, 1990), consistent with the known genetic interaction of *sec17* and *sec18* (Kaiser and Schekman, 1990). The role of sec22p remains unknown.

Mutations in another subset of yeast genes also disrupts ER to Golgi transport but without the concomitant accumulation of 50nm transport vesicles. Epistatic analysis demonstrates that these genes (*sec12*, *sec13*, *sec16*, *sec21* and *sec23*) are responsible for budding rather than fusion reactions (Kaiser and Schekman, 1990). Sec12p is an integral membrane protein that appears to be required for the recruitment of a small GTP-binding protein called sar1p from the cytosol (Nakano, *et al.*, 1988; d'Enfert, *et al.*, 1991). Transport from the ER to the Golgi in yeast has also been reconstituted *in vitro* and has in many cases confirmed the results of genetic analysis (Baker, *et al.*, 1988; Ruohola, *et al.*, 1988). GTPγS inhibition experiments in such *in vitro* systems confirms a role for GTP hydrolysis in vesicle budding from the ER (Rexach and Schekman, 1991). The close homology between sar1p and ARF fuels the speculation that sec12p may be responsible for nucleating the budding site on ER membranes by acting as a receptor for sar1p in the GTP-bound state, reminiscent of the mechanism proposed for recruitment of ARF from the cytosol to Golgi membranes to allow budding to occur. Once again, confirmation of these mechanisms would strengthen the notion that molecular mechanisms of vesicle-mediated traffic are conserved between organisms and also between different compartments of the secretory pathway.

Recently, sec21p has been shown to be a component of the yeast homologue of the mammalian coatomer (Hosobuchi, *et al.*, 1992) and is present in the cytosol as part of a 700-800 kD complex which also contains a family of other proteins of molecular weights 150, 110, 73, 35 and 25kD. Sec21p (105kD) and the 110kD subunit are the yeast homologues of γ-COP and β-COP respectively. The other proteins do not correspond to other characterised SEC gene products. Since *sec21* function is essential for ER to Golgi transport, this proves that coatomer proteins are essential factors in this step of the secretory pathway. Although sec13p and sec23p are also both present in yeast cytosol in large oligomeric complexes (Hicke and Schekman, 1989; Pryer *et al.*, 1993), their identity as yeast coatomer proteins has not yet been shown.

1.2.11 Glycosylation and the kinetics of transport

In the previous section, I outlined what is currently known of the molecular mechanisms underlying vesicle-mediated traffic through the secretory pathway. These mechanisms represent the basic machinery of transport which allows

secreted proteins to pass from one compartment to the next. It is now of interest to explore in more detail the extent of our understanding of the processing of the secretory proteins themselves; in other words, to review what is known of the reactions which occur within these secretory compartments, as opposed to what happens in transit between the compartments.

These reactions of interest include the assembly of secretory molecules in the ER, the factors affecting the rate of exit from the ER, and the post-translational modification of transported proteins as they pass through the ER and Golgi apparatus. Although there will be a number of general points made in the course of this discussion concerning these various reactions and processing steps, I propose to focus the main body of the following discussion on the assembly and processing of the Major Histocompatibility Complex (MHC) Class I proteins as examples of transported proteins since these molecules provided the main source of marker for monitoring biosynthetic protein transport in the work undertaken in this project.

1.2.12 Analysis of the glycosylation pathway

One of the most powerful tools for the study of the processing of secretory proteins along the secretory pathway has been the use of small lipid-enveloped viruses, such as Vesicular Stomatitus Virus (VSV), mentioned earlier in the context of the development of a cell-free system to monitor transport through the Golgi apparatus. These viruses have very restricted genomes and rely totally on the host cell biosynthetic machinery for the translation, processing and transport of their membrane proteins. The VSV genome, for example, encodes only five proteins: an integral membrane glycoprotein (G), a peripheral membrane protein (M), and three nucleocapsid-associated proteins (N,NS and L). As the virus shuts off the synthesis of the host cell proteins, the translational machinery is devoted entirely to synthesis of these proteins. Furthermore, the secretory pathway is deluged with these same proteins following their synthesis in the RER, facilitating the study of biosynthetic transport and post-translational modification of transported proteins as they make their way to the cell surface. Evidence points to the similarity in behaviour of these viral glycoproteins to that of endogenous proteins, thereby justifying their use as probes for the biosynthetic pathway; the kinetics, post-translational modification, and final delivery of G protein expressed following transfection with cDNA-encoded G protein is indistinguishable from those monitored in virally-infected cells (Rose and Bergmann, 1982, 1983; Gabel and Bergmann, 1985). Some of these viruses bud into intracellular compartments, and it is likely that the glycoproteins encoded by such viruses mimic the behaviour of the endogenous proteins that are targeted to these same compartments.

Localisation of the processing events which occur within each of the compartments of the secretory pathway has also relied on the subcellular fractionation of these compartments to demonstrate reproducible partitioning of enzymes responsible for post-translational modification of secretory proteins. Such fractionation and recovery of enzymatic function relies on density gradient centrifugation in which dense ER membranes may be separated from lighter Golgi membranes. Refinement of the fractionation and centrifugation procedures allows finer differentiation between subcompartments and more detailed allocation of enzyme-mediated processing reactions to each of these subcompartments. Distribution of modifying enzymes across sucrose gradients has been found to be entirely consistent with the known sequence of modifications across the Golgi stack (Goldberg and Kornfeld, 1983), confirming the close relationship between structure and function along the secretory pathway. A number of different lines of evidence suggest the presence of a cholesterol gradient along the secretory pathway which is the most likely explanation of the density gradient distribution of subcompartments from the ER to the *trans* Golgi.

One of the most rigorous methods of locating the processing enzymes of the secretory pathway is the use of immunocytochemical labelling of ultrathin frozen sections, allowing resolution of enzyme residency at the electron microscope level. Such techniques rely either on the direct tagging of primary antibodies raised against enzymes of the secretory pathway, or the tagging of secondary antibodies which may bind first to the specific primary antibodies. Using a variety of these techniques, enzymes such as α-mannosidase II, N-acetylglucosaminyltransferase I and galactosyltransferase have been localised to specific Golgi subcompartments in a number of different cell types.

Another method of mapping specifically the oligosaccharide processing steps is to label compartments with lectins that bind to specific carbohydrate moieties. Although lectin-binding studies have the disadvantage of only localising oligosaccharide products rather than the enzymes mediating these structural changes, they do confirm the conclusions of other types of study, demonstrating the polar distribution of resident enzymes within the Golgi apparatus, consistent with the sequential processing of proteins as they traverse the Golgi stack in a *cis* to *trans* direction.

1.2.13 Assembly and transport of the Major Histocompatibility Complex Class I proteins

Investigation of the passage of molecules encoded by the Major Histocompatibility Complex (MHC) region followed soon after the study of viral glycoproteins. Among the huge number of endogenous proteins which are constitutively secreted from

multicellular organisms, the MHC glycoproteins are of particular significance because of their critical role in the immune system, and in turn, of the cellular mechanisms of defence against invading organisms.

In order to understand the assembly and transport of the MHC glycoproteins, it is necessary first to briefly describe their structure and function.

1.2.13.1 The structure and function of the MHC Class I proteins

The defining hallmark of the immune system is the specific recognition of antigens. Specific receptors expressed on the surface of T lymphocytes are responsible for the recognition of short, linear peptides of around 8-10 amino acids presented in the "jaws" of an MHC glycoprotein on the surface of other non-lymphoid cells. Whereas the MHC Class II glycoproteins present peptides derived from exogenously synthesised proteins, the peptides presented by Class I molecules are derived from endogenously synthesised proteins.

The Class I molecule is a bound heterotrimeric complex, comprising a 45kD heavy chain, a 12kD light chain and the peptide antigen itself. All three of these components are required for the complex to assume its properly folded conformation in the ER.

The heavy chain is a type I integral membrane protein and is encoded within the MHC region. The extracellular portion of the molecule contains three domains, designated α1, α2 and α3, of 90, 92 and 92 amino acid residues respectively, and each encoded by separate exons (Malissen, *et al.*, 1982). The membrane-spanning region is about 25 residues long and there is a cytoplasmic tail of about 30 amino acids. The light chain, called β2-microglobulin (ß2m), is a non-MHC encoded protein and is invariant between all Class I complexes. The fact that the α3 domain sequence is relatively conserved and that most of the sequence variations between heavy chains are confined to the α1 and α2 domains, suggested that the peptide-binding function would reside in these latter two domains, which would therefore also mediate the interaction with the T cell receptor.

Since the heavy chains are highly polymorphic (more than one allele existing for each gene locus), an extensive nomenclature is required to uniquely describe each polymorphism. The heavy chains studied most extensively are those from mice and humans. The heavy chains in mice are designated "H2" molecules; those in humans are termed "HLA" molecules. The HLA molecules can be further subdivided into three main classes, HLA-A, B and C and further classification into distinct subtypes requires the addition of other numbers and letters.

The elucidation of the three dimensional structure of HLA-A2 at the 2.6Å level by X-ray crystallography has been of prime importance in unravelling the mechanisms of antigen presentation by Class I molecules (reviewed by Bjorkman and Parham, 1990). The structure is best described as two sets of homologous domains. The α3 domain along with ß2m constitute one set proximal to the membrane, while the α1 and α2 domains constitute the other, distal to the membrane. The membrane-proximal domains are folded into β-sandwich structures similar to immunoglobulin constant regions, connected by an internal disulphide bond. The membrane-distal domains together form a platform consisting of an eight-stranded β-pleated sheet, with two long α-helices which traverse the top of the sheet. The deep groove between these two α-helices forms the peptide-binding site (Bjorkman, *et al.*, 1987).

1.2.13.2 MHC Class I assembly

The co-translational translocation of the heavy chain into the ER follows the classically defined signal sequence-dependent pathway common to most secretory proteins. Assembly of the trimeric complex requires the initial folding of the heavy chain and ß2m into assembly-competent structures; association is rapid and follows 5-10 min after synthesis (Carlin and Merlie, 1986; Krangel, *et al.*, 1979; Owen, *et al.*, 1980). Association of the heavy chain with both ß2m and peptide is essential for the formation and subsequent transport of a stable Class I complex to the cell surface (Ploegh, *et al.*, 1979; Owen, *et al.*, 1980; Sege, *et al.*, 1981; Williams, *et al.*, 1989). The necessity of correct folding and oligomeric assembly prior to exit from the ER has been shown for many other secretory glycoproteins, including VSV G protein and hemagglutinin (HA) which must form trimers in the ER before the protein becomes transport-competent (Copeland, *et al.*,1986, 1988; Gething, *et al.*, 1986; Doms, *et al.*, 1987).

Pulse chase analysis reveals that not all the heavy chains and ß2m subunits are assembled into complexes within the ER (Neefjes and Ploegh, 1988). This is consistent with what is known of the retention of subunits of secretory oligomers within the ER for long periods, without the loss of assembly-competency. The retention mechanism is not known but must be able to prevent exit from the ER while still allowing subunit interaction and subsequent oligomerisation to occur. Retention via retrieval from a post-ER compartment is a possibility. Although the heavy chain requires oligomerisation to exit the ER, ß2m may be secreted in its monomeric state (Severinsson and Peterson, 1984). An excess of heavy chain and ß2m within the ER could be explained by the presence of peptide in limiting amounts. This makes good immunological sense; the antigen-presenting system would then be partially starved of peptide so that in the case of a viral infection,

there would be rapid, quantifiable binding of viral peptides leading to efficient cell surface presentation of these peptides and a correspondingly swift activation of cytotoxic T cells (Parham, 1990).

The *in vitro* reconstitution of Class I biosynthesis has been a major advance in the study of the early stages of assembly of the trimeric complex, and in the identification of the intermediates along the pathway (Kvist and Hamann, 1990; Schumacher, *et al.*, 1990; Townsend, *et al.*, 1990). These systems should also allow the determination of the particular residues responsible for subunit interaction and subsequent folding. *In vivo* studies have already shown that neither the cytoplasmic nor the transmembrane domain of the heavy chain is required for association with ß2m (Zuniga, *et al.*, 1983; Krangel, *et al.*, 1984).

The precise sequence of subunit association is not currently known. Evidence points to substantial conformational changes in the structure of the heavy chain upon ß2m binding (Krangel, *et al.*, 1984; Yokoyama, *et al.*, 1985; Allen, *et al.*, 1986), consistent with the finding that ß2m contacts all three domains of the HLA-A2 heavy chain. *In vivo* (Ljunggren, *et al.*, 1990) and *in vitro* (Townsend, *et al.*, 1990) studies point to the existence of heterodimers comprising the heavy chain and ß2m in a conformation which has an empty binding groove. Such heterodimers, in the so-called "open" state rapidly bind peptides forming stable "closed" heterotrimeric complexes in which the binding groove is virtually inaccessible (Chen and Parham, 1989). The transition from open to closed state is likely to involve another conformational change triggered by peptide-binding (Schumacher, *et al.*, 1990). Investigation of Class I assembly *in vitro* using detergent lysates of whole cells shows that addition of either ß2m or peptide promotes the formation of class I molecules which may be recognised by conformational specific antibodies (Townsend, *et al.*, 1990). Townsend and co-workers therefore favour a more general scheme of assembly involving equilibria between free subunits, heterodimers of heavy chain with ß2m or peptide and the fully assembled heterotrimer consisting of all three components. In this scenario, peptide binding would not be required for initial association of ß2m with heavy chain, but would stabilise the interaction. Equally, initial binding of peptide into the α1-α2 groove of the heavy chain would cause a conformational change which would favour and stabilise rapid association with ß2m. Such co-operative binding of the Class I components is consistent with the rapid rates of assembly of certain allelic forms of the heavy chain into transport-competent complexes (Krangel, *et al.*, 1979; Owen, *et al.*, 1980). Class I assembly is summarised in Figure 1.5.

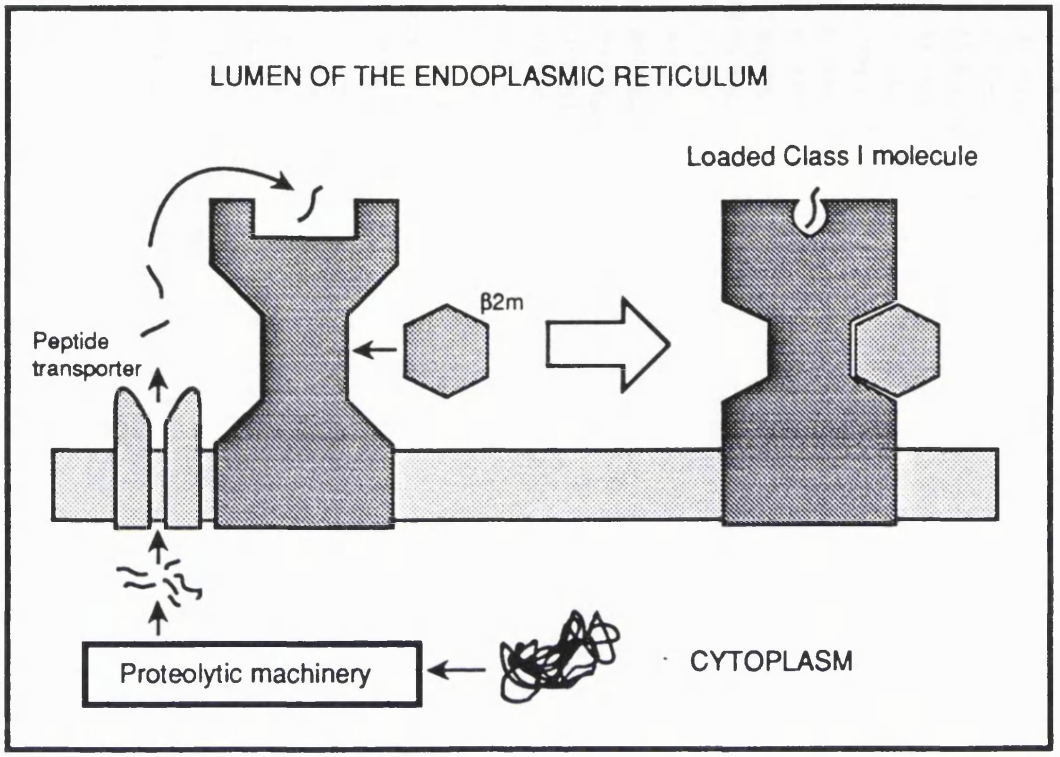

Figure 1.5 MHC Class I assembly in the endoplasmic reticulum.

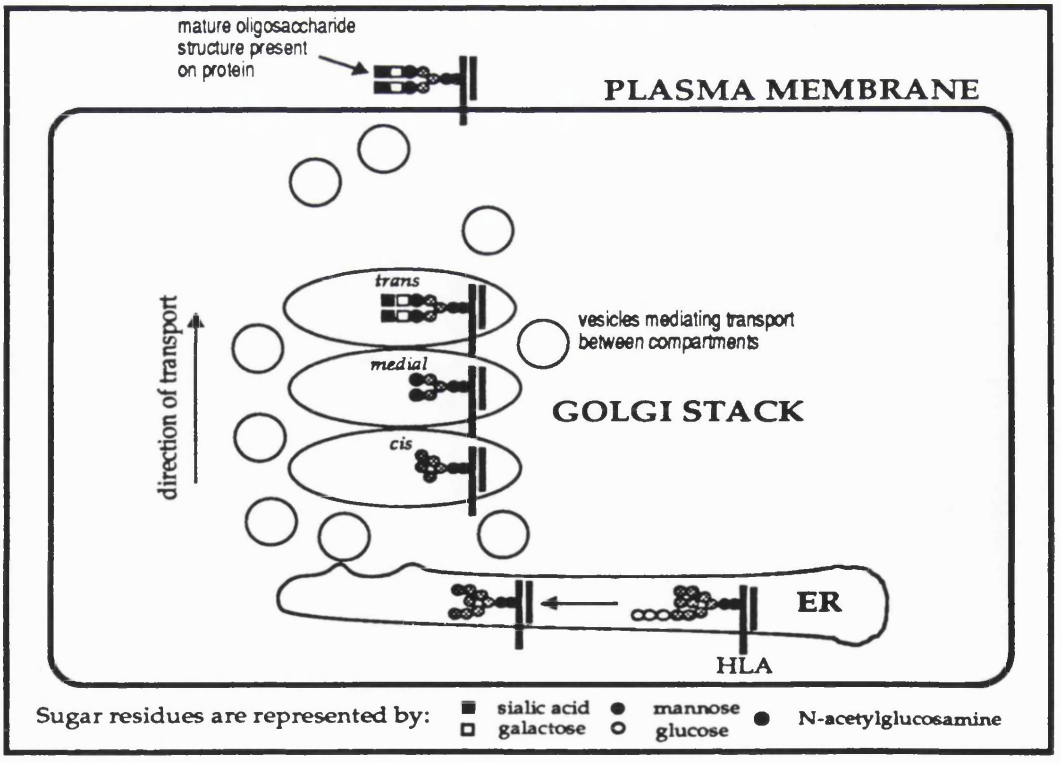

Figure 1.6 N-linked oligosaccharide processing of HLA during secretion.

1.2.14 Protein folding and chaperonins

It has recently become clear that cells possess a class of proteins which function as molecular chaperones to temporarily stabilise partially folded proteins and to prevent illicit interactions with other proteins. These "chaperonins" have been implicated at a variety of stages of cellular metabolism such as protein synthesis, translocation and folding, cycles of macromolecular assembly and disassembly, protection against environmental stress and targeting proteins for degradation.

One such chaperonin molecule has been identified in the process of Class I assembly in mice (Degen and Williams, 1991). The essential requirement for proper assembly of the Class I trimeric complex for transport and cell surface function necessitates adequate quality control within the ER lumen to ensure that only functional complexes enter the secretory pathway. This Class I chaperonin (p88) undergoes rapid and quantitative association with newly synthesised heavy chains in the ER. Dissociation is not triggered by ß2m binding to the heavy chain, but appears to occur while the complex is still in the ER. The rate of dissociation matches the rate of transport of the trimeric complex from the ER to the Golgi in each of the cell lines studied suggesting that p88 dissociation may be the rate-limiting step in the transfer of Class I in this step of the secretory pathway, consistent with a role in detaining "empty" heavy chains. As well as serving to stabilise and detain partially assembled Class I molecules, the p88 molecule may also have a role to play in directing peptide binding by physically interacting with the peptide transporters, though this theory remains speculative for the present.

1.2.15 Kinetics of protein transport out of the ER

Having considered the assembly of heavy chains, ß2m and peptide into transport-competent heterotrimeric complexes, it is of interest to consider the rate of transport from the ER to the Golgi apparatus (reviewed by Hurtley and Helenius, 1989; Pelham, 1989). The rate of secretion of different complexes shows allele-dependent variation (Williams, *et al.*, 1985; Neefjes and Ploegh, 1988). Evidence points to the transport of at least some Class I molecules in the bulk flow of membrane traffic through the secretory pathway once they are fully assembled; the fastest measured rates of transport to the Golgi apparatus for certain Class I molecules correspond to the fastest secretion rates for other proteins, with a half-time for export to the *medial* Golgi of about 15 to 25 min (Fries, *et al.*, 1984; Williams, *et al.*, 1985).

Investigation of the relative rates of Golgi-dependent glycosylation changes and cell surface arrival suggests that the rate-limiting step for secretion of H2 heavy chains in mice precedes the Golgi, and subcellular fractionation reveals the site of

accumulation of more slowly transported molecules to be the ER (Williams, *et al.*, 1985). Consistent with this finding, studies of the interaction of p88 with Class I complexes show that dissociation from this chaperonin may be the rate-limiting step for exit from the ER (Degen and Williams, 1991), which in turn, reflects the rate of proper assembly of the complex into a fully transport-competent conformation. Studies of human allelic differences in secretion rates suggest a relationship between the binding affinity of Class I subunits and the rate of transport to the Golgi apparatus (Neefjes and Ploegh, 1988); HLA-A and B have greater affinity for ß2m than HLA-C (Stam, *et al.*, 1986) and are transported more rapidly in assembled trimeric complexes than HLA-C, despite the fact that HLA-C is synthesised in comparable amounts to HLA-A and B.

Mutagenesis of the H2K allele in mice and the non-classical HLA-E and HLA-F alleles in humans have shown that while ß2m association is necessary for cell surface transport, it is not sufficient (Williams, *et al.*, 1988; Shimizu, *et al.*, 1988). It is clear that the peptides loaded onto Class I molecules also play a significant role in determining the overall conformation of the complex, and therefore the rate at which assembled complexes are transported to the cell surface (Townsend, *et al.*, 1989). As well as being an important observation with respect to the secretion rates of Class I molecules, the peptide-mediated inhibition of cell surface presentation has obvious implications for the possible strategies adopted by intracellular pathogens to evade immune surveillance.

Experiments with the glucosidase inhibitor 1-deoxynojirimycin shows that the lack of adequate oligosaccharide trimming prevents the export of certain glycoproteins from the ER (Gross, *et al.*, 1983; Lemansky, *et al.*, 1984; Lodish and Kong, 1984). These results suggest that carbohydrates might be involved in determining the native transport-competent conformation of the protein, highlighting the structural significance of oligosaccharide sidechains.

The variation in the rate of exit from the ER for many different secretory proteins has led to the suggestion that exit from the ER and more specifically, entry into transport vesicles destined for the Golgi apparatus is a receptor-mediated process (Lodish, *et al.*, 1983; Fries, *et al.*, 1984), involving complex protein interactions. However, since such delays may well be accounted for by oligomeric assembly and conformational changes within the ER which must precede the inclusion of secretory molecules into bulk flow carrier vesicles and since there has been no direct evidence to date for such receptors, the simpler explanation of conformational dependence seems more favourable.

1.2.16 Glycosylation of secretory proteins

The polypeptide backbone of newly synthesised secretory proteins is often modified in a series of glycosylation reactions as the proteins traverse the secretory pathway, sequential modifications occurring as the proteins pass from one compartment to the next in a vectorial fashion via vesicular-mediated traffic. Since glycosylation of the heavy chain of the Class I complex was the basis for monitoring transport along the secretory pathway in the work I have undertaken here, I propose to describe the classical oligosaccharide processing sequence and how such oligosaccharides may be used to determine which compartments have been visited along the secretory pathway.

1.2.16.1 The types of glycosylation

The function of oligosaccharide modification of proteins is not very well understood. In the case of plasma membrane proteins, these carbohydrate moieties may facilitate cell-cell interactions via the extracellular matrix, or may confer structural stability on the fully mature protein, perhaps by increasing resistance to proteolysis. In the case of secreted proteins, they may increase the solubility of proteins by increasing the net charge on the protein. Certain sugar residues are involved in the sorting of some lysosomal proteins. The use of tunicamycin, an antibiotic which inhibits the first stage of protein glycosylation, allows the secretion of some glycoproteins but not others; this indicates that the carbohydrate side chains of some but not all glycoproteins have an important function to play in allowing passage along the exocytic pathway.

The two main types of glycosylation are N or (asparagine)-linked and O (threonine or serine)-linked. The latter modifications are less common and have been less well characterised as a result. Moreover, the heavy chain of Class I does not have any O-linked sugars, and so I do not propose to discuss these modifications further.

Structural analysis of N-linked sugars using NMR has revealed that they fall into one of three classes, namely complex, hybrid or high mannnose. These three categories share a common pentasaccharide core. High mannose oligosaccharides generally have two to six additional mannose residues linked to the pentasaccharide core, while the hybrid structures contain elements of both the high mannose sugar structure and the complex type. The outer section of the sugar structure generally comprises two branched chains, and a highly variable set of sugar residues linked in sequence to the bi-antennary mannoses. The number of branches can however be highly variable too; structures with five outer chains have been found on certain proteins.

N-linked oligosaccharides are usually added to the structural motif Asn-X-Ser/Thr, where X may be any amino acid other than proline or aspartate (Marshall, 1972, 1974). The capacity of this tripeptide structure to assume an appropriate conformation in the context of short range interactions with nearby amino acids appears to have a major effect on the rate and extent of glycosylation. Such interactions would have the effect of altering the accessibility of the tripeptide motif to the oligosaccharyl transferases. The addition of the most proximal residues is a co-translational event, which therefore occurs while the protein is still in the process of folding. Once the protein assumes its fully native conformation , potential N-linked glycosylation sites may well be obscured, limiting the time during which such modifications may be initiated (Pless and Lennarz, 1977).

1.2.16.2 The sequence of oligosaccharide processing

The MHC Class I heavy chain has a single N-linked glycosylation site at Asn86 within the α1 domain. This glycosylation site is conserved between the 25 HLA-A, 35 HLA-B and 18 HLA-C sequences that have so far been determined. The sequence of oligosaccharide modifications of the heavy chain is summarised in Figure 1.6 (reviewed by Kornfeld and Kornfeld, 1985).

The trimming of the outer glucose residues and at least one of the outer mannose residues occurs in the ER, and have been shown to be co-translational events for the VSV G protein (Atkinson and Lee, 1984). Once within the *cis* Golgi, the high mannose sidechains are trimmed by the Golgi-specific mannosidase I to give a $Man_5GlcNAc_2$ structure. N-acetylglucosaminyltransferase I (NAGT I) catalyses the addition of N-acetylglucosamine (GlcNAc) to one branch of the bi-antennary sugar chain in the *medial* Golgi, and the two outer mannose residues of the other branch are then trimmed by mannosidase II. A second *medial* N-acetylglucosaminyltransferase (NAGT II) adds another N-acetylglucosamine residue to the second branch and fucosyltransferase may mediate the transfer of a fucose residue to the innermost GlcNAc residue. Galactosyltransferase (GT) and α2,6-sialyltransferase (ST) catalyse the addition of terminal galactose and sialic acid residues in the *trans* cisternae and TGN.

1.2.17 Using oligosaccharide processing to monitor glycoprotein transport

The spatial compartmentalisation of the oligosaccharide processing reactions and the fact that secretory glycoproteins visit these compartments in a defined order means that these reactions may be used to monitor the passage of these proteins through the secretory pathway. A particular oligosaccharide modification therefore signifies arrival at a certain compartment in which the specific enzyme carrying out that modification is localised. This technique may also be used to gain a kinetic

dissection of passage along the secretory pathway; the kinetics of biochemical modification provides a direct indication of the kinetics of transport.

Such an analysis requires that the location of processing enzymes be known precisely in order to be able to define the particular step of the pathway which is being monitored, and that there is no processing delay once proteins arrive within the correct processing compartment. With regard to the first assumption, the compartmental localisation of processing enzymes is already well-characterised (for a review of enzyme localisation see Dunphy and Rothman, 1985; Roth, 1987). Further refinements of our understanding of enzyme distributions are emerging all the time. A recent study by Nilsson and co-workers, for example, demonstrated that NAGT I is localised not only in the *medial* cisterna but also in the *trans* cisterna, thereby overlapping with GT (Nilsson, *et al.*, 1993a). This result indicates that the functional contents of each cisterna are specified not by discrete sets of enzymes, but by particular mixtures of these enzymes. However, for the purpose of measuring transport to a particular cisterna, the crucial requirement is that the arrival point under assessment is the *first* compartment frequented by the particular enzyme of interest; an extensive distribution of the same enzyme distal to that compartment does not weaken the assumption that that particular modification indicates passage to the first compartment containing that enzyme. The second assumption relating to the requirement for immediate enzymatic function is satisfied by evidence pointing to the direct nature of the processing reactions without any lag period; the secretion rates of extensively modified glycoproteins are indistinguishable from those of non-glycosylated proteins.

The localisation of ST to the *trans* Golgi and TGN (Roth, *et al.*, 1985) infers that the addition of terminal sialic acid residues mediated by this enzyme indicates transfer of a secretory glycoprotein to the *trans* cisterna of the Golgi apparatus. Moreover, the kinetics of sialylation are a useful indicator of the rate of transfer of glycoproteins to the *trans* region of the Golgi apparatus. It is worth reiterating that although most of the ST is present in the TGN, the localisation of at least some of the enzyme to the *trans* cisterna indicates that acquisition of sialic acid represents arrival at the *trans* cisterna, the *first* compartment in which the enzyme is located.

As well as using the acquisition or loss of particular residues, another means of monitoring passage to particular compartments is the acquisition or loss of sensitivity to specific exogenous glycosidases which cleave oligosaccharide sidechains in certain configurations but not others. One of the most widely used assays of this type is digestion with endoglycosidase H (endo H), an enzyme which cleaves the bond between the two proximal GlcNAc residues in oligosaccharide sidechains which have not yet been processed by NAGT I or

mannosidase II. Since these enzymes are known to be localised to the *medial* Golgi (Dunphy, *et al.*, 1985), N-linked sidechains will be sensitive to such digestion when isolated from compartments proximal to the *medial* Golgi. Once a glycoprotein has reached the *medial* Golgi however, the sidechain attains a configuration which is no longer sensitive to endo H digestion.

Assays based on glycosylation may also be used to determine arrival at the cell surface. Treatment of cells with exogenous glycosidases for example provides a direct means of establishing whether a protein has reached the cell surface or is still within the intracellular secretory pathway. The value of such assays in unravelling the complexities of the secretory pathway cannot be underestimated. These means of measuring the kinetics of transport have been extensively used in the work undertaken here.

1.3 Mitosis

The work I have undertaken focuses on intracellular transport during mitosis and it is therefore of some importance to review the current state of understanding of the control mechanisms underlying mitosis in eukaryotic cells in general, and in HeLa cells in particular. The last decade has seen very exciting progress in this field, and the elucidation of well-conserved mechanisms which co-ordinate not only the complexities of M phase itself, but ensure appropriate timing of M phase relative to the other phases of the cell cycle (for a review, see Jacobs, 1992).

A number of different approaches have been used to arrive at this current understanding, and one of the most satisfying aspects of this research has been the convergence of these different systems in identifying the key control components which show extensive homology to each other.

1.3.1 Mitotic control

The exploration of genes responsible for mitotic anomalies led to the identification of the *cdc2* and *CDC28* genes of fission and budding yeast respectively, functionally homologous genes encoding a 34kD protein kinase ($p34^{cdc2}$) required for entry into M phase (Simanis and Nurse, 1986). A parallel biochemical investigation of an unstable activity found in extracts from metaphase-arrested *Xenopus laevis* oocytes eventually revealed the identity of the catalytic component of this so-called "maturation promoting factor" or MPF as the homologous amphibian counterpart of the $p34^{cdc2}$ kinase. A third line of research focused on a family of proteins which demonstrated cell-cycle specific synthesis and degradation which came to be known as "cyclins." Together, these three approaches have allowed the elucidation of the basic mechanisms of cell cycle

control, though the similar patterns of control discovered in such diverse systems perhaps initially obscured the underlying complexities which are now becoming more apparent.

The key player in these complex networks of protein interactions is the $p34^{cdc2}$ -cyclin heterodimer, akin to other dimeric enzyme complexes comprising a catalytic subunit (here, the $p34^{cdc2}$ kinase) and a regulatory subunit (here the cyclin molecule). This complex is now recognised as the primary regulator of the G2-M transition in all eukaryotic cells, including HeLa cells.

The model of mitotic regulation in fission yeast currently favoured involves the activation of the $p34^{cdc2}$ serine-threonine kinase at the G2-M transition by association with newly synthesised cyclin B (p56) encoded by the *cdc13* gene, and specific dephosphorylation of the Tyr15 residue of $p34^{cdc2}$. This dephosphorylation is carried out by a protein phosphatase encoded by the *cdc25* gene termed $p80^{cdc25}$ (Russell and Nurse, 1986; reviewed by Millar and Russell, 1992). Exit from M phase occurs via the dissociation of cyclin B at the metaphase-anaphase transition and its ubiquitin-mediated degradation (Lee and Nurse, 1987; Booher, *et al.*, 1989). Cyclin stability is controlled by a specific sequence near the amino terminus of the molecule; fusion of this sequence to reporter molecules causes mitosis-specific degradation by the ubiquitin-pathway (Glotzer, *et al.*, 1991).

A refinement of this model involves another crucial residue of $p34^{cdc2}$, namely Thr167, which must be phosphorylated during S phase, and after cyclin B association. The kinase responsible in yeast remains unknown, but evidence points to this being the CAK kinase in *Xenopus* (Lee, *et al.*, 1991; Solomon, *et al.*, 1992). At the end of mitosis, Thr167 is dephosphorylated by the INH phosphatase in *Xenopus*, which comprises the catalytic domain of phosphatase 2A (Lee, *et al.*, 1991); its yeast and human homologues await discovery. A further refinement of the entry into M phase is the role played by the products of the *wee1/mik1* genes in fission yeast (Russell and Nurse, 1987; Lundgren, *et al.*, 1991). These proteins together interact to phosphorylate Tyr15 following cyclin B association, and Thr167 phosphorylation, but prior to the final Tyr15 dephosphorylation switch. Tyr15 phosphorylation generates the "pre-MPF" complex, still inactive, but committed to the final Tyr15 dephosphorylation switch.

The discussion above represent a consensus model of control of the G2-M transition, summarised in Figure 1.7. Although these control mechanisms share a striking degree of universality between organisms, the significant variations which have already been identified preclude the assumption that all such control mechanisms are identical until proven otherwise. Considerable caution needs to

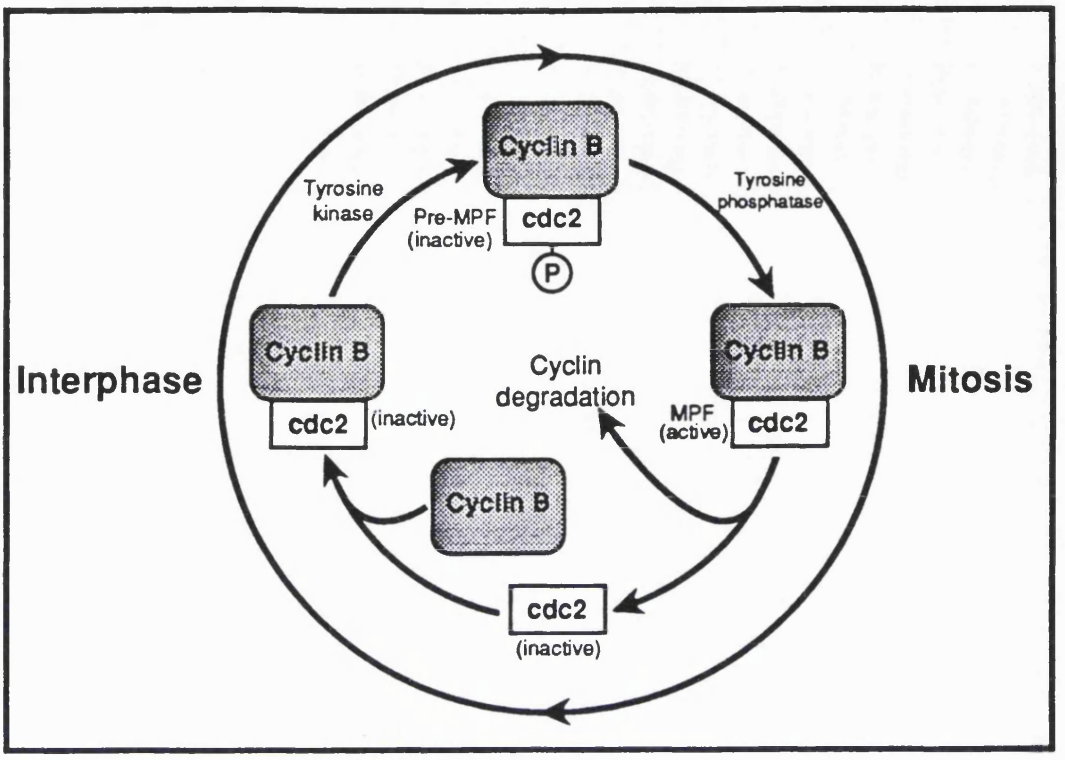

Figure 1.7 The cyclin-cdc2 cycle.

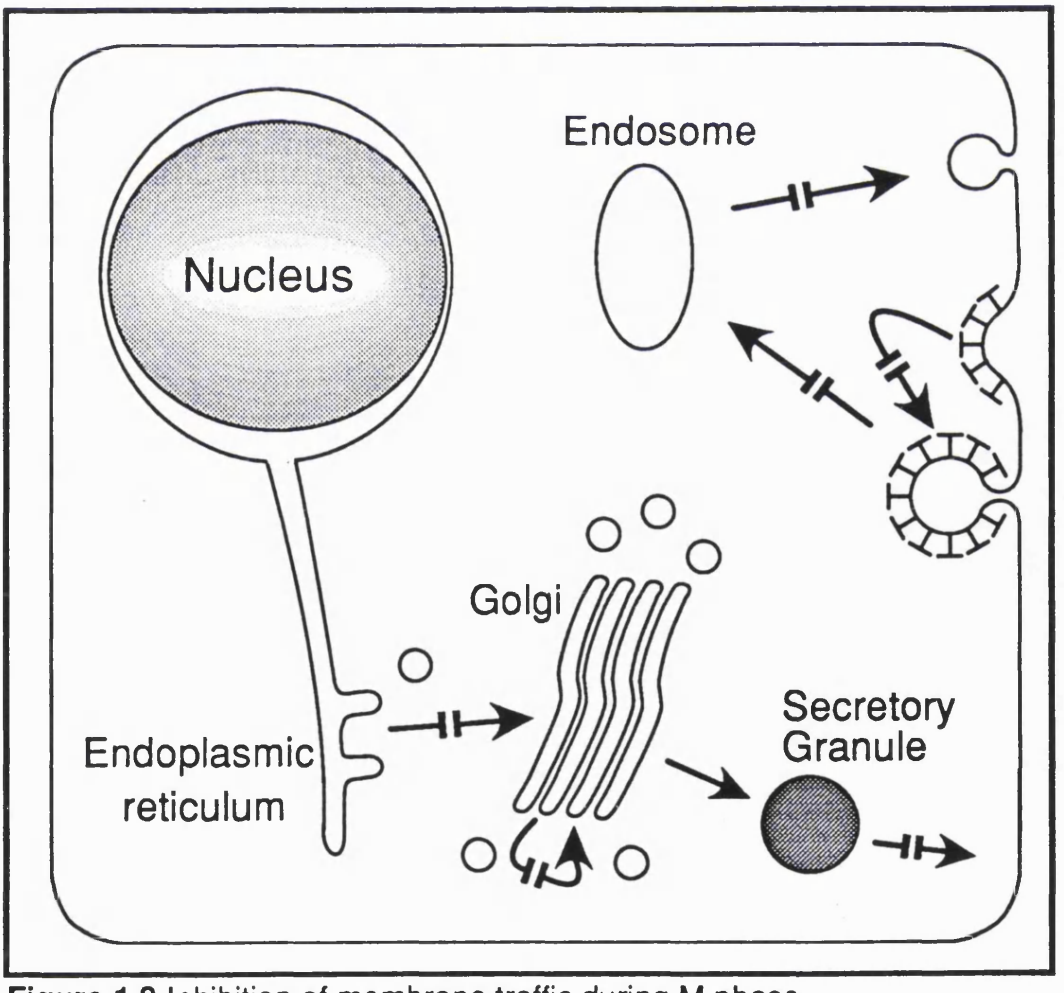

Figure 1.8 Inhibition of membrane traffic during M phase.

be exercised on this front. For example, cyclin levels rather than p34^{cdc2} dephosphorylation appear to be the major control checkpoint in early embryos of *Xenopus* (Murray and Kirschner, 1989). In addition, regulation of p80^{cdc25} in embryos appears to be different from that in post-embryonic cells. During the rapid divisions of the *Xenopus* embryo, p80^{cdc25} concentration remains constant throughout the cycle, while its activity is up-regulated by extensive hyperphosphorylation of its N-terminal region at the G2-M transition (Kumagai and Dunphy, 1992). In the contrasting case of post-embryonic *Drosophila* cells, p80^{cdc25} activity is directly related to synthetic levels of the phosphatase; both p80^{cdc25} mRNA and protein levels rise during interphase to a peak at the G2-M transition in yeast. Inactivation of p80^{cdc25} at the end of mitosis involves interaction with the product of the *suc1* gene (p13), which overcomes the activating influence of the p80^{cdc25} phosphatase, seemingly by direct physical contact (Hayles, *et al.*, 1986; Ducommun, *et al.*, 1991).

Activation of p34^{cdc2} in mammalian cells also demonstrates differences from the pathway elucidated for fission yeast. Tyr15 is phosphorylated in mice as in yeast, but the neighbouring residue (Thr14) is also phosphorylated (Norbury, *et al.*, 1991). Single and double mutant studies indicate that phosphorylation of either of these two residues restrains the p34^{cdc2} complex from activation during G2; the Thr residue therefore provides extra mitotic control. Both residues require to be dephosphorylated for full activation of p34^{cdc2}-cyclin B, and evidence points to p80^{cdc25} as the phosphatase that mediates both these reactions. Interestingly, a family of three cdc25 proteins have been identified in human cells (Sadhu, *et al.*, 1990; Galaktionov and Beach, 1991; Millar, *et al.*, 1991; Nagata, *et al.*, 1991), suggesting the possibility of multiple functions of this phosphatase family at different times and/or different locations within the cell.

These elegant refinements of the basic pathway of p34^{cdc2} activation together provide control checkpoints to prevent premature entry into M phase. The p34^{cdc2} molecule is therefore able to act as an intricate cell cycle clock which can co-ordinate the timing of M phase relative to the previous S phase, and each S phase relative to the previous M phase in turn. Recent studies have shown that the signal which prevents the onset of M phase before completion of the preceding S phase is mediated by the modulation of the p34^{cdc2} tyrosine phosphatase activity, which in turn occurs through a phosphorylation/dephosphorylation regulatory mechanism (Smythe and Newport, 1992). Each level of refinement represents a further means of attaining adequate "safety control" analogous to the interacting safety systems operating in complex and often potentially lethal situations in industry.

1.3.2 p34^{cdc2} substrates

The active p34^{cdc2}-cyclin complex appears to be both a "workhorse" kinase, phosphorylating substrates which are directly responsible for M phase changes in structure and function, and also a "regulatory" kinase, phosphorylating downstream regulatory molecules, thereby generating an intricate phosphorylation/dephosphorylation cascade.

Substrates of p34^{cdc2} so far identified include histones (Arion, *et al.*, 1988), possibly leading to chromosomal condensation, lamins (Heald and McKeon, 1990; Peter, *et al.*, 1990; Ward and Kirschner, 1990), causing nuclear lamina breakdown, and myosin II regulatory light chain, inhibiting interaction with actin and perhaps preventing cell cleavage until after completion of chromosomal segregation (Pollard, *et al.*, 1990). Regulatory substrates include pp60*src* and c-Abl, both tyrosine kinases (Shenoy, *et al.*, 1989; Kipreos and Wang, 1990), SWI5, a transcription factor in budding yeast (Moll, *et al.*, 1991) and cyclin B, its own regulatory subunit (Izumi and Maller, 1991).

These modifications are transient suggesting that p34^{cdc2}-mediated phosphorylation allows M phase specific alteration in function. Dephosphorylation at the end of mitosis allows these proteins to resume their normal interphase role within the cell.

1.3.3 Calcium and cell cycle control

The previous discussion has outlined what is known of the protein interactions which catalyse entry into M phase, and how cells terminate mitosis by returning to their interphase state. As well as elucidating the protein components of this mitotic machinery, it is also of interest to understand the signalling mechanisms involved in activating these "target" proteins at the right time. One of the cell's most potent signalling mechanisms is a rapid rise in intracellular calcium. The role of calcium in triggering mitotic events has been most extensively studied in amphibian cells, particularly sea urchin embryo cells, since signalling changes in these types of cell may be monitored much more easily than in somatic cells.

A distinctive rise in intracellular calcium ($[Ca]_i$) may be measured in eggs 1-2 min prior to the onset of mitosis from 250 to 350nM in monospermic eggs, and to about 700nM in polyspermic eggs. Premature entry into mitosis may be induced by the microinjection of $InsP_3$ or exogenous calcium (Twigg, *et al.*, 1988). Furthermore, entry into M phase may be blocked by the addition of a calcium chelator such as BAPTA or EGTA (Steinhardt and Alderton, 1988; Twigg, *et al.*, 1988). Such experiments demonstrate that this $[Ca]_i$ transient is both a necessary

and sufficient signal for entry into M phase. The immediate target of this rise in $[Ca]_i$ in sea urchin embryos is calmodulin and a type II calmodulin-regulated kinase (CAM-II kinase). Microinjection of antibodies against this kinase arrests the cell cycle at the mitotic entry point (Baitinger, *et al.*, 1990). $[Ca]_i$ transients have also been implicated as a mitotic exit signal in sea urchin embryos (Poenie, *et al.*, 1985). Microinjection of a calcium chelator into mitotic cells induces metaphase-arrest.

Although much more difficult to monitor, such $[Ca]_i$ transients at the exit from M phase have been also been measured in mammalian cells (Kao, *et al.*, 1990). Furthermore, calmodulin inhibitors cause arrest both at the M phase entry and exit points in mammalian cells (Chafouleas, *et al.*, 1982; Sasaki and Hidaka, 1982; ; Boder, *et al.*, 1983; Keith, *et al.*, 1983; Eilam and Chernichovsky, 1988). The fact that a number of key kinases and phosphatases implicated in M phase-specific phosphorylation and dephosphorylation are under rigorous $[Ca]_i$-dependent control lends further weight to the notion that $[Ca]_i$ has a primary role in cell cycle control in mammalian cells. The details of these networks of proteins and the precise role played by $[Ca]_i$ remain to be established.

1.3.4 Features of the animal cell during M phase

The mitotic animal cell manifests a number of features which distinguish the dividing cell from the interphase cell (reviewed by Warren, 1993b). Some of these changes apply to functional processes and some to the cytoarchitecture.

1.3.4.1 Functional changes during M phase

The importance of the sequence and timing of the various phases of the cell cycle has already been duly stressed. The intricate coupling of the timing of S phase and M phase is an absolute necessity for cell viability. The temporal separation of S and M phase necessarily implies that M phase is characterised by the absence of DNA synthesis. However, the synthesis of RNA and consequently protein is depressed considerably during M phase (Prescott and Bender, 1962; Preston, *et al.*, 1985; Hartl, *et al.*, 1993). Significantly, the reduction of protein synthesis is entirely due to the lack of message; protein synthesis may be restored to interphase levels by the supply of sufficient mRNA (Warren, *et al.*, 1983).

A further functional change during mitosis of central importance to the research I have undertaken is the cessation of membrane traffic. This arrest has been demonstrated to be a global phenomenon, affecting almost every step of intracellular membrane transport, including both the exocytic and endocytic pathways (see Figure 1.8).

With regard to the exocytic pathway, Warren and co-workers showed that VSV G protein was not transported to the cell surface of infected cells in M phase and that its transport did not resume until telophase (Warren, *et al.*, 1983). Immuno-EM and analysis of the oligosaccharide processing undergone revealed that the G protein had accumulated in the ER (Featherstone, *et al.*, 1985). Further investigation of the transport of two other secretory proteins, human growth hormone and truncated VSV G protein, have confirmed these initial observations (Kreiner and Moore, 1990).

Because of this inhibition of protein transport from the ER to the Golgi in mitotic cells, it has been necessary to resort to other secretory molecules which may progress through subsequent steps of exocytic transport by means other than vesicular traffic. Glycolipids provide such a marker, and the development of fluorescent analogues of ceramide in particular have been instrumental in generating informative data concerning intracellular traffic (see earlier section on lipid transport). During M phase, the transport of sphingomyelin and glucosylceramide (both ceramide derivatives) to the cell surface is inhibited by about 90% (Lipsky and Pagano, 1985a,b).

The *cis* location of the site of synthesis of these derivatives coupled with the absence of subsequent processing precludes any definitive conclusion about the exact location of the inhibited step of transport; inhibition could be localised to intra-Golgi traffic or a later stage of exocytic transport. This shortcoming may be overcome by the use of endogenous ceramide which is made in the ER and is then processed to glucosylceramide and lactosylceramide in the *cis* Golgi; further transport requires vesicle-mediated transfer (Wattenberg, 1990). Recent studies using this approach have focused on the M phase absence of synthesis of GA2, a further product of lactosylceramide. Since the site of GA2 synthesis is the *trans* Golgi or TGN, and GA2 synthesis is delayed until telophase, these results demonstrate the inhibition of intra-Golgi traffic during M phase; once Golgi transport resumes in telophase, the GA2 precursors can then be transferred to the *trans* Golgi (Collins and Warren, 1992). This result has also been confirmed *in vitro* using the cell-free intra-Golgi transport assay developed by Rothman and co-workers; transport of VSV G protein was found to be inhibited in the presence of mitotic but not interphase cytosol. Furthermore, this inhibition was reversible and $p34^{cdc2}$-dependent (Stuart, *et al.*, 1993).

Regulated secretion is also inhibited in mitosis. Appropriate stimulation of mitotic mast cells does not generate release of either histamine (Hesketh, *et al.*, 1984) or serotonin (Oliver, *et al.*, 1985).

In addition, many of the steps of endocytic transport have been shown to be inhibited during M phase. Fluid phase endocytosis is rapidly repressed by a factor of 30, within seconds of the onset of prophase (Berlin and Oliver, 1980). Phagocytosis is similarly repressed (Berlin, *et al.*, 1978). Formation of coated vesicles no longer occurs due to the inhibition of coated pit invagination (Fawcett, 1965; Pypaert, *et al.*, 1987, 1991). The observation that there is no accumulation of one particular intermediate along the endocytic route (Pypaert, *et al.*, 1987) strongly suggests the abrogation of every step on the pathway, including scission and uncoating. *In vitro* reconstitution shows the absence of endocytic vesicle fusion (Tuomikoski, *et al.*, 1989; Woodman, *et al.*, 1992). Studies of the recycling of the transferrin receptor during M phase show that inhibition of the various steps of the endocytic cycle are not synchronous but sequential. At the onset of M phase, the density of receptors at the plasma membrane falls, while at telophase, this density increases above interphase levels (Warren, *et al.*, 1984). These observations are most simply explained by the delayed arrest of receptor uptake with respect to receptor recycling at the start of mitosis, followed by the resumption of recycling ahead of uptake in telophase.

It is worth noting that these inhibitory measures which are such a striking feature of mitotic animal cells are confined to membrane traffic, and do not infer a global repression of function within all membrane-bound compartments connected operationally by vesicular traffic. The experiments outlined above have already demonstrated that glycosphingolipid biosynthesis is maintained during M phase as long as precursors are supplied to the appropriate compartment (Collins and Warren, 1992). Glycosyltransferases are also enzymatically active *in vitro* when isolated from mitotic cells (Lucocq, *et al.*, 1987; Nagata, *et al.*, 1990).

1.3.4.2 Species-specific differences in intracellular transport during M phase

The description above of the repression of intracellular transport demonstrates that this phenomenon is a widespread feature affecting many steps of membrane traffic. However, this inhibition is not completely universal between all steps and among all species. Two steps in particular have shown that differences do exist between species. The first concerns the transfer of protein from the ER to the Golgi. Although this has been confirmed in all mammalian cells so far studied, protein transfer is maintained in the case of *Xenopus* oocytes as far as the *medial* Golgi (Ceriotti and Colman, 1989; Leaf, *et al.*, 1990; Kanki and Newport, 1991).

A second step showing species-dependence is that from the TGN to the cell surface. Whereas the secretion of glycosaminoglycans (GAGs) from the TGN is virtually absent in *Xenopus* oocytes (Leaf, *et al.*, 1990; Kanki and Newport, 1991), the rate of transport of GAGs in mitotic mammalian cells is only reduced 2-fold

with respect to interphase levels (Kreiner and Moore, 1990). The reason for this discrepancy remains to be elucidated. Despite these differences however, it is clear that the exocytic pathway is inhibited overall in both *Xenopus* and mammals, regulation imposed early in the pathway in mammals and late in the pathway in *Xenopus*.

1.3.4.3 Molecular mechanisms of transport inhibition

The observation of transport inhibition has generated a healthy degree of interest in the molecular mechanisms responsible for this mitotic phenomenon. The key role of $p34^{cdc2}$ in the control of other mitotic events naturally led to the hypothesis that this kinase, either directly or indirectly through a downstream kinase, might be the regulator of transport arrest during M phase.

Evidence which fuels this speculation has come from a number of different laboratories. Studies of coated pit invagination *in vitro* showed that the reversibility of mitotically induced cessation of this process depended on protein phosphatase 2A (PP2A), indicating that the inhibitory state was induced by a phosphorylation reaction (Pypaert, *et al.*, 1991). Moreover, incubation of this cell-free assay with purified $p34^{cdc2}$ kinase partially mimicked the inhibition observed with mitotic cytosol. The fact that budding of coated vesicles does not require exogenous cytosol argues in favour of a membrane-bound target for the inhibitory phosphorylation; the enzyme responsible for coat rearrangement during invagination is one such candidate.

Cell cycle control of endocytic vesicle fusion has similarly been shown to be under the regulation of $p34^{cdc2}$ kinase (Tuomikoski, *et al.*, 1989), though whether this regulation is direct or mediated through a downstream kinase remains to be shown (Woodman, *et al.*, 1992). A possible target is rab4Ap, a member of the rab family of small GTP-biding proteins which have been implicated at a series of steps of intracellular transport. Rab4Ap is associated with endosomes (van der Sluijs, *et al.*, 1991) and is phosphorylated by $p34^{cdc2}$ kinase during M phase, leading to redistribution of the protein to the cytoplasm (Bailly, *et al.*, 1991; van der Sluijs, *et al.*, 1992). Another member of the same family, Rab1Ap, is also phosphorylated by $p34^{cdc2}$ (Bailly, *et al.*, 1991), but has no proven role in intracellular traffic. The observation that other members of the rab family are not specifically hyperphosphorylated in mitosis refutes the notion that these proteins are the common vesicular target of inhibitory phosphorylation in mitosis.

Use of okadaic acid, a specific inhibitor of PP1 and PP2A, has revealed a regulatory role for phosphorylation in ER to Golgi transport both *in vivo* (Lucocq, *et al.*, 1991) and *in vitro* (Davidson, *et al.*, 1992). A potential substrate for $p34^{cdc2}$

kinase at this step is a 62kD protein associated with the cytoplasmic face of membranes in the CGN and *cis* Golgi which is the most heavily palmitoylated protein in mitotic cells (Mundy and Warren, 1992). Palmitoylation of this protein is confined to M phase normally, but may be induced under conditions which arrest exocytic transport. Since acylation is required for intra-Golgi transport (Pfanner, *et al.*, 1989; Pfanner, *et al.*, 1990) this protein could possibly be a regulatory target molecule.

1.3.5 Morphological changes

As well as the distinctive functional changes which were outlined in the previous section, a number of morphological changes also occur in the animal cell during M phase. Within the context of the cell cycle, the main function of mitosis is the controlled segregation of the already replicated chromosomes to the two daughter cells which emerge from the division process. The machinery responsible for this segregation comprises the mitotic spindle and associated proteins. Since the spindle itself is made up of microtubules (MTs), one of the most distinctive structural changes associated with M phase is the disassembly of cytoskeletal MTs and their reassembly from the nucleation site of the centrosomes to form the spindle, aligned from one pole to the other. A number of other structural modifications also occur in animal cells to facilitate chromosomal segregation, including chromatin condensation during prophase and the disassembly of the nuclear envelope (NE).

1.3.5.1 The nuclear envelope and ER

Mitosis in animal cells is described as "open", indicating that the NE is disassembled at the onset of M phase. This is in marked contrast to the scenario in yeast in which mitosis is "closed": chromosomal segregation occurs within an intact, though elongating NE. Scission of the NE prior to septation generates the two interphase nuclei (Byers, 1981; Robinow and Hyams, 1989).

Disassembly of the nuclear envelope (NE) in animal cells begins in prometaphase after chromatin condensation, first of all generating large, flattened cisternae surrounding the chromatin. Accompanying this fragmentation process, the nuclear pores are dismantled (Roos, 1973; Zatsepina, *et al.*, 1977; Zeligs and Wollman, 1979; Hepler and Wolniak, 1984; Tamaki and Yamashina, 1991). In many cell types, further envelope disassembly occurs through metaphase and anaphase. By metaphase, the envelope fragments are distributed throughout the mitotic cytoplasm and are partitioned, apparently randomly, to the two daughter cells during telophase. NE disassembly is accompanied by breakdown of the nuclear lamina (Gerace, *et al.*, 1978), a meshwork of intermediate filament-like proteins

designated lamins A,B and C underlying the NE. This breakdown releases the NE from the protein matrix; lamin B remains attached to the NE fragments, while lamins A and C are dispersed throughout the cytoplasm as soluble 4.5S particles (Gerace and Blobel, 1980). The role of phosphorylation of the lamin proteins has already been noted in the preceding discussion of $p34^{cdc2}$ kinase substrates; each of the three lamins are phosphorylated on serine and threonine residues so that the level of phosphorylation is 4-7 fold higher during M phase than in interphase.

Reassembly occurs during telophase, initiated by binding of NE fragments to lateral chromatin surfaces most distant from the spindle poles (Roos, 1973). Lamin B molecules may act as nucleating sites for reassembly since they remain membrane-associated. Pore complexes are once again recruited to the reassembling NE, at first in low density, but by G1 assume their normal interphase frequency (Maul, *et al.*, 1972). Lateral fusion generates the familiar morphology of the interphase NE. Exclusion of cytoplasmic elements from the nucleus during telophase probably occurs through the tight association of NE membrane with the chromatin. Subsequent growth can occur by selective import of required components through pore complexes.

The precise mechanism of NE dynamics during M phase remains uncharacterised for the present; the lamina however is likely to be instrumental both in triggering breakdown of the NE and subsequent reassembly of the NE at the end of mitosis. Study of the NE dynamics in M phase should shed light on other cytoarchitectural changes since they involve both alteration in cytoskeletal conformation (lamina) and membranous structures. The development of *in vitro* systems for the investigation of NE disassembly and reassembly have paved the way for a detailed molecular understanding of these processes (for example, Lohka and Maller, 1985; Burke and Gerace, 1986).

The ER is also known to fragment during mitosis in certain cell types. In tissue cells for example, the ER breaks down extensively to yield large vesicular structures with diameters in the 0.3 to 1μm range (Zeligs and Wollman, 1979; Tamaki and Yamashina, 1991). In cultured cells, such breakdown is uncommon (Koch, *et al.*, 1988).

1.4 The Golgi apparatus

Similarly to the NE of animal cells discussed in the previous section, the Golgi apparatus undergoes extensive fragmentation and subsequent reassembly during M phase. Since this behaviour is intrinsically linked with the study of intracellular transport in mitotic HeLa cells undertaken in this project, I shall discuss the morphological behaviour of the Golgi apparatus in some detail. Insight into the

significance of the disassembly and reassembly of the Golgi apparatus in M phase assumes a detailed knowledge of the organelle's interphase structure. I therefore propose to precede a discussion of its mitotic behaviour with a review of its normal morphology.

1.4.1 The Golgi apparatus during interphase

1.4.1.1 An historical perspective

The Golgi apparatus was first discovered by Camillo Golgi in 1898 using light microscopy (Golgi, 1898), described as "il apparato reticulare interno". The pleiomorphic nature of the apparatus together with the fact that the structure was then thought to be present in only certain cell types meant that little progress was made for a considerable time after this initial description. Historically, 1954 represents a milestone in the evolution of our understanding of the fine structure of the Golgi apparatus. Prior to 1954, there had been considerable controversy surrounding light microscopic studies of the organelle due to suspicion that much of the details described were artifactual and did not represent *bona fide* Golgi features. The 1950's however saw the concerted application of electron microscopy to the refined investigation of subcellular ultrastructure. In particular, 1954 saw the publication of a spate of papers describing for the first time the distinctive "lamellar" structure of the Golgi apparatus, comprising a series of flattened cisternae. The work of Dalton and Felix (1954, 1956) especially deserves mention since they described not only the lamellar structure but also the associated vesicular structures, and the tissue diversity of the fine structure; accordingly, they coined the now familiar term Golgi "complex", indicating the growing degree of intricate facets of this organelle.

The decade following these pioneering studies was characterised by more and more detailed studies of the architecture of the Golgi apparatus as new and better EM techniques became available. Despite this progress in understanding the organisation of the complex in diverse tissue types, little was known of the function of the organelle at this stage, except its topographical contiguity with secretory granules.

During the late 1960's and early 1970's, a range of new methods were applied to the investigation of the Golgi apparatus, including phosphatase cytochemistry which revealed the diversity of subcompartments within the complex; autoradiography, generating the first data concerning the role of the organelle in protein secretion, glycosylation and sulphation; and subcellular fractionation techniques allowing the isolation of distinct subcompartments of the secretory pathway.

1.4.1.2 Interphase organisation of the Golgi apparatus

The accumulated insight into the fine structure of the Golgi apparatus has led to our current view of the organelle in animal cells as a series of smooth flattened cisternae, each cisterna characterised by a particular set of processing enzymes (Dunphy and Rothman, 1985; Nilsson, *et al.*, 1993a). These cisternae are organised into a series of stacks such that equivalent cisternae are interconnected between adjacent stacks (Rambourg and Clermont, 1990). These adjacent interconnected stacks form an intricate reticulum in mammalian cells which is localised to the juxtanuclear region of the cell (Novikoff, *et al.*, 1971; Lucocq and Warren, 1987; Rambourg and Clermont, 1990). The peripheral rims of each cisterna are dilated, and it is from these rims that the vesicles mediating intercisternal transport bud. These rims therefore represent domains which are structurally and functionally distinct from the membrane of the central regions of the cisternae. The appearance of the stacks demonstrates a clear polarity in the curvature of the membrane components, and the orientation of the transitional elements of the ER to the side termed the "*cis*" face. This polarity is confirmed by cytochemical labelling which often gives a heterologous pattern across the stack. In particular, *cis* cisternae show preferential staining with osmium while *trans* cisternae show specific labelling with thiamine pyrophosphatase or TPPase (Novikoff and Goldfischer, 1961).

A notable feature of the region occupied by the Golgi is the exclusion of other organelles such as mitochondria, peroxisomes and ER. This observation has led to the postulation of a "zone of exclusion" surrounding the Golgi (Mollenhauer and Morre, 1978), generated and maintained by some sort of protein matrix.

1.4.1.3 Protein interactions

The putative protein matrix implicated by the observed zone of exclusion has not received very much investigation and remains largely obscure. Studies from Slusarewicz and co-workers (1993) of the extraction of proteins from purified Golgi stacks with salt and Triton X-100 have led to the identification of a family of proteins which mediate the reassembly of stacked Golgi apparati after removal of the salt and Triton. These proteins may form part of the putative protein scaffold responsible for maintaining the zone of exclusion, analogous to the nuclear lamina.

Interaction of the Golgi with microtubules (MTs) is another key form of protein stabilisation of Golgi structure. The Golgi apparatus co-localises with the minus end of interphase MTs, which themselves are contiguous with the MT-organising centre (MTOC). Several lines of evidence point to this physical proximity of the

MTOC and the Golgi apparatus. Firstly, cell locomotion shows that the variation in the position of the MTOC and the Golgi apparatus are coupled (reviewed by (Singer and Kupfer, 1986). Secondly, co-ordinated migration of the MTOC and Golgi is observed during cell differentiation (Tassin, *et al.*, 1985; Bacallao, *et al.*, 1989). Thirdly, the depolymerisation of MTs at the onset of mitosis occurs concomitantly with the disassembly of the Golgi apparatus (see later discussion of the mechanism of this process). Finally, treatment of cells with MT-disrupting agents such as nocodazole or colchicine which cause complete depolymerisation of interphase MTs also leads to disassembly of the Golgi apparatus to form several hundred discrete stacks dispersed throughout the interphase cytoplasm, reminiscent of the initial step of Golgi disassembly at the onset of M phase (Moskalewski, *et al.*, 1975; Thyberg, *et al.*, 1980; Thyberg and Moskalewski, 1985). This disruption of interphase Golgi structure is completely reversible; removal of the drug restores normal Golgi morphology. Furthermore, analysis of this process of reclustering of dispersed stacks using C_6-NBD-ceramide as a vital stain shows that the discrete Golgi elements migrate along MTs toward their minus end; neither intermediate filaments nor microfilaments are involved in this process (Ho, *et al.*, 1989). These studies together indicate the profound role of MTs in generating and maintaining the interphase morphology of the Golgi apparatus. In particular, the role of MTs appears to be in the maintenance of clustered Golgi stacks rather than in the clustering of cisternae within individual stacks, i.e. in inter-stack interactions rather than in intra-stack interactions.

In vitro reconstitution of MT-based organelle transport has been of critical importance in unravelling the complexities of the process and in identifying the essential components. Such studies showed early on that cytoplasmic dynein was capable of generating movement of organelles along MTs in tissue cell extracts (Schroer, *et al.*, 1989). However, more recent studies have revealed that the interaction of Golgi membranes with MTs is mediated by NEM-sensitive cytosolic factors and membrane-associated receptors and not by dynein itself (Karecla and Kreis, 1992). Reconstitution of membrane motility along MTs using *Xenopus* extracts shows that cytoplasmic dynein maintains its capacity to drive MT-based transport throughout the cell cycle, suggesting that M phase arrest of such MT-based transport is regulated at the level of membrane attachment of other cytosolic factors or receptors (Allan and Vale, 1991).

In vitro systems should allow the identification of the molecules involved in MT association with the Golgi apparatus. The MT-associated proteins (MAPs) may include components responsible for such membrane interactions.

It is worth noting in passing that MTs have been implicated in other aspects of intracellular transport other than Golgi localisation. Lysosomes also co-localise with the MTOC suggesting a role of MTs in facilitating intercompartmental transport from the TGN to lysosomes. MTs have also been shown to provide transport tracks for the directed movement of endocytic compartments such as endosomes towards the MTOC and hence the Golgi apparatus (Matteoni and Kreis, 1987). In this light, the Golgi apparatus may be seen as the central hub of intracellular transport, lying at the crossroads of both exocytic and endocytic traffic. Furthermore, a role for MTs in directing secretory vesicular transport to the cell periphery in an anterograde direction (i.e. towards the plus end) has been shown, primarily in the case of regulated secretion and in axonal transport (reviewed in Whaley, 1975). The continuation of constitutive secretory transport in the presence of MT-disrupting agents such as nocodazole suggest that the role of MTs is limited to these particular cases rather than being a global phenomenon.

As well as Golgi matrix and MT interactions, a third form of interaction is essential to preserve the interphase morphology of the Golgi apparatus, namely, the protein interactions which "glue" the cisternae together to form stacks. These glue proteins are as yet largely uncharacterised. That there were particular molecules responsible for these adhesive interactions has been known for a long time; treatment of purified Golgi stacks with a crude lysosomal extract and physical disruption effected the unstacking of these complexes (Ovtracht, *et al.*, 1973; Morre, *et al.*, 1983). Recent attempts to unstack the cisternae of purified stacks using proteases have confirmed that stacking of cisternae is mediated by stable proteinaceous interactions, and have identified intercisternal bridges connecting closely apposed cisternal membranes (Cluett and Brown, 1992); they are absent from dilated rims, anastomosing tubules and Golgi-associated vesicles. These bridges were also protease-sensitive; intriguingly, proteases which did not alter stacking had no effect on the cisternal bridges. Investigation of disrupted Golgi stacks revealed the presence of rectangular nodules when viewed in cross-section which were identical in size to those seen between intact cisternae in non-disrupted stacks.

Despite the influence of these protein interactions in maintaining the structural stability of the Golgi apparatus, it is important to recognise that the organelle is a dynamic structure which can undergo rapid changes in morphology under certain conditions. Mitosis is one such "condition" which will be discussed in the following section, but a number of other conditions have been noted in which there is profound rearrangement of Golgi structure. BFA treatment (reviewed in Klausner, *et al.*, 1992), overexpression of the human ERD-2 receptor (Hsu, *et al.*, 1992) and certain secretory mutants in the so-called End4 complementation group (Zuber *et*

al., 1991; Kao and Draper, 1992) all give rise to loss of the usual Golgi morphology and concomitant retrograde transport of Golgi resident proteins to the ER. These effects are best explained in terms of an imbalance between anterograde bulk flow and retrograde recycling of membrane and demonstrate that the rate of forward and reverse flow of membrane between the ER and the Golgi is a major determinant of Golgi structure which should not be overlooked. It is all too easy to rely too heavily on electron micrographs to understand ultrastructure; the underlying dynamics of biochemical processes occurring within seemingly static structures may paint a very different picture. The Golgi apparatus is continually in a state of flux, receiving newly synthesised proteins and lipids at one face and sorting and packaging these molecules at the other face. It is the careful balancing of entry and exit to the organelle which makes an essential contribution to the maintenance of its overall integrity.

1.4.2 The Golgi apparatus during M phase

The previous discussion has provided a review of Golgi structure during interphase and an important platform on which to base a description of the disassembly of the organelle in M phase.

1.4.2.1 Disassembly of the Golgi apparatus

The disassembly process is initiated in prophase by the loss of the tight peri-centriolar location of the organelle and its dispersal to a peri-nuclear conformation. The finding that *Xenopus* oocytes in first meiotic prophase contain thousands of discrete Golgi stacks (Colman, *et al.*, 1985) suggests strongly that this initial rearrangement of Golgi structure occurs via scission of the inter-stack tubules, apparently stabilised during interphase by MTs.

Following the formation of these discrete stacks, a process of vesiculation yields "Golgi clusters" made up of groups of small vesicles embedded in some sort of matrix, perhaps equivalent to the matrix which generates the interphase zone of exclusion already discussed. These clusters are frequently associated with the transitional element region of the ER. The derivation of these clusters from Golgi stacks is demonstrated by their specific labelling with osmium and TPPase (Lucocq, *et al.*, 1987), previously described as *cis* and *trans* Golgi markers respectively. Furthermore, immuno-EM using antibodies raised against particular Golgi glycosyltransferases reveals the presence of resident Golgi proteins within these structures (Roth, 1987; Nilsson, *et al.*, 1993a). An important aspect of such labelling studies is the fact that these markers only label a certain subpopulation of the clustered vesicles indicating that disassembly appears to preserve the distinct functional compartmentalisation of each cisterna of the intact Golgi stack;

there appears to be no mixing of enzymes between subcompartments during fragmentation.

Quantitative EM studies confirm that by metaphase, HeLa cells contain anything from 10 to 300 clusters. There is an inverse proportional relationship between the number of clusters and the number of free vesicles dispersed throughout the cytoplasm strongly suggesting the shedding of Golgi vesicles from the clusters as passage through M phase continues (Lucocq, *et al.*, 1989). Figure 1.9 summarises these morphological changes during M phase.

1.4.2.2 Reassembly of the Golgi apparatus

Reassembly of the organelle in telophase occurs by reversing these steps of disassembly. Vesicles first congregate to generate about 300 Golgi clusters and fusion within these clusters generates a similar number of Golgi stacks. This value is remarkably consistent from cell to cell suggesting that this represents the basic copy number of Golgi stacks in each interphase cell. The mechanism of specifying such a copy number remains obscure, though presumably resides in nucleating components distinct from Golgi membrane itself, since the number of dispersed Golgi elements (about 10,000 free vesicles by anaphase) is so markedly different from the eventual number of copies of the dispersed Golgi stacks in telophase. Nucleating components may reside within the matrix underlying the zone of exclusion. These several hundred discrete stacks then congregate and fuse in the peri-centriolar region to restore the characteristic interphase morphology of the organelle (Lucocq, *et al.*, 1989). The migration of these dispersed stacks towards the centriolar region during telophase is likely to be driven by MT interaction (Moskalewski and Thyberg, 1992). After removal of nocodazole, a similar migration of dispersed stacks occurs and has been shown to occur along MTs (Ho, *et al.*, 1989) using dynein as a motor (Corthesy-Theulaz, *et al.*, 1992).

Interestingly, this process of fragmentation and reassembly during mitosis distinguishes animal cells from yeast and plants. In these organisms, Golgi stacks are dispersed throughout interphase and there is no evidence for any structural breakdown during M phase. Growth of Golgi stacks appears instead to be lateral, followed by scission of the elongated organelle down the middle (Noguchi, 1988; Garcia-Herdugo, *et al.*, 1988). The sites of such scission events show similar morphology to the tubules connecting equivalent cisternae during interphase in mammalian cells (Rambourg and Clermont, 1990). The organisation of the Golgi apparatus in interphase in yeast and plant cells is strikingly similar therefore to that of animal cells during prophase and telophase. A continuous mechanism of stack scission would ensure that copy number grows with cell size in plants and yeast.

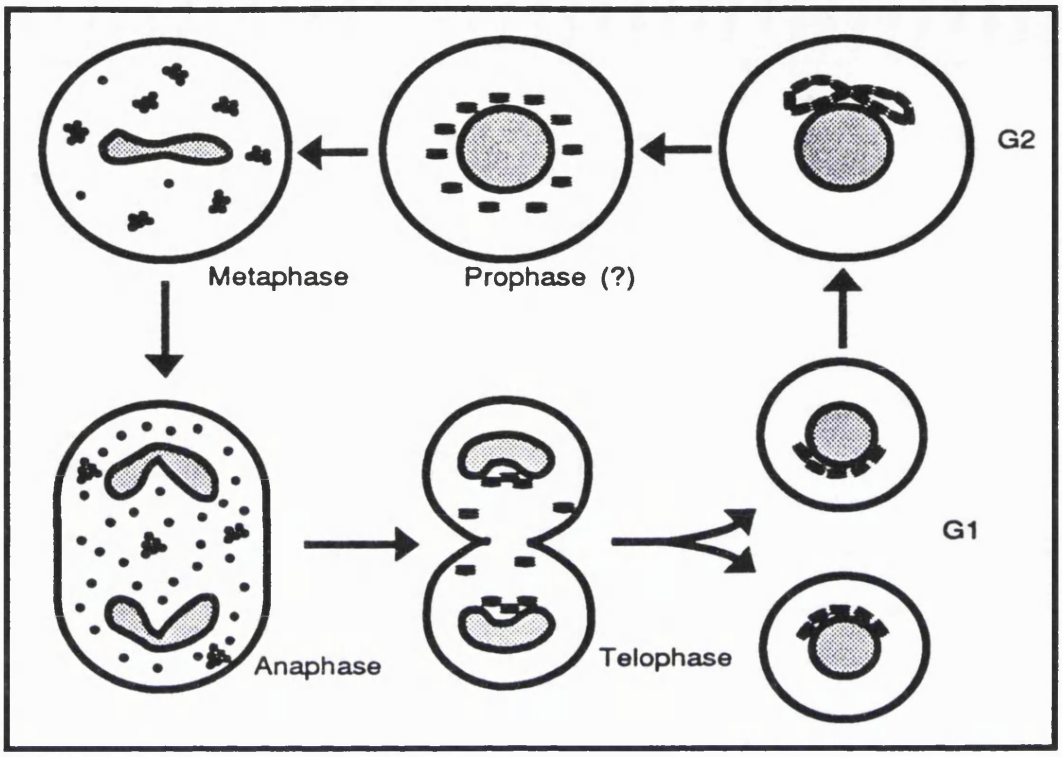

Figure 1.9 Behaviour of the Golgi apparatus during M phase.

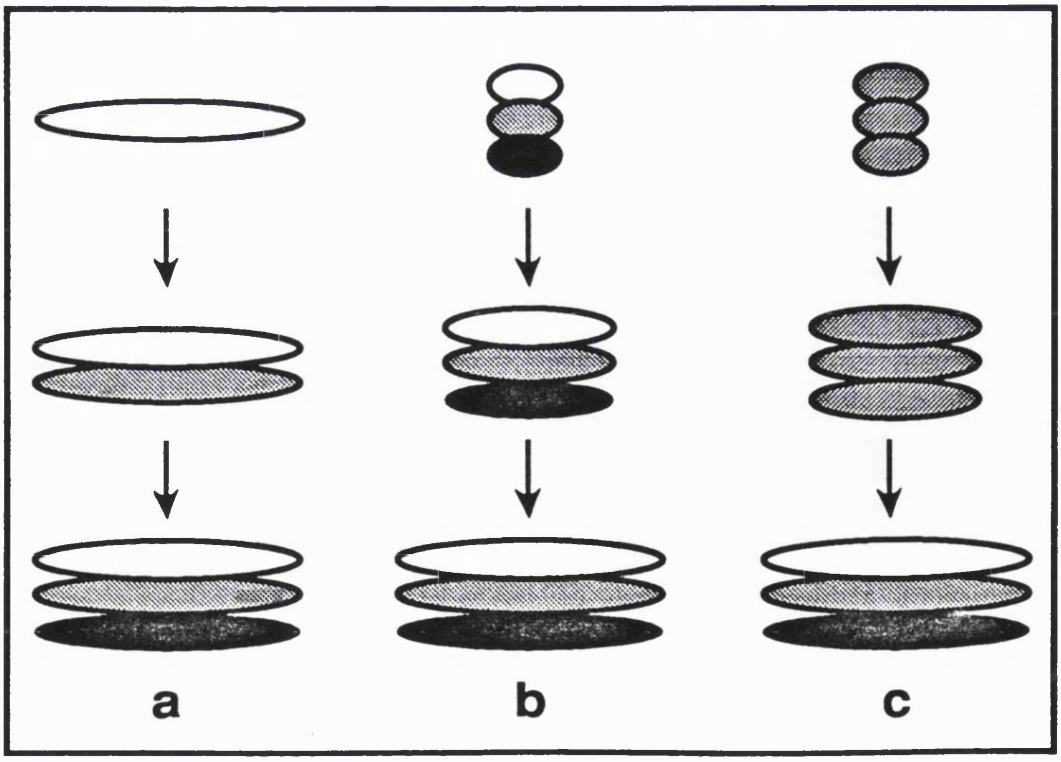

Figure 1.10 Three models of Golgi reassembly.

Since most other organelles exist in multiple copies in the animal cell, they do not require any fragmentation to ensure adequate organelle inheritance by the daughter cells; random partitioning by a stochastic process is sufficient.

1.4.2.3 Models of Golgi disassembly in M phase

The observations that the initial step of disassembly involves the scission of inter-stack tubules to generate several hundred discrete stacks, and that this effect may be mimicked by the incubation of cells with MT-disrupting agents such as nocodazole suggest that one possible mechanism to initiate Golgi fragmentation is the tubulin-dependent activation of a scission enzyme. The depolymerisation of the cytoskeletal MTs at the onset of M phase would generate the necessary tubulin gradient to trigger this process. A corollary of this observation is that the dispersal of Golgi stacks in prophase is not dependent on MT-based motility, consistent with the inactivation of MT-driven organelle transport in metaphase extracts (Allan and Vale, 1991).

With regard to the subsequent vesiculation of the organelle other mechanisms must be at work. One intriguing hypothesis was mentioned at the start of this chapter, namely the cessation of fusion of transport vesicles while vesicular budding was maintained (Warren, 1985, 1989). This would cause the sustained vesiculation of the Golgi stacks. Consistent with this notion is the observation that mitotic Golgi vesicles are the same size as Golgi transport vesicles during interphase (Melmed, *et al.*, 1973; Zeligs and Wollman, 1979; Lucocq, *et al.*, 1989; Tamaki and Yamashina, 1991). However, inhibition of membrane fusion during M phase has still to be shown.

In addition to a mechanism for vesiculation of the Golgi apparatus, there must be a means of initiating the "un-zipping" of stacked cisternae, presumably by modification of the adhesive function of the Golgi glue proteins. The previous discussion of the current understanding of these glue proteins showed that little is known of their structure and regulation of their function; extensive investigation of these putative cisternal adhesion proteins is necessary to address this aspect of Golgi disassembly.

Furthermore, the maintenance of the intracisternal organisation of resident Golgi processing enzymes must also be relaxed. Labelling studies have revealed the presence of these functional components of the Golgi apparatus in mitotic Golgi clusters (Roth, 1987; Nilsson, *et al.*, 1993a). Once again, there must be a means of repressing the mechanism responsible for maintaining this interphase structure. The existence of elongated protein "rafts" within the central portion of each cisterna responsible for kin recognition of resident Golgi proteins as has been

suggested (Nilsson, *et al.*, 1993a,b) would require extensive disruption of these protein aggregates during M phase to allow component proteins to enter mitotic Golgi vesicles. Interaction of the cytoplasmic tails of these resident Golgi enzymes with intercisternal glue proteins could be disrupted by the same M phase changes thereby regulating unzipping of adjacent cisternae. By analogy with the nuclear lamina, phosphorylation of the cytoplasmic tails of resident Golgi proteins might be a possible means of achieving such relaxation of matrix interactions. Such a mechanism however remains speculative for the present.

1.4.2.4 Models of Golgi reassembly in telophase

The reassembly of polarised stacks during telophase presents obvious morphological complexities which the cell must overcome to ensure efficient and accurate biogenesis of functional organelles which may be partitioned to the two daughter cells. Fusion of Golgi vesicles must be restricted to two dimensions in order to generate a stack of flattened cisternae. A number of different models have been postulated to explain the mechanism of reassembly. These include the notion of sequential cisternal nucleation and lateral growth so that each cisterna is fully reassembled before the next one is nucleated (Figure 1.10a); initial fusion of nucleating subcompartments to form a polarised template, followed by simultaneous growth of all cisternae (Figure 1.10b); and an initial reassembly of randomly stacked cisternae, followed by intercompartmental sorting of resident proteins to form the polarised stack (Figure 1.10c).

1.4.3 The role of fragmentation in animal cells

In the case of cellular components which exist as single copies during interphase, two forms of partitioning are used by the cell: either controlled segregation using intricate machinery to do so, as in the case of chromosomal partitioning under the control of the mitotic spindle, or stochastic (i.e. random) partitioning following fragmentation into a sufficient number of constituent pieces. It is this latter method which the cell appears to use for the partitioning of the Golgi apparatus, present as a single copy (or at most a few copies) in animal cells. Clearly, organelles present in sufficient copy number during interphase such as mitochondria or lysosomes (approximately 1000 copies) may partition stochastically without further fragmentation. The binomial distribution predicts that for a 1000 copies, there is a 99.99% chance of the two daughter cells inheriting 500±60 copies (Warren, 1993b). Since there are about 10,000 Golgi fragments in anaphase HeLa cells, the partitioning accuracy will be even greater.

Although the extensive vesiculation seen in animal cells is largely explained by the requirement for equal partitioning of the Golgi apparatus to the two daughter cells,

it appears that the process may serve a second function. This conclusion follows from the observation that despite the fact that the number of dispersed stacks in *Xenopus* oocytes arrested in first meiotic prophase is at least an order of magnitude greater than the number of Golgi-derived vesicles in mammalian cells during anaphase (i.e. 10,000), and therefore sufficiently large to allow efficient redistribution of these dispersed stacks to the daughter cells, these stacks undergo subsequent vesiculation as they progress into second meiotic metaphase (Colman, *et al.*, 1985). Since this vesiculation is statistically redundant, it must serve some other useful biological function, and an intriguing possibility is the remodelling of the Golgi stack. In this way, the cell might make use of the opportunity afforded by the breakdown of the Golgi into vesicular fragments to undertake the fine tuning of the stack's precise structure and function which occurs during differentiation in multi-cellular organisms. This is consistent with the absence of vesiculation in unicellular eukaryotes such as yeast. However, it is inconsistent with the need for differentiation in higher plant cells where there is also no Golgi fragmentation during M phase; an alternative mechanism would have to be at work in these cells.

Equal partitioning by a stochastic process is conditional upon the division of the parent cell into two equally-sized daughter cells, which in turn is conditional on there being a mechanism to ensure the accurate positioning of the cleavage furrow at the onset of cytokinesis. Such mechanisms await discovery.

1.5 Summary and Aims

The study of intracellular protein traffic has provided many insights into the generation and maintenance of compartmentalisation within the cell. In particular, the exocytic pathway demonstrates the elegant specialisation of function which is necessary to ensure the efficient synthesis, assembly, processing and targeting of molecules. A series of mechanisms exist to target proteins to their correct destination, and to retrieve them in the event of illicit exit from their correct location. The precise segregation of enzymes responsible for post-translational modification of secretory proteins to distinct subcompartments maximises the efficiency of the processing; sequential processing steps occur in sequential compartments, so that structure and function within the secretory pathway are intrinsically linked. Such functional compartmentalisation also means that post-translational modifications of secretory proteins provide a useful means of establishing which compartments have been visited and monitoring the kinetics of transfer between these compartments.

The combined power of genetic analysis and *in vitro* reconstitution of protein transport has already shed much light on the molecular mechanisms underlying

intercompartmental transport, and has led to the identification of a number of key components which are not only conserved between different steps of the pathway but also between species.

During M phase, almost all stages of intracellular protein transport are arrested, and transport does not resume until telophase. The mechanism of inhibition of transport remains obscure for the present. Concomitant with the cessation of protein transport are a number of striking changes in cell architecture. One of the most startling of these changes is the vesiculation of the Golgi apparatus; disassembly yields about 10,000 vesicles scattered throughout the mitotic cell cytoplasm. These vesicles are then partitioned to the two daughter cells and form the building bricks for the generation of a single copy of the organelle within each daughter cell. Although the pathways of disassembly and reassembly are known in considerable detail, the molecular mechanisms remain uncertain.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Overview

This presentation of methods used in the work I have undertaken will combine both references to published methodology and a detailed description of the techniques I have used. In many cases, I found it necessary or preferable to adapt the published methods to suit the requirements of the task in hand. The sequence of methods generally follows the sequence of experiments described in the text of Chapters 3 to 5 inclusive.

2.2 Media and reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co., BDH Chemicals Ltd, or Boehringer Mannheim. Cell culture reagents came from Northumbria Biologicals Ltd, Flow Laboratories or Gibco.

2.3 Cell Culture

Monolayer HeLa cells (ATCC CCL 185) were grown at 37°C in an atmosphere of 95% air/ 5% CO_2 in MEM supplemented with 10% fetal calf serum, penicillin, streptomycin and non-essential amino acids.

2.4 Production of populations of G1 and prometaphase HeLa cells

The method for generating synchronous HeLa cells followed essentially that of Zieve *et al.*, (1980). HeLa cells were grown in 150cm^2 tissue culture flasks until they reached 90% confluency and were then trypsinised and transferred into plastic roller bottles containing 100 ml of growth medium (GM) as described above. The bottles were then gassed with a mixture of 95% air/ 5% CO_2 and the bottles sealed tight. The cells were allowed to grow on the roller bottle surfaces at 37°C while being rotated at 0.25rpm until they reached 60-70% confluency (about two days).

The growth medium from the roller bottles was then replaced with GM containing 5mM thymidine (added to the GM as a powder) and the cells were incubated in this medium for 16 hours. The roller bottles were then spun at 200rpm for 5 min to release any dead cells from the monolayer and the medium was aspirated. The monolayers were then washed with 25ml of fresh GM and were incubated in 50 ml of fresh GM for 9.5 hours. 0.5ml of methyl (5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) carbamate or "nocodazole" (4μg/ml in DMSO) was added to the roller bottles to give a final concentration of 40ng/ml and the cells were pre-incubated in this medium for 20 min. The bottles were then spun once more for 5 min at 200rpm to

release any dead cells. The medium was aspirated and 50ml of fresh GM containing nocodazole at 40ng/ml (using a 200µg/ml stock in DMSO kept at -80°C) were added. The cells were incubated for a further 4 hours. At each stage of the procedure when the bottles were opened, they were gassed for approximately 15 seconds with 95% air/ 5% CO_2.

Prometaphase HeLa cells were selectively detached by a harvest spin of 5 min at 200rpm and the number of cells harvested was estimated by haemocytometry.

2.5 Labelling mitotic cells

The mitotic cells were spun down at 1400rpm for 4 min at room temperature (RT) and were then resuspended in labelling medium (methionine-free RPMI containing 0.2% dialysed fetal calf serum, nocodazole (40ng/ml) and 10mM HEPES pH 7.2) such that the concentration of cells was 2-3 x 10^6/ml. The cells were spun at 1400rpm for 2 min at 4°C and this labelling medium wash was repeated once more. The pellet was then resuspended in 1ml of labelling medium and was transferred to a 1ml screw-capped Eppendorf. The cells were spun at 2000rpm for 2 min at RT in a benchtop Eppendorf centrifuge and were then resuspended in 1ml of pre-warmed labelling medium containing 5mCi of ^{35}S-methionine (Amersham). The cells were pulsed for 5 min at 37°C and were then spun at 2000rpm for 2 min at 4°C in a benchtop Eppendorf centrifuge to recover the labelling medium[1]. The pellet was then resuspended in E4 medium containing 10% fetal calf serum, 10mM HEPES pH7.2 and 10µg/ml cycloheximide to a final cell density of 3-4x10^6/ml. The cells were spun for 2 min at 1400rpm at 4°C and the E4 wash was repeated once more. The cells were then resuspended in chase medium (MEM modified for suspension cultures containing 10% fetal calf serum, penicillin, streptomycin, non-essential amino acids, 10mM HEPES pH7.2 and cycloheximide at a final concentration of 10µg/ml) so that the final cell density was 10^6/ml. The suspension was divided into 1.4ml aliquots[2] in screw-capped Eppendorfs and incubated at 37°C on a spiromix.

2.6 Labelling G1 cells

The protocol for labelling G1 cells followed that of the mitotic cells outlined in the previous section except that the cells were first allowed to progress through mitosis into G1 before being pulsed. In order to allow this mitotic progression, the nocodazole arrest had first to be relieved; after the initial 1400rpm, 4 min, RT spin,

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[1]Labelling medium was used three or four times before being discarded.
[2]Since the pre-chase washes were carried out in a 15ml Falcon tube, it was found easier to resuspend the pellet in 0.5ml of chase medium, transfer this to an Eppendorf with a glass pipette, and then wash out the Falcon tube with another 0.5ml of chase medium. The 1ml suspension of cells could then be accurately and safely divided into aliquots for each of the chase time samples.

the cells were washed twice in chase medium omitting the cycloheximide at a density of 2-3x10^6/ml and were then incubated in chase medium (again without the cycloheximide) for 2 hours at 37°C at a density of 10^6/ml. This incubation gave a population of HeLa cells with a mitotic index typically less than 10%. These cells were then washed twice in the labelling medium and the rest of the procedure followed that of the mitotic protocol above (including the presence of nocodazole in the labelling medium so that the labelling conditions for both the mitotic and G1 cells were identical).

2.7 Nocodazole controls

In the case of the controls in which the chase was carried out in the presence of nocodazole, the labelled suspension of cells was divided into two equal aliquots. One half was washed in the nocodazole-free E4 medium and then the nocodazole-free chase medium, prior to chase incubation in nocodazole-free chase medium as described above. The other half was washed and chased in parallel, except that the nocodazole concentration was maintained throughout at 40ng/ml. This regime was used for both the mitotic and G1 controls.

2.8 Cell surface antibody binding

Binding of antibody to MHC Class I molecules at the cell surface was used to establish transfer of protein to the cell surface during the course of the pulse chase period. At the end of each chase period, the cells were washed once in ice-cold CMF-PBS[3], and were resuspended in 200µl of pre-spun (50,000rpm, 5 min, 4°C) W6/32 1x hybridoma supernatant. The cells were incubated on ice for 30 min with resuspension every 10 min. After 4 washes in ice-cold CMF-PBS, the cells were lysed in a 5-fold excess of unlabelled HeLa cell lysate in order to quench any free antibody that had not been washed away. The rest of the lysis procedure followed the regime described below.

2.9 Pre-condensation of Triton X-114

Pre-condensation of Triton X-114 (TX-114) followed the procedure of Bordier (1981). 20g of TX-114 (Boehringer Mannheim) containing 16mg butylated hydroxytoluene were dissolved in 980ml 10mM Tris-HCl pH7.4, 150mM NaCl at 4°C. The solution was incubated at 30°C to condense the detergent, and was allowed to separate into a detergent phase and an aqueous phase overnight. The aqueous phase was discarded and replaced by the same volume of 10mM Tris-HCl pH7.4, 150mM NaCl. The detergent was once again dissolved at 4°C, and the condensation repeated at 30°C. This condensation procedure was repeated twice

[3]Calcium and magnesium-free PBS

(three rounds in all). The final TX-114 concentration was measured by absorbance at 275nm and found to be 11%. The precondensed TX-114 was used as a stock for lysis buffer preparation.

2.10 Preparation of cell lysates

Cell lysis and immunoprecipitation were carried out as described by Yang (1989). At the end of each chase period, the cells were spun for 2 min at 2000rpm at 4°C in a benchtop Eppendorf centrifuge and were washed once in 1ml ice-cold CMF-PBS. The cells were then lysed in 1ml lysis buffer (50mM Tris-HCl pH7.5, 5mM EDTA, 25mM iodoacetamide containing 0.5% pre-condensed TX-114) for at least 30 min at 4°C while being rotated end-over-end. The lysate was then centrifuged for 10 min at 14,000rpm at 4°C in a benchtop Eppendorf centrifuge and the supernatant transferred to a fresh screw-capped Eppendorf.

2.11 Cloud-point separation

Following a 5 min incubation at 37°C, the lysates were spun for 5 min at 5000rpm at RT in a benchtop Eppendorf centrifuge to separate the aqueous phase from the lower detergent phase. The upper phase was discarded and 450µl of TNEN buffer (20mM Tris-HCl pH 7.6, 100mM NaCl, 10mM EDTA, 0.5% NP40) were added to the detergent phase.

2.12 Pre-clearing lysates prior to immunoprecipitation

7.5µl rabbit anti-mouse IgG and 50µl of a 10% suspension of *Staphylococcus Aureus*[4] *(Staph. A.)* cells were added to the lysates which were then allowed to preclear overnight at 4°C while being rotated end-over-end.

2.13 Antibodies

MHC Class I heavy chain molecules were immunoprecipitated using the monoclonal antibody W6/32 (Barnstable, *et al.*, 1978). The antibody was added to lysates in the form of 1x supernatant from the W6/32 hybridoma culture, gratefully received from Dr. Julia Bodmer, ICRF. The control immunoprecipitation of the heavy chain using an anti-ß2m antibody was carried out with the affinity purified K355 polyclonal antibody, donated from Dr. Per Peterson, La Jolla. The transferrin receptor was immunoisolated with the affinity purified OKT9 antibody (Sutherland, *et al.*, 1981) provided by Dr. Richard Hunt, University of South Carolina. Western

[4]The *Staph. A.* cells were washed three times in ice-cold CMF-PBS, and then once in Wash Buffer A (see section on immunoprecipitate washing for recipe), before being resuspended to give a 10% cell suspension. The suspension was finally sonicated for a few seconds to ensure adequate dispersion of any remaining clumps. Between washes, the cells were spun at 9K for 2min at RT. Resuspension was best achieved by running the tubes rapidly along the holes of a plastic Eppendorf rack.

blotting of galactosyltransferase was carried out with polyclonal antibody N11, donated by Dr. Eric Berger, Zurich (Watzele, *et al.*, 1991). Myc tagged N-acetylglucosaminyltransferase I was blotted with affinity-purified monoclonal antibody 9E10 from Dr. Gerard Evan, ICRF (Evan, *et al.*, 1985).

2.14 Immunoprecipitation

The lysates were spun at 14,000rpm for 10 min at 4°C in a benchtop Eppendorf centrifuge and the supernatants were then transferred to fresh screw-capped Eppendorf tubes. HLA was immunoprecipitated by the addition of 50 µl of pre-spun (5 min, 50,000rpm, 4°C) W6/32 monoclonal antibody to each lysate; the transferrin receptor was immunoprecipitated by the addition of 2.5µl of affinity purified OKT9 antibody along with 2µl of rabbit anti-mouse IgG. The tubes were incubated on ice for 3.5 hours. 50µl of *Staph. A.* suspension were added to each and the tubes were rotated end-over-end at 4°C for 30 min.

2.15 Washing the immunoprecipitates

The immunoprecipitate pellets were then washed three times, twice in TNEN buffer containing 0.1% SDS, 0.5% deoxycholate and 1% BSA, and once in a ten-fold dilution of TNEN containing 0.5M NaCl. Between washes the *Staph. A.* pellets were spun at 4000rpm for 4 min at 4°C in a benchtop Eppendorf centrifuge and the pellets were resuspended by running the tubes rapidly along the holes of a plastic Eppendorf rack.

2.16 Enzyme digestions

2.16.1 Cell surface neuraminidase digestion: The cells were washed once in 1ml of ice-cold CMF-PBS and resuspended in 33µl of ice-cold CMF-PBS containing 1mM $CaCl_2$. 17µl of CMF-PBS/$CaCl_2$ (mock-treated samples) or of CMF-PBS/$CaCl_2$ containing neuraminidase (Sigma Type VIII) at 5U/ml were added to each and the suspension was left on ice for 30 min with tapping every 10 min. After four ice-cold CMF-PBS washes, the cells were then lysed in 1ml of lysis buffer.

2.16.2 Endoglycosidase H digest of immunoprecipitated HLA:
Endoglycosidase H (endo H) digestion was carried out as described by Sege *et al.* (1981). 30µl of 50mM Na Acetate pH5.0 containing 0.3% SDS were added to the immunoprecipitate pellets. The pellets were vortexed and left for 5 min at RT. After a 4000rpm spin for 2 min at RT in a benchtop Eppendorf centrifuge, the pellets were once again resuspended on a whirlimix and left for a further 5 min at RT. The pellets were then spun for 20 seconds at 14,000rpm at RT in a benchtop Eppendorf centrifuge and were heated at 95°C for 3 min. After further vortexing,

the tubes were spun again for 20 seconds at 14,000rpm and the supernatants divided into two aliquots of 15 µl each. 10µl of 50mM Na Acetate pH5.3 containing 1µl of endo H or of Na Acetate alone (mock-treated samples) were added to each and the tubes were incubated at 37°C for at least 8 hours or overnight. The reaction was terminated by the addition of 25µl of two-fold concentrated SDS-PAGE sample buffer (200mM Tris-HCl pH8.8, 30% sucrose, 4% SDS and bromophenol blue) containing 10mM DTT.

2.16.3 Endoglycosidase D digest of immunoprecipitated HLA: Endo D digestion was carried out as described by Beckers *et al.*, (1987).The immunoprecipitate *Staph. A.* pellets were resuspended in 1ml of ice-cold digestion buffer A (25mM NaH_2PO_4, 0.1% TX-100; 5mM EDTA pH 6.5) and this volume was then divided into two 0.5ml aliquots. Following a 4 min, 4000rpm spin at 4°C, the supernatant was aspirated and 30µl of digestion buffer were added to each tube. 10µl of digestion buffer B (10 mM Tris-HCl pH7.4, 200mM NaCl) alone (mock-treated samples) or digestion buffer B containing endo D at 0.1U/ml were added to each and the samples incubated for 4 hours at 37°C, with occasional resuspension by tapping. 1ml of TNEN was added to each and the tubes were spun for 4 min at 4000rpm at RT in a benchtop Eppendorf centrifuge. The supernatant was aspirated and 50µl of SDS-PAGE sample buffer were added to each.

2.16.4 Neuraminidase digest of immunoprecipitated HLA: The immunoprecipitate *Staph. A.* pellets were resuspended in 1ml of ice-cold digestion buffer (300mM NaCl, 100mM Na Acetate, 14mM $CaCl_2$ pH 5.5) and this volume was then divided into two 0.5ml aliquots. Following a 4 min, 4000rpm spin at 4°C, the supernatant was aspirated and 30µl of digestion buffer containing 1.33mM PMSF were added to each tube. 10µl of digestion buffer alone (mock-treated samples) or digestion buffer containing neuraminidase (Sigma Type VIII) at 5U/ml were added to each and the samples were incubated for 4 hours at 37°C, with occasional resuspension by tapping. 1ml of TNEN was added to each and the tubes were spun for 4 min at 4000rpm at RT in a benchtop Eppendorf centrifuge. The supernatant was aspirated and 40µl of isoelectric focusing sample buffer were added to each.

2.17 Analysis of immunoprecipitated protein

SDS-PAGE followed the procedure of Blobel and Dobberstein (Blobel and Dobberstein, 1975). The endoglycosidase treated samples were heated at 95°C for 3 min and allowed to cool to RT before 5µl of 0.5M iodoacetamide were added to each. The samples were left at RT for at least 20 min before being loaded on a 10-15% gradient gel. SDS-PAGE was carried out overnight at about 6mA per gel

(constant current) and the gels were then fixed for 30 min in 40% methanol/10% acetic acid (v/v) in water. After 30 min in Amplify, the gels were dried and were then fluorographed using pre-flashed Kodak XAR film, according to Laskey and Mills (1975). In the case of Coomassie Blue R-250 staining, the cells were stained for 10-15 min in a 0.1% (w/v) stain solution and destained in fix solution for 30 min before drying.

The neuraminidase treated samples were shaken at RT in IEF sample buffer for 30 min. The samples were spun for 5 min at 14,000rpm at RT, and the supernatants transferred to fresh Eppendorf tubes in order to avoid loading any *Staph. A.* cells onto the IEF gel. The method of IEF followed the protocol described in Yang (1989). Gel fixation, treatment with Amplify and fluorography followed the procedure used for SDS-PAGE gels. Because of the fragility of the IEF gels, it was found easier to keep the gels resting on a hard surface at all stages of gel manipulation; transfer to the gel dryer was most easily carried out by pressing dry filter paper onto the gel while it was still in a plastic container box, and then lifting the paper and gel together to the gel dryer.

2.18 Laser densitometry

Fluorographs were scanned using an LKB Ultroscan laser densitometer. Protein bands were quanitified by generating a printed profile of track intensities followed by cutting out and weighing the relevant peaks. The extent of transport to the *medial* Golgi was measured as the proportion of HLA present in the endo H-resistant band relative to total HLA. The extent of transport to the *trans* Golgi was measured as the proportion of HLA-A containing either one or two sialic acid residues relative to the total HLA-A.

Transport to the plasma membrane was measured as the extent of desialylation of HLA-A treated with exogenous neuraminidase. This was calculated as 1-(ratio of sialylated HLA-A after digestion to sialylated HLA-A before digestion). This method of quantitation meant that the protein levels were normalised relative to total protein within each track before comparisons were made between tracks, eliminating any error due to variation in protein amounts in different tracks. Since transport to the *medial* and *trans* Golgi relied on comparison of protein bands within the same track, there was no risk of error due to such protein variation between tracks.

2.19 Determination of protein synthesis levels

The level of protein synthesis in mitotic and G1 populations was measured by means of TCA precipitable counts (Mans and Novelli, 1961). 10µl from a 1ml

labelled lysate were spotted onto 2.4cm round Whatman 3MM filters. The filters were transferred to several hundred ml of ice-cold 10% TCA and left for an hour. After being boiled in 5% TCA for 10 min, the filters were washed in a 1:1 ethanol:ether mixture, rinsed in ether alone and then allowed to air-dry. The dry filters were immersed in scintillation cocktail and the amount of radioactivity incorporation was determined by scintillation counting in a Beckman counter.

2.20 Determination of cell latency

Cell latency was measured by means of a lactate dehydrogenase assay. Triton X-100 was added to both sample cell pellets and supernatants to 0.1%. A suitable volume of sample (10-100µl of cell suspension or supernatant) was added to 933 µl of reaction mix (5ml of 1 M sucrose, 0.66ml of 10 mM Na pyruvate, 6.6 ml of 20mM HEPES pH7.2, 6.4 ml of H_2O) in a glass cuvette. 66µl NADH solution (prepared by adding 1.43 ml 1 mM $NaHCO_3$ to a pre-weighed vial of NADH) were added and the cuvette inverted several times. The kinetics of LDH activity were then measured in duplicate using a spectrophotometer and appropriate enzyme kinetics programme. The percentage permeabilisation was determined by calculating the proportion of LDH activity in the supernatant fraction.

2.21 Mitotic index scoring

Calculation of the mitotic index followed the procedure of Berlin *et al.* (1978). An aliquot of about 5-10x10^5 cells was removed from chase samples after 0, 30, 60, 90 and 120 min. The cells were spun for a few seconds at 14,000rpm at RT and were resuspended in 3% paraformaldehyde containing 0.2% TX-100 and bis-benzamide (Hoechst 33258) at 2µg/ml. The cells were inspected and scored according to mitotic phases using a Zeiss Axiophot immunofluorescence microscope. Quantitation of the mitotic index at each time point involved scoring an integral number of fields of view such that the total number of cells counted was in excess of 200. The fixed aliquots of cells could be kept at 4°C for several days without affecting the accuracy of mitotic index determination.

Because of the characteristic staining of dead cells with Hoechst 33258, mitotic index evaluation also allowed scoring of cell viability over the same time period, and was typically greater than 90%.

2.22 Histone H1 kinase assay

The procedure for establishing the H1 kinase activity followed the method of Felix *et al.* (1989). Mitotic HeLa cells were aliquotted into Eppendorf tubes at a density of 10^6/ml (1.4x10^6 cells in each tube) and after 0, 30, 60, 90 and 120 min the cells were spun for 2 min at 2000rpm at 4°C in a benchtop Eppendorf centrifuge and

then washed once in 1ml of ice-cold EBS buffer (80mM beta-glycerophosphate buffer pH7.2 containing 100mM sucrose, 15mM $MgCl_2$, 20mM EGTA, 2mM ATP, 1mM DTT and 1mM PMSF). The cells were spun again, aspirated and 30µl of the EBS buffer were added to each. The cells were then snap-frozen in liquid nitrogen and stored in liquid nitrogen until the histone H1 kinase assay was carried out. After freezing, the samples were rapidly thawed at 37°C and 5µl of each were added to 15µl of a reaction mix containing 107mM betaglycerophosphate, 67mM NaF, 1.33mM $MgCl_2$, 1.33mM ATP and 20µCi of ^{32}P-ATP. The reaction mix was either made up in KEHM buffer (50mM KCl, 10mM EGTA, 50mM HEPES pH7.2 and 1.92mM $MgCl_2$) alone (-histone control) or KEHM buffer containing histones at 3.45mg/ml. The mixture was incubated at 37°C for 15 min and then returned to ice, 12µl of the sample volume were spotted onto 1.5cm^2 grid squares on P81 phosphocellulose paper. The sample grid was washed three times in 150mM H_3PO_4 (20ml per sample) for 15 min each, soaked briefly in 95% ethanol and air-dried. The samples were immersed in scintillation cocktail and counted in a Beckman scintillation counter.

Evaluation of the final H1 kinase activity involved the calculation of pmol phosphate transferred per min per mg of protein present in the assay, corrected for histone-independent counts and background.

2.23 Electron microscopy

Non-radioactive mitotic cells were treated in parallel with the radioactively-labelled cells and chased for up to 180 min at 37°C. Samples were prepared for electron microscopy following the method of Lucocq *et al.* (1989).

For each of the time points, ten fields containing cells were sampled at random and photographed at x11,500. These fields were then scanned at higher magnification and every Golgi stack or cluster was photographed at x28,500. Golgi clusters were defined as groups of at least five vesicles (Lucocq *et al.* 1989). Photographs were printed at a final magnification of x102,560.

Surface densities of Golgi membrane per cell volume (S_{go}/V_{cell}) were calculated using the following equation :

$$S_{go}/V_{cell} = S_{go}/V_{go} \times V_{go}/V_{cell} \qquad \text{(Equation 1)}$$

where S_{go}/V_{go} is the surface density of the Golgi membrane within individual Golgi stacks and V_{go}/V_{cell} is the fraction of cell volume occupied by Golgi stacks.

S_{go}/V_{go} was estimated from the high magnification pictures using a square lattice grid with 20 mm spacing using the following equation:

$S_{go}/V_{go} = \Sigma I/\Sigma P \times d$ (Equation 2)

where ΣI is the sum of vertical and horizontal intercepts of the grid with Golgi membranes, ΣP is the sum of the points lying on Golgi cisternae, associated vesicles and intercisternal space and d is the distance separating the vertical and horizontal lines of the grid.

A cisterna was defined as any structure within the Golgi region of which the length was at least four times the width (Lucocq, *et al.*, 1989). A cisterna was further defined as stacked if a line perpendicular to the cisterna at the point of lattice intersection also intersected an adjacent parallel cisterna within one cisternal width. The number of cisternae within a stack was defined as the number of these adjacent parallel cisternae.

V_{go}/V_{cell} was estimated directly from the lower magnification negatives using a double square lattice grid (16 and 2 mm spacings). Points lying over the cell were counted using the 16 mm grid lines and those over the Golgi using 2 mm grid lines. V_{go}/V_{cell} was then given by the ratio of the number of points over the Golgi to the points over the cell, multiplied by 64 to allow for the different line spacings.

Cisternal length was measured by counting intersections of a grid with 13 mm spacings with cisternal membranes of Golgi stacks sampled at random, photographed, and printed at a final magnification of x102,560. The total length of cisternae for each Golgi was calculated, and this was then divided by the number of cisternae in the stack.

2.24 Golgi fractionation from tissue culture cells

Golgi fractionation followed the procedure for preparing "donor" membranes as described by Beckers and Rothman (1992). 4/12 HeLa cells were grown on 576cm^2 plates in growth medium (GM) until they reached 80 to 90% confluency. The cells were detached from the plates using trypsin/EDTA , resuspended in ice-cold GM, spun at 1500rpm for 5 min at 4°C and washed twice in 0.25ST buffer (0.25M sucrose in 10mM Tris-HCl, pH7.4). The cell pellet was resuspended in an equal volume of 0.25ST buffer using a 1ml Gilson pipette and the suspension homogenised with a ball-bearing homogeniser. About 15 strokes were required to break the cells without excessive fragmentation of the nuclei. The level of cell breakage was determined using Hoechst 33258 staining. The volume of the homogenate was made up to 6ml with 0.25ST buffer. Equal volumes of the homogenate were mixed with 2.3ST buffer (2.3M sucrose in 10mM Tris-HCl, pH7.4) to give a final concentration of 1.4M sucrose. EDTA was added to give a final concentration of 1mM, the mixture added to SW27 tubes (about 12ml per

tube) and overlaid with 14ml 1.2ST buffer (1.2M sucrose in 10mM Tris-HCl, pH7.4) and 8ml of 0.8ST buffer (0.8M sucrose in 10mM Tris-HCl, pH7.4). Following centrifugation at 25,000rpm for 2.5h at 4°C, the Golgi membranes were harvested from the 0.8/1.2M interface by tube puncture using a needle and syringe in the smallest possible volume. The membranes were frozen in liquid nitrogen in 60µl aliquots and stored at -80°C. Each aliquot was thawed once only.

2.25 Chloroform-methanol precipitation of TX-114 lysates

Chloroform-methanol precipitation followed the method of Wessel and Flugge (1984). The TX-114 detergent phase (about 50µl) was thawed from -80°C storage and 200µl methanol were added. After vortexing, the mixture was centrifuged for 10 seconds at 11,500rpm on the benchtop centrifuge. 100µl of chloroform were added and the tube vortexed. The same centrifuge step was repeated. After the addition of 150µl of water, the tube was vortexed and spun at the same speed for 1 min. The upper phase was discarded and 150µl of methanol were added to the lower phase. The tube was vortexed, spun for 2 min and the supernatant discarded. The protein pellet was allowed to air dry for about 30 min before being dissolved in the appropriate sample buffer.

2.26 Two dimensional gel electrophoresis

Two dimensional gel electrophoresis followed the protocol of O'Farrell (1975) with a few minor changes.

2.26.1 Preparation of the 1st. dimensional gel

The gel tubes were washed in concentrated nitric acid overnight, rinsed well with water, rinsed once in ethanol and dried in an oven for 1 hour. The tubes were then placed in an appropriate cylinder (e.g. a 50ml plastic syringe from which the top has been cut and replaced with a rubber bung). 10 ml of gel solution (5.7g urea, 2ml of 10% (v/v) NP40, 1.33ml of 28.4% acrylamide/ 1.6% bis solution, 400µl pH5-7 ampholines, 100µl pH3.5-10 ampholines, 1.95ml water) were prepared by incubating the mixture at 37°C until the urea had dissolved (about 1h). 5µl TEMED and 10µl 10% ammonium persulphate were added, the solution mixed and then carefully poured down the side of the tubes. The gel solution was overlaid to the top of the syringe with water. The tubes were lifted occasionally to allow the gel solution to enter the tubes. As the solution travelled up the tubes, more water was overlaid so that the gel solution eventually reached the tops of the tubes. The tubes were overlaid with 0.02% NP40 sprayed over the tops of the tubes and left to polymerise for 1 hour on the bench. The NP40 overlay was removed and replaced with 25µl sample buffer (2.85g urea, 1ml of 10% (v/v) NP-

40, 200µl pH5-7 ampholines, 50µl pH3.5-10 ampholines, 76mg DTT, 1.5ml water, dissolved at 37°C for 1 h). The ends of the tubes could then be wrapped in parafilm and stored in the fridge for up to a week.

2.26.2 Pre-focusing the 1st dimension gel

Tubes with the same length of gel were selected and left at RT for 1h to allow the urea to dissolve. The gel apparatus was assembled, the anolyte (10mM H_3PO_4) added to the lower chamber and the catholyte (20mM NaOH) added to the upper chamber. The sample buffer was aspirated from the top of the tube gels and carefully replaced with 25µl of fresh sample buffer to avoid any air bubbles in the sample. 10µl of sample overlay (2.7g urea, 100µl pH5-7 ampholines, 25µl pH3.5-10 ampholines, 2.6ml water) were placed over the sample buffer and the tubes were then filled to the top with NaOH. Having lowered the tubes under the surface of the catholyte, the upper tank assembly was carefully lowered into the main tank to avoid spilling any of the alkali into the acid. Air bubbles were removed from underneath the tube gels. The pH gradients within the tube gels were pre-focused by applying 200V for 15 min, then 300V for 30 min and finally 400V for 30 min.

2.26.3 Running the sample on the 1st. dimensional gel

The inner tank assembly was removed from the outer tank and the tube gels pushed up out of the catholyte. The NaOH, overlay and sample buffer were aspirated. 25µl of the sample and then 10µl of overlay were placed on top of the gel, and the tubes then filled to the top with alkali. The tube position was adjusted as before and the inner tank assembly replaced into the outer tank. The gels were run for 16 hours at 300V or 12 hours at 400V. The voltage was increased to 800V for 1 hour at the end of the run to sharpen the bands. The gels could then be stored in their glass tubes at -20°C until required.

2.26.4 Running the 2nd. dimension

The 2nd dimension gel (1.5mm thickness) was poured. A 2D gel comb was used for the stacking gel. Using a syringe filled with water, the tube gel was extruded onto a sheet of parafilm and soaked in 1.4ml of sample buffer containing 200µl 20% SDS and 200µl 0.5M DTT for 30 min. The positive and negative ends of the gel were marked carefully. Having washed out the top of the 2nd dimension gel, the sample buffer was drained from the 1st dimension gel and the tube gel then carefully placed along the top of the gel with the positive end next to the molecular weight marker slot. Care was taken to remove any air bubbles under the gel. The gel was run at about 4mA per gel overnight (constant current).

2.27 Western blotting

Protein from SDS-PAGE gels and IEF gels was wet blotted in CAPS buffer pH11.0 for 3 h at 300mA at 4°C onto Hybond-C filters. Prior to this transfer, IEF gels were washed five times in 50% methanol, 1% SDS, 5mM Tris-HCl pH8.0 to remove NP-40 . Each wash consisted of a 30 min incubation at RT in 200ml of solution. After the transfer, the binding and detection of antibodies on the Hybond C filters followed the Amersham Enhanced Chemiluminescence (ECL) protocol. The filters were incubated for 1 h at RT in blocking solution (5% (w/v) dried milk powder, 2% (v/v) Tween-20 dissolved in CMF-PBS) and then usually left overnight at 4°C. The filters were incubated in primary antibody (1:1000 dilution in blocking solution of either N11 or 9E10) for 1 h at RT, rinsed 3 times in CMF-PBS containing 2% Tween-20, washed 3 times for 30 min each in blocking solution, and then two times for 5 min each in CMF-PBS containing 2% Tween-20. The secondary antibodies were affinity-isolated goat anti-mouse (for use with 9E10) or anti-rabbit (for use with N11) antibodies conjugated to horseradish peroxidase. Secondary antibody incubation was for 45 min, and all the washing steps were identical to the post-primary washes. An ECL kit was used for the detection of the secondary antibodies and the filters were exposed to Kodak XAR film for varying lengths of time.

CHAPTER THREE: ESTABLISHING A SYSTEM TO MONITOR INTRACELLULAR PROTEIN TRANSPORT

3.1 Aims and objectives

Since the overall goal of this investigation was the way in which the resumption of protein transport through the Golgi apparatus relates to the reassembly of the organelle during telophase in HeLa cells, it was clearly necessary to first establish a system with which to monitor the kinetics of transport along the secretory pathway during interphase. Such a system would have to allow the quantitative analysis of the rate of protein transfer to different compartments of the exocytic pathway. Secondly, the system would have to be capable of monitoring transport not only in interphase cells, but also in a synchronised population of HeLa cells progressing through M phase, since it was this kinetic comparison which formed an integral part of the project undertaken. Establishing this system was the aim of the work described in this first experimental section.

I shall first outline the reasons for the choice of marker protein for this work and then discuss the various biochemical assays which have been employed in parallel to quantify the interphase kinetics of protein secretion from the ER to the cell surface.

3.2 Choice of marker protein

Establishing the relationship between protein traffic and reassembly requires the use of a distinctive marker protein to monitor the kinetics of transfer through the secretory pathway. Much of the early investigation of protein transport in mitotic animal cells relied on the use of virally-infected cells (Warren, *et al.*, 1983; Featherstone, *et al.*, 1985). While such systems have been extremely informative with respect to demonstrating mitotic inhibition of transport, they are of rather more limited use in the study of the resumption of transport at the end of mitosis; viral infection puts the cell under considerable stress which undermines the capacity to progress through M phase effectively. To avoid these technical problems, I decided to use an endogenous protein to monitor the resumption of transport. Specifically, I chose MHC Class I heavy chain for these studies, for a number of reasons. Firstly, these molecules provide excellent probes for the secretory pathway since they are expressed at the cell surface, and therefore are transported along the default pathway following their synthesis in the ER. Secondly, the structure of the human heavy chain (HLA) is well-characterised. Thirdly, there is a single N-linked glycosylation site at Asn86, facilitating quantitative analysis of oligosaccharide processing as a means of monitoring

passage through the secretory pathway. Fourthly, the HLA molecules are expressed at suitable levels in HeLa cells for immunoprecipitation. Finally, antibodies against the Class I complex are readily available for biochemical analysis.

3.3 Monitoring transport from the ER to the medial Golgi cisternae

One of the most fully characterised means of establishing transport from the ER to the *medial* Golgi is the use of endoglycosidase H (endo H). N-linked oligosaccharide side-chains which have been modified by mannosidase I, a resident of the ER and *cis* Golgi, but not by mannosidase II, a resident of the *medial* Golgi are sensitive to digestion by this exogenous glycosidase. In the case of glycoproteins which undergo full oligosaccharide processing from high mannose to complex sidechains, digestion with this enzyme therefore provides a means of establishing whether such a glycoprotein has reached the *medial* Golgi or has only visited pre-*medial* compartments such as the ER or *cis* Golgi. This digestion assay therefore provided the first means of dissecting the kinetics of transport in HeLa cells. Although the digestion may be carried out on cell lysates, I elected to treat immunoprecipitated HLA with endo H.

3.4 Use of G1 rather than interphase cells

The initial experiments were performed using interphase populations of HeLa cells. However, it was advantageous to consider synchronised G1 populations as soon as possible so that the methodology of the early experiments was kept as similar as possible to the subsequent investigation of synchronised cells progressing through M phase. In this way, any differences in the kinetics between mitotic cells (i.e. cells progressing through M phase) and interphase (G1) cells could be attributed to differences in cell physiology rather than methodological artefacts. A later control demonstrated that the intracellular transport kinetics of G1 cells adequately reflected those of interphase cells (see section 3.11 on control experiments, page 80).

3.5 Cell synchronisation: production of a G1 population

Since passage past prometaphase requires the rearrangement of the interphase MTs of the cytoskeleton into the mitotic spindle, any MT disrupting agent will preclude this spindle construction and have the effect of arresting the cell population in prometaphase. These pulse-chase studies relied on the use of one such MT-disrupting drug to synchronise HeLa cells in prometaphase, namely, methyl (5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) carbamate, also termed "nocodazole".

Rather than simply using this drug on its own, it was possible to refine the synchronisation procedure very simply by first arresting the cell population in S phase or at the G1/S transition with thymidine. The population was released from the thymidine arrest and allowed to progress through S phase and most of G2 phase. A nocodazole arrest was then imposed when most of the cells were in late G2. The combined use of thymidine and nocodazole produced a population with a mitotic index typically in excess of 98%, with a viability index over 95%. The exact incubation times were worked out to give an optimal yield of prometaphase cells; these times and concentrations of the agents used are outlined in Materials and Methods (Chapter 2).

In order to investigate protein transport in mitotic HeLa cells, the prometaphase population could be pulsed immediately with ^{35}S-methionine and then chased through the rest of M phase into G1 phase; this was the method used in the work described in the next chapter on the resumption of protein transport at the end of mitosis. Alternatively, the arrested population could be immediately washed free of nocodazole and incubated for 2 h so that the entire population entered G1. This G1 population provided the "interphase" cells depleted of mitotic cells which were used in the experiments described in the present chapter.

3.6 Labelling and isolating HLA molecules

The most advantageous means of performing these pulse chase experiments was a 5 min pulse with ^{35}S-methionine, followed by a chase for up to 90 min. Early extraction of protein was achieved using Triton X-100 but gave rise to an unacceptable level of background labelling on SDS-PAGE gels. Triton X-114 (TX-114) however gave much cleaner gels because of the possibility of cloud point phase separation with this detergent at physiological (i.e. non-denaturing) temperatures; this allowed the enrichment of membrane proteins and the exclusion of soluble cytosolic proteins present in the cell lysate prior to immunoprecipitation. Since the heavy chain forms part of an integral membrane complex, this cloud point separation step provided a very straightforward ten fold purification of the heavy chain into the detergent phase.

Following TX-114 extraction, HLA-A, B and C antigens were immunoprecipitated using the W6/32 monoclonal antibody (Barnstable, *et al.*, 1978). Although a number of other anti-heavy chain antibodies were tried, W6/32 was used in preference because of its high affinity for the heavy chain. The intensity of protein bands immunoprecipitated with this antibody were 5 to 10 times greater than those resulting from immunoprecipitation with the best alternative antibodies (data not shown). Moreover, W6/32 binding is conformation-dependent; the antibody only binds the heavy chain once it is part of a fully assembled and therefore

transport-competent Class I complex (Barnstable, *et al.*, 1978; Parham, *et al.*, 1979).

3.7 Transport of HLA to the medial Golgi

As described above, transport to the *medial* Golgi cisterna was monitored by the acquisition of resistance to endoglycosidase H. Immunoprecipitated protein was digested with endo H for at least 8 h or overnight, and the protein was then fractionated by SDS-PAGE (10-15% gradient gel), and analysed by fluorography. The results of one such pulse chase experiment are shown as examples in Figure 3.1; two time points are represented. In the absence of a chase, all of the HLA was sensitive to endo H; after 60 min of chase, virtually all of it was resistant, indicating transfer during the chase period from the ER to the *medial* Golgi or beyond.

The minor, more slowly migrating protein band, most easily seen in lane b of Figure 3.1 is likely to be HLA-C. Although there is only a very minor molecular weight difference between the HLA alleles (only several hundred Da), there could be other factors responsible for producing the gel shift of this particular allele such as phosphorylation. This protein remained sensitive to endo H throughout the chase period (seen more clearly in later gels, e.g. Figure 4.5) suggesting that the protein is not reaching the *medial* Golgi. Previous work has demonstrated the weak affinity that HLA-C has for ß2m, causing a much slower rate of trimeric assembly within the ER and hence a slower rate of transfer to the cell surface (Stam, *et al.*, 1986; Neefjes and Ploegh, 1988). This depression of cell surface transport clearly means that despite the fact that HLA-C is synthesised in similar amounts relative to both HLA-A and B, only small amounts of HLA-C are detectable on the cell surface relative to both HLA-A and B. Attempts to chase this minor band into an endo H-resistant form were unsuccessful. Even after a 4 h chase, this minor band remained endo H-sensitive (data not shown) suggesting that its rate of transport out of the ER is more than one order of magnitude slower than that of other forms of HLA.

Such allelic differences in the rate of transfer of Class I heavy chains to the cell surface are not limited to humans; work on two mouse heavy chain alleles in the same cell line has demonstrated a similar striking contrast in the rate of secretion. Whereas newly synthesised H-2K^k is transported to the cell surface within 1 h, H-2D^k takes 4-5 times longer to do so (Williams, *et al.*, 1988). The similar rate of transfer from the Golgi to the cell surface for these two mouse alleles suggested that the rate-limiting step was transfer from the ER to the Golgi, entirely consistent with the notion that the rate of subunit assembly determines the overall rate of cell surface transfer (discussed in the Introduction).

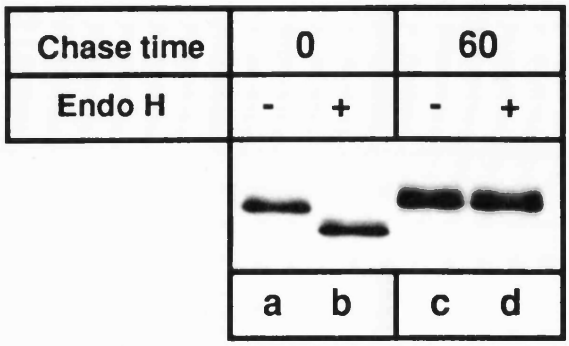

Figure 3.1 Transport of HLA in G1 cells. Cells were pulsed for 5 min with ^{35}S-methionine and chased for 0 or 60 min. HLA-A, B and C were immunoprecipitated from lysed cells using the W6/32 monoclonal antibody, fractionated by SDS-PAGE and fluorographed. HLA was treated (lanes b and d) or mock-treated (lanes a and c) with endo H.

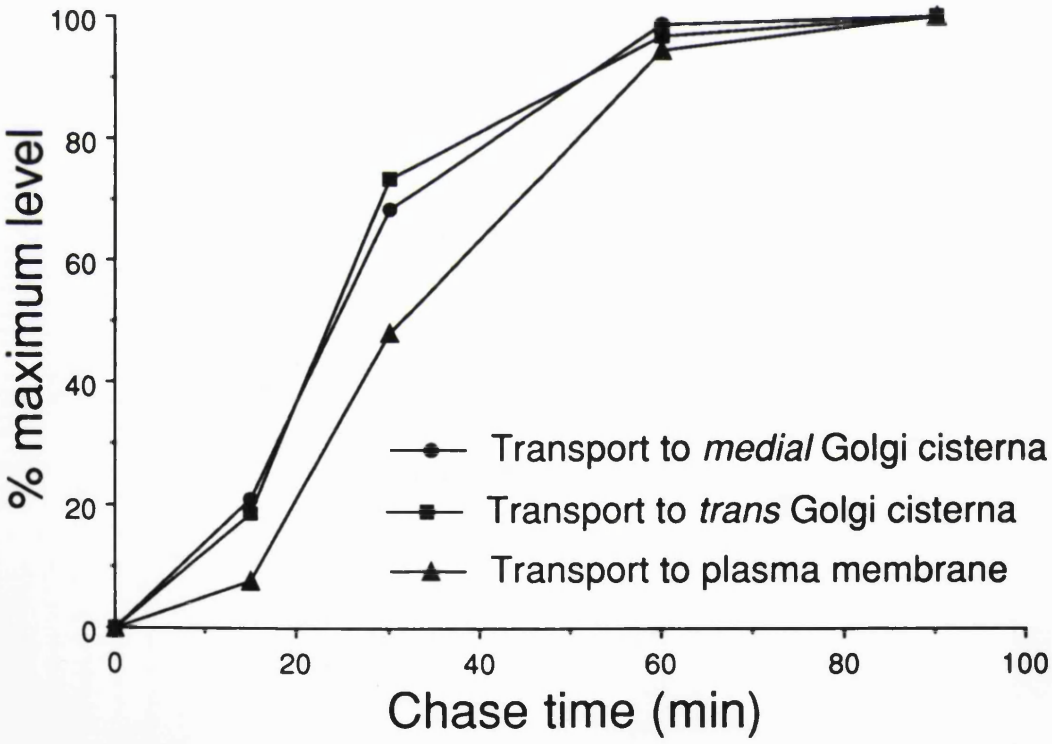

Figure 3.2 Kinetics of HLA transport in G1 cells. Cells were pulsed for 5 min with ^{35}S-methionine, chased for varying times and HLA analysed for resistance to endo H (arrival at *medial* Golgi cisterna), acquisition of sialic acid residues (arrival at *trans* Golgi cisternae) and sensitivity to exogenous neuraminidase (arrival at the plasma membrane). Fluorographs were scanned by laser densitometry and the results presented are the average of two experiments.

Acquisition of resistance to endo H-digestion was therefore quantified for the major HLA bands present (HLA-A and B), but not for this minor band.

The only other protein band which appeared on the gel reproducibly was that of ß2m which ran well down the gel as a 12kD monomer, and does not therefore appear in the panel shown in Figure 3.1.

3.8 Quantitation of enzymatic digests

The investigations of Laskey and Mills (1975) have shown that provided photographic film is pre-flashed and that the patterns appearing on the film are not over-exposed (grey rather than black) , there is a linear relationship between the quantity of labelled protein present in the gel exposed to the film and the intensity of the bands which appear on the film. This therefore means that accurate measurement of the relative intensities of protein bands on the exposed film will provide an accurate means of determining the relative quantities of protein present on the gel.

The most widely used form of fluorographic quantitation is laser densitometry. Such densitometric scanning may be readily used to plot a profile of protein intensity within a particular track of a gel. Although it is possible to determine relative amounts of protein by an integration function of the scanner itself, the close proximity of other bands made accurate use of such automated functions more difficult. I decided therefore to integrate the amount of protein present by obtaining a printed protein profile, cutting out the relevant protein peaks and weighing them on an accurate balance. In one experiment, both the automated method and the "cut and weigh" method were used in parallel and gave the same T1/2 value to within 1 min. This degree of similarity demonstrated that the manual method was providing an accurate means of quantitation, coupled with a greater control of the area to be integrated. The "cut and weigh" method was employed for all subsequent fluorographic quantitation.

By applying this densitometry method to each of the tracks corresponding to the various time points during the chase period, the relative amounts of endo H-sensitive and resistant forms of HLA were quantitated. The results from two experiments were averaged and are plotted in Figure 3.2 (closed circles).

An important point to make at this stage is that these values have been normalised to 100%; in other words, the % of endo H-resistant HLA at any time point is evaluated relative to the amount of resistant protein at 90 min when the profile has reached a plateau level. In the case of endo H-resistance, this plateau

level was typically in excess of 90%. The need for doing so is discussed later in the context of determining cell surface transport.

The most valid way of being able to compare and contrast such profiles representing dynamic processes such as protein transport is the determination of the "half-time". In the case of endo H digestion, this time corresponded to the 50% level of endo H-resistance once the proportion of endo H-resistant protein present at each time point has been normalised relative to the endo H-resistance acquired after 90 min of chase. This technique therefore allowed valid comparison of the same parameter between different experiments, and also between different parameters measured for the same experiment. In the case of Figure 3.2, the half-time for acquisition of resistance to endo H was 24 min.

3.9 Transport of HLA to the trans Golgi

Sialylation of N-linked oligosaccharides occurs in the *trans* Golgi cisternae and the *trans* Golgi network since this is the localisation of sialyltransferase which catalyses this process (Roth, *et al.*, 1985). This modification therefore provided a useful means of monitoring transport of secreted protein to these compartments; given the fact that the oligosaccharide processing of a particular glycoprotein includes the addition of terminal sialic acid (SA) residues, a protein which has reached these compartments will be sialylated.

Because of the very slight change in molecular weight after sialylation, the resolution of SDS-PAGE is insufficient to permit differentiation between sialylated and non-sialylated forms of HLA. A better means of identifying the change on exposure to sialyltransferase activity is separation on the basis of charge. Iso-electric focusing lent itself as a suitable means of such charge-dependent separation in these experiments.

3.9.1 Isoelectric focusing of HLA

The technique of isoelectric focusing (IEF) relies on the separation of proteins according to their isoelectric point. Once the proteins have migrated to their pI, there is no further protein movement through the gel matrix. The pattern of bands resulting from IEF separation of an immunoprecipitate with W6/32 is shown in Figure 3.3. These bands correspond to a mixture of different HLA polymorphisms and ß2m, as well as differentially glycosylated heavy chains which have different charges.

In order to identify the different polymorphic forms of HLA on these IEF gels I sought assistance from Dr. Martin Guttridge of the Human Tissue Antigen group at the UK Transplant Support Service in Bristol. Dr. Guttridge employed a

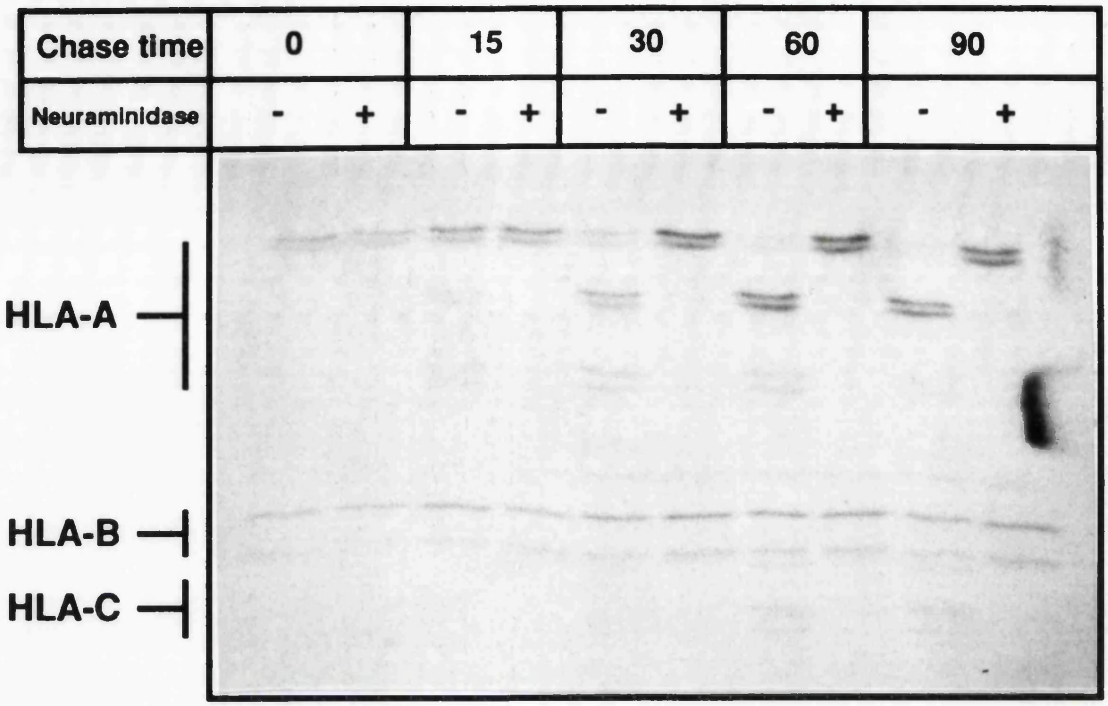

Figure 3.3 IEF analysis of HLA in G1 HeLa cells. Cells were pulsed for 5 min with ^{35}S-methionine and chased for up to 90 min. HLA-A, B and C were immunoprecipitated from lysed cells with the W6/32 monoclonal antibody, fractionated by IEF and flurographed. The positions of HLA-A, B and C are indicated. HLA-A is present as a doublet.

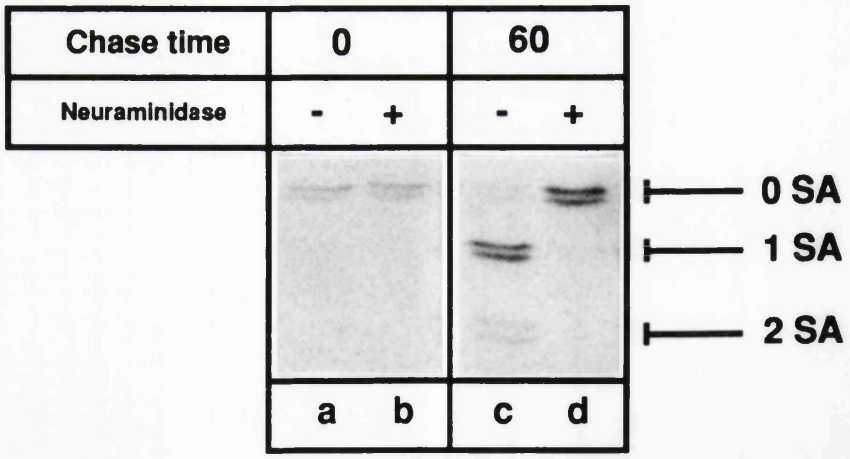

Figure 3.4 IEF analysis of HLA-A immunoprecipitated from G1 HeLa cells. Immunoprecipitated protein was either treated (b and d) or mock-treated (a and c) with neuraminidase before being fractionated by IEF and fluorographed. The positions of HLA-A with 0, 1 or 2 sialic acid (SA) residues are indicated.

standard technique for the identification of Class I molecules which involves the modification of the heavy chain cytoplasmic tail using a series of proteases such as chymotrypsin and carboxypeptidase B. He was able to confirm that the pattern of bands at the top of the gel is that of HLA-A (probably HLA-A2) which often has a characteristic doublet appearance after IEF separation. The reason for this doublet pattern is not known; it is due to neither glycosylation nor phosphorylation (Hidde Ploegh, personal communication). HLA-B and C migrated further down the gel and were too close together for densitometric analysis. Interestingly, the patterns of HLA-C showed resistance to neuraminidase treatment, suggesting that HLA-C had not been sialylated, consistent with the notion that this form of HLA remained in the ER. Further analysis of the HLA patterns was therefore confined to investigation of HLA-A. Examples of the pattern of HLA-A bands at two time points are given in Figure 3.4. In the absence of a chase, a single doublet of HLA-A was observed (lanes a and b). After a 60 min chase two more doublets appeared (lane c) which collapsed to a single band after digestion with the desialylating enzyme neuraminidase (lane d) confirming their identity as HLA-A bearing either one or two SA residues. The structure of HLA has been well-documented (reviewed by Bjorkman and Parham, 1990) and this pattern of sialylation is consistent with the presence of a single N-linked bi-antennary oligosaccharide on the protein.

The extent of sialylation at different times of chase was quantitated by laser densitometry as in the case of SDS-PAGE analysis of endo H digested HLA. The results were again normalised to % of the total amount of sialylation (including both single and double sialylated forms) which occurs at 90 min. The plateau region for sialylation at 90 min was typically in the range from 80 to 90 % prior to normalisation. The average values from two experiments are plotted in Figure 3.2 (closed squares); the half time for acquisition of SA residues was 24 min.

Given the rate of transfer of HLA-A to the *medial* Golgi of 24 min, this sialylation time suggests inordinately rapid protein transfer from the *medial* to the *trans* Golgi cisternae. The work of Green *et al.* (1981) suggested that viral glycoproteins require about 15 min to traverse the mammalian Golgi stack, a figure which therefore implied an interval of about 4-5 min for each intercisternal transfer. However, such intercisternal rates were not measured directly and therefore the precise timing of *medial* to *trans* Golgi transfer remains uncertain. Given the variation between IEF experiments, the half-times are probably only accurate to within 5 min, so the similarity of the timing for passage from the ER to both the *medial* Golgi and *trans* Golgi is consistent with the interpretation that the step from the *medial* to the *trans* Golgi takes a few min.

One point of interest is the lack of complete maturation of HLA-A to the form containing 2 SA residues. Even after 90 min of chase, the form with only a single SA seems to predominate over the double-sialylated form. The reason for this observation is unclear. The same pattern was also true of interphase HeLa cells, so cannot be explained as a G1 anomaly. The extent and rate of glycosylation however appears to be very allele-dependent and is presumably a distinctive feature of this particular allele of HLA. Since this pattern of sialylation was reproducible between experiments, the absence of complete processing to the fully sialylated form should not affect the fluorographic quantitation.

3.10 Transport of HLA to the cell surface

Two methods were used to monitor arrival of HLA at the cell surface. The first involved using exogenous neuraminidase to digest intact cells which will only remove sialic acid from HLA that has reached the cell surface; internal HLA will be protected from digestion. The second method involved the binding of W6/32 to cell surface HLA, followed by cell lysis in the presence of a five-fold excess of unlabelled cell extract to quench any unbound antibody.

3.10.1 Exogenous neuraminidase method

The results from one pulse chase experiment using exogenous neuraminidase is shown in Figure 3.5. After 60 min of chase, most of the sialylated HLA-A was accessible to neuraminidase, which collapsed the pattern down to the non-sialylated form. The level of desialylation at each time point during the chase period was quantified by laser densitometry, and the results normalised to % maximum desialylation at 90 min. The average results from two experiments are plotted in Figure 3.2 (closed triangles) and show that the half-time for transport from the ER to the plasma membrane was 31 min. This value indicates that it took approximately 7 min for HLA-A transport from the *trans* Golgi cisternae to the cell surface.

3.10.2 Normalisation of transport profiles

The comparison of the profiles for transport of HLA from the ER to the *medial* Golgi, *trans* Golgi and cell surface requires the normalisation of the levels of digestion measured to the % of the maximum level at the end of the chase period (90 min). The endo H-resistant levels typically reached 90% to 100% of total HLA at the end of a 90 min chase, so the degree of adjustment was very small. The levels of sialylation of HLA-A were also found to be high; experiments generally showed an 80 to 90% level of sialylation, again meaning that the adjustment necessary was minor. In the case of cell surface digestion, however, the % levels

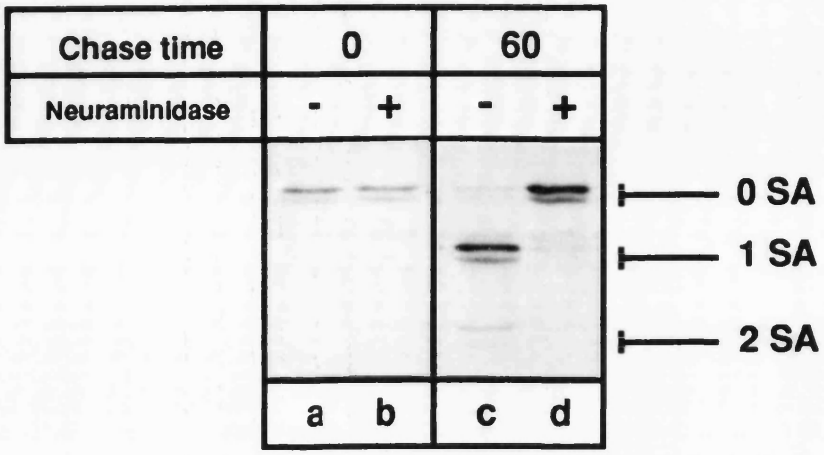

Figure 3.5 IEF analysis of HLA-A immunoprecipitated from G1 HeLa cells after either cell-surface treatment (b and d) or mock-treatment (a and c) with neuraminidase prior to lysis. The positions of HLA-A with 0, 1 or 2 sialic acid (SA) residues are indicated.

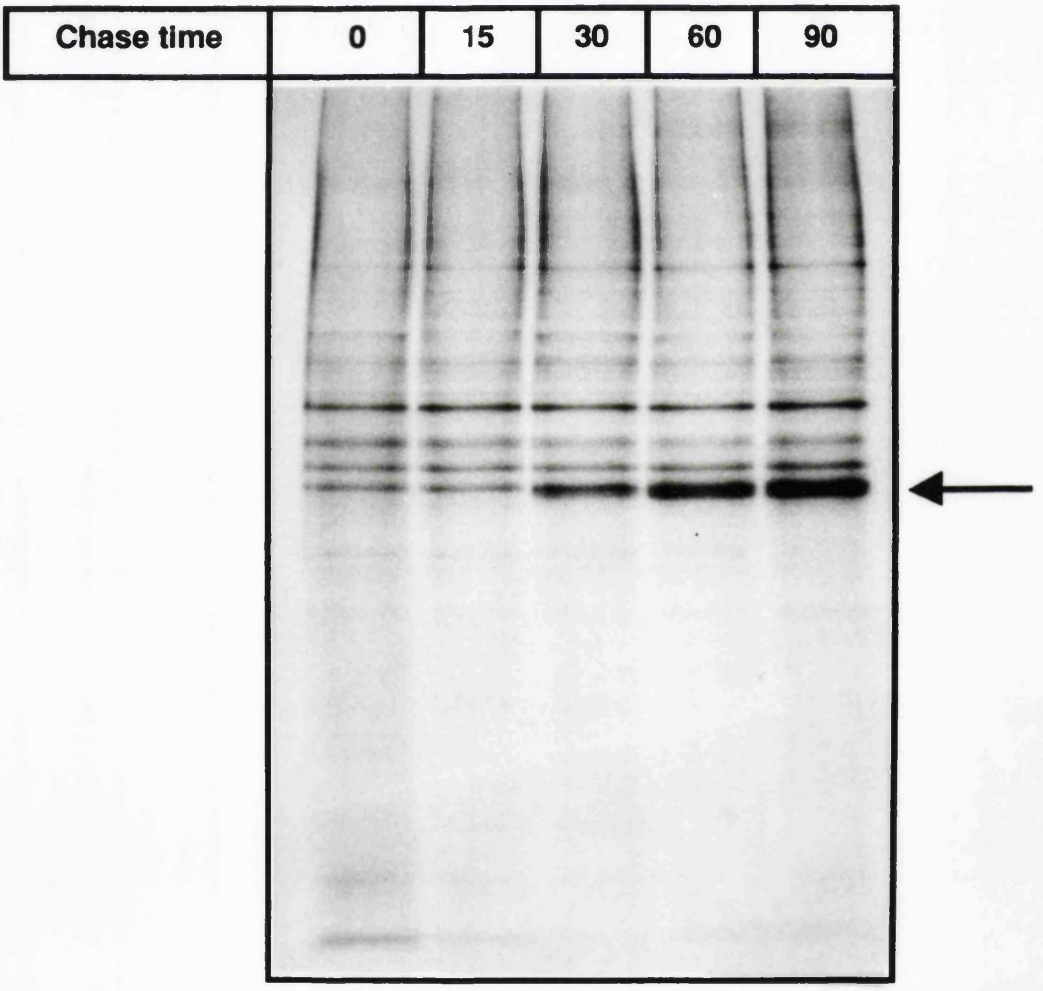

Figure 3.6 Immunoprecipitation of cell surface HLA from G1 HeLa cells. Cells were pulsed for 5 min with ^{35}S-methionine, chased for up to 90 min and incubated for 30 min at 4°C with monoclonal antibody W6/32 before lysis in the presence of a five-fold excess of unlabelled cell lysate. The immunoprecipitated protein was fractionated by SDS-PAGE and fluorographed. The position of HLA is indicated by the arrow.

for digested HLA-A only reached the 60 to 70% mark, meaning a fairly large correction factor to compare these figures to the % maximum value. Whereas the submaximal levels of endo H digestion and sialylation might be explained by a 10% loss of cell viability during the chase period (see later discussion of cell viability), the lower levels of desialylation are too large to be adequately accounted for by cell death. The reason for these lower levels was probably due to sub-optimal conditions for neuraminidase digestion in this assay. Whereas digestion of immunoprecipitated protein was carried out for 4 h at 37°C at pH5.5, the necessary restrictions of time, temperature and pH for the cell surface digestion (i.e. 30 min, 4°C, pH7) presumably mean that digestion could never reach 100% levels. Such normalisation was essential however to allow valid calculation of T1/2 values for these different parameters of exocytic transport.

3.10.3 Cell surface antibody method

Figure 3.6 shows the results from an experiment using the technique of binding antibody (here, W6/32) to cell surface antigen. It was important that the binding was carried out on ice to ensure that there was no further transport of HLA occurring. During the course of the pulse chase, there was a striking increase in the amount of HLA present in the immunoprecipitate (marked with an arrow).

A major disadvantage of this technique was the fact that since the immunoprecipitating antibody is added prior to cell lysis, there was limited scope for carrying out a pre-clearing step as in the case of the previous experiments described. This had the obvious effect of making the background much higher, as is clear from Figure 3.6, and more importantly, made accurate quantitation of the results extremely difficult. Initial attempts to quantitate these fluorographs did not provide satisfactory results. Similarly, attempts at carrying out pre-clearing steps using *Staph. A.* cells pre-coated with rabbit IgG met with limited success. It was therefore decided to pursue the technique of exogenous neuraminidase digestion for the purpose of determining cell surface rates of transport.

It is of interest to note that the HLA band which appears during the chase period appeared to be a single band and that there was no associated minor band as was clearly visible in the immunoprecipitation of intracellular HLA required for the analysis of transport of HLA to the *medial* Golgi (see Figure 3.1). This observation lends further weight to the suggestion that the minor band seen in earlier experiments was an intracellular form of HLA (most probably HLA-C) which was retained in the ER and was not transported to the cell surface.

3.11 Control experiments

These results using G1 populations raised a number of questions. Firstly, the kinetics of transport in G1 cells might not necessarily have been an accurate reflection of the rate of intracellular transport in non-synchronised "interphase" cells. Secondly, the use of nocodazole in the synchronisation and labelling procedure might have affected the kinetics of intracellular transport and led to anomalous results. Thirdly, the use of exogenous neuraminidase required the preservation of the plasma membrane at all points during the chase period. The last question was addressed in the context of investigation of protein transport in mitotic HeLa cells and will be discussed in the following chapter. The first two questions were answered by the following control experiments.

3.11.1 Comparison of transport kinetics of HLA in interphase and G1 cells

In order to assess any differences between the kinetics of HLA transport from the ER to the Golgi in interphase and G1 cells, a non-synchronised population of suspension HeLa cells was pulsed and chased identically to the G1 populations of HeLa cells described above. HLA was immunoprecipitated as before and digested with endo H. The resulting fluorography yielded the profile of endo H-resistance shown in Figure 3.7. Comparison of this profile with that shown for G1 cells (as shown in Figure 3.2) demonstrates that the interphase kinetics of transport from the ER to the *medial* Golgi was not significantly different from that of a semi-synchronous G1 population (T1/2 of 22 min rather than 24 min). This result lends support to the conclusion that comparison of G1 kinetics with those of mitotic cells was valid in all subsequent work.

3.11.2 Nocodazole controls

In order to address the question of whether nocodazole was adversely affecting the transport system, two populations of cells were set up, one pulsed and chased in the presence of nocodazole and the other pulsed in the presence of nocodazole, but chased in the absence of nocodazole. The samples were immunoprecipitated in parallel and digested with endo H. Laser densitometric analysis of the fluorographs from these two sets of samples showed strikingly similar profiles of endo H-resistant protein; in the presence and absence of nocodazole, the half-times were 24 and 23 min respectively. Nocodazole therefore had no effect on the rate of transport from the ER to the *medial* Golgi and the use of this drug for synchronisation purposes did not interfere with the kinetic results. The relative levels of methionine incorporation into G1 and mitotic cells in the presence and absence of nocodazole as measured by TCA precipitation of labelled protein from detergent lysates are shown in Figure 3.8. The results

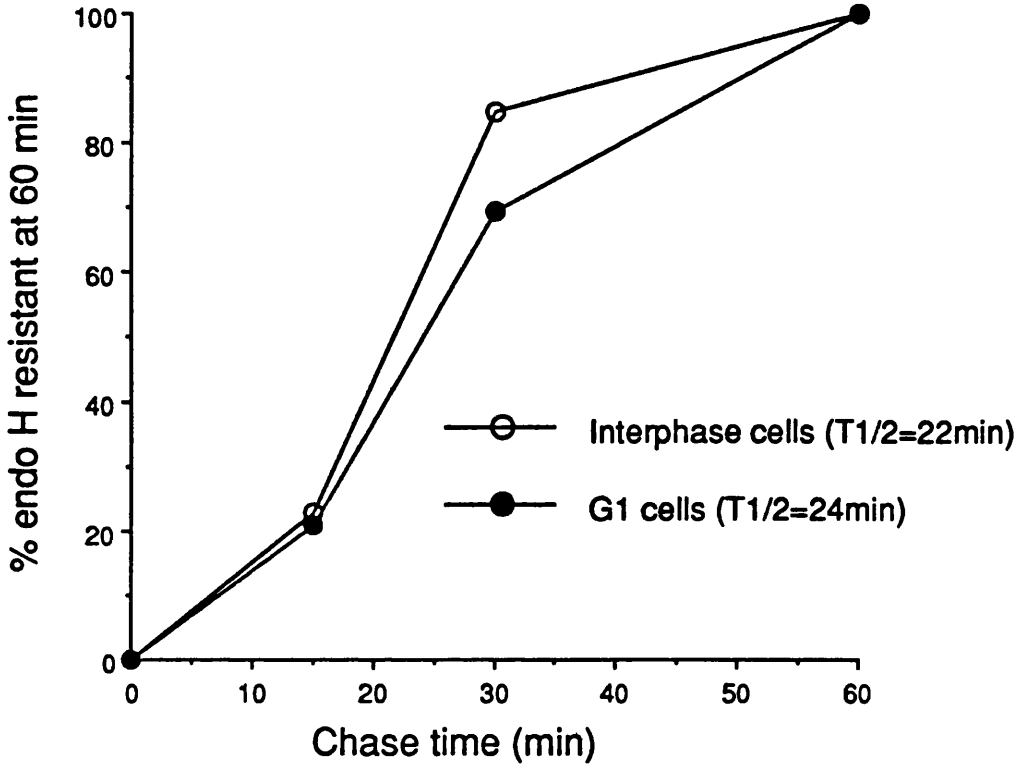

Figure 3.7 Comparison of HLA transport in interphase and G1 HeLa cells. The profiles represent the level of endo H-resistant HLA isolated from interphase and G1 HeLa cells in order to determine the rate of transfer to the *medial* Golgi.

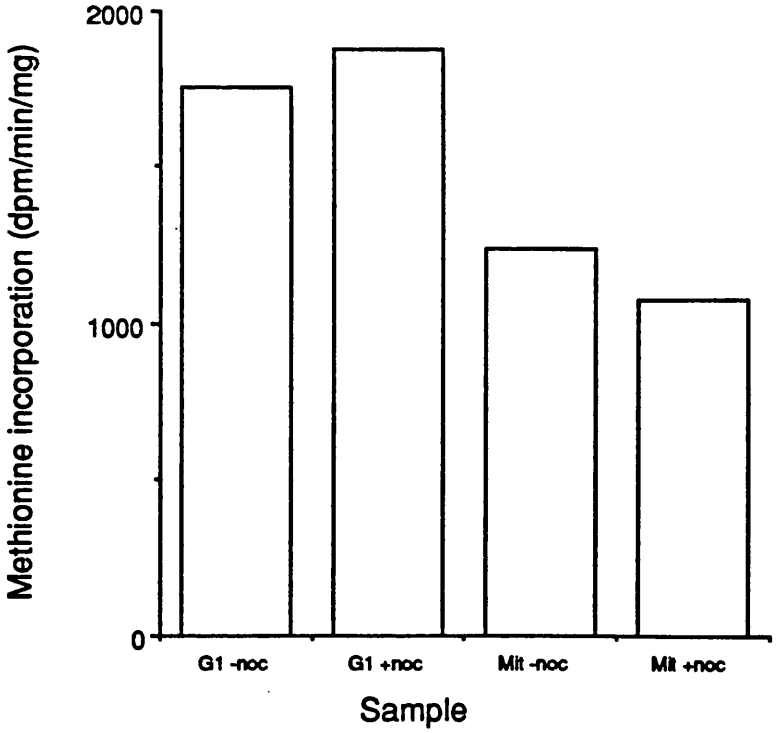

Figure 3.8 Effect of nocodazole (noc) on the incorporation of methionine label into protein in G1 and mitotic HeLa cells. The level of radioactive protein was measured by TCA precipitation of a cell lysate.

demonstrate that nocodazole had no effect on the level of ^{35}S-methionine incorporation into newly synthesised protein. This was also found to be true of interphase populations of HeLa cells (data not shown).

3.12 Summary and conclusions

By using HLA as a marker protein for the intracellular transport pathway, it has been possible to monitor the kinetics of transfer of this protein from the ER to the *medial* Golgi, *trans* Golgi and cell surface. Laser densitometrical quantitation of the fluorographs from a series of experiments with G1 populations of HeLa cells has demonstrated that HLA protein took about 24 min to reach the *medial* Golgi and that it was rapidly transferred from there to the *trans* Golgi. Subsequent transfer from the *trans* Golgi cisternae to the cell surface required a further 7 min approximately.

The half times for transport of HLA through the Golgi to the cell surface were similar to those obtained for Class I molecules of other mammalian cell lines (Owen, *et al.*, 1980; Neefjes and Ploegh, 1988; Williams, *et al.*, 1988). In particular, the work of Owen and co-workers (1980) demonstrated that the N-linked sugars of HLA-A and HLA-B underwent a transition from the high mannose to the complex form 20 to 25 min after synthesis, which would correspond to the delivery of the protein to the Golgi cisternae, and that these antigens were expressed at the cell surface between 30 and 40 min after synthesis. These values show a striking degree of similarity to the rates of transfer calculated here.

Control experiments showed that nocodazole had no effect on the rate of protein transport from the ER to the Golgi apparatus. Since nocodazole has the effect of breaking up the juxtanuclear reticulum which constitutes the interphase Golgi organelle in mammalian cells (Thyberg and Moskalewski, 1985), preservation of this morphology is not necessary to allow protein transport to continue. The interconnection of small Golgi stacks must serve some other purpose than the maintenance of intracellular protein transport.

In conclusion, the methods employed in these experiments were therefore ones which could be used reproducibly to quantify the rate of intracellular protein transport between various stages of the secretory pathway. Having established the methodology in G1 cells, it was of interest to employ these same methods to investigate the identical steps of intracellular transport in HeLa cells progressing through mitosis into G1 phase. This investigation formed the subject of the next chapter.

CHAPTER FOUR: INVESTIGATION OF TRANSPORT IN MITOTIC CELLS

4.1 Aims

The overall aim of this investigation was the reassembly of the Golgi apparatus in telophase and how this process of reassembly relates to the resumption of protein transport through the organelle. The transport assays described in the last chapter provided a means of dissecting the kinetics of protein transport through the secretory pathway in interphase HeLa cells. In order to be able to address the question of Golgi reassembly and the resumption of protein transport at the end of M phase, it was first necessary to apply these transport assays to characterise transport kinetics in mitotic cells. The aim of the work discussed in this chapter therefore was the investigation of intracellular protein transport in mitotic HeLa cells.

Early studies of virally infected cells demonstrated the inhibition of protein transport from the ER to the Golgi apparatus and cell surface during mitosis (Warren, *et al.*, 1983; Featherstone, *et al.*, 1985). Systems relying on viral infection are of restricted use in the study of the resumption of protein transport at the end of mitosis since infected cells have only very limited capacity to progress through M phase.

The assays employed in the previous chapter do not share this limitation since the marker used (HLA) is an endogenous protein. These assays therefore lend themselves to adaptation for the study of protein transport in mitotic HeLa cells.

Of particular interest was the exploration of the timing of transport resumption, and how this relates to other mitotic parameters, most notably the different phases of mitosis, and also the histone H1 kinase activity. In addition, there was the intriguing question as to whether transport resumption resumed simultaneously between the various stages of the secretory pathway or whether the resumption process exhibited any polarity.

4.2 Characterising mitotic progression

It was of interest to monitor various mitotic parameters which could be used to quantifiably describe the passage of HeLa cells through M phase. These same parameters could also be used to compare and contrast the kinetics of protein transport resumption at the end of M phase. The two parameters most easily measured are the various phases of mitosis and the level of histone H1 kinase activity.

The methodology used to generate a synchronous population of HeLa cells relied on the nocodazole-induced disruption of the cytoskeletal MTs. This disruption has the effect of preventing mitotic spindle construction and arresting the population in prometaphase. The cells can then be released from the nocodazole block and allowed to progress through mitosis into G1 phase. Both the mitotic phase and the H1 kinase activity undergo a distinctive progression once the mitotic block is released. This sequential progression means that these parameters can be reproducibly quantified and used together to describe the mitotic progression in detail.

4.2.1 Mitotic index

The quantitation of the mitotic index and more specifically the allocation of HeLa cells to the various stages of M phase was carried out using the DNA stain bis-benzamide (Hoechst 33258). Aliquots of cells were fixed at various time points (0,30,60,90,120 min after the nocodazole washout) with formaldehyde and permeabilised with 0.2% TX-100 to allow nuclear penetration of the stain. These stained cells could be easily allocated to one of the mitotic phases (prometaphase, metaphase, anaphase, telophase) or G1 phase through inspection under a Zeiss axiophot immunofluorescence microscope.

Figure 4.1 shows a panel of representative photographs revealing the characteristic DNA staining patterns of HeLa cells in various stages of cell division (i.e. mitosis and cytokinesis together), ranging from prometaphase cells to early G1 cells.

Since most of these stages are well-defined and easily recognisable phases of the mitotic progression, such cells could be allocated unambiguously. The demarcation of late telophase cells relative to early G1 cells however posed a greater degree of complexity and therefore warrants more discussion here. A number of distinctive features relating to nuclear structure were eventually used to uniquely define each pair of cells so that they could be reproducibly scored as either late telophase or early G1 phase (Table 4.1).

Feature	Telophase	G1 phase
Nuclear staining	Non-homogeneous (DNA still partially condensed)	Homogeneous (DNA now fully decondensed)
Nuclear periphery	Rough; "ball of wool"	Smooth
Nuclear position	Small central portion of cell	Fills complete cell volume
Nuclear shape	"Half-moon"	Circular
Nucleoli	Absent	Visible

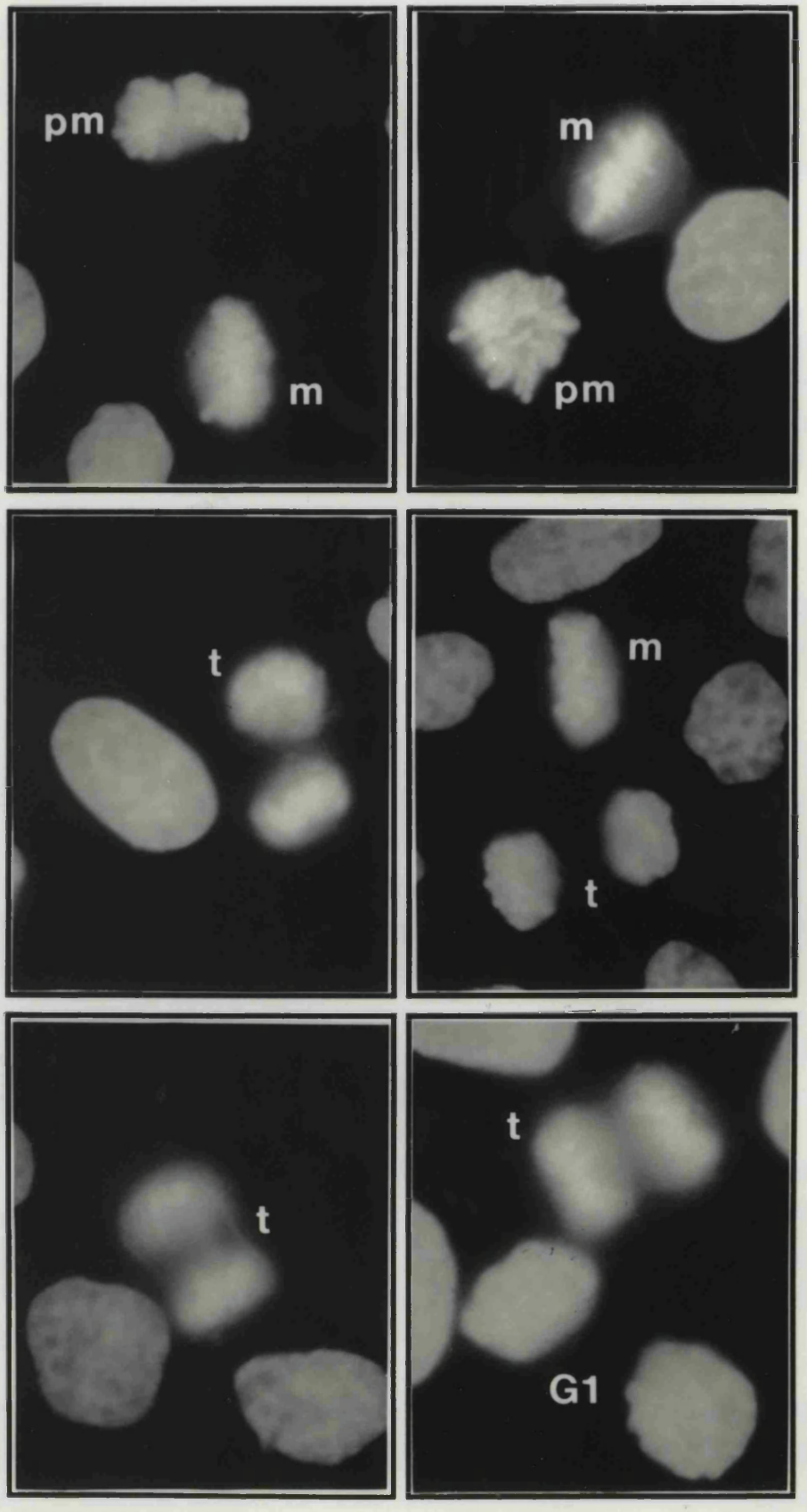

Figure 4.1 Visualisation of mitotic phases. HeLa cells have been stained with Hoechst 33258 to reveal the pattern of chromosomes during cell division. The panel shows examples of prometaphase (pm), metaphase (m), telophase (t) and G1 cells.

Some of these criteria (e.g. presence of nucleoli and nuclear position) required visualisation of cells under bright field as well as under dark field optics.

4.2.2 Mitotic anomalies

Mitotic phase inspection routinely revealed the presence of mitotic anomalies amongst cell populations as they progressed through M phase. These anomalies generally took the form of tripolar spindles and became evident as anomalous to a lesser extent at metaphase (tripolar orientation of chromosomes along the metaphase plate) and more so during anaphase as the chromosomes were seen to diverge in three rather than the usual two directions. These anomalies typically only amounted to 10% of the populations and were not included in the process of allocation to phases.

4.2.3 Quantitation of progression through M phase

Having established the criteria by which cells could be allocated to particular phases, it was possible to begin the process of scoring mitotic populations in detail and generating graphic profiles of the mitotic progression.

With regard to this process of scoring cells, it is important to note that the doubling in cell number which occurs as cells enter G1 phase is not matched by a doubling in cell volume or cell protein levels. Comparison of mitotic indices with protein digestion emphasises the importance of scoring total amounts rather than numbers. For this reason, G1 cells were counted as "half" cells in order not to bias % G1 profiles in favour of G1 rather than mitosis. Even up to 120 min most of the G1 cells were present as pairs of cells, often with the cytoplasmic bridge still evident. These G1 pairs were therefore scored as single cells in line with the scoring of late telophase dumb-bell-shaped cells being scored as single cells.

The profiles in Figure 4.2 represent the percentage of mitotic cells within the population in either the presence or absence of nocodazole. Progression through M phase was clearly dependent on release from the nocodazole arrest since the population remained in M phase (specifically prometaphase) if the nocodazole was maintained.

The profiles of the various phase transitions (e.g. prometaphase to metaphase) are plotted in Figure 4.3, and represent the average results from 9 experiments ±SEM values. These profiles are cumulative in the sense that they represent the percentage of the cell population in a particular phase or in an earlier phase. For example, the anaphase to telophase transition represents the summed percentage of the population in prometaphase, metaphase or anaphase. The telophase to G1 transition can therefore be seen to be equivalent to the mitotic

index since this profile represents the summed percentage of all cells in a mitotic phase (including telophase).

Since the population of cells loses synchrony rapidly after release from nocodazole, at any one time there will be a mixture of different phases present. As in the case of the profile comparisons discussed in the last chapter relating to protein transport, the most convenient means of comparing the mitotic phase transitions of such a semi-synchronous population is that of the half-time values. These values are given alongside the transition profiles of Figure 4.3. The most significant figure to note is the half-time for progression into G1 phase which in these experiments was 82 min.

Mitosis usually takes about 60 min in HeLa cells so the slower rate of mitotic passage measured in these experiments indicates that the imposition of a nocodazole arrest slows down the rate of progression through M phase once the block is released. Presumably, cells require a certain period after the washout of nocodazole to recover their capacity to progress through the subsequent stages of M phase. Such recovery will involve the construction of the mitotic spindle.

The rate of mitotic progression is very much dependent on the duration of the nocodazole block. The experiments described here relied on a 4 h nocodazole block. In the case of a 24 h block which may be employed in the production of a mitotic population of HeLa cells grown in suspension culture, recovery is considerably delayed. The half-time for such arrested cells to progress into G1 phase is about 4 h (Deborah Pryde, personal communication). An increase of approximately 30% in the time taken to enter G1 phase above normal physiological rates seems an acceptable delay when the mitotic index of the starting population is typically in excess of 98% and the progression into G1 phase is over 90% after 2 h of incubation. The 4 h nocodazole arrest was therefore used throughout these experiments.

Another way of quantitating these data is to plot the percentage of the cell population in each phase at each time point, rather than phase transitions. Such phase "peaks" for the same series of experiments are plotted in Figure 4.4. In contrast to previous graphic profiles where straight line plots have been used, here an interpolation function has been employed. In the case of a maintained rise or fall in a particular parameter, as in all previous plots described, straight line plots provide the least subjective analysis, since there is no room for interpolating one curve shape in preference to another. However, in the case of peaks there is a requirement for establishing the time corresponding to the maximum peak height. Such times will be more accurately determined if curves are plotted in

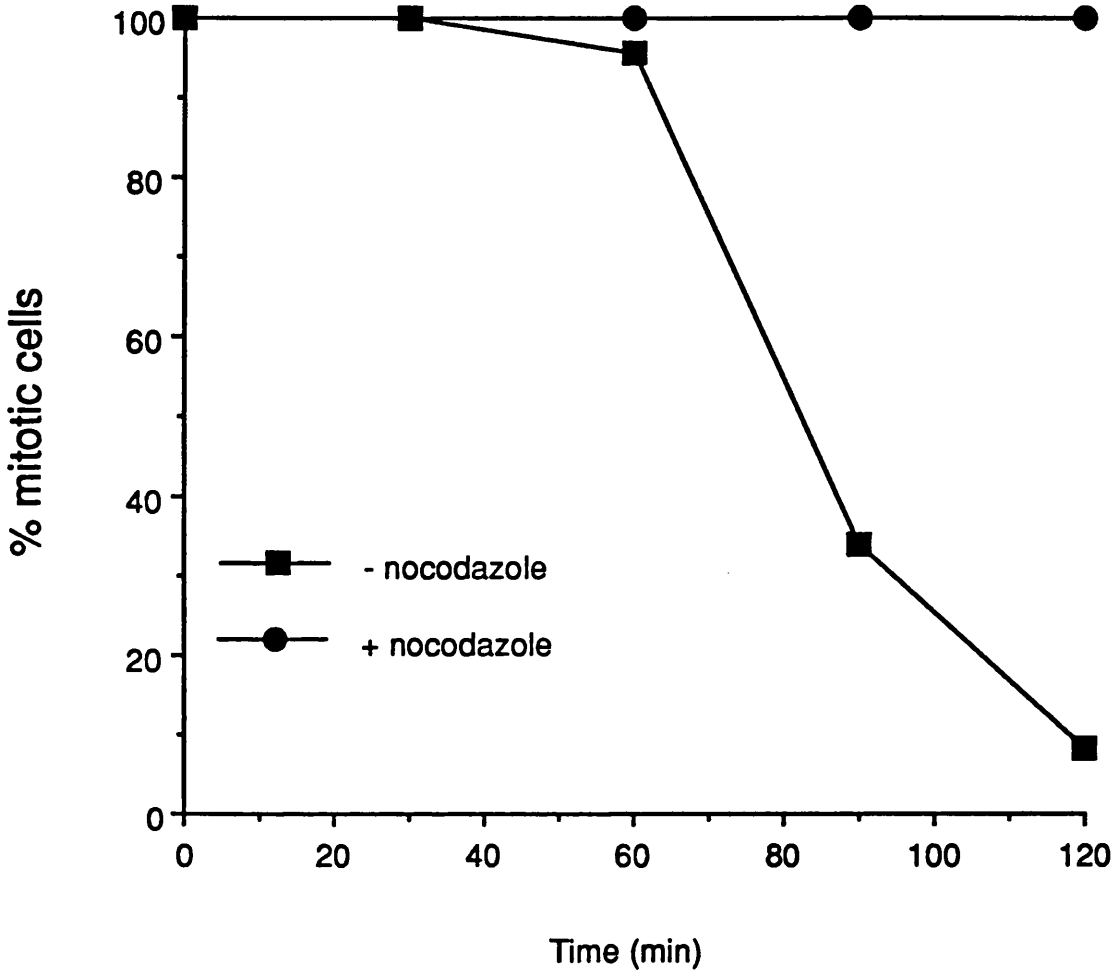

Figure 4.2 Mitotic progression depends on release from nocodazole. HeLa cells arrested in prometaphase with nocodazole were divided into two populations, one of which was released from nocodazole, the other maintained in nocodazole-containing medium. The cells were incubated for up to 2 h at 37°C, and the mitotic index measured every 30 min using Hoechst 33258 staining.

preference to straight lines since often maximum values do not correspond to a distinct time point.

The height of each peak provides a measure of the length of each phase. The low accumulation of anaphase cells therefore denotes the short duration of this phase, while metaphase and telophase both take appreciably longer to complete. The maximum levels of metaphase, anaphase and telophase cells occurred approximately 40 min, 60 min and 70 min after release from nocodazole respectively.

4.2.4 Cell density and mitotic progression

Early experiments demonstrated that the kinetics of mitotic progression were density-dependent. Increasing the cell density from 10^6/ml to $2x10^6$/ml delayed the half-time for entry into G1 phase from 80 min to 95 min, and at a density of $4x10^6$/ml, the mitotic index was nearly 70% even after 120 min (data not shown). Decreasing the density below 10^6/ml did not accelerate mitotic progression so this cell density was maintained throughout all subsequent experiments, including the investigation of protein transport resumption.

4.2.5 H1 kinase activity

The second assay used to characterise the mitotic progression was that of the histone H1 kinase activity. This activity provides a convenient means of monitoring the related activity of the primary mitotic kinase, $p34^{cdc2}$ kinase (Hunt, *et al.*, 1992).

4.2.5.1 Characterisation of the H1 kinase assay

The basic assay methodology had been previously employed to measure the H1 kinase activity of mitotic cytosol and was adapted to allow measurement of H1 kinase activity within mitotic HeLa cells.

In order to release the H1 kinase activity from whole cells, the plasma membrane must first be disrupted. A convenient means of breaking the cells open was required which would reproducibly release all the soluble kinase from the cytosol of the cell. The method which was utilised was that of rapid freezing in liquid nitrogen since it provided not only an easy means of breaking cells open, but also frozen cells could be left in liquid nitrogen until a suitable time for carrying out the kinase assay. Sequential assays of the H1 kinase activity of the same population of mitotic HeLa cells frozen in liquid nitrogen for 1, 4 or 7 days showed no significant deterioration in activity (data not shown). Since all kinase assays were

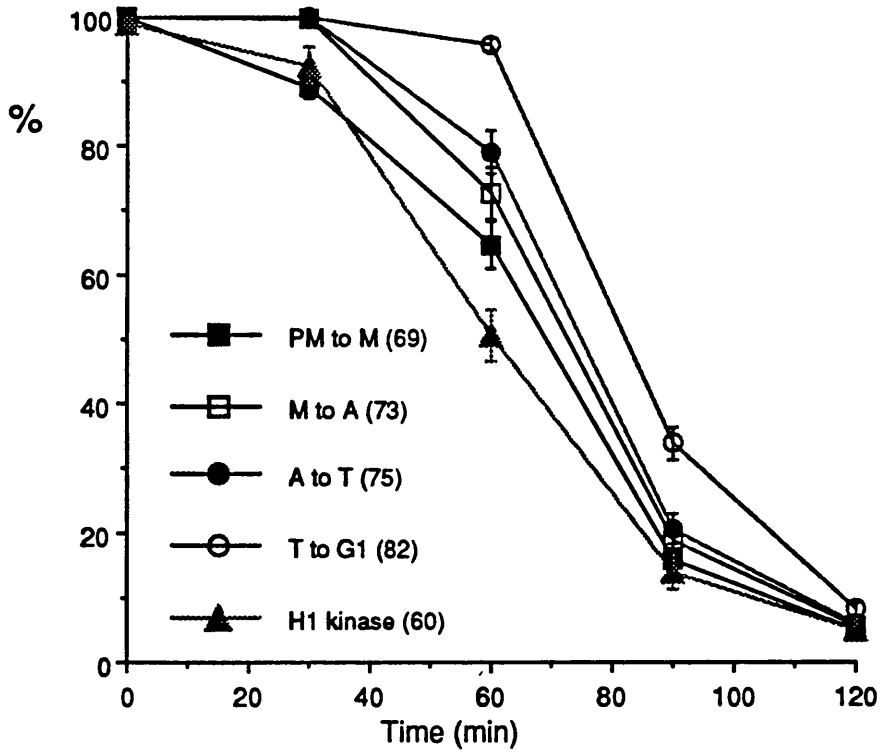

Figure 4.3 Mitotic phase transitions and H1 kinase activity. HeLa cells were blocked in prometaphase, released out of nocodazole and allowed to progress through M phase. The mitotic phases and H1 kinase activity were assayed every 30 min. The data represent the averages of 9 experiments ±SEM. The half-times for each transition are given in brackets.

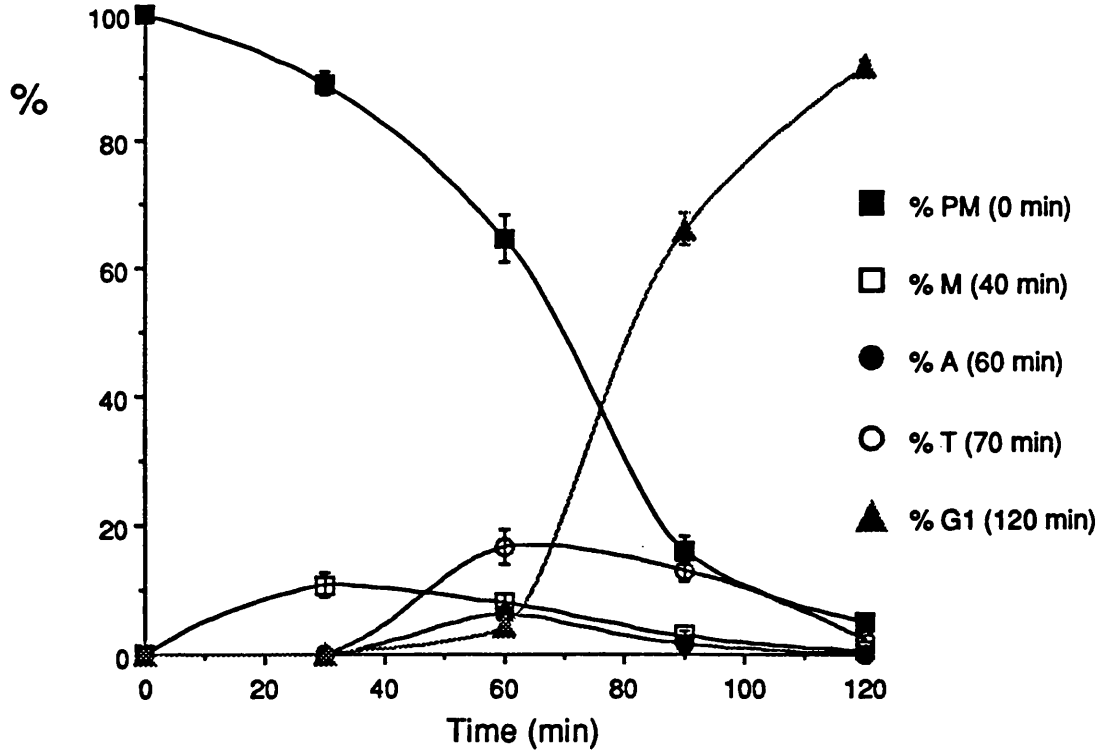

Figure 4.4: Peaks of the mitotic phase. HeLa cells were blocked in prometaphase, released out of nocodazole and allowed to progress through M phase. The profiles represent the average percentage in each phase during the mitotic progression from 9 experiments ±SEM. The times corresponding to the maximun peak height are given in brackets.

undertaken within a week of initial freezing, there could be no variation between different assays due to enzymatic degradation during cell storage.

Characterisation of the assay requirements showed the importance of adequate stabilisation of the mitotic activity. One key means of achieving such stabilisation is the inhibition of phosphatase activity. Since betaglycerophosphate is a potent phosphatase inhibitor and Ca^{2+}-chelation has the effect of inhibiting Ca^{2+}-dependent phosphatases, EBS (EGTA, betaglycerophosphate, sucrose) buffer provided such stabilisation of H1 kinase activity. The cells were therefore frozen in this buffer prior to assaying the kinase activity.

4.2.5.2 Quantitation of the H1 kinase assay

The assay relied on the H1 kinase-mediated transfer of labelled phosphate to histone H1, and the subsequent quantitation of this transfer to allow calculation of the specific activity of the H1 kinase. Figure 4.3 shows the profile of H1 kinase relative to the mitotic transition profiles, plotted as the percentage of the maximum H1 kinase level at zero time, and representing the average from 6 experiments ±SEM values. The profile shows the progressive loss of activity from the initial maximal level as the cells progress through M phase. The half-time for this loss of activity was 60 min.

Direct comparison of the H1 kinase profile with the phase transitions clearly indicates that the fall in this activity precedes even the prometaphase to metaphase transition. This result is unexpected in view of the published data concerning the timing of $p34^{cdc2}$ kinase deactivation which occurs at the metaphase to anaphase transition in clam oocytes (Hunt, *et al.*, 1992). The most likely explanation of this non-physiological relationship of H1 kinase activity to phase progression is the methodology of prometaphase arrest using nocodazole. The elongation of M phase following release from the nocodazole block has already been noted, presumably arising from the need for the cell to recover from the disruption of MT growth. A distinction could however be made between the retarding effect of nocodazole on processes involving structural involvement of MTs and the less severe effect on processes involving soluble components of the mitotic cytosol which might continue with accordingly less retarded kinetics. In contrast to phase progression which clearly requires structural changes, the deactivation of the mitotic kinase involves the interaction of soluble components. The differential effect of nocodazole on structural and soluble components of the mitotic machinery might explain the kinetic uncoupling of phase transitions and the fall in H1 kinase activity in the post-nocodazole arrested cell. Even though the sequence is unexpected, the relative timing of the fall in H1 kinase activity and the

phase transitions is reproducible and therefore does not undermine the usefulness of this assay as a means of monitoring mitotic progression.

4.2.6 Conclusions about mitotic parameters

These two parameters therefore provide quantifiable markers for the kinetics of the progression through M phase into G1 phase. Both the mitotic index and the H1 kinase activity have been used throughout the following investigations into the resumption of intracellular protein transport in HeLa cells progressing through M phase in order to compare and contrast different experiments. From the discussion of the protein transport assays in the previous discussion of transport in interphase HeLa cells, it will be clear that these assays require a considerable input of time. In contrast, the two parameters described above are relatively rapid and easily performed assays and have therefore provided a quick "diagnostic" check of the mitotic progression. By plotting these parameters, it is possible to assess whether there has been a significant deficiency in M phase progression causing delay in the rate of passage through M phase. In the event of such inconsistent rates of mitotic progression, these experiments could be aborted early on without having to expend large amounts of time investigating the protein data. This time-saving aspect of these two assays has been very beneficial to the work undertaken.

4.3 Monitoring transport in mitotic cells

Having used the mitotic index and H1 kinase activity to characterise the progression through M phase, the stage was now set for the investigation of the resumption of intracellular protein transport at the end of M phase. The labelling procedure, HLA isolation and the various assays used were identical to those described in the previous chapter.

4.3.1 Protein synthesis during M phase

Previous work has shown depression of protein synthesis during M phase (Prescott and Bender, 1962; Preston, *et al.*, 1985), primarily due to the absence of mRNA from the cytoplasm. There is also a mitotic decrease in the uptake of amino acids, so that metabolic labelling is further impaired during M phase. Although in certain cell lines levels of synthetic depression around 75% have been reported (Preston, *et al.*, 1985), comparison of the incorporation of ^{35}S-methionine into newly synthesised protein in HeLa cells showed that the decrease from interphase to M phase levels was only about 30 to 40% (see Figure 3.8). This level therefore meant that there was no requirement for modifying the labelling conditions for mitotic cells, and the conditions were maintained as a 5 min pulse with pre-warmed labelling medium. Following this pulse, the cells were washed

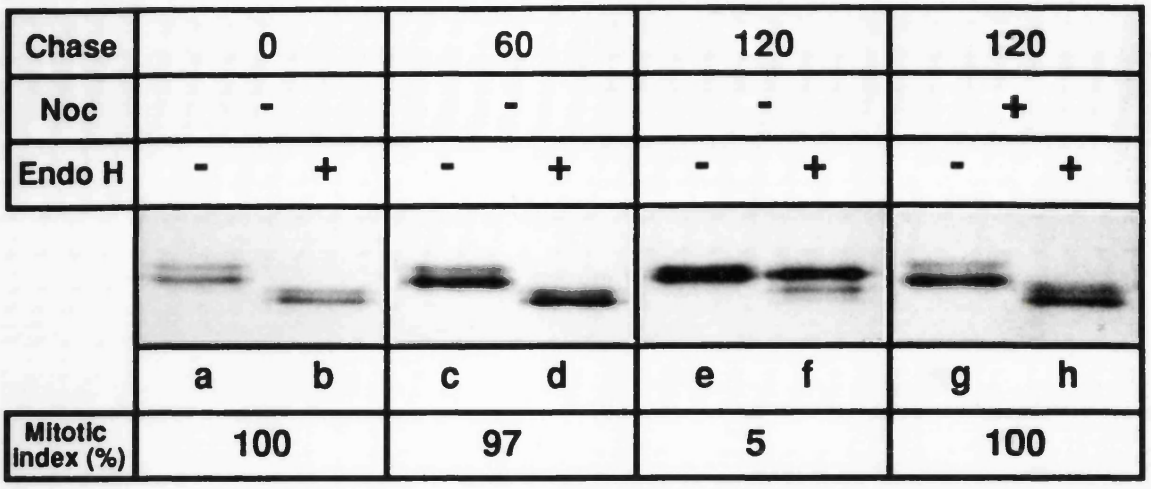

Figure 4.5 Transport of HLA in mitotic cells. Prometaphase cells were pulsed for 5 min with ^{35}S-methionine and chased for 0, 60 or 120 min in the absence (lanes a to f) of nocodazole (noc) or for 180 min in its presence (lanes g and h). HLA-A, B and C were immunoprecipitated from lysed cells using the W6/32 monoclonal antibody, fractionated by SDS-PAGE and fluorographed. HLA was treated (lanes b, d, f and h) or mock-treated (lanes a, c, e and g) with endo H. The minor band is most likely HLA-C and remains endo H-sensitive.

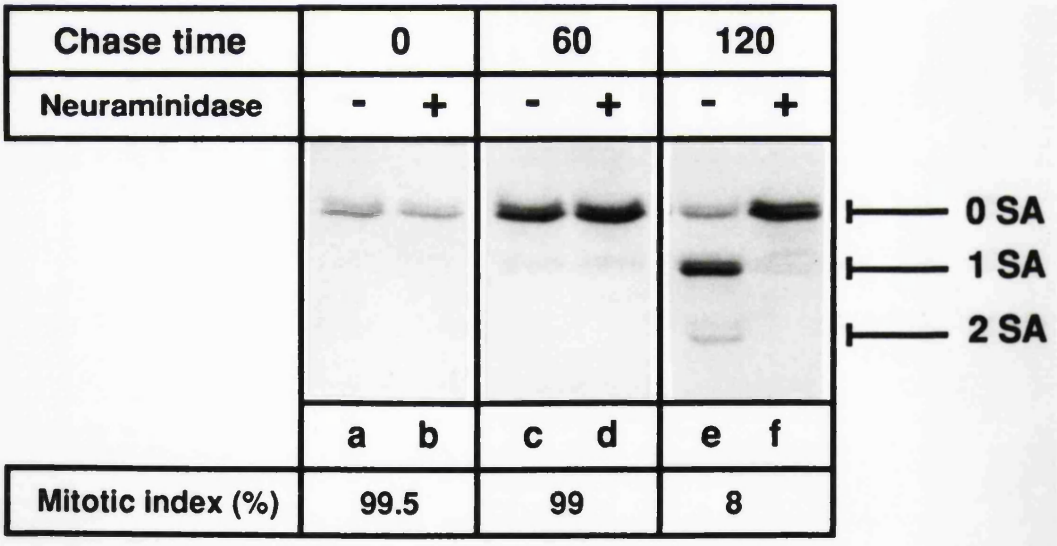

Figure 4.6 IEF analysis of HLA-A immunoprecipitated from mitotic HeLa cells. Prometaphase cells were pulsed for 5 min with ^{35}S-methionine and chased for 0, 60 or 120 min. HLA was immunoprecipitated from lysed cells using the W6/32 monoclonal antibody, treated (lanes b, d and f) or mock-treated (lanes a, c and e) with neuraminidase, fractionated by IEF and fluorographed. The positions of HLA-A containing 0, 1 or 2 sialic (SA) acid residues are indicated.

free of nocodazole, and were allowed to progress through M phase into G1 phase. The chase period in these experiments is therefore coincident with M phase progression.

4.3.2 Monitoring transport from the ER to the medial Golgi apparatus

The results from one such pulse chase experiment are shown in Figure 4.5, together with the mitotic indices for each of the time points shown. The patterns of HLA bands subjected to endo H digestion indicate much slower kinetics of transport from the ER to the *medial* Golgi in these mitotic cells. Whereas in the case of G1 cells, 60 min was sufficient to confer endo H-resistance to virtually all of the immunoprecipitated protein, here HLA remained almost entirely sensitive to endo H digestion after the same period of chase (lanes c and d). By 120 min of chase, when the mitotic index had fallen to 5%, the protein had acquired endo H-resistance (lanes e and f). The minor band which remained endo H-sensitive throughout the chase is likely to be HLA-C, and was discussed in Chapter 3.

The panel in Figure 4.5 also reveals the importance of the nocodazole removal for subsequent resumption of protein transport from the ER to the *medial* Golgi. If the cells were chased in the presence of nocodazole, cells remained in prometaphase (mitotic index of 100%) and HLA remained endo H-sensitive even after 120 min (lanes g and h), consistent with the protein remaining in the ER.

These data are therefore consistent with what is known of the cessation of intracellular protein transport out of the ER during M phase (Featherstone, *et al.*, 1985)

4.3.3 Monitoring transport from the ER to the trans Golgi and cell surface

Figure 4.6 shows the pattern of HLA-A bands isolated from mitotic HeLa cells 0, 60 and 120 min after the nocodazole washout and then analysed by IEF. The panel indicates that after 60 min of chase, when almost all the immunoprecipitated protein isolated from G1 cells was sialylated, the level of sialylation of HLA-A remained very low (lane c). The relative lack of sialylation at this time point therefore demonstrates that HLA reached the *trans* Golgi at a much slower rate in mitotic HeLa cells. After 120 min of chase, HLA-A had been extensively sialylated (lane e) indicating that protein transport through the Golgi apparatus had resumed by this time.

These findings are confirmed by the analysis of cell surface transport of HLA in Figure 4.7 and 4.8. Chase times up to 60 min are insufficient to detect any digestion of HLA-A by exogenous neuraminidase in Figure 4.7 (lanes c and d). Later arrival of newly synthesised protein at the cell surface is evidenced by SA

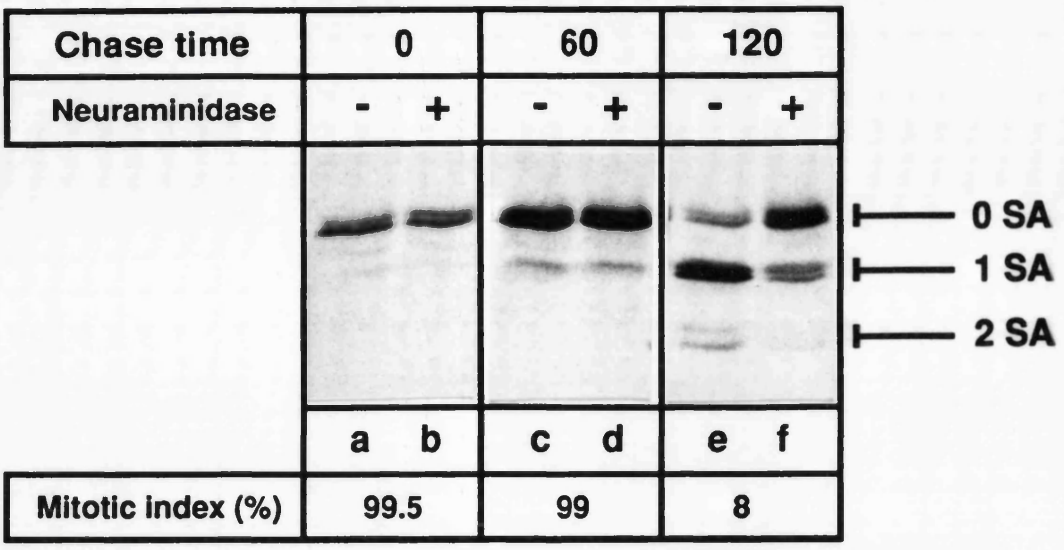

Figure 4.7 IEF analysis of HLA-A immunoprecipitated from mitotic HeLa cells either cell-surface treated (lanes b,d and f) or mock-treated (lanes a,c and e) with neuraminidase prior to cell lysis. The positions of HLA-A with 0, 1 or 2 sialic acid (SA) residues are indicated.

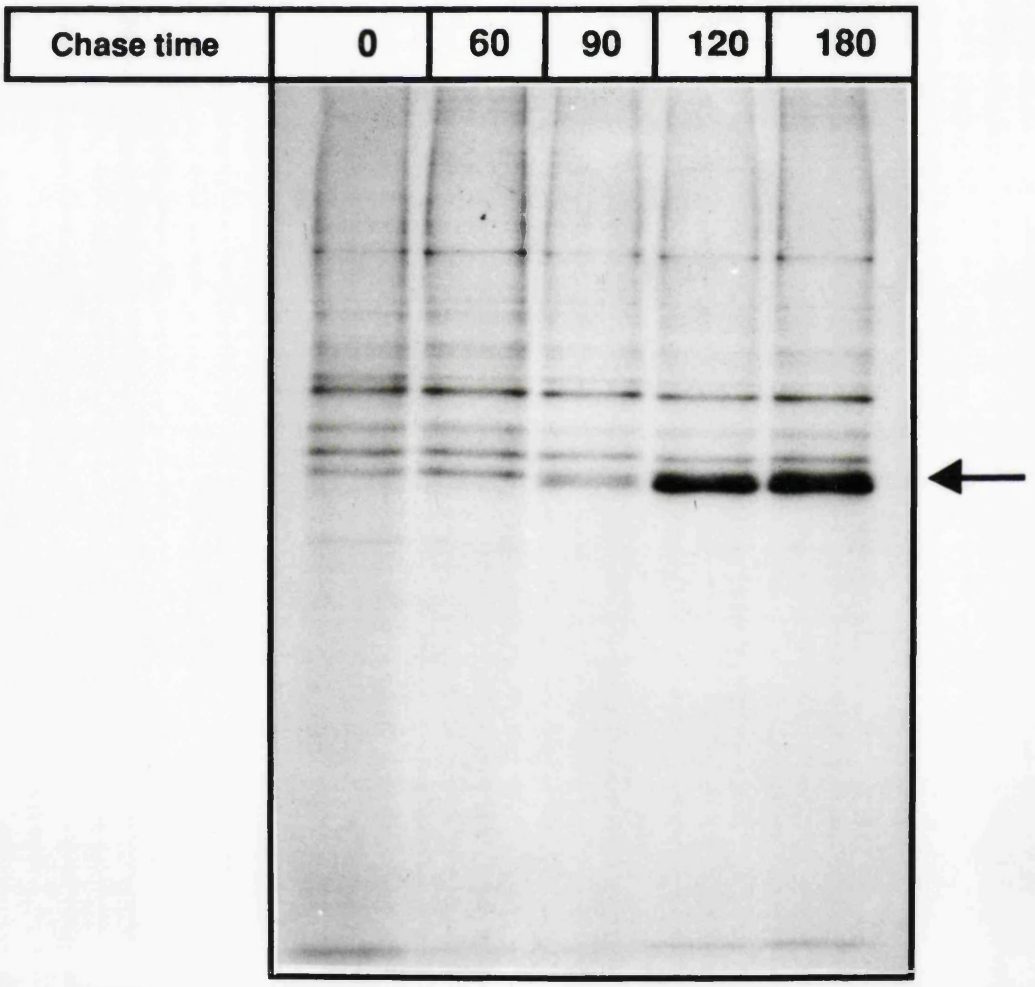

Figure 4.8 Immunoprecipitation of cell surface HLA from mitotic HeLa cells. Cells were pulsed for 5 min with ^{35}S-methionine, chased for up to 180 min and incubated for 30 min at 4°C with monoclonal antibody W6/32 before lysis in the presence of a five-fold excess of unlabelled cell lysate. The immunoprecipitated protein was fractionated by SDS-PAGE and fluorographed. The position of HLA is indicated by the arrow.

removal at the 120 min time point when the mitotic index had fallen to 8% (lanes e and f). Similarly, there is no sign of HLA immunoprecipitated by cell surface bound antibody until 90 min of chase in Figure 4.8.

4.3.4 Quantitation of HLA transport In mitotic HeLa cells

These fluorographic patterns were analysed by laser densitometry and the resulting profiles are plotted in Figure 4.9, along with associated profiles of the mitotic index and H1 kinase activity. The number of experiments (n) represented in each of these profiles is shown in the legend.

The kinetic profiles are strikingly similar in form and pattern to those of G1 cells. Comparison of the half-times for transport to the *medial* and *trans* Golgi and to the cell surface showed that once protein transport had resumed, the kinetics of passage through the secretory pathway were indistinguishable from those of G1 cells; arrival at the *medial* and *trans* cisternae occurred within a minute of each other (the half-time for arrival at the *medial* Golgi was 94 min), and arrival at the cell surface took about 5 min longer. Statistical analysis using the *t*-test indicated the intervals between the G1 half-times for *medial* , *trans* and cell surface arrival were not significantly different from the intervals between the mitotic half-times at the 95% confidence level.

The overwhelming difference between these profiles and those for HLA transport in G1 cells is the observation that the half-times were about 70 min longer in mitotic cells, corresponding to a 70 min period during which intracellular protein transport was inhibited in M phase HeLa cells.

The half-time for H1 kinase activity was found to be 60 min, 10 min before the onset of protein transport. This 10 min lag period is therefore kinetically consistent with the notion that the resumption of secretory transport occurs as a consequence of the loss of mitotic kinase activity.

4.3.5 Resumption of protein transport is independent of protein synthesis

The inclusion of cycloheximide in the chase medium had no effect on transport kinetics demonstrating that the restoration of transport was independent of protein synthesis (data not shown). Furthermore, both the mitotic phase progression and the fall in H1 kinase activity were also insensitive to cycloheximide. Once these facts had been established, cycloheximide was included in all further chase incubations so that the pulsed pool of protein would not be diluted by unlabelled protein synthesised during the chase period.

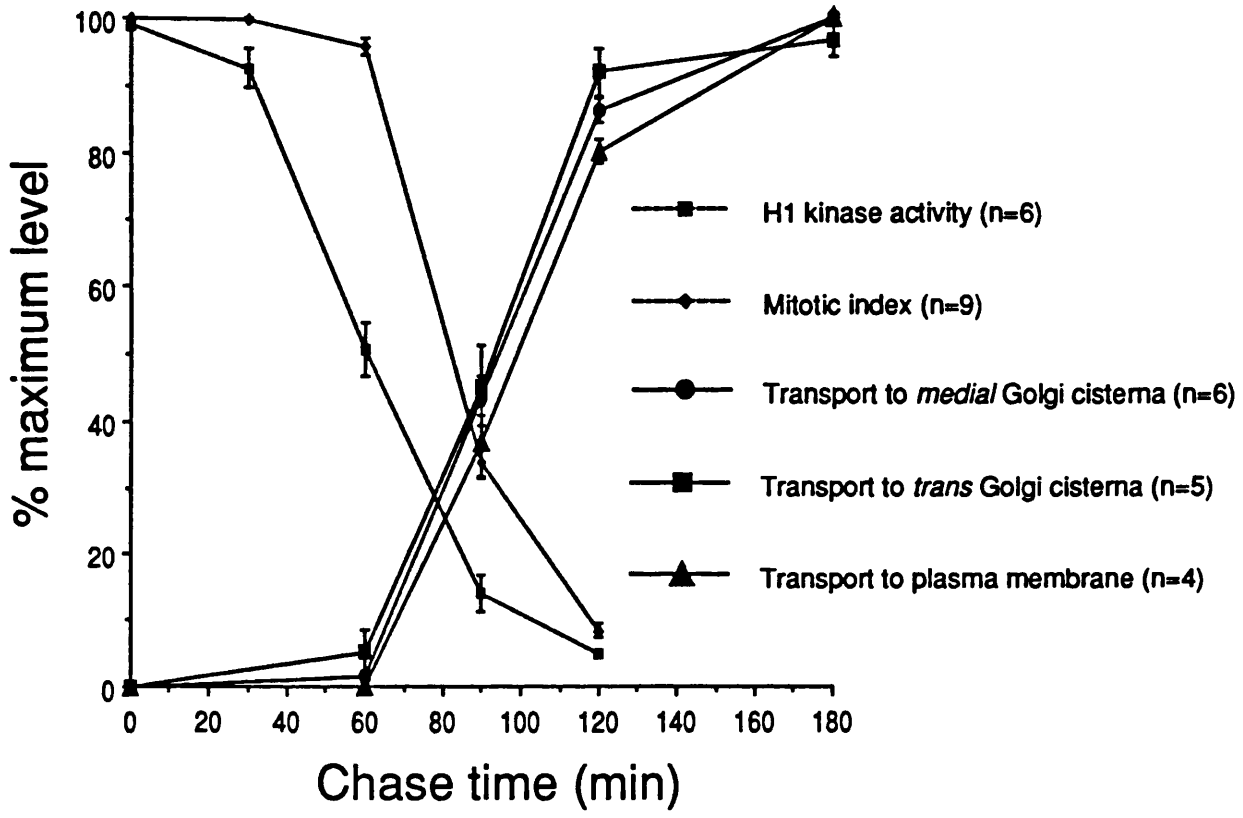

Figure 4.9 Kinetics of HLA transport in mitotic cells. Prometaphase cells were pulsed for 5 min with ^{35}S-methionine, chased for varying times in the absence of nocodazole and HLA analysed for resistance to endo H (arrival at *medial* Golgi cisterna), acquisition of sialic acid residues (arrival at *trans* Golgi cisternae) and sensitivity to exogenous neuraminidase (arrival at the plasma membrane). Fluorographs were scanned by laser densitometry and the results are presented as the average of "n" experiments ±SEM. Cells were also scored for mitotic index using Hoechst 33258 and assayed for the H1 kinase activity of mitotic kinase.

4.4 Control experiments

The data presented so far indicate an inhibition of intracellular protein transport through the secretory pathway in mitotic HeLa cells. However, it was important to confirm that this observation was independent of the particular methodology employed in these studies, and particularly, that the phenomenon of mitotic delay in transport rates was not peculiar to HLA. These questions were addressed by the following series of control experiments.

4.4.1 Independence of the timing of the pulse

In the experiments described so far, the pulse labelling was carried out immediately prior to the release from the nocodazole block. In order to show that the results were not dependent on the time of the pulse, we varied the pulse time to either 30 min before or 30 min after the release from the nocodazole block. Analysis of the kinetics of HLA transport demonstrated that despite a 60 min interval between pulse times, there was only about a five minute difference in the time of arrival of labelled HLA at the *medial* Golgi. This result also demonstrated that there was no preferential transport of any particular protein pool once protein transport resumed at the end of mitosis; protein synthesised 30 min before the release from nocodazole was transported at almost the same rate as protein synthesised 30 min after this release.

4.4.2 Establishing transport-competence of immunoprecipitated HLA

In order to conclude that the lag period observed in mitotic cells corresponds to the inhibition of protein transport, it was essential to ascertain that the heavy chain was isolated from transport-competent complexes. Absence of protein exit from the ER during M phase could be attributed to protein assembly inhibition rather than transport inhibition. Cessation of peptide generation or peptide translocation into the ER could equally lead to the retention of Class I complexes in the ER during mitosis, since complete assembly is required for secretion of the heavy chain (Ploegh *et al.,* 1979; Owen *et al*; 1980; Sege *et al.,* 1981; Williams *et al,. 1988*). Such an explanation is however not valid in the light of the fact that the antibody used in the experiments described above was W6/32, a monoclonal antibody which binds the heavy chain in its fully assembled form, complexed with ß2m and peptide (Barnstable *et al.*, 1978; Parham *et al.*, 1979). Thus, heavy chain isolated with this antibody is clearly derived from transport-competent complexes. In order to establish this point with further certainty, pulse chase analysis of the heavy chain was undertaken by immunoprecipitation with a polyclonal antibody (K355) raised against ß2m. Any heavy chain isolated with this antibody must already be tightly associated with the light chain. Since the assembly of the

complex is co-operative, and binding of the peptide is required to maintain the tight interaction of HLA with ß2m, it may be assumed that HLA isolated with this antibody forms part of a Class I complex fully capable of exit from the ER.

Quantitative analysis of heavy chain immunoprecipitated with K355 showed that the kinetics of transport to the *medial* Golgi in mitotic HeLa cells were identical to those already established using the monoclonal antibody W6/32; the average half-time from two experiments was 92 min, compared to a value of 94 min when HLA was immunoprecipitated with W6/32. This therefore provides conclusive evidence that the kinetics of transport observed in these experiments were independent of the means of immuno-isolating the heavy chain from mitotic cells. Furthermore, this control confirms that the lag period observed in the transport of HLA in mitotic HeLa cells was due to cessation of protein transport through the secretory pathway and was not due to inhibition of Class I assembly during M phase.

4.4.3 Transport of the transferrin receptor

Analysis of the transport kinetics of another protein other than HLA was of interest in verifying that the observed inhibition was not peculiar to Class I complexes, but was a global phenomenon affecting all secreted proteins. The protein chosen for this control was the transferrin receptor.

The transferrin receptor has 3 N-linked glycosylation sites at Asn 251, 317 and 727. Only one of these N-linked sugar chains however is fully processed to the complex oligosaccharide structure; the others remain in the simple high mannose form (Omary and Trowbridge, 1981; Schneider, *et al.*, 1982). Figure 4.10 shows the results of a pulse chase experiment using mitotic HeLa cells, in which the transferrin receptor was immunoprecipitated with monoclonal antibody OKT9. At zero time, all three N-linked sugar chains were in the high mannose form, characteristic of proteins isolated from the ER. During the chase a second heavier band appeared representing the modified receptor which now had two simple sugar chains and one complex sugar chain. By the end of the chase the heavier band predominated. Endo H digestion showed that the lighter chain remained completely sensitive, while the heavier chain became partially resistant, consistent with one oligosaccharide chain being complex and two remaining simple. Sequential immunoprecipitation of the two proteins using OKT9 and W6/32 provided a simple and convenient means of directly comparing the kinetics of transport from the ER to the *medial* Golgi in both G1 and mitotic HeLa cells.

Quantitation of the fluorograph shown in Figure 4.10 revealed that the transferrin receptor is transported to the *medial* Golgi more slowly than HLA in G1 cells. The

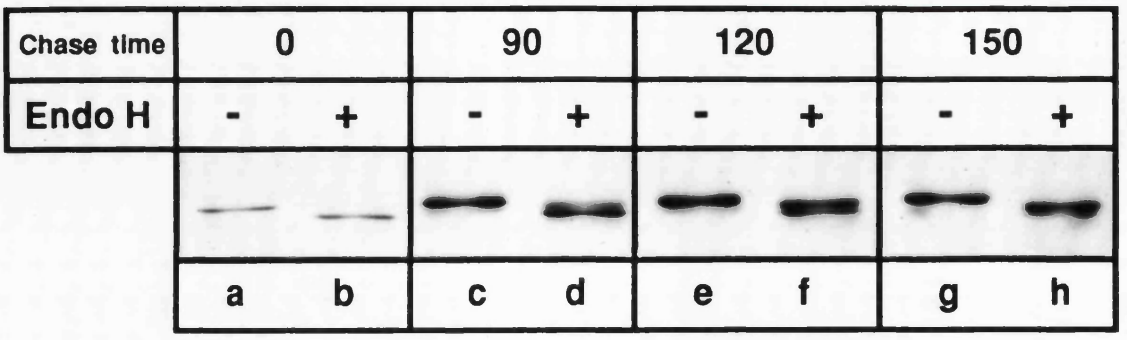

Figure 4.10 Analysis of transport of the transferrin receptor in mitotic HeLa cells. HeLa cells were pulsed, washed out of nocodazole and chased through M phase. The transferrin receptor was immunoprecipitated with OKT9 antibody and either treated (lanes b, d, f and h) or mock-treated (lanes a, c, e and g) with endo H before being analysed by SDS-PAGE and fluorography.

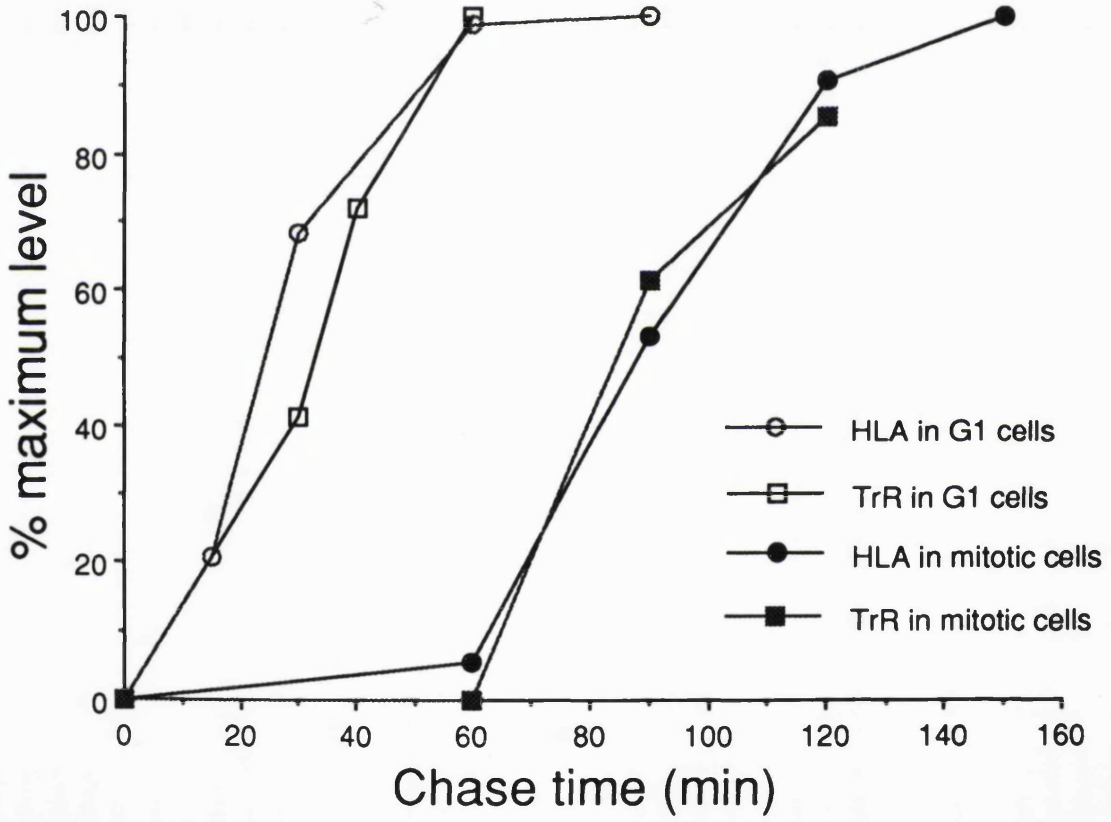

Figure 4.11 Comparison of transport of HLA and the transferrin receptor from the ER to the *medial* Golgi in G1 and mitotic HeLa cells. Cells were pulsed for 5 min with ^{35}S-methionine, chased for up to 150 min, lysed in TX-114, and HLA and the transferrin receptor were serially immunoprecipitated with W6/32 and OKT9 antibodies respectively. The samples were fractionated by SDS-PAGE and the resulting fluorographs quantified by laser densitometry.

half-time for this transfer was about 33 min, about 9 min longer than that of HLA (Figure 4.11). Similar quantitation of the mitotic transport data demonstrated that the half-time for transferrin receptor transport was coincident with that of HLA demonstrating that the inhibition of secretory transport in these experiments was a general phenomenon in mitotic HeLa cells and was not restricted to HLA.

The simultaneous arrival at the *medial* Golgi at the end of M phase was of interest in the light of the fact that there was a window of approximately 10 min between the equivalent G1 half-times. This finding argues that the point of inhibition of protein transport in mitotic HeLa cells is downstream of the rate-limiting step for protein transport from the ER to the Golgi apparatus. Since this rate-limiting step is likely to be the assembly of transport-competent complexes in the ER (assembly of the trimeric complex in the case of HLA and dimerisation in the case of the transferrin receptor), the data presented here suggest that this assembly is able to continue throughout M phase. Newly synthesised proteins are therefore continually assembled into their transport complexes during the mitotically imposed arrest of protein exit from the ER, so that they are ready to be released once transport is resumed at the end of M phase. Proteins which under interphase conditions exhibit widely divergent rates of secretion to the Golgi therefore enter transport vesicles simultaneously at the end of M phase and consequently arrive at the *medial* Golgi at the same time.

4.4.4 Endo D digestion

Since proteins do not acquire endo H-resistance until they reach the *medial* Golgi, the assay system used here cannot distinguish the various steps of transport between the ER and the *medial* Golgi cisterna. The data are equally consistent with the arrest of protein exit from the ER during mitosis or maintenance of this transport and cessation of transport between a downstream compartment and the *medial* Golgi. Digestion of immunoprecipitated HLA with another endoglycosidase (endo D) may be used to determine if the *cis* cisterna rather than the ER is the site of mitotic accumulation. Proteins which have been exposed to mannosidase I in the *cis* Golgi but not NAGT I in the *medial* Golgi are sensitive to endo D digestion. This control however demonstrated that during the mitotic lag period during which protein transport is arrested, HLA remained endo D-resistant, ruling out the possibility that protein had accumulated in the *cis* cisterna (data not shown). This control does not exclude other compartments which might act as mitotic accumulation sites, including the intermediate compartment and the transitional element region. Firm identification of this compartment would require quantitative immuno-EM analysis.

4.4.5 Preservation of cell integrity during chase period

The methodology used to establish cell surface transport of HLA, namely exogenous neuraminidase and surface binding of antibody, necessitates the preservation of plasma membrane integrity at all points during the chase period. The integrity of the plasma membrane was established by means of a lactate dehydrogenase assay. The proportion of the LDH activity contained within the cell pellets remained high throughout the chase period (Figure 4.12), indicating that cell latency was maintained. Neuraminidase-digested protein must therefore have been on the cell surface rather than representing promiscuous digestion of intracellular HLA exposed via disruption of the plasma membrane and membrane-bound compartments within the cell. Similarly, none of the HLA immunoprecipitated with cell surface bound antibody represented contamination from the intracellular pool.

Measurement of cell viability by means of Hoechst 33258 staining indicated that the viability index remained in excess of 95% during the chase period. This confirms that there was no significant deterioration of the cell population as would be expected if excessive cell manipulation was causing extensive plasma membrane damage.

4.5 Timing of the resumption of protein transport

The half-time for transport of protein to the *medial* Golgi was found to be about 94 min. Protein transport from the ER resumed 24 min before the half-time for arrival at the *medial* Golgi, indicating that resumption of transport occurred 70 min after the nocodazole washout. This figure correlates with the telophase peak in Figure 4.4, consistent with previous studies indicating that protein transport resumes during telophase (Warren *et al.*, 1983).

The fact that the half-time for the mitotic index profile was 82 min shows that progression into G1 phase precedes arrival of protein at the *medial* Golgi by about 10 min.

4.6 Summary and conclusions

The experiments undertaken in this section are consistent with what is known of the inhibition of intracellular protein transport from the ER to the Golgi apparatus during mitosis. Quantitation of the immunoprecipitation data has demonstrated that the kinetics of transport in cells progressing through M phase lag behind interphase kinetics by about 70 min. Since many of the early studies of this mitotic arrest used viral proteins, the investigation here lends important verification of these observations using endogenously synthesised protein.

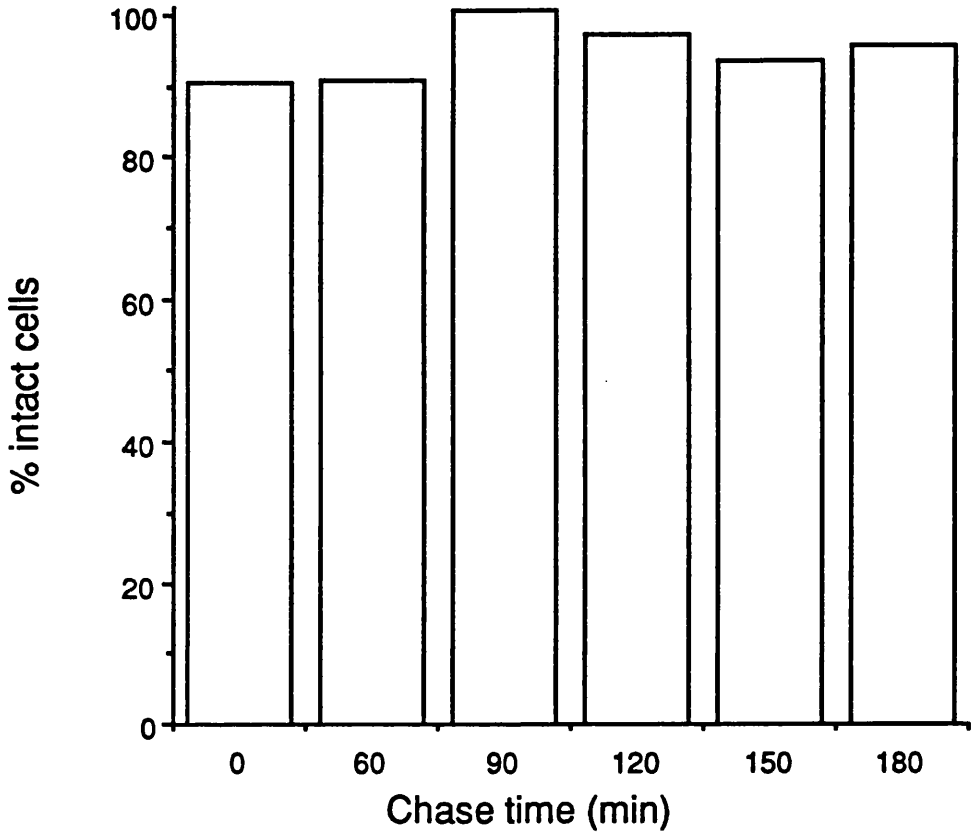

Figure 4.12: Cell viability during chase period. Mitotic HeLa cells were assayed for release of the cytosolic marker lactate dehydrogenase as a measure of cell integrity.

The fact that identical transport kinetics were observed with a different antibody argues that the effect was not due to the methodology of the experiment. Furthermore, analysis of the kinetics of transport of the transferrin receptor revealed the same delay in protein transport from the ER to the Golgi during mitosis. The simultaneous arrival of HLA and the transferrin receptor at the *medial* Golgi indicates that the point of inhibition of protein traffic from the ER to the Golgi was downstream of the rate-limiting step for this transfer during interphase. Proteins therefore continue to accumulate into transport complexes during M phase and leave the ER as a wave of newly synthesised protein once protein transport is restored.

The form and pattern of protein transport at the end of mitosis were very similar to those of G1 cells. This preservation of the interphase rates of transfer from *medial* to *trans* Golgi and from *trans* Golgi to the cell surface strongly argues that once protein transport is reinitiated, transport continues at the same rate as that of interphase; there is not therefore a gradual restoration of transport kinetics, rising to an interphase level. The half-time for the fall in H1 kinase activity was 60 min, 10 min before the resumption of HLA transport. Since the $p34^{cdc2}$ activity is thought to be the major control point during mitotic progression, this 10 min window is at least kinetically consistent with the hypothesis that protein transport is under the control of the predominant mitotic kinase. This observation therefore supports the theory that transport arrest and resumption is regulated by a phosphorylation-dephosphorylation mechanism.

Progression into G1 phase preceded the arrival of HLA at the *medial* Golgi by about 10 min. Since earlier investigation of the reassembly of the Golgi apparatus has demonstrated that this process occurs during telophase (Lucocq *et al.*, 1989), this 10 min window between entry to G1 phase and arrival of newly synthesised protein at the *medial* Golgi suggested that once protein reaches the Golgi apparatus, the stack was already fully reassembled.

The relative timing of the morphological reassembly of the Golgi stack and the resumption of protein transport through the organelle forms the subject of the experiments discussed in the following chapter.

CHAPTER FIVE: KINETIC RELATIONSHIP BETWEEN PROTEIN TRANSPORT AND GOLGI REASSEMBLY IN TELOPHASE

5.1 Overview

The experimental work of the first two chapters compared the intracellular protein transport through the secretory pathway in interphase and mitotic HeLa cells. More specifically, these assay systems facilitated dissection of the resumption of protein transport at the end of M phase. This work paved the way for the investigation of the morphological reassembly of the Golgi apparatus during telophase and the kinetic relationship between this reassembly and the onset of secretory traffic through the organelle.

The work of this chapter addressed this kinetic relationship. The experimental strategy involved the parallel investigation of radioactively labelled cell populations to dissect protein transport and of unlabelled cell populations to dissect Golgi reassembly. The biochemical analysis was identical in format to the work of the last chapter, while the morphological investigation made use of quantitative stereological EM methods. The use of mitotic phase progression and the fall in H1 kinase activity provided further means of comparing these experiments with previous work and also of relating transport data with EM data. Using these methods together, it was possible to build up a comprehensive picture of the resumption of protein traffic and Golgi reassembly.

I am indebted to Dr. Marc Pypaert for undertaking the EM studies in this section of the work, including the extensive stereological EM quantitation required to be able to adequately compare the structural and biochemical data.

5.2 Visualisation of morphology

The electron microscope has been of invaluable importance in the evolution of our understanding of the fine structure of the Golgi apparatus in mammalian cells and the development of powerful stereological techniques has allowed detailed analysis of this structure. Such stereological methods have proven essential in unravelling the reassembly pathway of the Golgi apparatus, including the identification of the various structural intermediates along the pathway. These studies have also demonstrated that reassembly occurs during telophase (Lucocq *et al.*, 1989).

Similar stereological techniques have been used in the work discussed here and are outlined in Chapter 2.

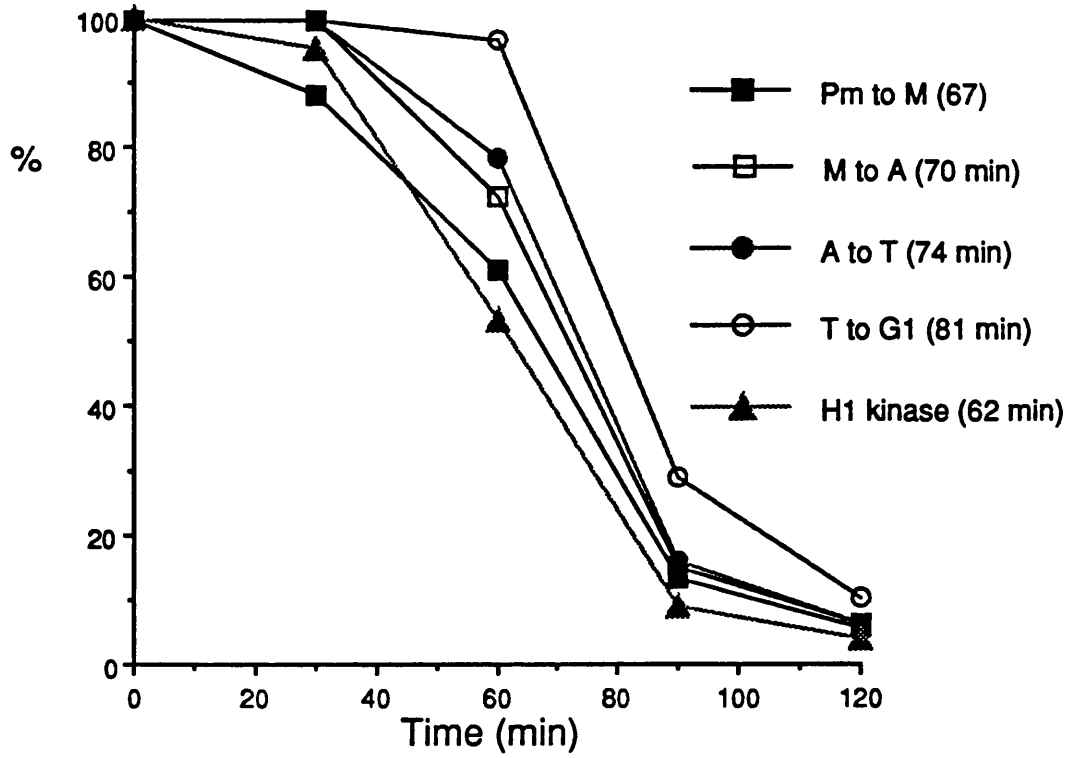

Figure 5.1 Mitotic phase transitions and H1 kinase activity. HeLa cells were blocked in prometaphase, released out of nocodazole and allowed to progress through M phase. The mitotic phases and H1 kinase activity were assayed every 30 min. The data represent the average of 2 experiments. The half-times for each transition are given in brackets.

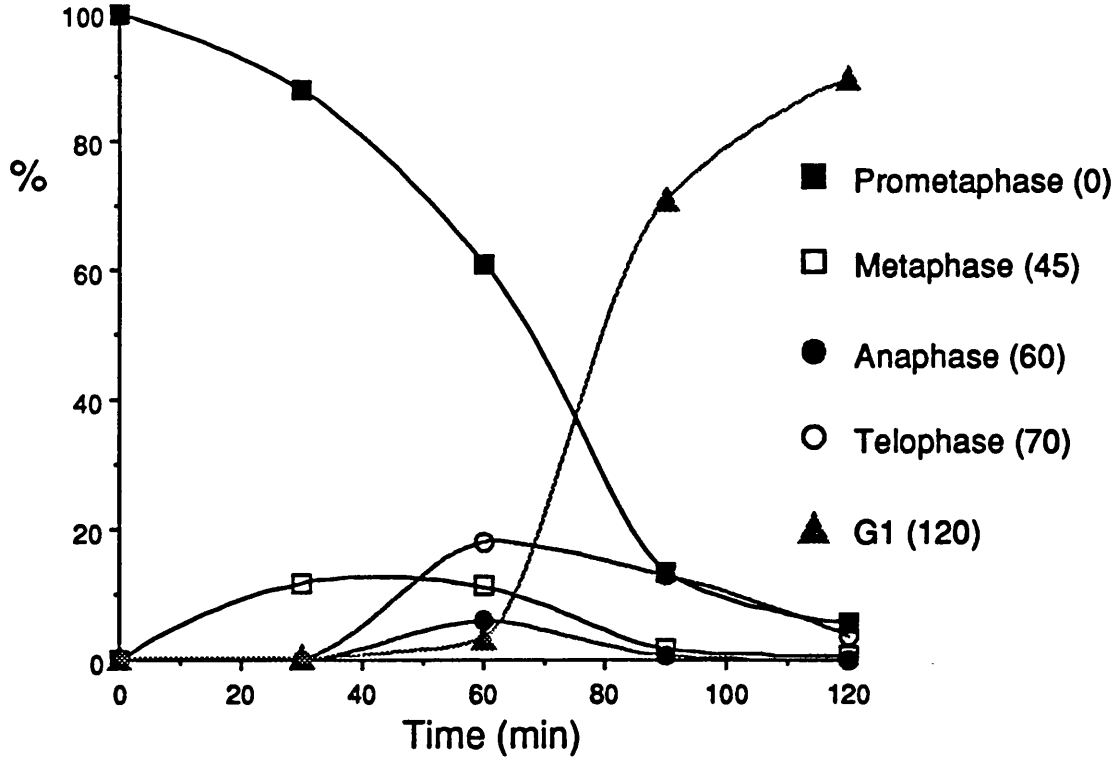

Figure 5.2 Peaks of the mitotic phases. HeLa cells were blocked in prometaphase, released out of nocodazole and allowed to progress through M phase. The profiles represent the average percentage in each phase during the mitotic progression from 2 experiments. The times corresponding to the maximun peak height are given in brackets.

5.3 Experimental strategy for comparing the kinetics of protein transport and Golgi reassembly

In the initial experiments, the time points for sampling were spaced at 30 min intervals. These early experiments provided a picture in outline form of the kinetics of Golgi reassembly and how these related to protein traffic kinetics. This picture was then filled in with much greater detail in later experiments where the time points for sampling were more closely spaced.

5.3.1 Characterisation of the mitotic progression in preliminary experiments

The results from two identical experiments were averaged and the phase transition profiles along with the H1 kinase activity are shown in Figure 5.1. The half-times for each of these profiles (shown in brackets) demonstrate that the mitotic progression is consistent with previous experiments (see Figure 4.3). This observation is further confirmed by the mitotic phase peaks plotted in Figure 5.2; the approximate times after nocodazole release corresponding to the maximum phase peak heights (given in the legend) are strikingly similar to those derived from earlier mitotic experiments (see Figure 4.4). Having established that the mitotic progression was consistent with previous investigations, the biochemical data relating to HLA transport were quantified.

5.3.2 Quantitation of HLA transport

The fluorographs corresponding to the transport of HLA to *medial* and *trans* Golgi cisternae and to the cell surface were quantitated by laser densitometry and the results plotted in Figure 5.3. The half-time values are given in brackets. Comparison of half-times for arrival at the *medial* and *trans* Golgi cisternae (92 and 90 min respectively) reveals the technical difficulty in performing IEF analysis reproducibly, and argues that the error margin on these values is ±5 min. The data here represent the average from two experiments, whereas the corresponding *medial* and *trans* Golgi transport profiles in Figure 4.9 represent the average from 6 and 5 experiments respectively. The increasing degree of consistency between rates of transport with increasing number of experiments is not unsurprising given the complex methods of quantitation used here. As in earlier experiments, the arrival of HLA at the cell surface (98 min) followed about 5 min after arrival at the *medial* Golgi.

5.3.3 EM analysis of Golgi reassembly

During M phase in animal cells, the Golgi apparatus undergoes extensive fragmentation. The vesicles generated by this fragmentation are dispersed throughout the mitotic cell cytoplasm. Reassembly of the organelle during

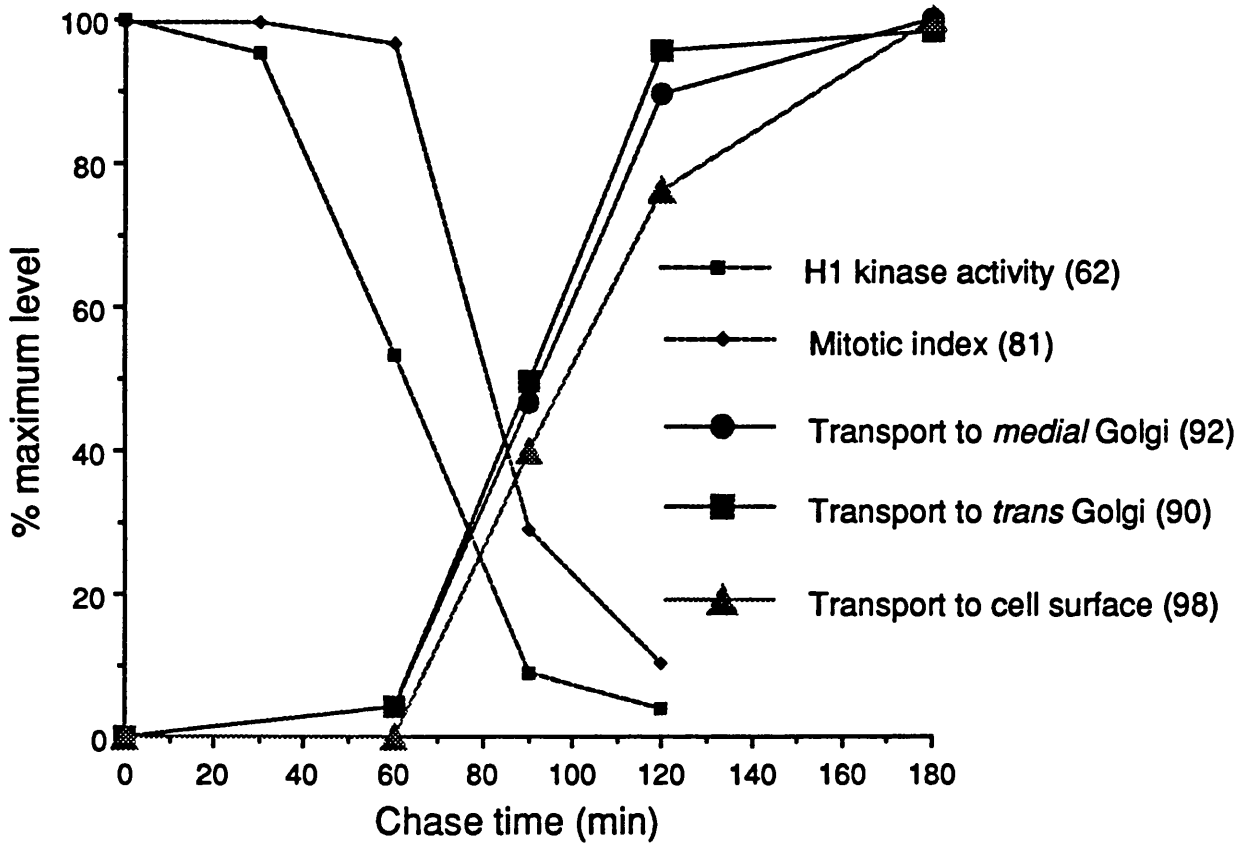

Figure 5.3 Kinetics of HLA transport in mitotic cells. The profiles for arrival at the *medial* Golgi, *trans* Golgi and cell surface represent acquisition of endo H-resistance, sialylation and sensitivity to exogenous neuraminidase respectively. Cells were also scored for mitotic index and histone H1 kinase activity. The results represent the average of two experiments. The half-times for each of the profiles is indicated in brackets.

telophase requires the congregation and fusion of these vesicles to form discrete stacks, and finally congregation and fusion of these stacks to form the peri-centriolar reticulum, characteristic of the mammalian Golgi apparatus during interphase. In order to dissect the kinetics of the reassembly process, it is necessary to be able to visualise structural intermediates along the reassembly pathway and subject these intermediates to stereological analysis.

Figure 5.4 shows a panel of four electron micrographs showing various stages of the reassembly pathway. The first structural intermediate on the reassembly pathway is the so-called "Golgi cluster", which forms as a result of the congregation of dispersed Golgi vesicles. Around 300 such clusters are generated throughout the mitotic cell cytoplasm. For the purposes of quantitation, a Golgi cluster was defined as a group of five or more vesicles (Lucocq *et al.,* 1989); an example is illustrated in Figure 5.4a, indicated by an arrowhead. These clustered Golgi vesicles then fuse with one another to generate dispersed Golgi stacks, each stack arising from a discrete cluster. Figure 5.4b illustrates an immature stack structure, comprising both reassembling cisternae and associated vesicles. More mature stacks are shown in Figure 5.4c and 5.4d, indistinguishable apart from cisternal length (see later discussion of Figure 5.9). Such micrographs provided the "starting material" for stereological quantitation.

5.3.4 Quantitation of electron micrographs

Generating graphic profiles of Golgi reassembly required the calculation of the surface density of stacked cisternal membrane at each of the time points during the chase period. The surface density of Golgi membrane provides a means of estimating the amount of surface area within a particular three-dimensional structure from a two-dimensional picture of the structure. In the same way that the density of a substance represents the mass per unit volume, so the surface density corresponds to the amount of surface area per unit volume. Surface density therefore represents a mathematical abstraction generated from two-dimensional analysis of a three-dimensional structure. The surface densities of Golgi membrane per cell volume were calculated by measuring the ratio of surface density of Golgi membrane within each reassembling stack to the volume fraction per cell occupied by Golgi stacks. The formulae used in calculating these surface densities are described in more detail Chapter 2 Section 2.23, along with the definitions of cisternae and stacked cisternae.

The results of the stereological analysis of the two experiments described above with respect to HLA transport are plotted in Figure 5.5. The profile of stacked cisternal membrane indicates that reassembly is a rapid process occurring between 60 and 90 min after release from the nocodazole arrest with a half-time

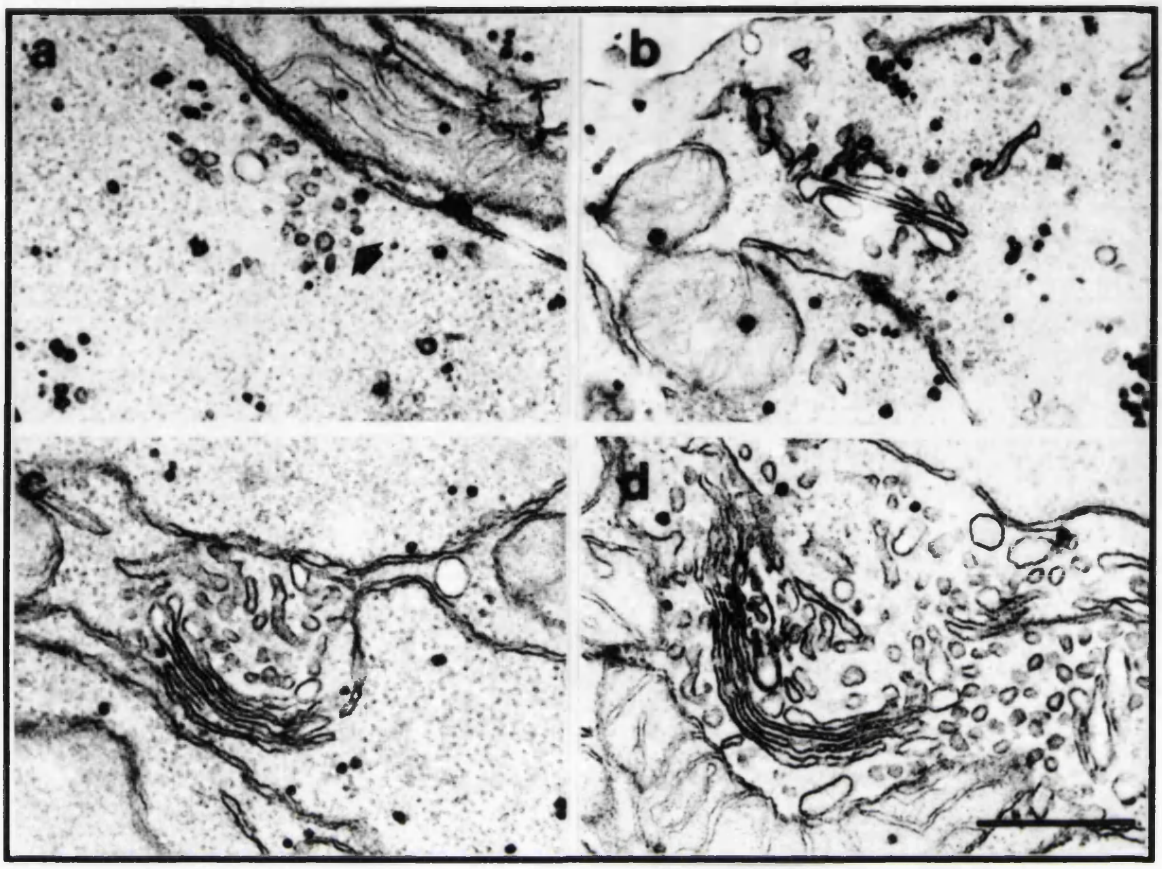

Figure 5.4 Reassembly of Golgi stacks. Representative micrographs from the samples used for stereological analysis in Figures 5.5 to 5.9. Prometaphase cells were incubated in the absence of nocodazole for (a) 50min, (b) 60min, (c) 70min and (d) 180min. Note the Golgi clusters (arrowhead in a), the early Golgi stacks (b) and the fully reassembled Golgi stacks (c and d). The cisternal length increased from c to d. Bar = 0.5µm.

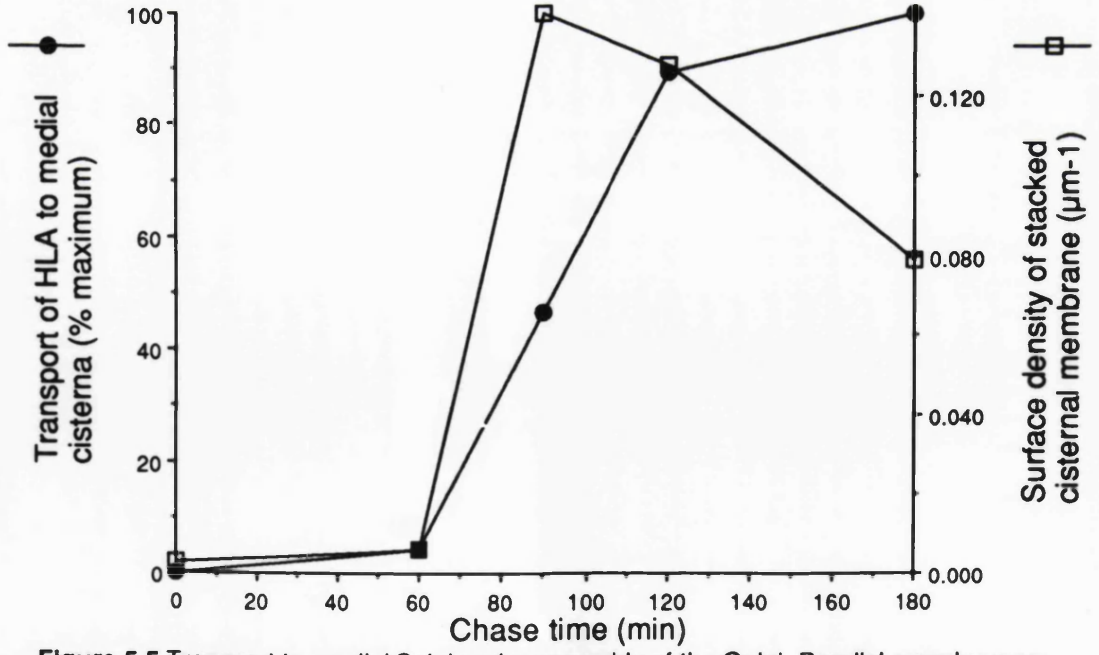

Figure 5.5 Transport to *medial* Golgi and reassembly of the Golgi. Parallel samples were analysed to determine the rate of HLA transport to the *medial* Golgi and the structural reassembly of the Golgi using stereological techniques.

of 74 min, coinciding with the telophase peak (Figure 5.2). This result is therefore consistent with the previous demonstration that Golgi reassembly is a telophase event (Lucocq *et al., 1989*). Comparison of the profiles of stacked cisternal membrane with arrival of HLA at the *medial* cisternae reveals that after 90 min of chase, when the Golgi stacks were fully reassembled, only half of the HLA had become endo H-resistant. This suggests that arrival at the *medial* Golgi lags behind structural reassembly of the organelle.

The analysis of HLA transport in interphase HeLa cells (Chapter 3) demonstrated that HLA leaves the ER about 24 min before arrival at the *medial* Golgi. The HLA transport profile of Figure 5.5 therefore indicates that the onset of HLA exit from the ER occurs about 70 min after the release from the nocodazole block. The onset of this transfer of protein roughly coincides with the half-time for Golgi reassembly as measured by the amount of stacked cisternal membrane (74 min). By the time HLA transport resumes therefore, half of the Golgi stacks are fully reassembled, lending further weight to the notion that resumption of protein transport through the Golgi apparatus lags behind its morphological reassembly.

The reason for the fall in stacked cisternal membrane at later time points is unknown. The data represent the mean from two experiments. In one of these two experiments there was a 20% decrease in stacked cisternal membrane between 90 min and 180 min of chase; in the second experiment, the fall was 70%. The large decrease in the level of stacked cisternal membrane in Figure 5.5 is therefore largely due to this excessive fall in the second experiment. Although the scoring of mitotic indices using Hoechst 33258 staining in this second experiment did not reveal large quantities of dead cells at later time points, inspection of cells at low magnification under the EM suggested that the cell population was not particularly healthy. Although the reason for this deterioration remains unidentified, this observation may provide at least a partial explanation for the unexpected fall in surface density after 90 min of chase.

The fact that stacked cisternal membrane was negligible at the 60 min time point, but had reached a maximum by the next time point (90 min) pointed to the requirement for intermediate time points to refine the analysis of reassembly kinetics within this 30 min window. Without interpolation, it was unclear whether reassembly was occurring steadily throughout this 30 min period, rapidly after the 60 min time point or rapidly just before the 90 min time point. A further experiment was therefore undertaken in which additional time points were included (70 and 80 min) with the aim of distinguishing between these three possibilities. Since it was possible that there was further reassembly occurring just after the 90 min time point, a 100 min sample was also included.

5.4 Refinement of the analysis of Golgi reassembly kinetics

The quantitation of reassembly kinetics was again carried out in parallel with the analysis of mitotic phase progression, H1 kinase activity and HLA transport to the *medial* Golgi. Profiles of the mitotic phase peaks are plotted in Figure 5.6, and again show striking similarity to previous results (see Figure 5.2). Transport to the *trans* Golgi and cell surface was assumed to follow the kinetic profiles already well-characterised in earlier work and these steps were not assayed in this experiment.

5.4.1 Stacked cisternal membrane analysis

Having established consistent mitotic kinetics, EM quantitation was again carried out. Figure 5.7 shows the profile of stacked cisternal membrane during the chase period along with the profile of endo H-resistant HLA. The graph shows that the reassembly of the Golgi was even more rapid than earlier experiments had indicated (Figure 5.5), and occurred essentially within a 20 min window from 60 to 80 min, consistent with the telophase peak plotted in Figure 5.6. Negligible amounts of HLA were endo H-resistant at a time when Golgi reassembly was halfway complete, consistent with earlier analysis of reassembly kinetics (Figure 5.5). The half-time for reassembly was 69 min, 22 min before the half-time for delivery of HLA to the *medial* Golgi (91 min). There was therefore a few min at most between the onset of HLA transport out of the ER and the half-time for reassembly of the Golgi apparatus as measured by stacked cisternal membrane. The more detailed kinetic analysis carried out in this experiment therefore provides clear evidence for the fact that Golgi reassembly precedes the onset of transport of newly synthesised protein through the organelle.

5.4.2 Analysis of the number of cisternae within each stack

Three reassembly models were presented in the Introduction (section 1.4.2.4), namely: sequential cisternal reassembly; simultaneous cisternal reassembly with conservation of subcompartmental specificity; and simultaneous cisternal reassembly without conservation of subcompartmental specificity, requiring subsequent sorting of resident Golgi enzymes.

With respect to the number of cisternae within a reassembling stack, a clear distinction may be seen between the first model and the second two. The first model predicts that the number of cisternae within a reassembling stack increases steadily during the reassembly process at a rate more or less consistent with the increase in stacked cisternal membrane. In contrast, the second and third models both predict that the number of cisternae increases very rapidly early on, and then

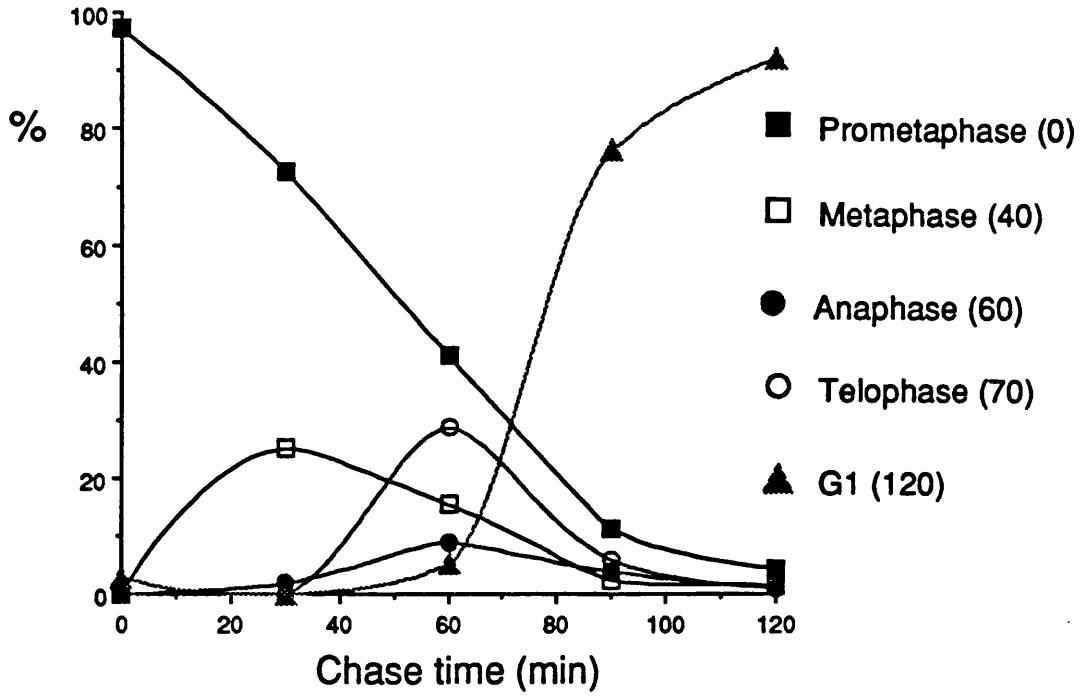

Figure 5.6 Mitotic phase peaks. The profiles represent the average percentage in each phase during the mitotic progression of the more detailed experiment where transport and morphology were measured every 10 min rather than every 30 min. The times corresponding to the maximun peak height are given in brackets.

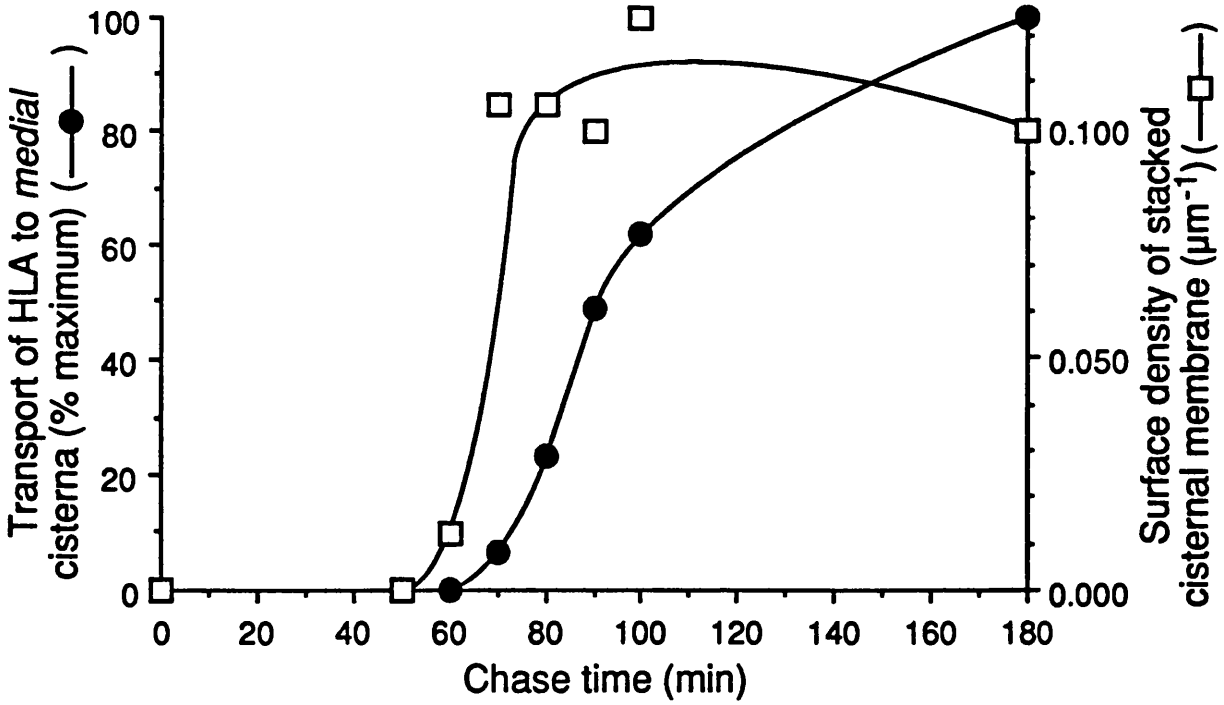

Figure 5.7 Kinetics of transport of HLA and reassembly of Golgi stacks. Parallel samples were analysed to determine the rate of HLA transport (T1/2=91 min) and to measure the surface density of stacked cisternal membrane (T1/2=65 min).

remains more or less constant during later stages of the reassembly pathway. This distinction may therefore be readily explored by scoring the number of cisternae present in each stack at each time point. Figure 5.8 shows the results from this quantitation and reveals that the increase in the number of cisternae was considerably faster than that of stacked cisternal membrane (half-time 58 min). This observation therefore favours the second two models of reassembly described in the Introduction (Figure 1.10b and c) rather than the first (Figure 1.10a) and suggests that the process of reassembly involves nucleation of stacks comprising multiple subcompartments, each subcompartment corresponding to a discrete cisterna. Simultaneous lateral growth of each subcompartment or cisterna would ensue rather than complete reassembly of one cisterna followed by the nucleation and growth of an adjacent one and so on. Distinction between the second and third models is beyond the scope of the data presented here. Such a distinction would require immuno-EM techniques to demonstrate the presence or absence of specific compartmentalisation of resident Golgi enzymes during the cisternal growth period.

5.4.3 Analysis of cisternal length

Figure 5.4c and 5.4d show typical Golgi stacks within cells isolated after 70 min and 180 min of chase, respectively. The stacks display a clear degree of similarity in overall morphology and in the number of cisternae. There is however a discernible increase in the average length of cisternae between the two time points and this difference suggested that it was of interest to quantitate cisternal length during the chase period. Since the final stage of Golgi reassembly is the congregation of dispersed Golgi stacks and their fusion to form the Golgi reticulum in the peri-centriolar region of the interphase cell (Lucocq *et al.*, 1989), one would expect that this lateral fusion of stacks would result in continued growth in average cisternal length during the later stages of the reassembly process.

The results of Figure 5.9 showing average cisternal length (half-time of 63 min) were entirely consistent with this late process of stack fusion; the profile clearly indicates that cisternal length continued to grow after 80 min when the profile of stacked cisternal membrane was more or less constant (Figure 5.7). Lateral fusion of dispersed stacks does not cause an increase in the surface density of Golgi membrane per stack since such fusion events do not alter the overall surface area to volume ratio.

5.5 Relative rates of transport resumption, reassembly and H1 kinase activity

The half-time for reassembly of the Golgi apparatus as measured by stacked cisternal membrane was measured to be 69 min in this experiment. Comparison

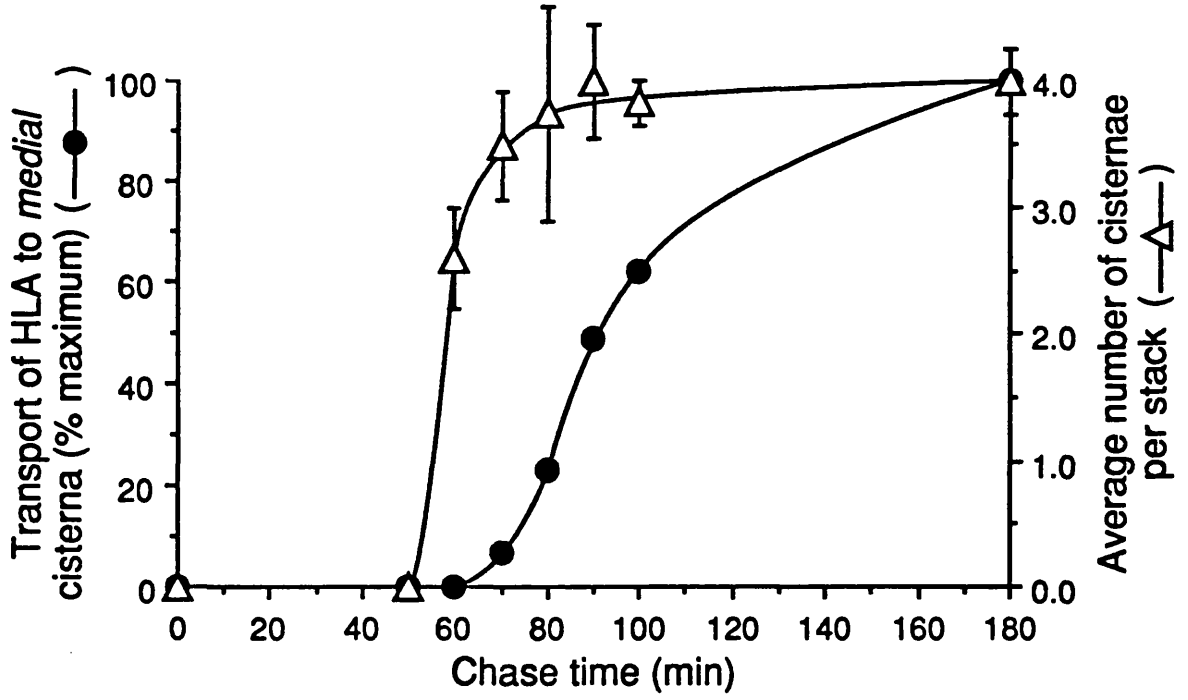

Figure 5.8 HLA transport and reassembly of the Golgi apparatus. Comparison of HLA arrival at the *medial* cisterna (T1/2= 91 min) with the average number of cisternae per stack during the reassembly process (T1/2=58 min).

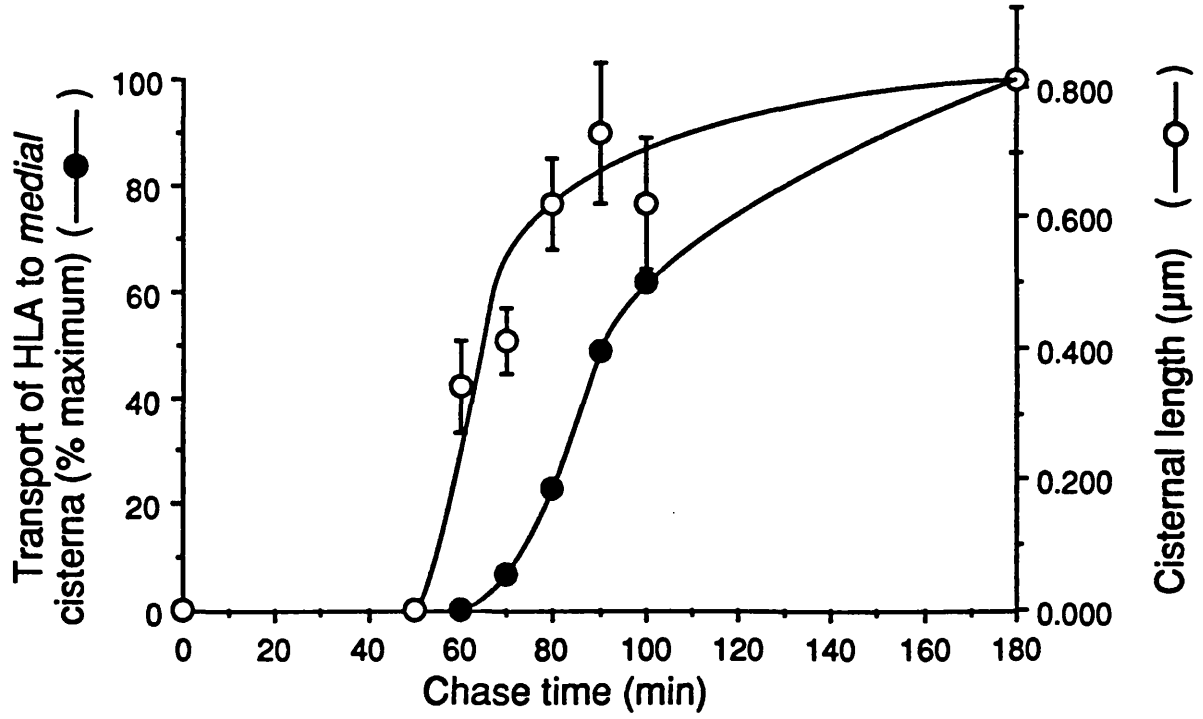

Figure 5.9 HLA transport and reassembly of the Golgi apparatus. Comparison of HLA arrival at the *medial* cisterna (T1/2= 91 min) with the average cisternal length during the reassembly process (T1/2=63 min).

of this half-time with that of the H1 kinase activity reveals that reassembly lagged the fall in mitotic kinase activity by about 7 min. This window fuels the speculation that Golgi reassembly is under the regulatory control of the mitotic kinase $p34^{cdc2}$, and that both the disassembly of the organelle and its subsequent reassembly in telophase are mediated by phosphorylation and dephosphorylation of key structural components. Such a hypothesis of $p34^{cdc2}$-induced changes in Golgi structure has clear precedence in the known involvement of $p34^{cdc2}$-induced phosphorylation in the behaviour of the nuclear lamina during M phase (Heald and McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990).

5.6 Summary and conclusions

The work described in this chapter has shown that the morphological reassembly of the Golgi apparatus in telophase precedes the resumption of HLA transport through the organelle. The reassembly is virtually complete after 90 min following release from nocodazole, while only half of the HLA has reached the *medial* Golgi. The half-time for Golgi reassembly falls within a few min of the onset of HLA transport out of the ER. By the time HLA reaches the *medial* cisterna, reassembly is complete.

The average number of cisternae within each stack rises even more rapidly than that of stacked cisternal membrane suggesting that the reassembly pathway involves nucleation of stacks and simultaneous cisternal growth throughout the stack, rather than growth of each cisterna comprising the stack in turn. The fact that the average length of cisternae continues to rise while the surface density of stacked cisternal membrane remains constant is consistent with subsequent stack migration to the peri-centriolar region and lateral fusion during the later stages of reassembly.

Both resumption of HLA transport and Golgi reassembly follow the fall in histone H1 kinase activity by 5 to 10 min, suggesting that both these processes could be under the control of $p34^{cdc2}$ kinase, either directly or indirectly, and lends weight to the notion that these processes are mediated by phosphorylation-dephosphorylation reactions.

CHAPTER SIX: DISCUSSION

The work I have described in the previous chapters has focused on the comparison of the kinetics of intracellular protein transport in G1 HeLa cells and in cells leaving M phase, and how these kinetics relate to the rate of reassembly of the Golgi apparatus in telophase.

6.1 Investigation of intracellular protein transport

Importantly, the analysis of intracellular protein transport has made use of an endogenous protein, rather than a viral protein to circumvent the technical limitations of viral systems with respect to mitotic progression. Since completion of mitosis is a prerequisite for investigation of transport resumption and Golgi reassembly, use of an endogenous marker proved invaluable.

Investigation of the restoration of protein transport at the end of M phase has relied on the pulse chase analysis of synchronised HeLa cells such that the chase period coincided with the progression of the population from prometaphase into G1 phase as a semi-synchronous wave. A series of *in vivo* assays were employed to dissect the rate of secretion from the ER to the cell surface which may readily be applied to both G1 and mitotic HeLa cells.

The kinetics of secretion following release of the mitotic arrest are independent of the timing of the pulse relative to release from nocodazole. Protein synthesised 30 min before and 30 min after the release from nocodazole arrive at the *medial* Golgi with very similar kinetics. This observation is a necessary criterion for asserting that pulse chase analysis accurately reflects protein transport in cells emerging from mitosis. Selective transport of one particular protein pool in preference to another would be entirely missed by the pulse chase investigations undertaken here.

These investigations have shown that newly synthesised HLA takes approximately 25 min to pass from the ER to the *medial* Golgi in G1 and interphase HeLa cells. The same step of the secretory pathway takes about 95 min in M phase cells, indicating a 70 min lag period during which HLA transport is inhibited in HeLa cells. This lag period cannot be due to the use of nocodazole to arrest cells in prometaphase, since analysis of G1 protein transport in the presence of nocodazole had no effect on transport kinetics. The delay is also not a consequence of delay in assembling the Class I complex, since the monoclonal antibody W6/32 only recognises the heavy chain as part of the tightly folded complex (Barnstable *et al.*, 1978; Parham *et al.*, 1979), and it is the completion of assembly which confers transport-competence upon the Class I complex.

Furthermore, transport rates evaluated via HLA immunoprecipitation with W6/32 antibody were indistinguishable from those measured after co-precipitation of HLA with an anti-ß2m antibody, indicating that the results were independent of the antibody used.

Comparison of rates of transfer of HLA from the ER to the *trans* Golgi and cell surface in G1 and mitotic HeLa cells gave kinetic profiles of similar form and pattern, with the mitotic half-times delayed by about 70 min. The parity of these profiles strongly suggests that the arrest of protein transport is relieved at all inhibited steps of the secretory pathway simultaneously. There does not appear to be a gradual acceleration of transport at the end of mitosis; once vesicular traffic is triggered, the rate of protein transport through the secretory pathway proceeds at interphase levels.

The delay in HLA transport observed during M phase was also true of the transferrin receptor. Interestingly, after the mitotic delay both marker proteins were delivered to the *medial* cisterna of the Golgi apparatus together, despite the fact that the transferrin receptor took about 10 min longer than HLA to travel from the ER to the *medial* Golgi in G1 cells. This observation argues that the most proximal step of transport inhibition between the ER and the Golgi apparatus is distal to the rate-limiting step for newly synthesised secretory proteins arriving at the Golgi. A likely candidate for this rate-limiting step is the folding of nascent polypeptide backbones and associated assembly into transport-competent oligomers within the ER. If this is the major rate-limiting step, the transport data indicate that these assembly events continue throughout M phase, so that proteins accumulate in transport-competent forms immediately prior to the first inhibited step of transport. Once protein transport is triggered at the end of mitosis, secretory proteins are therefore able to be secreted together as a wave of newly synthesised protein; widely divergent rates of secretion in interphase cells are therefore not a feature of cells emerging from mitosis.

Earlier work using viral infection strongly suggested that the point of accumulation was the ER; the oligosaccharide processing of viral proteins during mitosis was characteristic of ER enzymes but not Golgi enzymes (Featherstone *et al.*, 1985). Furthermore, immuno-EM revealed that viral protein could not be chased out of the ER in the presence of cycloheximide. Interestingly, this viral work suggested that the site of accumulation during M phase was distal to the site of protein synthesis, consistent with the simultaneous entry of HLA and transferrin receptor into vesicle carriers at the onset of protein transport.

Because the acquisition of endo H-resistance occurs in the *medial* Golgi, mitotic transport to intermediate steps are not taken into consideration by such an assay.

Although ER to Golgi transport is inhibited overall, the results are consistent with inhibition of a transport step distal to exit from the ER. The results of an endo D digest however indicated that the point of accumulation is not the *cis* cisterna. This leaves the transitional element region and the intermediate compartment as two other possible mitotic accumulation sites. Distinguishing between these compartments cannot be achieved with the present system on a kinetic basis. Such a distinction would require immuno-EM analysis of the secretory pathway in mitotic HeLa cells. Since EM-compatible anti-HLA antibodies are available, such an investigation could be readily undertaken. Clearly, such an experiment requires the use of cycloheximide to chase out any HLA synthesised during the previous G2 phase. The most heavily labelled compartment would therefore indicate the point of accumulation during M phase.

6.2 Reassembly of the Golgi apparatus during telophase

Quantitative morphometric analysis of Golgi membranes during the chase period allowed a kinetic dissection of the reassembly process during telophase. Reappearance of stacked cisternal membrane was found to be a rapid process which occurred within a 20 min window with a half-time of about 70 min, coincident with the resumption of HLA transport. Because the Golgi reassembly process is so much more rapid than the rate of transfer of newly synthesised HLA to the Golgi, by the time HLA reaches the complex, the stack is virtually complete. At 75 min, when reassembly is 80% complete, only 15% of the HLA had reached the *medial* cisterna.

Parallel quantitation of the number of cisternae suggested that this parameter rose even earlier than stacked cisternal membrane. This observation is strongly suggestive of early nucleation of all subcompartments of the Golgi stack, followed by lateral growth of all cisternae simultaneously and argue against an alternative model of sequential cisternal nucleation and growth.

Treatment of cells with the fungal metabolite brefeldin A (BFA) causes disruption of the Golgi apparatus and redistribution of Golgi resident proteins to the ER. Alcalde and co-workers (1992) have investigated the sequence of events underlying the restoration of normal interphase Golgi morphology. They found that the reassembly was a sequential process and that *cis* and *medial* cisternae reappeared before *trans* cisternae. It is unclear to what extent these results are in contradiction to the results reported here. It is certainly worth noting that recovery from BFA treatment may not necessarily reflect reassembly of the Golgi in telophase; a significant difference lies in the fact that Golgi proteins are not relocalised to the ER during M phase. Evidence points to the asymmetrical disruption of the Golgi by BFA (Chege and Pfeffer, 1990; Alcalde *et al.*, 1992) so

perhaps asymmetric recovery is not too surprising. Moreover, the investigation of BFA treated cells relied on the immunofluorescent localisation of Golgi markers. Although such immunofluorescent studies may be used to determine the sequence of correct positioning of resident Golgi proteins, they are of limited use in the dissection of structural reassembly of Golgi cisternae; EM quantitation is essential for such analysis. While the data presented here suggest that all cisternae grow simultaneously, they do not indicate whether there is any symmetrical aspect in the correct sorting or location of Golgi resident proteins. If there was such a polarised adjustment of Golgi protein location, then the findings of Alcalde and colleagues might well be consistent with a reassembly model predicting simultaneous reappearance of cisternae. Accurate distinction between simultaneous cisternal growth and protein sorting and initial cisternal growth followed by sorting awaits quantitative double or triple label immuno-EM studies to assess whether compartmental markers remain distinct during all stages of reassembly or whether they are first mixed and then segregated to their appropriate destinations.

The length of cisternae was also measured and in contrast to the amount of stacked cisternal membrane and the number of cisternae was found to continue to increase after 80 min. This later increase in cisternal length is consistent with what is known of the peri-centriolar migration of discrete stacks, followed by fusion of these stacks to form the single copy Golgi reticulum which constitutes the interphase Golgi apparatus in animal cells.

6.3 Conclusions from the correlation of transport and reassembly data

Since the biochemical investigation of protein transport used in this work relied on pulse chase analysis, only the transport of newly synthesised protein could be monitored. Because of this limitation, the absence of newly synthesised protein from the reassembling Golgi apparatus does not infer that intra-Golgi transport is not occurring during the reassembly process. With regard to the fate of previously synthesised protein immobilised during M phase between the ER and the Golgi or within Golgi vesicles, one could envisage two models. In the first case, transport of such previously synthesised protein could be arrested during the reassembly process and only resume once reassembly was complete (Figure 6.1a). The alternative scenario would be the resumption of transport of this protein during Golgi reassembly (Figure 6.1b). This second model would seem to be the most likely one for two reasons. Firstly, such a model would predict that all mitotically-arrested steps of vesicle traffic would resume together, precluding the need for staggered control mechanisms as cells emerge from M phase. The mechanism of triggering vesicle transport would therefore be correspondingly simpler. Secondly,

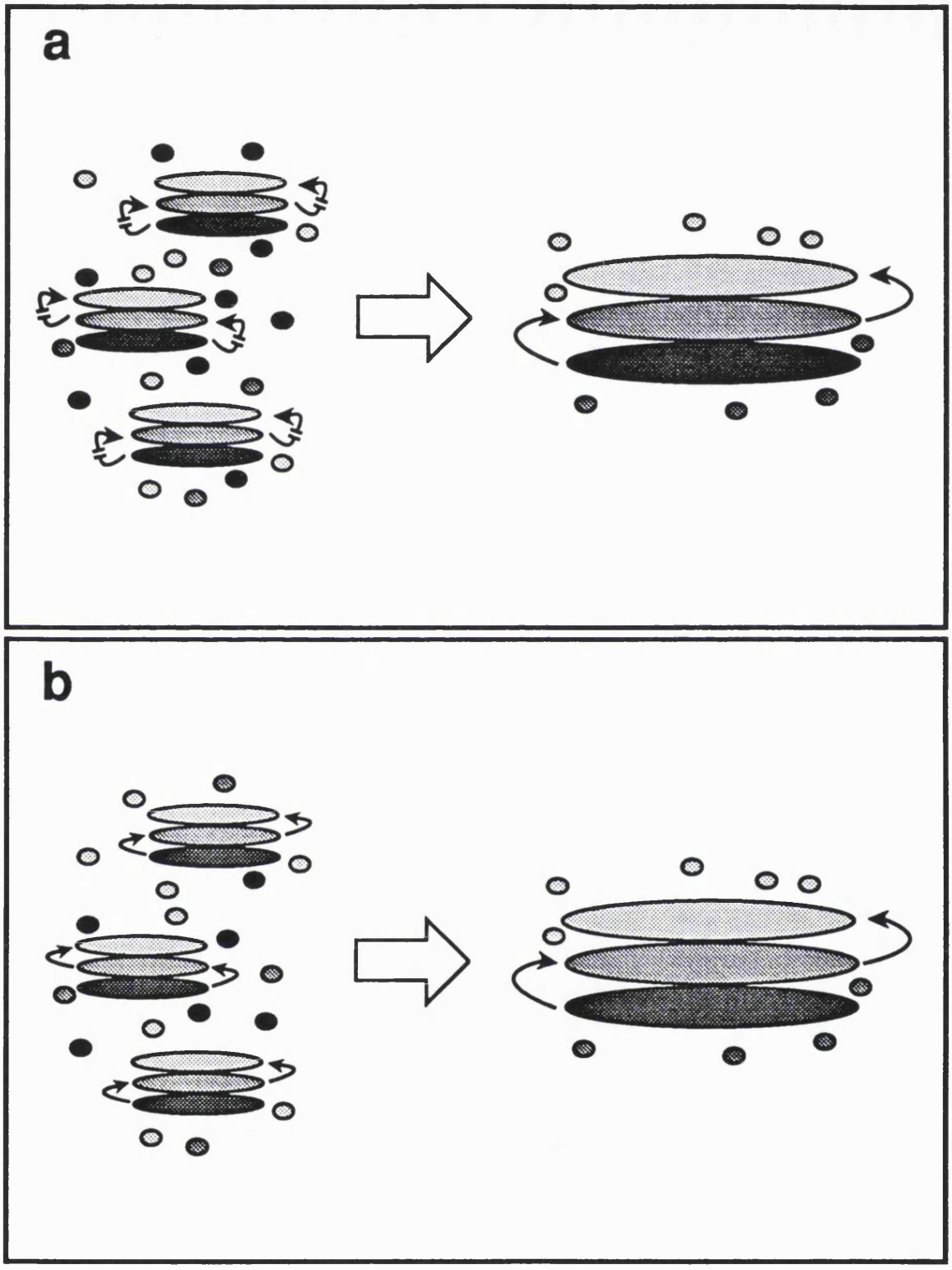

Figure 6.1 Intra-Golgi transport and the reassembly of the Golgi apparatus. Transport could be either inhibited during stack reassembly (a) or could resume during reassembly (b).

there is evidence for vesicle-mediated glycosphingolipid traffic through the Golgi during the reassembly process; the conversion of lactosylceramide made in an early Golgi compartment to GA2, made in a late Golgi compartment, is most closely coincident with the anaphase to telophase transition in mitotic HeLa cells (Collins and Warren, 1992). This result indicates that transport of lactosylceramide from early to late Golgi occurs during telophase, concomitantly with reappearance of the Golgi stack. This finding combined with the simultaneous trigger of protein secretion from the ER and Golgi reassembly suggests that vesicle-mediated traffic at all M phase-arrested stages of the secretory pathway resumes at the same time as fusion of Golgi-derived vesicles to form the Golgi stack.

The inhibition of vesicle fusion and maintenance of vesicle budding was postulated some time ago as a possible model to at least partially account for Golgi fragmentation (Warren, 1985). Cessation of membrane fusion would facilitate the efficient fragmentation of the Golgi quickly and preclude the possibility of random fusion events opposing the disassembly process. Moreover, the cessation of vesicle fusion during mitosis would prevent any non-specific fusion of donor transport vesicles with acceptor Golgi vesicles which might otherwise lead to anomalous oligosaccharide processing since the Golgi enzymes maintain their function during M phase (Lucocq, *et al.*, 1987; Nagata, *et al.*, 1990). Such membrane fusion inhibition would have to allow for the fact that fusion of vesicles derived from the TGN with the plasma membrane is only partially inhibited in mammalian cells (Kreiner and Moore, 1990). The mechanism of arresting membrane fusion would therefore have to permit compartmental specificity of application.

If inhibition of membrane fusion proves to be a mitotic phenomenon, then clearly restoration of fusion is a prerequisite for both the restoration of protein transport and the regeneration of the Golgi apparatus. The apparently simultaneous trigger of both Golgi reassembly and protein transport could well signify the release from the arrest of membrane fusion. Although this simultaneous trigger of transport and reassembly indicates that transport or "heterotypic" fusion is occurring at the same time as the structural or "homotypic" fusion events required to rebuild the stack, it is clear that the rate of these latter fusion events would have to greatly exceed the budding of Golgi transport vesicles if the stack is to be regenerated as rapidly as the data indicate.

Different steps of the secretory pathway are differentially inhibited during M phase. Although ER to Golgi transport is arrested in mammalian cells in mitosis, protein transport from the ER to the TGN is maintained in the case of *Xenopus* (Ceriotti and Colman, 1989). Furthermore, despite the maintenance of transport from the

TGN to the plasma membrane in mammalian cells (Kreiner and Moore, 1990), this stage of secretion ceases during mitosis in *Xenopus*. Thus, the early secretory pathway is mitotically regulated in mammals, while the later pathway is regulated in *Xenopus*.

These findings raise a number of intriguing questions concerning the control of transport. One possible means of triggering the resumption of ER to Golgi transport in mammalian cells might be a Ca^{2+} transient since this step is exquisitely sensitive to μM levels of Ca^{2+} during interphase (Beckers and Balch, 1989), and Ca^{2+} transients have a major role to play in mitotic control (see Whitaker and Patel, 1990 for a review). The absence of such Ca^{2+} transients in the *Xenopus* embryo (Cork, *et al.*, 1987) combined with the maintenance of ER to Golgi transport in this species adds further weight to a putative role of Ca^{2+} in the species-specific regulation of this step of secretion. Since intra-Golgi transport is insensitive to Ca^{2+} (Fries and Rothman, 1980), another trigger mechanism must be at work in the restoration of transport within the Golgi apparatus. Mitotic phosphorylation and subsequent dephosphorylation of a transport component might be one means of achieving such compartmental specificity in transport control. There is *in vitro* evidence for $p34^{cdc2}$ kinase being involved in the mitotic inhibition of intra-Golgi transport (Stuart, *et al.*, 1993) which clearly favours such a mechanism. Even though successive stages of transport through the secretory pathway could be triggered at the same time, the mechanisms underlying these trigger processes could be different.

Measurement of the fall in histone H1 kinase activity showed that the half-time for this fall was 60 min, 10 min before both the half-time for the resumption of protein exit from the ER and the half-time for Golgi reassembly. The sequence of these timings therefore is consistent with the loss of $p34^{cdc2}$ kinase activity providing the common trigger which causes the onset of both protein transport and Golgi reassembly. This correlative observation adds further weight to the theory that the inhibition of transport and the disassembly of the Golgi apparatus is mediated by a phosphorylation reaction, and conversely, that restoration of transport and reassembly of the Golgi are under the control of dephosphorylation reactions.

There has been considerable focus on the targets of phosphorylation during M phase in recent years, both with respect to functional and structural changes. The demonstration that the lamins were phosphorylated by $p34^{cdc2}$ kinase and that this phosphorylation was essential for nuclear lamina disassembly (Heald and McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990) provides a clear example of the way in which such post-translational modification can be a major means of regulating structural changes during mitosis. It is tempting to speculate

that similar post-translational modification is involved at some or all levels of Golgi disassembly and reassembly, including relaxation of the protein matrices within cisternal membranes which mediate kin recognition of resident Golgi enzymes, intercisternal glue interactions and also the matrix which preserves the zone of exclusion around the interphase complex.

The mechanism underlying the inhibition of protein transport during mitosis remains obscure. Compartment-specific modification of key components of the transport machinery is obviously an attractive hypothesis but awaits careful exploration. There is some evidence for phosphorylation of some of the rab proteins, but the physiological significance of this finding is as yet unknown.

6.4 The purpose of Golgi fragmentation

One likely reason for Golgi fragmentation in animal cells is the accurate partitioning of Golgi membrane to both daughter cells so that each cell emerges from M phase with a viable Golgi apparatus. In fission yeast, where there are already a sufficient number of discrete stacks, random partitioning of these stacks alone will suffice to ensure adequate segregation of Golgi stacks. However, the observation of Golgi disassembly in multicellular organisms which is so strikingly absent in unicellular higher eukaryotes suggests that disassembly might also fulfil the purpose of differentiation of the stack during the proliferative stage of cell development. If the cell were to make use of Golgi fragmentation to implement the fine tuning of the distribution of processing enzymes within different cisternae, then this might also account for the restoration of protein transport during reassembly, rather than after reassembly is complete. As well as the usual transport events occurring within this window, it is conceivable that there may also be other more "unusual" transport events allowing the shuffling of certain Golgi enzymes so that the next cell generation is slightly better adapted for the processing tasks it requires to carry out. By such a process of repeated adjustments of the enzymatic complement of each cisterna, by the time the cell is fully differentiated and in a quiescent G_0 state, its Golgi apparatus will be adjusted to the functional requirements of the specific tissue in which the cell now resides. Since plant and fungus cells must clearly differentiate, and yet do not undergo Golgi vesiculation during mitosis, other mechanisms must exist in this case should Golgi differentiation be required for refining Golgi function.

6.5 Future prospects

The interaction of protein transport and Golgi reassembly during telophase is a field of enquiry still riddled with unanswered questions. With respect to the regulation of protein secretion during mitosis, the variety of steps of transport

inhibited in different animal cells necessitates a series of mechanisms for regulating exocytic transport. These mechanisms must therefore be extremely specific, and will provide many challenges for future years of investigation.

The establishment of this assay system in semi-permeable cells is an additional challenge worth pursuing. Intracellular protein transport has been reconstituted in permeabilised cells in a number of laboratories with the aims of investigating a series of different stages of transport, including ER to Golgi transport (Davidson and Balch, 1993) and Golgi to cell surface transport (de Curtis and Simons, 1989). These systems are proving to be of undoubted value in the molecular dissection of intracellular transport. Reconstitution of the mitotic assay system employed in these present experiments could also prove of immense value in the identification of crucial factors responsible for both the resumption of protein transport and the reassembly of the Golgi apparatus. Once these factors have been identified, immunological techniques could be employed to undertake specific immunodepletion experiments to verify their participation in these changes.

In addition, *in vitro* reconstitution of the study of post-translational modification of resident Golgi proteins would be a richly profitable field of exploration, both in terms of defining more clearly the precise nature of these modifications and also in establishing the molecules involved in the catalysis of these mitotically-induced changes.

APPENDIX A: INVESTIGATION OF CELL-CYCLE DEPENDENT POST-TRANSLATIONAL MODIFICATION OF RESIDENT GOLGI PROTEINS

A.1 Overview

The work described in the main body of the thesis focused on the analysis of intracellular protein transport in mitotic and G1 cells, and relating the kinetics of such transport to the reassembly of the Golgi apparatus during telophase. These experiments have each relied on the combined use of thymidine and nocodazole to generate a synchronous population of HeLa cells, arrested in prometaphase. This methodology of synchronising a population of HeLa cells is extremely useful in the investigation of cell-cycle dependent aspects of cell physiology in general, and in the comparison of various intracellular processes during M phase and G1 phase in particular. Further to the previous exploration of protein transport and Golgi reassembly, it was of interest to use this same system of synchronising cells to investigate the nature of the post-translational modification of resident Golgi proteins during mitosis. This Appendix describes the preliminary work undertaken in addressing this question.

A.2 Relaxation of intracisternal protein interactions within the Golgi apparatus during M phase

The result of the mitotic disassembly of the Golgi apparatus is the dispersal of Golgi derived vesicles throughout the anaphase cytoplasm. Such vesiculation of the organelle clearly requires relaxation of the mechanism responsible for keeping resident Golgi proteins out of membrane regions from which vesicles are budding.

Although the basis for the exclusion of resident proteins from dilated rims during interphase is not fully understood, there is increasing evidence for a "kin recognition" model, in which lateral interaction of resident Golgi proteins including processing enzymes both serves the purpose of retaining these proteins in the correct compartment and also precludes them from entering the dilated rims of each cisterna (Nilsson, *et al.*, 1993b). A prediction of this model is the existence of linear matrices or "rafts" of resident Golgi proteins, embedded in the central portion of each cisternal membrane.

The specific aim of the work described here was to look for evidence of post-translational modification of resident Golgi enzymes during M phase which might play a role in the relaxation of protein-protein interactions at the onset of mitosis. Since phosphorylation presented itself as an obvious choice of mitosis-specific modification, the methodological approach adopted was the detection of charge

shifts of proteins as they move from a non-phosphorylated state to a phosphorylated one, or indeed from a phosphorylated state to a hyperphosphorylated state.

Two resident Golgi enzymes, namely N-acetylglucosaminyltransferase I (NAGT I) and galactosyltransferase (GT), were studied in these experiments due to the ease of identifying these two enzymes by immunological techniques. A previously generated HeLa cell line (termed 4/12) in which NAGT I had been tagged with a myc epitope facilitated detection of the first enzyme by blotting with the anti-myc monoclonal antibody 9E10 (Nilsson *et al.*, 1993a). GT was detected using a polyclonal antibody N11.

Preliminary investigations with N11 antibody showed that GT could be easily detected on Western blots of both SDS-PAGE and IEF gels. However, no significant differences could be discerned between mitotic, G1 or interphase samples analysed either by IEF or two-dimensional gel electrophoresis (data not shown). Further experimentation was therefore limited to the consideration of NAGT I.

A.3 Characterising antibody detection of NAGT I with 9E10 antibody

In order to demonstrate NAGT I staining, Golgi membranes were fractionated from non-synchronised 4/12 HeLa cells, solubilised and separated by SDS-PAGE, and the gel then Western blotted and probed with 9E10 antibody. Figure A.1 shows the resulting staining pattern. A major protein band is detected in Golgi membranes from the 4/12 cell line (lane b) which is absent from a lysate from wild-type HeLa cells (lane a). The identity of the minor band of lower molecular weight (lane b) is not known, but its appearance presumably results form non-specific binding of the 9E10 antibody. Since this minor band can also be seen in suspension HeLa lysate (lane a) it cannot represent a proteolytically clipped form of NAGT I still bearing the myc epitope.

A.4 Analysis of NAGT I in synchronised cells

Having shown the specificity of staining achieved with the 9E10 antibody, it was of interest to look for changes in this pattern as cells progressed through M phase. 4/12 HeLa cells were therefore synchronised with thymidine and nocodazole as in the experiments described in Chapters 3 to 5. Prometaphase-arrested cells were harvested, released from the nocodazole block and were allowed to progress through to G1 phase. Samples were taken at hourly intervals following the nocodazole washout at zero time, lysed in TX-114 and the protein from the detergent phase chloroform-methanol precipitated. Despite numerous attempts,

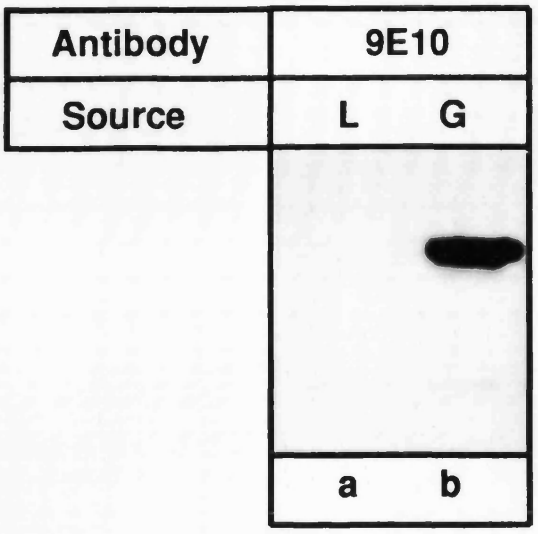

Figure A.1 Characterisation of 9E10 antibody for Western blot detection of myc-tagged NAGT I. The detergent phase of a TX-114 lysate of 4/12 HeLa cells (L) or Golgi membranes from wild type HeLa cells (G) were fractionated by SDS-PAGE and Western blotted.

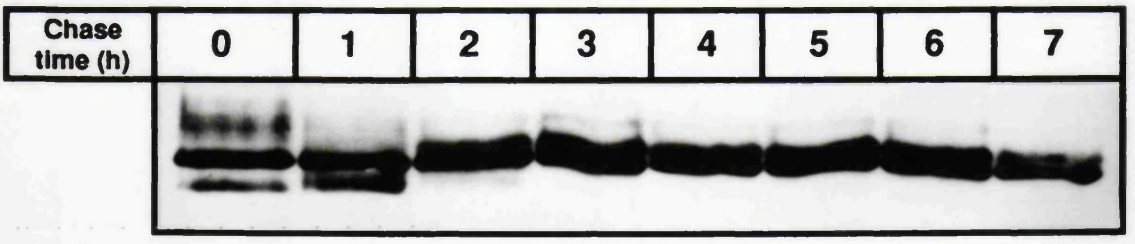

Figure A.2 Analysis of NAGT I during progression from mitosis into G1 phase. HeLa (4/12) cells were synchronized in prometaphase with nocodazole, released from this mitotic arrest at zero time and allowed to progress through M phase into G1 phase. Samples were lysed in TX-114 hourly, the detergent phase separated by SDS-PAGE, western blotted and probed with 9E10 antibody to detect NAGT I. A distinct band shift occurs between zero time and the 2 hour time point by which time the cells have entered G1 phase.

no staining of NAGT I on Western-blotted IEF or two-dimensional gels was achieved. Analysis of the 4/12 lysates was therefore undertaken by one-dimensional SDS-PAGE. The results from this experiment are shown in Figure A.2 and demonstrate a clear shift in the pattern of bands with time. In particular, the shift of the minor form to an apparently higher molecular weight form from the 1 h to the 2 h time points is clearly suggestive of a progression in protein modification. Since charge changes rather than molecular weight changes can account for some shifts in protein mobility on SDS-PAGE gels, this shift could indicate post-translational modification of NAGT I during M phase.

It is worth noting in passing that care must be taken in the interpretation of cell-cycle dependent patterns observed with immunological reagents, particularly in the case of Western blotting and probing with antibodies. This is because certain epitopes can show cell-cycle dependent structural alterations so that antibody binding becomes cell-cycle dependent accordingly. Such alterations can be misleading and suggest protein modifications other than changing epitope motifs.

The limitations of time precluded the repetition of this experiment to show the reproducibility of these band shifts or to extend these studies further. However, this intriguing result at least paved the way for a more detailed study of post-translational modifications of NAGT I during M phase.

The investigation of such post-translational modifications *in vitro* is also likely to be a fruitful avenue to pursue. With regard to mitotic-dependent phosphorylation, incubation of purified Golgi membranes with radioactively labelled phosphate in the presence of either mitotic or interphase cytosol would be another avenue worth exploring. The combination of *in vivo* and *in vitro* experimentation should allow a detailed analysis of the role of post-translational modification in general and phosphorylation in particular in the relaxation of resident protein interactions during Golgi disassembly at the onset of M phase.

APPENDIX B: KINETICS OF HLA TRANSPORT IN TRANSFECTED HeLa CELLS WITH DISTURBED GOLGI MORPHOLOGY

B.1 Overview

The investigation of Golgi retention signals and mechanisms by Tommy Nilsson and co-workers in our laboratory has led to the generation of a series of stable HeLa transfectants in which certain resident enzymes of the Golgi apparatus have been modified in some way. A number of these transfectants also manifest altered Golgi morphology as a result of the changes which have been made in these Golgi enzymes. It was therefore intriguing to determine if the transport of proteins along the secretory pathway in these cell lines was altered as a result. The system developed in the course of the thesis work described here lent itself to analysis of transport in these transfected cell lines and the results from these experiments are reviewed briefly in this second Appendix.

B.2 Description of cell lines

The first stably transfected HeLa cell line to be investigated was the "10:40" transfectant which expresses murine mannosidase II (mann II), a *medial* Golgi enzyme, genetically manipulated so that its lumenal domain is replaced with the lumenal domain of the human invariant chain (p31), a component of the MHC Class II complex. Indirect immunofluorescence shows that the localisation of the resulting hybrid molecule is not affected. However, the overall morphology of the Golgi is affected. In particular, there is a disruption of the stack and a proliferation in the number of Golgi coated vesicles, similar to the effect of treating cells with GTPγS or AlF (Melançon, *et al.*, 1987).

The second transfectant studied was the "448" cell line in which the cytoplasmic domain of NAGT I has been replaced with the cytoplasmic domain of the invariant chain Iip33, a protein retained in the ER. The result of this transfection is the relocalisation of about 80% of the total NAGT I activity to the ER. Furthermore, about 70% of the endogenous mannosidase II and 25% of the endogenous galactosyltransferase activities are also translocated to the ER providing evidence for the kin-recognition model for retention of resident enzymes within the correct cisternae of the Golgi apparatus (Nilsson *et al.*, 1993b). As well as the profound redistribution of Golgi enzymes in this cell line, the Golgi cisternae are seen to be dilated under the EM, and there is an associated increase in tubulo-vesicular profiles.

B.3 Kinetics of HLA transport in the stable transfectants

The question of the kinetics and sequence of glycosylation of transported proteins in these two cell lines was clearly an important one because of the striking alteration in Golgi morphology. HLA was again used to monitor the glycosylation along the secretory pathway and the methodology of these experiments followed the regime adopted in Chapter 3 for measuring the transport of HLA in G1 HeLa cells. In the case of the 10:40 cell line, the cells were pulsed and chased as a monolayer population and not as a suspension culture. Lysis, immunoprecipitation, enzymatic digestion, analysis and quantitation of fluorographs were otherwise identical.

B.3.1 HLA transport in the 10:40 cell line

Figure B.1 shows the profiles representing transfer of HLA to the *medial* (endo H-resistance) and *trans* (sialylation) Golgi in the 10:40 cell line (dark symbols) relative to wild type G1 cells (open symbols). The profiles indicate slightly faster transport in the 10:40 cell line (half-time for arrival at the *medial* Golgi of 17 min rather than 24 min). This difference could be partly due to the fact that the pulse chase was carried out on monolayers where quenching transport processes on ice may be less rapid; the G1 data refer to suspension cultures which probably take a correspondingly shorter period to reach 4°C, thereby suggesting slower rates of transfer. However, given the increased number of Golgi coated vesicles, the data could also indicate a faster rate of transport due to more budding events occurring in this cell line. The reason for the proliferation of coated vesicles is not known. One possibility is the perturbation of the oligomeric protein rafts, suggested by Nilsson *et al.*, (1993b) as the basis of Golgi retention. Since the invariant chain is known to trimerise in the ER whereas Golgi enzymes usually interact as dimers, the hybrid molecule may be less able to interact with other *medial* enzymes thereby precluding the formation of tightly bound rafts which might normally specify the regions of cisternal membrane from which budding may occur. Fragmentation of these rafts might mean that more cisternal membrane is available for the nucleation of coated buds and hence increase the number of coated vesicles. Furthermore, dispersal of the enzyme oligomers might also have the effect of increasing the amount of illicit Golgi enzyme entry into transport vesicles thereby necessitating recycling events to return enzymes to their proper location. Such recycling would in turn necessitate more budding events in order to maintain the rate of secretion of transported proteins, and therefore explain the rise in the number of coated vesicles seen near the Golgi stack under the EM.

Whatever the underlying mechanism, the data indicate that the altered morphology of the Golgi apparatus does not hinder transport, confirming the

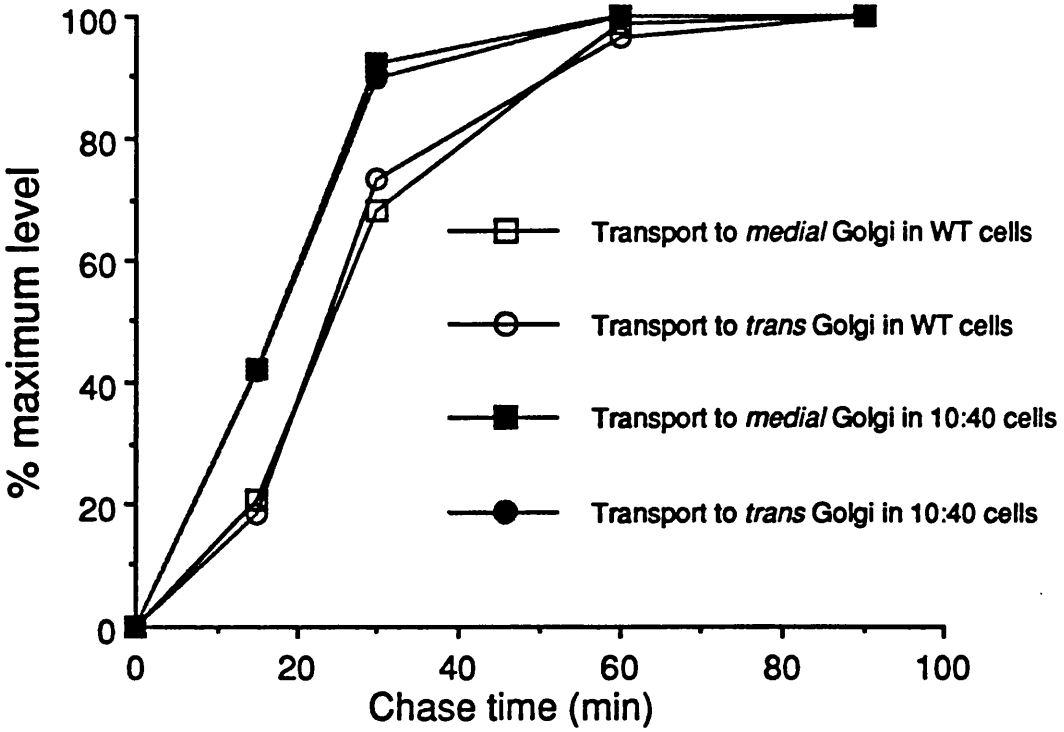

Figure B.1 Comparison of transport kinetics in 10:40 HeLa cells relative to wild type (WT) HeLa cells. Transport from the ER to the *medial* and *trans* Golgi was assayed by acquisition of endo H-resistance and sialylation respectively.

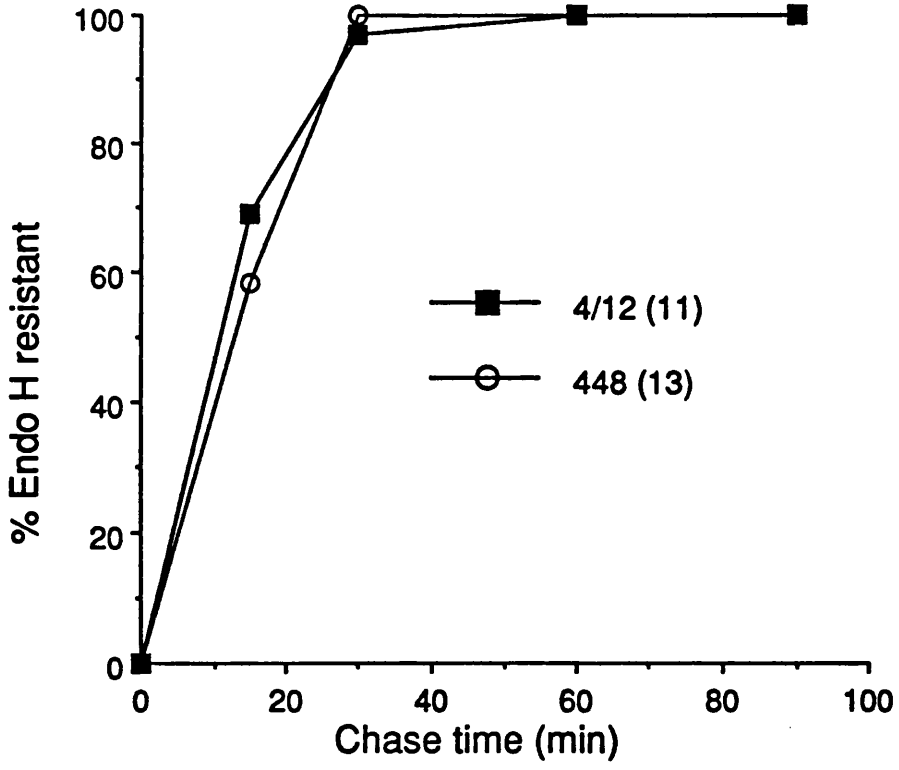

Figure B.2 Comparison of HLA transport from the ER to the *medial* Golgi in 448 cells and 4/12 cells. The half-times are given in brackets.

dynamic nature of the organelle. The overall morphology can clearly become much less tightly organised without adversely affecting the rate of protein transport through the organelle. This observation is consistent with what is known from *in vitro* reconstitution of intra-Golgi transport in which stacking of cisternae is much less rigidly preserved than *in vivo*. Moreover infection with Uukuniemi virus disrupts the stack but not protein transport through the apparatus (Gahmberg, *et al.*, 1986; Gahmberg and Peltonen, 1987). The physiological reason for the stacking of the cisternae has yet to be fully established.

B.3.2 HLA transport in the 448 cell line

The p33-NAGT I hybrid molecule transfected in the 448 cell line had been tagged with a myc epitope for ease of immunological analysis with 9E10 antibody. The most appropriate control for analysis of HLA transport in this transfectant was therefore another transfectant (the 4/12 cell line) in which NAGT I is also myc-tagged and yet is maintained in its correct location in the Golgi apparatus. Both these cell lines were pulse chased in suspension in parallel so that any differences in the form or rate of HLA glycosylation could be attributed to the anomalous expression of NAGT I in the ER rather than any methodological disparity.

Profiles representing acquisition of endo H-resistance for the two cell lines are shown in Figure B.2. The results show that there is no significant difference in the rate of glycosylation and therefore indicate that processing of HLA to an endo H-resistant form in the 448 cell line was not occurring prematurely in the ER. The most likely implication of these data is that HLA must first be modified by *cis* Golgi enzymes before it can be modified by *medial* Golgi enzymes, confirming the sequence-specific fidelity of oligosaccharide processing. Thus, even if the *medial* enzymes have been translocated to the ER, the form and rate of glycosylation of transported proteins will remain unchanged if *cis* enzymes such as mannosidase I remain in their correct location. Without any retrograde recycling of transported protein from the *cis* Golgi to the ER to gain endo H-resistance through mannosidase II processing, the second implication is that the 20 or 30% of the NAGT I and mannosidase II respectively which remain in the *medial* Golgi cisterna must be sufficient to process the N-linked oligosaccharides present on the HLA transported through the organelle.

Figures B.3 and B.4 reveal that HLA sialylation and transfer to the cell surface proceeded with very similar kinetics in these two cell lines. The glycosylation patterns were similarly preserved indicating that the form of oligosaccharide processing was unaffected. Taken together, these data therefore indicate that the

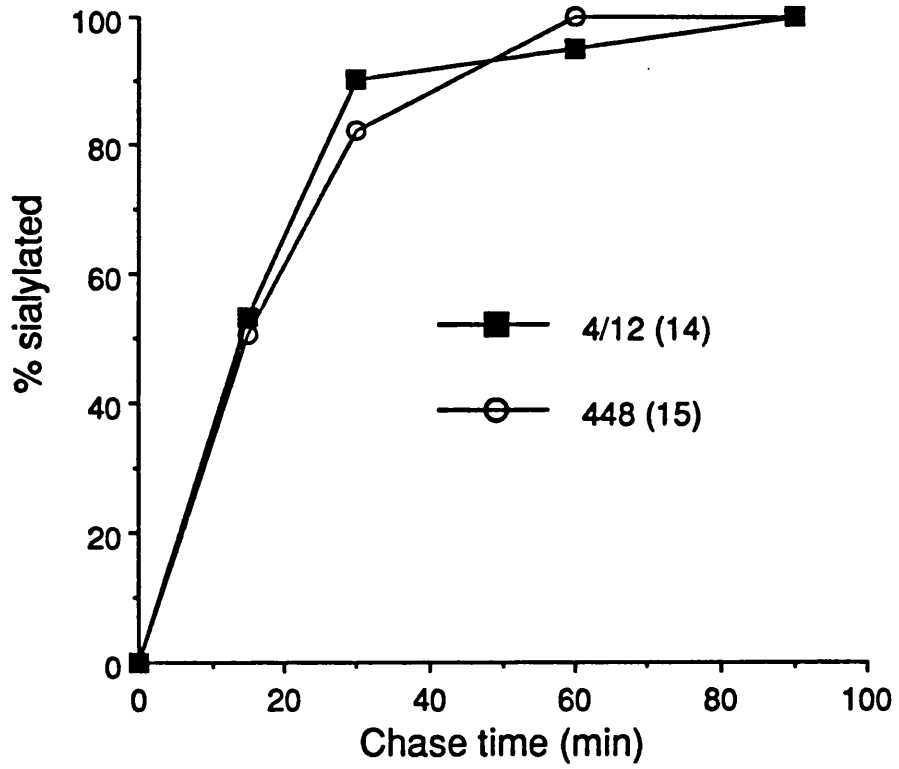

Figure B.3 Comparison of HLA transport from the ER to the *trans* Golgi in 448 cells and 4/12 cells. The half-times are given in brackets.

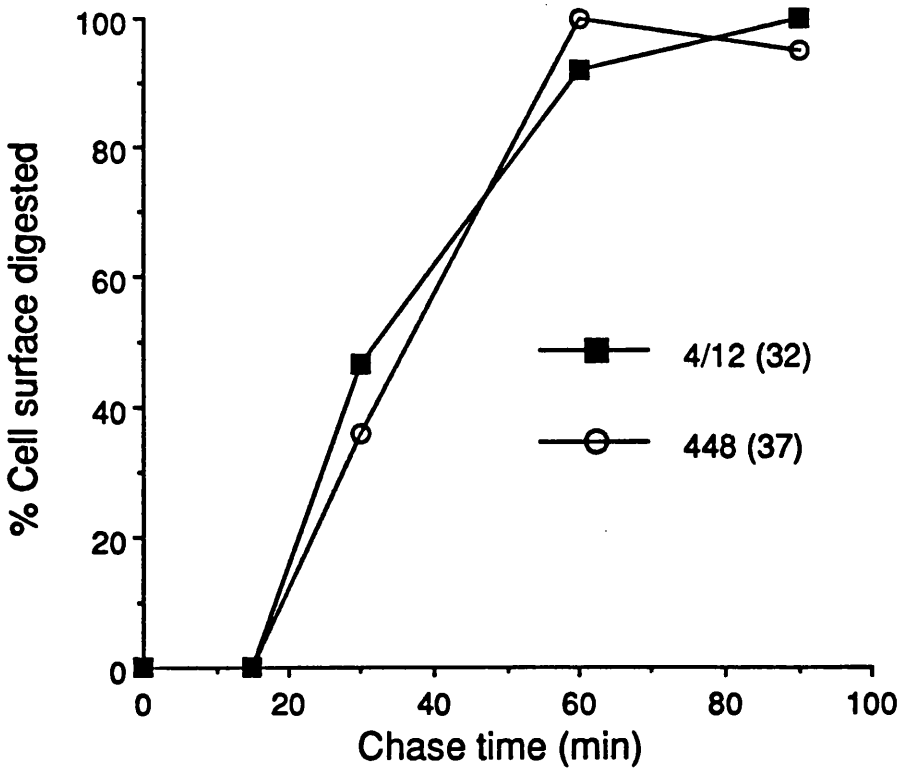

Figure B.4 Comparison of HLA transport from the ER to the cell surface in 448 cells and 4/12 cells. The half-times are given in brackets.

redistribution of Golgi enzymes in the 448 cell line does not adversely affect the processing or rate of transport of molecules along the secretory pathway.

The half-times for acquisition of resistance to endo H and sialylation are considerably shorter for these two cell lines than for wild type interphase cells. This more rapid rate of transport in the early part of the secretory pathway may be connected to the faster rate of cell growth seen in these transfectants relative to wild-type HeLa cells (Tommy Nilsson, personal communication).

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