Isolation of an intercisternal matrix that binds *medial*-Golgi enzymes

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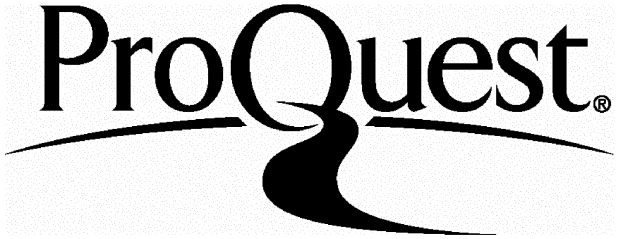

Abstract

Purified rat-liver Golgi stacks were extracted in a buffer containing 2% (w/v) TX-100, 50mM MOPS pH7.0, 0.1mM $MgCl_2$, 1mM DTT and 10% (w/v) sucrose, and centrifuged to produce an insoluble pellet which contained the majority of three *medial*-Golgi enzymes, mannosidase II and N-acetylglucosaminyltransferases I and II. Proteins from other regions of the Golgi stack were mostly solubilised. A further extraction of this pellet in the TX-100 buffer containing 150mM NaCl led to complete solubilisation of these *medial*-Golgi enzymes.

After the salt extraction, a second insoluble pellet was produced which was termed the Golgi matrix. The salt-solubilised *medial*-enzymes could rebind the matrix upon dialysis, while an enzyme from the *trans*-Golgi could not. Scatchard analysis revealed that rebinding was saturable and occurred with a high affinity, suggesting that the matrix contained a fixed number of receptors which specifically bound the *medial*-enzymes. This suggested that the enzyme insolubility in detergent was due to their interaction with the matrix.

Digestion of intact Golgi membranes with proteinase K greatly increased the detergent-solubility to the *medial*-enzymes, suggesting that components of the matrix were present on the cytoplasmic, intercisternal face of the Golgi membranes. This topological orientation suggested that the matrix might play a role in stacking the Golgi cisternae. Binding of the enzymes did not, however, occur via their cytoplasmic or membrane-spanning domains, suggesting that the matrix is a complex structure, containing components on both sides of the Golgi membrane.

Because of its topology and its binding capacity for *medial*-enzymes, the matrix may function in aiding the retention of Golgi proteins, maintaining the flattened cisternal morphology or in connecting adjacent cisternae to form the characteristic Golgi stack.

To Those I Love:

Mother, Peter,
Tracy and Bill

Lives of great men are reminders,
We may make our lives sublime,
And in parting leave behind us,
Footprints in the sands of time.

- H.W. Longfellow

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Abbreviations

ARF	ADP-Ribosylation Factor
BiP	Immunoglobulin Heavy Chain Binding Protein
BFA	Brefeldin A
CAPS	3-[Cyclohexylamino]-1-propanesulphonic acid
CGN	Cis Golgi Network
CHO	Chinese Hamster Ovary
CoA	Coenzyme A
ConA	Concanavalin A
COP	Coat Protein
CT-Mann II	Chymotryptic Mann II fragment
DMEM	Dulbecco's Modified Eagle Medium
dMM	1,5-dideoxy-1,5-imino-D-mannitol; Deoxymannojirimycin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
FFS	Fast Flow SP Sepharose
FucT	α1,6-Fucosyltransferase
G-418	Geneticin sulphate
Gal	Galactose
GalT	β1,4-Galactosyltransferase
GlcNAc	N-Acetylglucosamine
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
HDEL	His-Asp-Glu-Leu
HEPES	N-[Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IP	Immunoprecipitation; Immunoprecipitate
KDEL	Lys-Asp-Glu-Leu
Man	Mannose
Mann I	α1,2-Mannosidase I
Mann II	α1,3-1,6-Mannosidase II
MES	2-[N-Morpholino] ethanesulphonic acid
MII-C	Mann II cytoplasmic tail peptide

MII-CT	Mann II cytoplasmic/partial membrane-spanning domain peptide
MII-SCR	Scrambled Mann II cytoplasmic tail peptide
MMP	Methylmannopyranoside
MOPS	3-[N-Morpholino] propanesulphonic acid
MTOC	Microtubule Organising Centre
NA-C	NAGT I cytoplasmic tail peptide
NA-SCR	Scrambled NAGT I cytoplasmic tail peptide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAG	p-Nitrophenyl-N-acetyl-β-D-glucosaminidine
NAGT I	β1,2-N-acetylglucosaminyltransferase I
NAGT II	β1,2-N-acetylglucosaminyltransferase II
NANA	N-acetylneuraminic acid; Sialic acid
NEM	N-ethylmaleimide
NRK	Normal Rat Kidney
NSF	NEM-Sensitive Fusion Protein
OD	Optical Density
PAS	Protein A-Sepharose
PBS	Phosphate Buffered Saline
PIC	Protease Inhibitor Cocktail
PIPES	Piperazine-N-N'-bis [2-ethanesulphonic acid]
PMSF	Phenylmethylsulphonyl fluoride
PNM	p-Nitrophenol-α-D-mannopyranoside
PNS	Post Nuclear Supernatant
PTA	Phosphotungstic acid; Dodecatungstophosphoric acid
PVDF	Polyvinylidine difluoride
RNA	Ribonucleic acid
SNAP	Soluble NSF Attachment Protein
TCA	Trichloroacetic acid
TEA	Triethanolamine
TPPase	Thiamine pyrophosphatase
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SialylT	α2,6-Sialyltransferase
SPITC	4-Sulphophenyl isothiocyanate
SRP	Signal Recognition Particle
TGN	Trans Golgi Network

Tween 20	Polyoxyethylenesorbitan monolaurate
TX-100	Triton X-100
TX-114	Triton X-114
WGA	Wheat Germ Agglutinin
VSV	Vesicular Stomatitis Virus

Buffer Acronyms

AMC	50mM Sodium acetate pH 5.5, 2mM $MnCl_2$, 2mM $CaCl_2$
FFS (10x)	200mM Tris pH7.5, 1.0% (w/v) TX-100
HMS	50mM HEPES pH8.0, 0.1mM $MgCl_2$, 10% (w/v) sucrose
IP Lysis (2x)	40mM Tris pH8.0, 300mM NaCl, 1.0% (w/v) TX-100
IP Wash	20mM Tris pH8.0, 300mM NaCl, 0.1% (w/v) TX-100
KEHM	50mM KCl, 10mM EGTA, 50mM HEPES pH7.4, 1.92mM $MgCl_2$
MMS	50mM MOPS pH7.0, 0.1mM $MgCl_2$, 10% (w/v) sucrose
PBS	10mM sodium phosphate pH7.2, 150mM NaCl, 3mM KCl
PM	0.5M potassium phosphate pH6.7
SDS-PAGE Electrode	50mM Tris, 380mM glycine, 0.1% (w/v) SDS
SDS-PAGE Loading	200mM Tris pH8.8, 30% (w/v) sucrose, 5mM EDTA, 0.004% (w/v) bromophenol blue
TG	0.2M Tris, 1.5M glycine
TMC	20mM Tris pH 7.4, 2mM $MnCl_2$, 2mM $CaCl_2$
TMG	20mM Tris pH 7.4, 2mM $MnCl_2$, 2mM $CaCl_2$, 250mM MMP, 250mM GlcNAc
TMMDS	2% (w/v) TX-100, 50mM MOPS pH7.0, 0.1mM $MgCl_2$, 1mM DTT, 10% (w/v) sucrose
TMMS	2% (w/v) TX-100, 50mM MOPS pH7.0, 0.1mM $MgCl_2$, 10% (w/v) sucrose
TTMDS	0.25% (w/v) TX-100, 10mM TEA pH7.0, 0.1mM $MgCl_2$, 1mM DTT, 10% (w/v) sucrose

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Publications

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

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Nilsson, T., P. Slusarewicz, M.H. Hoe and G. Warren. 1993. Kin Recognition. A model for the retention of Golgi enzymes. *FEBS Lett.* **330:** 1-4.

Nilsson, T., M.H. Hoe, P. Slusarewicz, C. Rabouille, R. Watson, F. Hunte, G. Watzele, E.G. Berger and G. Warren. 1994. Kin recognition between *medial* Golgi enzymes in HeLa cells. *EMBO J.* **13:** 562-574.

Nilsson, T., M. Pypaert, M.H. Hoe, P. Slusarewicz, E. Berger and G. Warren. 1993. Overlapping Distribution of Two Glycosyltransferases in the Golgi Apparatus of Hela Cells. *J. Cell Biol.* **120:** 5-13.

Chapter 1

Introduction

1.1 Overview

The Golgi apparatus plays a crucial role in the eukaryotic exocytic pathway. Its major known functions are the post-translational modification of proteins in transit through the secretory pathway and the targeting of these molecules to their final subcellular destinations. Structurally, the Golgi apparatus is visualised by electron microscopy as consisting of a series of flattened cisternae which are closely apposed to each other to form a stack. Apposed to the stack are two tubulo-vesicular structures, the *cis*- and *trans*-Golgi networks which respectively receive and target newly synthesised proteins.

In this introduction, I will attempt to describe what is known about some of the molecular processes which occur in this organelle, and its unique architecture. I shall begin by giving a brief overview of the secretory pathway and of the way in which proteins are transported through it by discussing the major secretory organelles in turn from the site of protein synthesis in the ER to their final targeting in the *trans*-Golgi network. Additionally, there will be a discussion of the mechanisms by which proteins, whose functions are carried out in the organelles of this pathway, are selectively retained against the forward flow of membrane.

I shall then go on to describe the current state of knowledge regarding the molecular mechanisms which underlie the process of vesicular-mediated transport through the Golgi apparatus, the method by which newly-synthesised proteins pass from one compartment to the next, and the way in which compartmental identity is maintained against the continual exchange of membrane between different organelles. An elucidation of these fundamental mechanisms is crucial if we are to obtain a full understanding of the functions of the Golgi apparatus.

Having established this framework, I will describe the morphology of the Golgi apparatus, and in particular, discuss what little is known about the mechanisms which maintain the characteristic stacked structure of this organelle. Additionally, I shall describe in detail the best-characterised post-translational modifications which occur in the Golgi apparatus, those of N-linked glycosylation. Furthermore, a review will be presented of what is currently known about the sub-compartmentalisation which occurs within stack, particularly in the process of N-glycan processing, and assess the biochemical and immuno-electron microscopic data which have contributed to our understanding of this phenomenon.

In conclusion I shall describe the aims of this project and explain the approach taken in attempting to isolate a matrix which might be involved in maintaining the most characteristic feature of this organelle, the Golgi stack. Identification of such a matrix

would for the first time allow the design of experiments aimed at the elucidation of the mechanisms involved in the production of the Golgi stack and its function.

1.2 The Secretory Pathway

The secretory pathway is the term used to describe the intracellular route taken by newly-synthesised proteins from their site of production at the endoplasmic reticulum (ER), to their final subcellular destinations. The pathway was originally defined by Palade and co-workers (Palade, 1975), and it was postulated that the newly synthesised proteins are carried vectorially between discrete membrane-bound organelles in small vesicles which bud from the first compartment and fuse with the next. The route taken by these vesicles is depicted schematically in figure 1.1.

After synthesis the proteins move to the Golgi complex via an intermediate compartment, termed the *cis*-Golgi network or CGN (Hauri and Schweizer, 1992). The proteins then pass vectorially through the cisternae of the stack by successive events of vesicular budding and fusion (Pfeffer and Rothman, 1987), before emerging in a compartment named the *trans*-Golgi network (TGN). From here, proteins are targeted to their final cellular destinations, such as the cell surface and endosomes/lysosomes (Griffiths and Simons, 1986). In specialised cells proteins are also targeted to other organelles, such as synaptic vesicles and secretory storage granules. The functions of these various organelles during protein synthesis and secretion are described individually below.

1.2.1 The Endoplasmic Reticulum

The ER is the major biosynthetic organelle of eukaryotic cells. Protein synthesis is initiated in the cell cytoplasm on free ribosomes. After the initiation of translation, a cytoplasmic, multi-subunit protein termed the Signal Recognition Particle (SRP) recognises and binds to a portion of the nascent polypeptide chain known as the signal sequence (see Walter and Lingappa (1986) for a review). There is no apparent consensus for the signal sequences of various proteins, however statistical analyses have shown that they generally consist of a hydrophobic domain flanked by a charged N-terminal and polar C-terminal region (von Heijne, 1985).

Once the SRP has bound, further translation is arrested until the ribosome binds to the ER membrane after which the SRP dissociates and translation resumes with the nascent peptide chain being co-translationally translocated into the ER lumen (see Simon (1993) for a review).

The ER provides a physiochemical environment conducive to the folding of the nascent chains into their final tertiary structures (Gaut and Hendershot, 1993) and also for

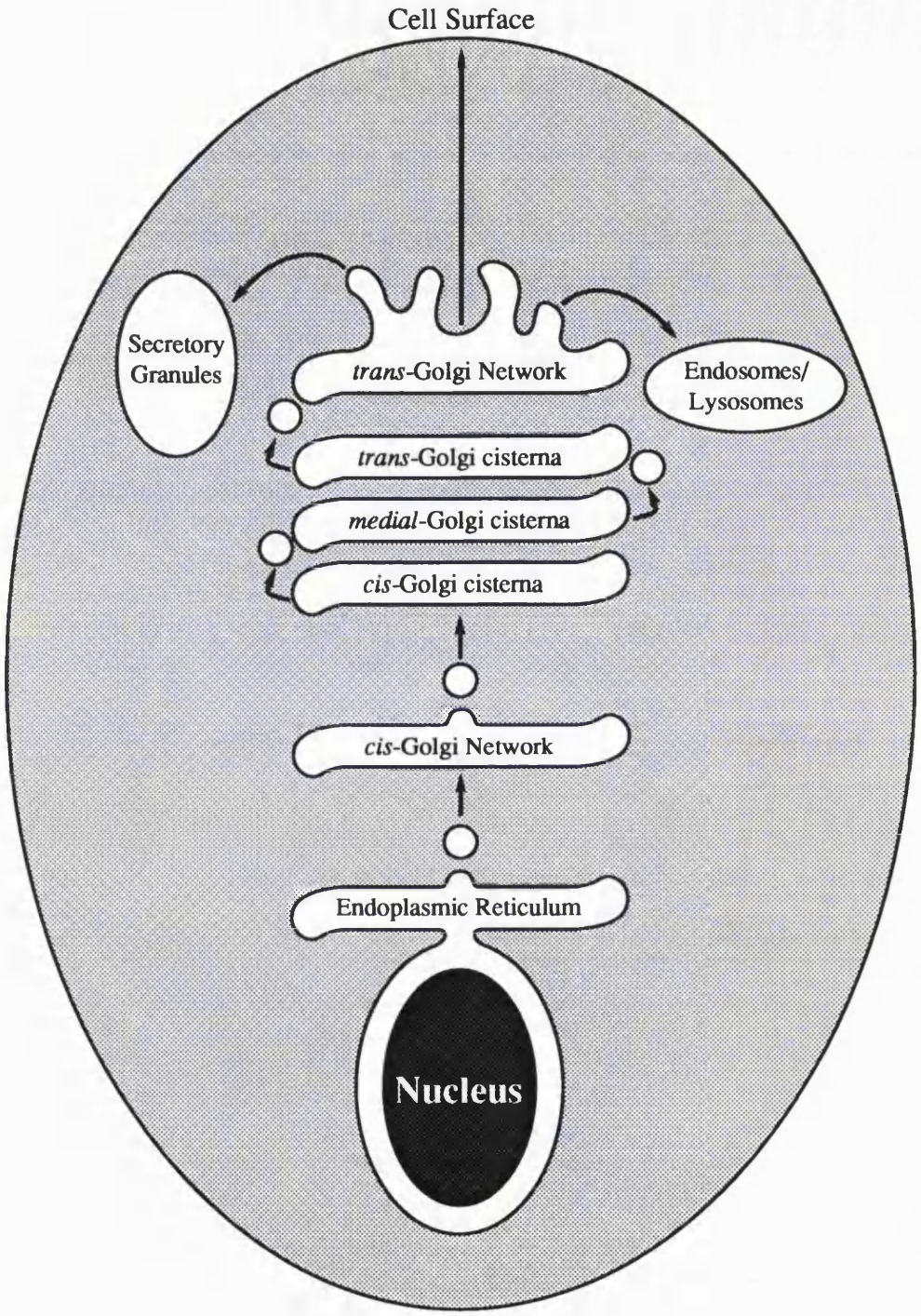

Figure 1.1: Schematic diagram of the secretory pathway. The diagram depicts the movement of vesicles from the ER, through the CGN and sequentially through the cisternae of the Golgi stack. After arrival at the TGN, proteins are transported to the cell surface by default, or targeted to organelles such as the endosome/lysosome system or to secretory granules in cells which exhibit regulated secretion.

the oligomerisation of proteins into their final quaternary forms (Hurtley and Helenius, 1989). Such folding and assembly is often mediated by resident ER proteins (see Pelham (1989) for a review). Correct protein folding and oligomerisation of newly synthesised proteins is crucial to allow them to exit the ER (Gething *et al.*, 1986; Kreis and Lodish, 1986). Misfolded proteins are retained either because they form large aggregates which cannot enter transport vesicles, or because they remain bound to proteins such as BiP which are residents of this organelle. Such proteins seldom reach the cell surface and are degraded intracellularly (Hurtley and Helenius, 1989; Pelham, 1989; Rose and Doms, 1988). Correctly-folded proteins are thought to exit the ER via specialised structures termed the transitional elements (Palade, 1975). From these, transport vesicles bud and move on to the next stage of the secretory pathway, the Golgi apparatus.

Transport of correctly-folded proteins is now widely thought to occur by default - the bulk flow hypothesis (see Pfeffer and Rothman (1987) for a review). Proteins are non-specifically incorporated into transport vesicles and then move forward through the pathway. Specific signals are required to divert them from the default destination of the plasma membrane, such as the mannose-6-phosphate signal present on the N-glycans of lysosomal enzymes (Dahms *et al.*, 1989), or to retain them at specific locations within the pathway (see later). Two lines of evidence favour this hypothesis.

Firstly, expression of the bacterial protein β-lactamase in *Xenopus* oocytes showed that this protein was efficiently secreted from the cell (Wiedmann *et al.*, 1984). Such a prokaryotic protein would not be expected to possess transport signals required for secretion in eukaryotic cells, and the fact that it is still secreted argues that such signals do not exist. Secondly, the rate of bulk flow has been measured for transport to the cell surface from both the ER (Wieland *et al.*, 1987), and the *cis*-side of the Golgi apparatus (Karrenbauer *et al.*, 1990), and the rate of secretion is equal to that of even the fastest secreted proteins. This indicates that the rate of bulk flow is fast enough to account for the secretion of all proteins.

An alternative view is that all proteins require specific signals to be incorporated into transport vesicles (Lodish *et al.*, 1983), since the secretion rates of different proteins have been observed to vary widely. If transport were occurring by bulk flow, all proteins would be expected to be secreted at the same rate. The differential rates of secretion of different proteins can be simply explained by differential rates of folding in, and therefore exit from, the ER. Evidence of positive protein sorting has, however, recently been presented where it has been shown that the VSV G protein is concentrated 5-10 fold upon exit from the ER (Balch *et al.*, 1994), though no further concentration is observed after entry to the Golgi. Such an increase in protein concentration after transport from the ER to the Golgi has been previously reported (Griffiths *et al.*, 1984; Quinn *et al.*, 1984) but

the mechanism by which this concentration occurs has not been identified. Thus bulk flow may not be the sole method by which proteins move along the secretory pathway, and some positive selection of secretory proteins may occur at their point of exit from the ER.

1.2.2 The *cis*-Golgi Network

Once the vesicles carrying the newly-synthesised proteins leave the ER, they fuse with the *cis*-Golgi network (CGN) en route to the Golgi stack. The CGN consists of a set of tubulo-vesicular structures located in between the transitional elements and the *cis*-face of the Golgi stack. The CGN has also been termed the intermediate compartment and salvage compartment, and is defined as containing a variety of marker proteins (p53, p58 and p63) to which antibodies have been raised (Hauri and Schweizer, 1992). It is best visualised when cells are incubated at 15°C. At this temperature delivery of proteins from the ER to the Golgi is inhibited, and the CGN expands. Secretory proteins accumulate in the CGN and colocalise with p58 (Saraste and Kuismanen, 1984; Saraste and Svensson, 1991). The CGN may act as a salvage compartment, responsible for the recycling of resident proteins that have escaped the ER (Hauri and Schweizer, 1992; Pelham, 1989; Warren, 1987).

1.2.3 The Golgi Stack

The Golgi stack is responsible for the post-translational modification of the proteins that pass through it. The modifications which occur are highly ordered and occur in a strict sequential manner. Vesicles fuse with the first (*cis*) cisternae and the proteins which they contain pass through the middle (*medial*) and last (*trans*) cisternae by successive steps of vesicular budding and fusion (see Pfeffer and Rothman (1987) for a review).

The enzyme compositions of these different cisternae vary, and this generally reflects the sequential nature of the post-translational modifications which occur in this organelle. These modifications include carbohydrate phosphorylation (Hasilik, 1980), sulphation (Niehrs and Huttner, 1990; Baeuerle and Huttner, 1987), acylation (Bonatti *et al.*, 1989) and the processing of O- and N-linked glycans (for reviews see Kornfeld and Kornfeld (1985) and Roth (1987)).

Since the Golgi stack is the subject of this thesis, its structure, sub-compartmentalisation and function are reviewed in greater detail later in this chapter.

1.2.4 The *trans*-Golgi Network

The *trans*-Golgi network (TGN) is a set of reticular membrane structures which are closely apposed to the *trans*-face of the Golgi apparatus. It contains significant amounts

of the *trans*-Golgi enzymes sialyltransferase (Roth *et al.*, 1985) and galactosyltransferase (Nilsson *et al.*, 1993a), as well as a protein of unknown function, TGN38, which is not present in the Golgi stack (Luzio *et al.*, 1990).

The TGN is responsible for the targeting of proteins to their final destinations (see Griffiths and Simons (1986) for a review). Vesicles budding from the TGN utilise clathrin coated vesicles, and it is these vesicles that are thought to be responsible for intracellular targeting to secretory granules and lysosomes (Pearse and Robinson, 1990).

1.2.5 Protein Localisation

With the constant flow of membrane and proteins through the secretory pathway, mechanisms must exist which allow proteins whose functions are required in the ER and Golgi to remain in their correct intracellular locations. Such mechanisms would presumably function by the recognition of specific signals on molecules which are to be retained within the secretory pathway. The search for such signals has been the subject of intensive investigation in the past few years and the results of such studies are discussed below.

The maintenance of proteins in their correct locations in the secretory pathway is currently thought to occur by a balance of two mechanisms, retention and retrieval. Protein are retained in the correct organelle by mechanisms which exclude them from anterograde transport vesicles. Such mechanisms fail occasionally, however, and proteins that have accidentally been lost from a compartment are retrieved by a secondary safety mechanism (see Nilsson and Warren (1994) for a review).

1.2.5.1 Retrieval Signals

Soluble mammalian ER proteins are localised to this organelle by a tetrapeptide motif at their C-termini (Munro and Pelham, 1987; Pelham, 1988), with the sequence Lys-Asp-Glu-Leu (KDEL). Deletion of this sequence causes loss of such proteins from the ER, albeit rather slowly. The KDEL sequence is recognised by a membrane-bound receptor (Lewis *et al.*, 1990) which has been identified in both *Saccharomyces cerevisiae* (Semenza *et al.*, 1990) and humans (Lewis and Pelham, 1992), though the yeast receptor recognises a slightly different sequence (HDEL) to the mammalian protein.

Because KDEL-tagged marker proteins obtain sugar modifications characteristic of the *cis*-Golgi, and because the KDEL-receptor has been localised to a post-ER compartment, it is thought the KDEL-receptor specifically binds KDEL sequences in a post-ER environment (probably the CGN) and returns them to the ER in retrograde transport vesicles (see Pelham (1989) and (1990) for reviews) i.e. KDEL constitutes a retrieval signal.

Type I integral-membrane residents of the ER are localised to this organelle by a signal in their cytoplasmic tails (Pääbo *et al.*, 1987; Nilsson *et al.*, 1989), and this signal consists of the C-terminal consensus sequence K(X)KXX (Jackson *et al.*, 1990). This motif is also a signal for protein retrieval since suitably tagged reporter molecules can still obtain post-ER modifications (Jackson *et al.*, 1993). Type II proteins, have similarly been shown to be localised to the ER by a cytoplasmic double-arginine motif (Schutze *et al.*, 1994).

Removal of cytoplasmic domains from ER resident proteins causes them to be secreted only slowly (Jackson *et al.*, 1993), in a manner analogous to removal of the KDEL sequence from soluble ER proteins (Munro and Pelham, 1987). This suggests that in addition to the retrieval signal, ER proteins are also retained, and that retrieval is a compensatory mechanism to recover proteins that have inadvertently been lost, though the mechanism of this retention has yet to be determined.

1.2.5.2 Retention Signals

All the Golgi enzymes that have been cloned to date are type II proteins, and can be retained by their membrane-spanning domains, and their flanking sequences (Burke *et al.*, 1992; Aoki *et al.*, 1992; Colley *et al.*, 1992; Russo *et al.*, 1992; Tang *et al.*, 1992; Teasdale *et al.*, 1992; Wong *et al.*, 1992; Munro, 1991; Nilsson *et al.*, 1991). Additionally, the first membrane-spanning domain of the M glycoprotein of avian coronavirus is also responsible for its retention in the *cis*-Golgi (Swift and Machamer, 1991). Membrane-spanning domains appear not only to confer Golgi localisation, but also do so to the correct region of the Golgi apparatus (Nilsson *et al.*, 1991; Burke *et al.*, 1992).

The effect of the membrane-spanning domain in retention also seems to be modulated by the flanking amino acids in the cytoplasmic and lumenal domains, though the nature of this modulation is far from clear. For example, efficient retention of galactosyltransferase requires both the cytoplasmic and lumenal domains of the enzyme. Removal of the cytoplasmic tail reduces retention efficiency but substitution of a cytoplasmic tail from a non-Golgi protein restores the retention efficiency (Nilsson *et al.*, 1991). The involvement of stalk regions in retention has been implicated in another experiment. A sialyltransferase molecule whose cytoplasmic and membrane-spanning domains have been removed by the introduction of a signal-peptidase cleavage site just after the membrane-spanning region is still retained in the Golgi (Colley *et al.*, 1992). Since the soluble catalytic domain of sialyltransferase lacking the stem region is secreted by cells, this implies that the stem region is involved in some aspect of retention. In another study, replacement of the membrane-spanning domain of sialyltransferase with

17 leucine residues did not qualitatively affect retention (Munro, 1991). However, increasing the length to 23 leucines abolished retention, suggesting that this may affect the position of the flanking amino acids on the cytoplasmic and lumenal sides relative to the lipid bilayer, and again implicating these regions in contributing to the retention phenomenon. Alternatively, this suggests that the length of the membrane spanning domain affects the enzyme's retention.

Two hypotheses have been put forward to explain the mechanism by which a membrane-spanning domain, embedding in a lipid bilayer, can function as a retention motif. In the first, Golgi enzymes are proposed to oligomerise via their membrane-spanning domains into structures which are too large to enter transport vesicles, thus preventing their loss from the cisterna(e) in which they reside (Machamer, 1991; Machamer, 1993; Nilsson *et al.*, 1993b). The second hypothesis invokes the observation that a cholesterol gradient exists across the Golgi stack, with a higher concentration occurring at the *trans* side relative to the *cis* (Orci *et al.*, 1981). This gradient has the effect of increasing the width of the lipid bilayers of membranes with higher cholesterol content and has led to the suggestion that early Golgi enzymes are excluded from compartments further upstream because their membrane-spanning domains are too short to allow their incorporation into thicker lipid bilayers to be thermodynamically favourable (Bretscher and Munro, 1993).

Of these hypotheses, that of protein oligomerisation has stronger experimental support. It has been suggested that residents of a cisterna can oligomerise with each other and that this oligomerisation could only occur between residents of the same cisterna, because premature oligomerisation of late Golgi enzymes in an earlier compartment would not allow them access to their final destination. This is supported by the fact that expression of the *medial*-Golgi enzyme N-acetylglucosaminyltransferase I (NAGT I) in the ER, by the addition of a cytoplasmic ER retrieval signal, can cause the redistribution of another *medial*-Golgi enzyme, mannosidase II (Mann II) but not the *trans*-Golgi enzyme galactosyltransferase (Nilsson *et al.*, 1994). This effect has been shown to be due to the membrane-spanning domain of NAGT I because an ER retained construct which carried the GalT membrane-spanning domain fused to the NAGT I lumenal domain failed to redistribute Mann II to the ER. This study did not directly demonstrate the existence of large oligomers, since interaction of Mann II and NAGT I could be interpreted as being the formation of a multi-enzyme complex, but suggested that their existence *in vivo* was feasible. Furthermore, cells which expressed high levels of the ER-form of NAGT I were shown to display an altered Golgi morphology. More specifically, the cisternal length appeared to decrease and there was an increase in the amount of tubular-vesicular profiles associated with the stack. This suggested that the presence of *medial*-enzymes within the

Golgi apparatus was crucial for maintaining Golgi structure. One possibility is that the enzymes may be anchored to a structural matrix which is responsible for maintaining cisternal shape. Loss of the enzymes could lead to the reduction of the Golgi membrane's ability to interact with such a matrix and thereby lead to a fragmentation of the cisternae.

Evidence for the formation of oligomers *in vivo* was obtained in another study. In this, the first membrane-spanning domain of the M glycoprotein of avian coronavirus was transplanted into the Vesicular Stomatitis Virus G protein (Weisz *et al.*, 1993). This molecule had been shown to be retained in the Golgi previously (Swift and Machamer, 1991). The later study demonstrated that this construct formed large oligomers *in vivo*, and that these oligomers only formed after exit from the ER. However, due to the non-physiological nature of this construct, the validity of these results could be questioned, though the data do show that oligomer formation mediated by the membrane-spanning domain can occur.

The main evidence in favour of the hypothesis of retention solely mediated by lipid bilayer width is based on the ability of a 17 amino acid poly-leucine sequence to retain sialyltransferase while a 23 amino acid sequence cannot (Munro, 1991). However, this hypothesis requires *medial*-Golgi enzymes to have shorter membrane-spanning domains than those of the *trans*-region in order to exclude them from the late cisternae of the stack, and this does not appear to hold true in all cases, for the enzymes that have to date been cloned (see Weinstein *et al.* (1987) and Kumar *et al.* (1990) for examples).

In conclusion, retention of Golgi enzymes is mediated by the membrane spanning domain, and the adjacent amino acids. The mechanism by which retention is achieved is still not clear and the hypotheses that have been presented are probably both over-simplistic. Since membrane-spanning domains are completely embedded in lipid bilayers, it seems unlikely that the surrounding lipids will exert no effect on the retention of the enzymes, though their being the sole effector seems unlikely given the data available to date. It is perhaps more likely that retention is mediated by protein oligomerisation and that this is modulated by the context of the lipid bilayer in which the enzymes are located. The over-simplicity of these models is highlighted by a recent study which has shown that although the membrane-spanning of NAGT I alone can confer Golgi retention, retention efficiency is increased by the presence of either the cytoplasmic or lumenal domains (Burke *et al.*, 1994). This suggests not only that these domains may also be involved in protein oligomerisation but also that interaction of these domains with cytoplasmic or lumenal structural elements of the Golgi cisternae may also contribute to protein localisation within the stack. Such interaction could also then contribute to stacking of the cisternae and/or maintenance of cisternal shape.

Although the retention motif of Golgi enzymes has now been identified, no direct evidence has been put forward to indicate that a recycling mechanism also exists for these proteins to retrieve molecules that have evaded the retention system. However, some indirect evidence that resident Golgi proteins do recycle does exist. A 160kD protein of unknown function named MG160 contains N-glycans which are sialylated, a modification of the *trans*-Golgi, while the molecule has been localised to the *medial*-Golgi cisternae by immunoelectron microscopy (Gonatas *et al.*, 1989; Croul *et al.*, 1990). This discrepancy in the protein localisation and its post-translational modifications suggests that the protein is able to reach the *trans*-cisternae and is retrieved to its normal location in the *medial*-Golgi (Johnston *et al.*, 1994). It is not yet known whether MG160 is retained in the Golgi by its membrane-spanning domain or whether it is maintained in the *medial*-Golgi solely by a recycling mechanism. Such studies await the isolation of the protein's cDNA, but these data do indicate that Golgi proteins could, in addition to their retention signals also possess retrieval motifs.

1.2.5.3 Retention and Retrieval Signals in a Single Protein

Although circumstantial evidence exists that soluble and membrane-spanning proteins of the ER and the membrane-spanning proteins of the Golgi stack posses both retention and recycling motifs, there are as yet no known examples where these signals have been identified in the same protein. A good example of how it is possible for such signals to exist in the same molecule, comes from studies on the intracellular distribution of a protein of the *trans*-Golgi network, TGN38.

TGN localisation has been shown to be conferred by a tetrapeptide motif, YQRL, on the TGN38 cytoplasmic tail (Bos *et al.*, 1993). This represents a retrieval signal because a reporter molecule bearing this tag is expressed at the cell surface at low levels and this cell surface population can re-enter the cell (Bos *et al.*, 1993). Recycling occurs via endosomes because it is sensitive to drugs which neutralise endosomal pH (Chapman and Munro, 1994). However, the membrane-spanning domain of this molecule can also confer TGN localisation (Ponnambalam *et al.*, 1994), demonstrating that TGN38 possesses two, non-overlapping localisation signals and suggesting that the membrane spanning domain confers retention, while the cytoplasmic tail is used for the retrieval of molecules that had inadvertently escaped to the cell surface.

As already stated, TGN38 is the only example of a protein to date in which both the retention and retrieval signals have been identified, and further work will determine whether this is a general case for the other molecules which reside in compartments within the secretory pathway.

1.3 The Mechanisms of Intra-Golgi Transport

It is widely accepted that transport of material through the secretory pathway is mediated by discrete carrier vesicles which bud from one compartment and fuse with the next to deliver their cargo of proteins and lipids. The use of discrete vesicles means that compartments need not fuse directly with each other while material is transferred between them, and thus maintains the high degree of ordered subcompartmentalisation within the cell. Other mechanisms for protein transport have been proposed such as transport via tubules which connect different organelles (Mellman and Simons, 1992), or by a process referred to as cisternal maturation (Lippencott-Schwartz, 1992; Brown and Romanovicz, 1976) where entire cisternae are proposed to move through the stack in a *cis* to *trans* direction. The evidence for such processes, however, is not as compelling as that for vesicular transport.

Vesicular transport was demonstrated to occur *in vivo* when it was shown that the secretory proteins within the Golgi stacks of two different cells could mix after the cells had been fused (Rothman *et al.*, 1984*b*), even though the stacks themselves remained distinct, as judged by immunofluorescence (Rothman *et al.*, 1984*a*). The simplest explanation for these results was that mixing had occurred by vesicular transport. The mechanisms by which these vesicles bud, are targeted to the correct compartment and then fuse have been the subject of intense study in the past few years.

The molecules involved in these processes have been identified by a mixture of biochemical and genetic approaches. The genetic approach has isolated many secretion deficient yeast mutants (*sec* mutants) and allowed the identification of proteins involved in these lesions and the points at which they act. Initially, this led to the identification of mutants which fell into 23 separate complementation groups (Novick *et al.*, 1980), indicating that many molecules were involved in the secretory process, as would be expected in such a complex process. Unfortunately, the lack until recently of a suitable *in vitro* system for the study of these proteins in yeast has not fully facilitated the elucidation of the molecular mechanisms of their action. The mutants were only characterised morphologically, and their lesions could be narrowed down to particular compartments and classed as mutations which caused vesicle accumulation or compartmental dilation (see Pryer *et al.* (1992) and Hicke and Scheckman (1990) for reviews).

This has not been a problem for the group of Rothman, which has taken a purely biochemical approach to studying this problem, utilising an *in vitro* assay which reconstitutes transport between the *cis*- and *medial*- Golgi (Balch *et al.*, 1984a; Balch *et al.*, 1984b). This has gone a long way to identifying and determining the functions of

many of the proteins involved in the transport process (see Rothman and Orci (1991) and Rothman and Orci (1992) for reviews). Some of the molecules that have been identified by this approach have been shown to be mammalian homologues of the proteins which are deficient in the *sec* mutants (see later), and this has strengthened the argument that these molecules play a true role in the transport process *in vivo*.

1.3.1 Vesicle Budding

Intra-Golgi transport occurs via vesicles which are covered by a non-clathrin-based coat (Orci *et al.*, 1986). Vesicle formation is dependant not only on the presence of cytosol ATP but also on acyl-Coenzyme A (acyl-CoA) (Pfanner *et al.*, 1989), and such vesicles accumulate *in vitro* in the presence of $AlF4^-$ or GTPγS, reagents which also block transport (Melançon *et al.*, 1987). The vesicles are quickly consumed upon removal of an $AlF4^-$ block, suggesting that they are viable transport intermediates (Orci *et al.*, 1989).

Analysis of the protein composition of the purified vesicles has led to the identification of the individual coat protein (Malhotra *et al.*, 1989; Serafini *et al.*, 1991b). These proteins have been termed α-, β-, γ- and δ-COP (Coat Protein). The cloning of β-COP showed that it displayed significant homology to β-adaptin (Duden *et al.*, 1991), a constituent of the coats of clathrin coated vesicles. β-COP exists in a membrane-bound form as well as a soluble cytosolic, hetero-oligomer (Duden *et al.*, 1991; Waters *et al.*, 1991), which contains α-, β-, γ- and δ-COP and two other low molecular-weight components, ε-COP (Hara-Kuge *et al.*, 1994) and ζ-COP (Kuge *et al.*, 1993). This complex constitutes the basic building block of the Golgi-vesicle coat, the coatomer ("coat promoter"). This role has been confirmed by immunodepletion of coatomer from cytosol, which inhibits the transport assay by preventing vesicle formation (Orci *et al.*, 1993b). Additionally, cloning of γ-COP has revealed it to be a homologue of the protein which is defective in the *sec21* yeast secretory mutant (Stenbeck *et al.*, 1992), thus confirming the role the coatomer in the transport process. One function of the COP-coat is thought to be the prevention of non-specific fusion of membranes (Elazar *et al.*, 1994) by obscuring components required for fusion which are present on the membranes themselves. Thus the coat uncouples vesicular budding and fusion by preventing membrane fusion until after a vesicle has pinched off.

Coatomer binds to Golgi membranes via ARF (ADP-ribosylation factor), a small monomeric GTP-binding protein (Kahn and Gilman, 1984), which is present in Golgi transport vesicles (Serafini *et al.*, 1991a). Upon exchange of GDP for GTP, cytosolic ARF binds to Golgi membranes and promotes the binding of coatomer to form the vesicle coat (Serafini *et al.*, 1991a; Palmer *et al.*, 1993; Orci *et al.*, 1993a). Hydrolysis of the GTP then causes uncoating of the vesicles (Tanigawa *et al.*, 1993), which is thought to

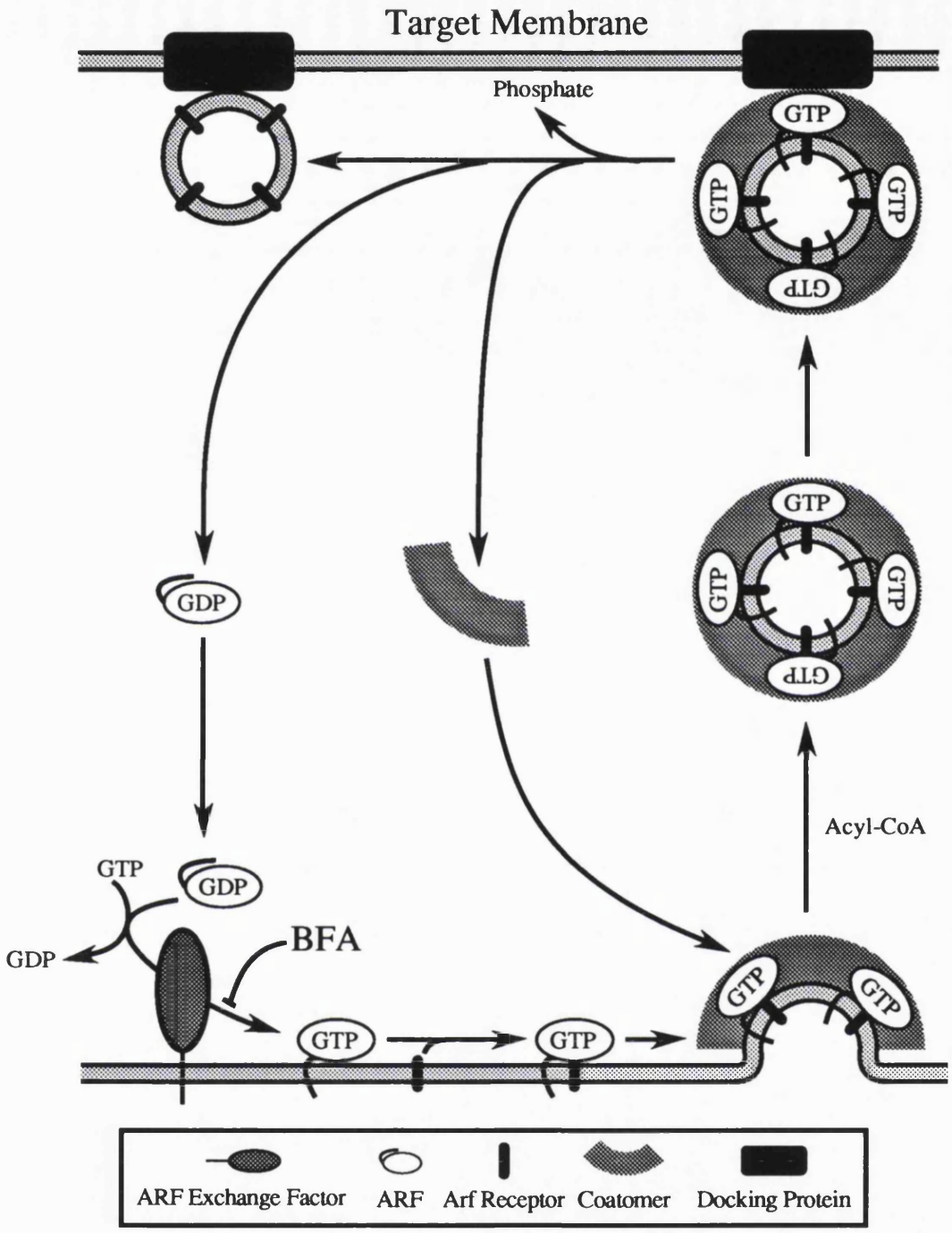

Figure 1.2: Schematic diagram of the mechanism of vesicle budding. ARF is inserted into the donor membrane after enzyme catalysed exchange of GDP for GTP. After meeting a membrane bound receptor, ARF promotes formation of the COP coat by recruiting coatomer from the cytosol. After pinching off, which requires only acyl-CoA, the vesicle docks with the target membrane via a specific receptor. Hydrolysis of GTP by ARF removes the coat and leaves a docked, uncoated vesicle which will go on to fuse. ARF and coatomer are recycled by diffusion through the cytoplasm.

occur after docking at the target membrane (Orci *et al.*, 1989). Exchange of GDP for GTP on ARF is catalysed by an, as yet, unidentified exchange factor on Golgi membranes (Donaldson *et al.*, 1992; Helms and Rothman, 1992), and it is this protein that is thought to be sensitive to Brefeldin A (see later). ARF binds to Golgi membranes in both a saturable and unsaturable manner (Helms *et al.*, 1993). Unsaturable binding of ARF represents binding directly to the membrane, while the saturable binding represents a pool of ARF which interacts with a specific membrane-bound receptor, and is responsible for coatomer recruitment. More recently, the formation of COP-coated vesicles has been dissected to show that the only requirements are ARF, GTP, coatomer and acyl-CoA (Ostermann *et al.*, 1994).

The culmination of these studies has led to a model for the formation of intra-Golgi transport vesicles which is depicted schematically in figure 1.2. Cytosolic GDP-bound ARF interacts with a Golgi-membranes associated exchange factor and ARF attains a GTP bound state which facilitates its insertion into the Golgi bilayer via its covalently attached myristylate, to join the unsaturable pool of membrane bound ARF. When this molecule encounters its membrane receptor it binds to it and becomes part of the saturable ARF pool, at which point it begins to promote the binding of coatomer. Successive rounds of coatomer binding form a bud which pinches off to form a vesicle, a process requiring only acyl-CoA. The vesicle diffuses away to the acceptor compartment. After docking at the acceptor membrane to a specific receptor (see below), hydrolysis of the GTP leads to uncoating leaving a vesicle which will fuse with the acceptor compartment. Coatomer and ARF are then recycled for further rounds of vesicle budding.

An interesting point arising from studies of vesicle budding is that, even in the presence of GTPγS, there does not appear to be a complete loss of Golgi cisternae, as would be expected when fusion was prevented (Orci *et al.*, 1989). Instead, the cisternal length does not appear to change significantly. Furthermore, three-dimensional imaging of platinum replicas of isolated Golgi cisternae has shown that cisternals rims, the site at which vesicles exclusively bud, exhibit a different surface texture to the central portion of the cisternal plate (Weidman *et al.*, 1993). This suggests that budding from the central region is somehow prevented, and it is tempting to speculate that this is due to the presence of an intercisternal matrix on the surface of the cisternal membranes, represented by the difference in texture in the different cisternal subdomains. Such a structure would also be a candidate for the material which stacks the cisternae.

1.3.2 Vesicle Targeting

One of the most fundamental questions regarding vesicular transport regards the maintenance of specificity. Vesicles which bud from one compartment must fuse to, and

only to, the next compartment in the pathway to preserve the fidelity of intracellular compartmentalisation.

Light was recently shed upon this mechanism by the identification of a family of proteins which have been termed SNAREs (SNAP Receptors) (Söllner *et al.*, 1993b), though they had been previously identified independently as components of synaptic membranes and termed syntaxin A and B (Bennett *et al.*, 1992), SNAP25 (Oyler *et al.*, 1989) and synaptobrevin (Baumert *et al.*, 1989). These proteins all have the same topology, with the bulk of the protein being cytoplasmically oriented. Furthermore tetanus and botulinum-B neurotoxins have both been shown to be specific proteases for synaptobrevin (Schiavo *et al.*, 1992), and block the release of neurotransmitter, implicating these proteins in the process of membrane traffic.

The fact that SNAP-25 and the syntaxins are present in the synaptic membranes and synaptobrevin is present in synaptic vesicles led to the proposal that these molecules are involved in the specificity of membrane fusion - the SNARE hypothesis (see Warren (1993a) and Rothman and Warren (1994) for reviews). Each compartment is postulated to possess two sets of SNAREs. The v-SNAREs are incorporated into secretory vesicles and this programs them to fuse only with the next compartment in the secretory pathway. The second set, the t-SNAREs label the acceptor compartment and interact only with the v-SNAREs of the previous compartment. Thus budding vesicles incorporate one or more v-SNAREs from their compartment which act as tags to identify their point of departure. These v-SNAREs can only recognise the complimentary t-SNAREs on the target membrane. This is shown schematically in figure 1.3. After vesicle fusion, v-SNAREs are depicted as being recycled to their previous compartment to be used in a new round of vesicular transport, though no direct evidence that this occurs has yet been presented, and it is also possible that these v-SNAREs are inactivated to prevent their incorporation into the incorrect transport vesicles.

A subsequent study demonstrated that v-SNAREs (synaptobrevin; VAMP) and t-SNAREs (syntaxin and SNAP-25) could form complexes with each other (Söllner *et al.*, 1993a) and that another protein, synaptotagmin, was associated with the SNARE pair and could be displaced by the addition of SNAPs. Synaptotagmin is thought to be a calcium-sensing protein which induces fusion of synaptic vesicles with the synaptic membrane upon receipt of the appropriate signal (DeBello *et al.*, 1993; Elferink *et al.*, 1993). These data led to the suggestion that synaptotagmin acts as a fusion clamp and thus prevents fusion of docked vesicles with the synaptic membrane until they are required to do so by the prevention of SNAP binding.

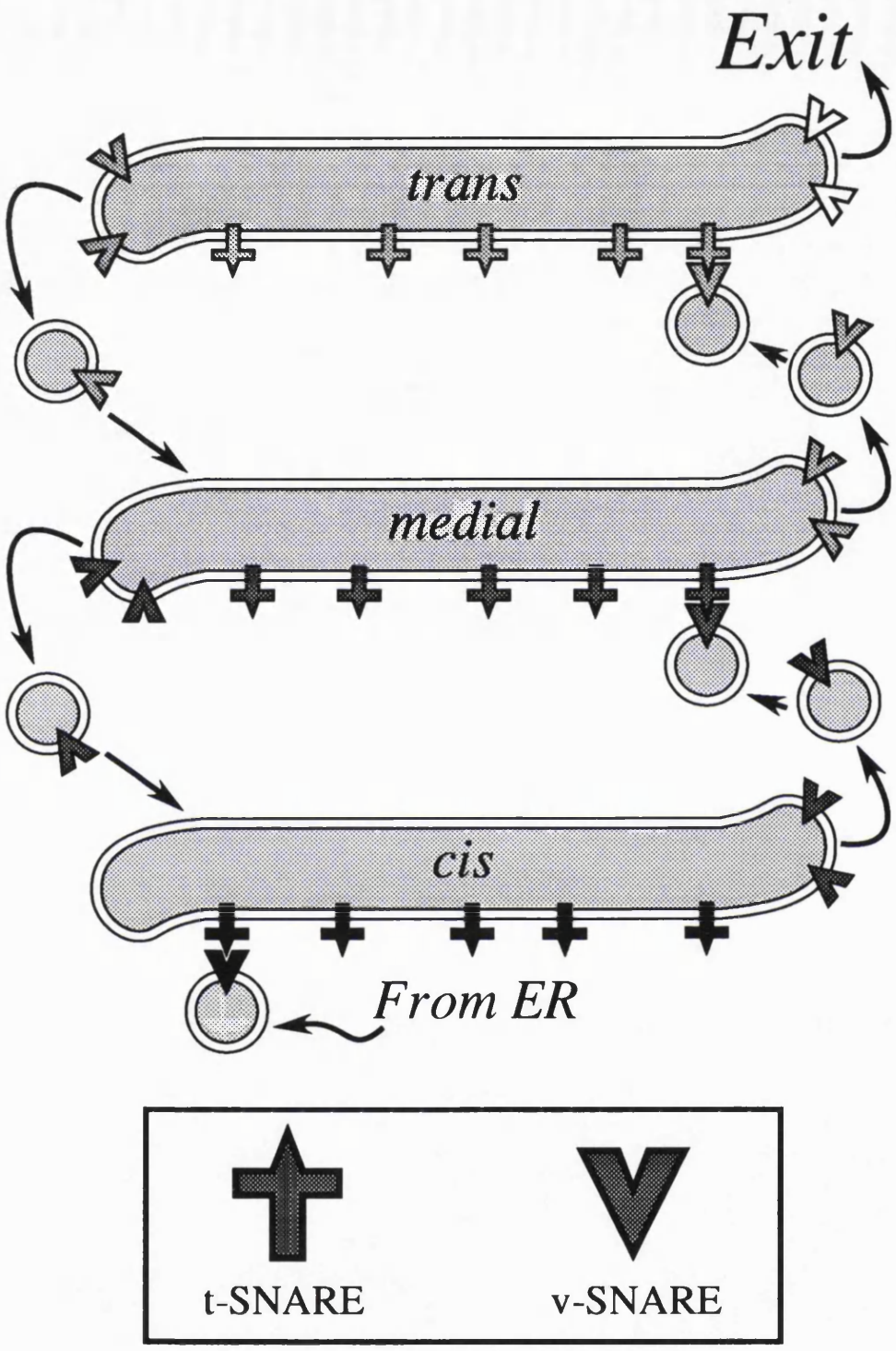

Figure 1.3: Schematic diagram of SNARE-mediated intra-Golgi vesicle targeting. Each cisterna is defined by the t-SNARE which it contains. A vesicle associated v-SNARE from one compartment recognises the next by association with a cognate t-SNARE in the target membrane. After fusion, v-SNAREs have to be recycled to their original compartment.

1.3.3 Vesicle Fusion

The first protein to be biochemically isolated and identified as being involved in the fusion of transport vesicles with their target membrane was NSF (NEM Sensitive Fusion Protein). The protein was first identified by the fact that its activity was sensitive to treatment with N-ethylmaleimide (NEM), an alkylating agent (Balch *et al.*, 1984b). NSF is a 76kD protein which forms a homotetramer (Block *et al.*, 1988), though more recent evidence suggests that it is actually a homotrimer (Whiteheart *et al.*, 1994). NSF is thought to act after budding has occurred, suggesting that it is involved in the fusion process (Malhotra *et al.*, 1988). It is related to the yeast protein Sec18p (Wilson *et al.*, 1989), thus demonstrating its function *in vivo*.

NSF binds to Golgi membranes, and this process requires both cytosolic components termed SNAPs (Soluble NSF Attachment Proteins), and a component in the Golgi membrane (Weidman *et al.*, 1989). The SNAPs have been purified and named α-, β- and γ-SNAP (Clary and Rothman, 1990), though β-SNAP has since been shown to be a brain-specific isoform of α-SNAP (Whiteheart *et al.*, 1993). The protein Sec17p is the functional yeast equivalent of α-SNAP, again confirming its function *in vivo* (Clary *et al.*, 1990).

SNAPs and NSF interact with each other and with a membrane-bound SNAP receptors (or SNAREs - see above) to form a detergent-soluble complex with a sedimentation coefficient of 20S (Wilson *et al.*, 1992), which is postulated to constitute the "fusion complex". The 20S particle disassembles upon ATP hydrolysis by NSF (Wilson *et al.*, 1992), a process which is thought to drive membrane fusion, though acyl-CoA is also required in an, as yet, unknown role (Pfanner *et al.*, 1990).

The model for the fusion of transport vesicles with their acceptor compartment that has been proposed is depicted schematically in figure 1.4. A docked, uncoated vesicle is attached to the acceptor membrane by interaction of cognate v- and t-SNAREs in the vesicle and target membranes. Cytosolic SNAPs bind to the SNAREs and promote the binding of NSF to form the fusion complex. Hydrolysis of ATP by NSF initiates membrane fusion which requires acyl CoA. The fusion complex disassembles and the SNAPs and NSF are recycled to promote a fresh round of vesicle fusion.

1.3.4 GTP-Binding Proteins

The large Rab family of small Ras-like GTP-binding proteins has long been implicated in being involved in the transport process. Before the identification of the SNAP receptors, the Rab proteins were considered to be the most likely candidates for the proteins that encoded targeting specificity. The main evidence for their involvement in this process

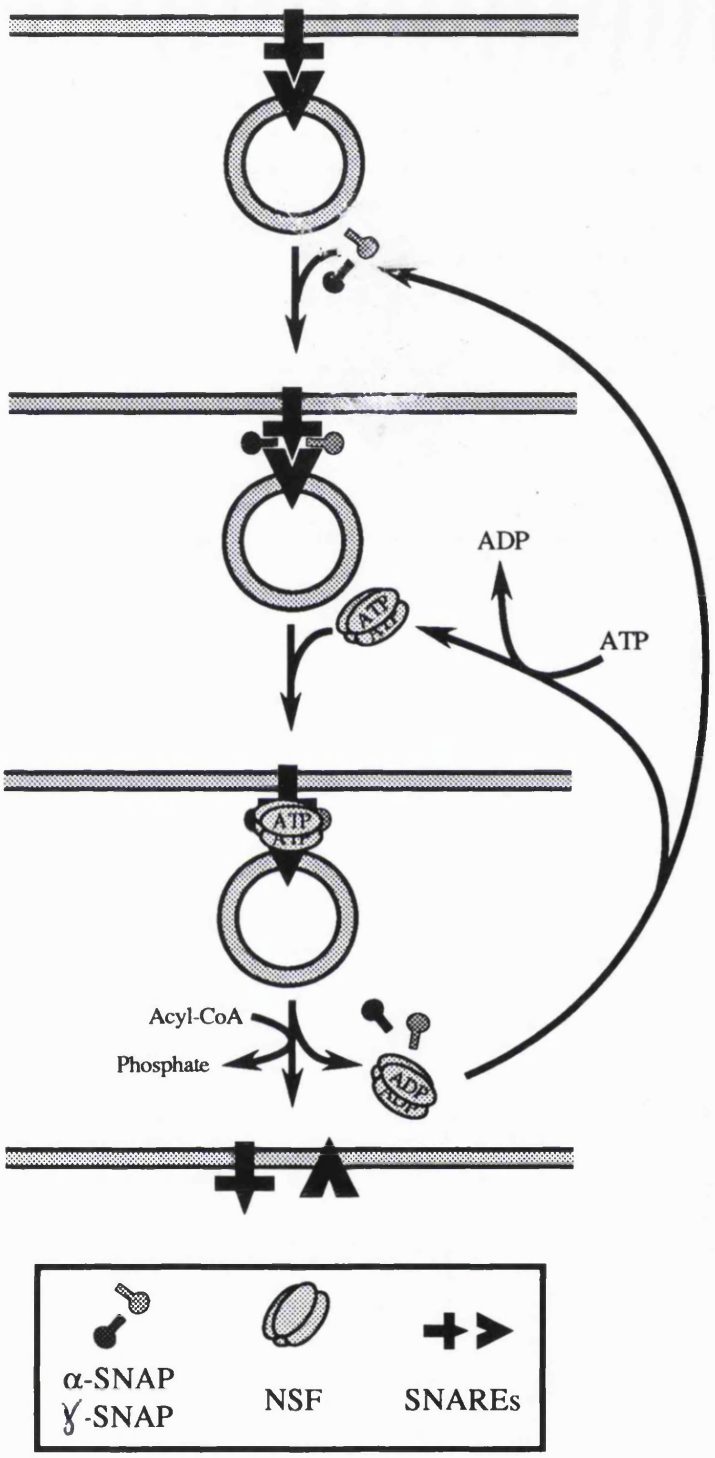

Figure 1.4: Schematic diagram of the mechanism of vesicle fusion. After uncoating, α- and γ-SNAP bind to the docked SNAP receptors (SNAREs). This promotes binding of ATP-bound NSF. Hydrolysis of ATP by NSF in the presence of acyl-CoA initiates fusion and NSF and SNAPs dissociate. These are recycled for successive rounds of fusion.

was indirect and was based on the fact that these proteins seemed to be required for transport, and because each stage in membrane transport seemed to possess its own unique Rab protein (see Zerial and Stenmark (1993) and Balch (1990) for reviews). With the advent of the SNARE hypothesis, the role of the Rab family in vesicular transport has been rethought. In addition other lines of evidence have emerged which indicate that the Rab proteins do not encode specificity.

Firstly, a chimeric protein of Yptlp and Sec4p has been shown to be capable of performing the roles of both wild type proteins in yeast (Brennwald and Novick, 1993; Dunn *et al.*, 1993). No missorting of proteins was detected, suggesting that Yptlp and Sec4p are not involved in targeting specificity. Secondly, several genes have been isolated which, in high copy number, can suppress the effect of the loss of the *YPT1* gene (Dascher *et al.*, 1991; Ossig *et al.*, 1991). These have been termed the *SLY* family (Suppresser of Loss of *YPT1*) and Sly2p and Sly12p have been shown to be related to the synaptobrevins or v-SNAREs. It is possible that over-expression of these v-SNAREs allows the fusion of vesicles with target membranes, even in the absence of Yptlp.

These observations suggest that *ras*-like GTP-binding proteins may play a more ancillary role in the transport process than previously thought. Two possibilities have been suggested. Firstly, the Rabs may act as "molecular clocks" and regulate the recognition of a cognate v-SNARE/t-SNARE pair. This would be analogous the GTPase activity of the elongation factor, EFTu, in the ribosome (Thompson, 1988). This GTPase has a constant turnover rate and can only hydrolyse GTP if a tRNA is present in the t-site of the ribosome. Hydrolysis promotes chain elongation and GTP hydrolysis can only occur when the correct tRNA is present in the t-site of the ribosome, due to its longer residence time. In a similar way, the Rab proteins may act as clocks which determine whether a SNARE-pair have interacted for long enough for the interaction to be deemed specific before allowing the initiation of fusion.

Another possibility is that the Rabs may specify the directionality of vesicular transport. Since the SNARE hypothesis implies that retrograde transport occurs to facilitate v-SNARE recycling, the direction in which a vesicle is travelling may be determined by the associated Rab protein. Further work will be needed to elucidate the true role of the Rab proteins in vesicular transport.

1.4 The Golgi Apparatus

The previous section has summarised the current thinking regarding the secretory pathway through which proteins pass on their way to their final subcellular destinations. In this section, I will elaborate on the structure of the Golgi apparatus and its functions in this pathway more specifically.

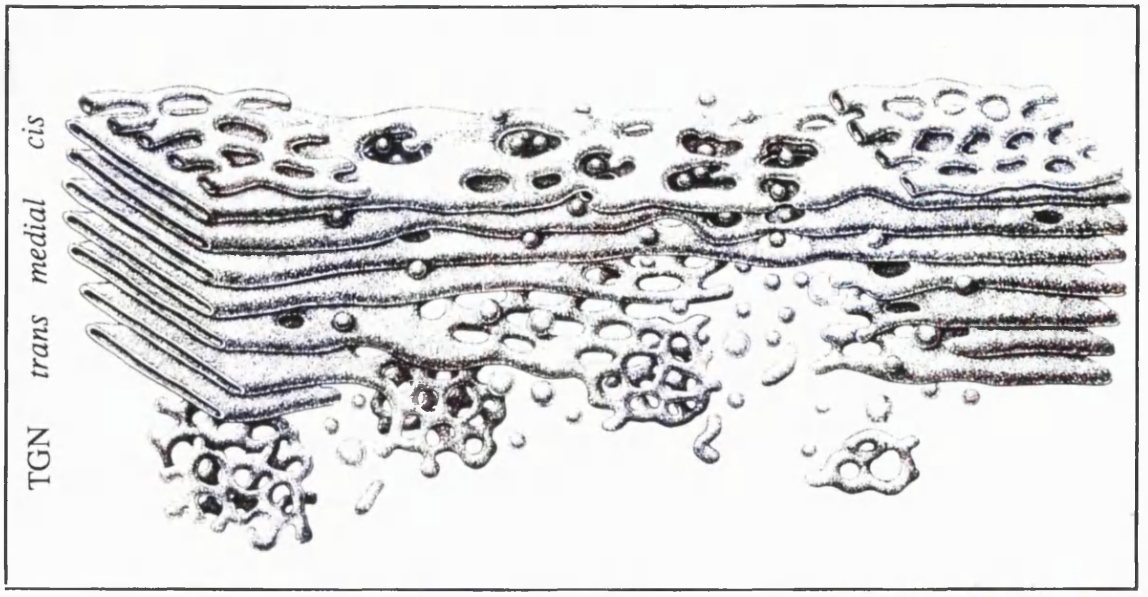

Figure 1.5: Reconstruction of the structure of the Golgi apparatus. The diagram is adapted from Rambourg and Clermont (1990) and shows the morphology of the various subcompartments of the Golgi apparatus.

1.4.1 Morphology

The Golgi stack consists of a set of closely apposed cisternae which are located in a juxta-nuclear location within the cell. It is thought to be a single copy organelle, though several stacks can be observed by examination of a single section by electron microscopy. More thorough studies using serial sectioning (Lucocq *et al.*, 1987; Lucocq *et al.*, 1989) or high-voltage electron-microscopy (Rambourg and Clermont, 1990; Rambourg *et al.*, 1981) have revealed, however, that the organelle undulates throughout the cytoplasm and consists of many stacks connected by tubular extensions, thus giving the impression of being a multi-copy organelle in a single section.

The Golgi stack is flanked by the *cis*-Golgi and *trans*-Golgi networks respectively (see earlier) and the number of cisternae in the stack varies from cell-type to cell-type (see Whaley (1975) for a comprehensive discussion). Although usually thought of as consisting of at least three cisternae (*cis*, *medial* and *trans*), this number can increase greatly with, for example, the alga *Euglina gracilis* possessing a stack of some 20 cisternae (Osafune *et al.*, 1991). The morphology of the cisternae also varies depending on their position in the stack, with the *cis*-cisterna being highly fenestrated. In addition, the *medial*- and *trans*- cisternae also contain fenestrations but these are localised to the edges of the cisternae while the central plates remain free of fenestrations (Rambourg *et*

al., 1981; Rambourg and Clermont, 1990). A three-dimensional representation of the Golgi apparatus is depicted in figure 1.5.

It has been suggested that this fenestration at the cisternal edges is due to the continuous budding and fusion of vesicles which occurs in this area (Warren, 1985). Vesicles contain a larger ratio of lipids in their outer compared to their inner leaflets than planar membranes, and thus vesicle fusion at the cisternal rims would result in a change in this ratio of the cisternal membranes in this region and thereby cause fenestration at the sites of vesicular budding and fusion.

The production of fenestrations has also been suggested to occur by a process termed periplasmic fusion (Rothman and Warren, 1994). In this hypothesis, it is assumed that membrane fusion occurs stochastically whenever two lumenal faces of membranes meet. For example, a vesicle coat would deform the membrane until the lumenal faces touch and the vesicle would automatically bud. As a consequence of such a system, periplasmic fusion would occur at random whenever two membranes touched each other and such fusion in a planar system such as a cisterna would cause the production of a fenestration. The production of fenestrations would be reduced if membranes were protected from the turbulence of the cytoplasm, such as being in the centre of the stack, or by a protective layer of proteins such as the ribosomes and nuclear lamina of the nucleus. Since the *cis*-cisterna is not protected in this way, fenestration would be produced more frequently. No evidence yet exists to directly support this hypothesis, though it does proffer some elegantly simple mechanisms to explain the morphology of various cellular organelles.

The juxta-nuclear location of the Golgi apparatus coincides with that of the microtubule organising centre (MTOC). Depolymerisation of microtubules leads to a fragmentation and redistribution of the Golgi apparatus to the cell periphery (Sandoval *et al.*, 1984), suggesting that microtubules play an important role in this intracellular localisation. The dispersion involves the loss of microtubule attachment to the MTOC since treatment with non-hydrolysable analogue of GTP causes bundling of microtubules which are still attached to the MTOC and no Golgi dispersion, while treatment with taxol causes bundling without MTOC attachment and a concomitant dispersion of Golgi membranes. Thus juxta-nuclear Golgi localisation appears to be due to the presence of intact microtubules which must be attached to the MTOC. Electron microscopic examination of nocodazole-treated cells showed that dispersion was due to a fragmentation of the Golgi stack to yield many small stacks dispersed in the cytoplasm (Thyberg and Moskalewski, 1985), in agreement with the observation that the Golgi apparatus consists of many stacks linked by tubular connections. This redistribution did not have any effect on the rate or extent of secretion or sialylation of secretory proteins as judged by the measurement of transport properties of VSV G protein (Rogalski *et al.*,

1984), and could be reversed upon reassembly of microtubules (Rogalski and Singer, 1984; Sandoval *et al.*, 1984).

A more thorough study of this phenomenon showed that redistribution occurred in three distinct steps (Turner and Tartakoff, 1989). Firstly, microtubule disassembly occurred but fragmentation could be inhibited by the addition of metabolic inhibitors or by incubation at 4°C, showing that removal of microtubules alone could not cause the fragmentation and suggesting that membrane-fission may also be required in this process, perhaps to enable the severing of the interconnecting tubules. In absence of the metabolic inhibitors the Golgi dispersed but the fragments remained in a perinuclear location for 30 minutes, from which they slowly diffused to the cell periphery in the final stage 2 hours after microtubule disassembly. Re-formation of the Golgi apparatus after repolymerisation of the microtubules occurred in the same stages, but more swiftly, with the scattered elements beginning to re-cluster in a perinuclear position within 15 minutes with coalescence to re-form an intact Golgi apparatus occurring within 90 minutes of microtubule reassembly. This re-clustering was also sensitive to metabolic poisons. In a further study of the re-clustering, the Golgi apparatus was labelled with the fluorescent lipid analogue C_6-NBD-ceremide and re-clustering upon microtubule reassembly examined in real-time using video-microscopy. The Golgi fragments appeared to migrate to the MTOC in a saltatory fashion and along tracks defined by microtubules (Ho *et al.*, 1989). The re-clustering was not dependant on the presence of intermediate or micro-filaments since micro-injected anti-vimentin antibodies and cytochalasin D did not inhibit the effect. Further studies have demonstrated that this reclustering is dependent on the microtubule-motor protein dynein (Corthésy-Theulaz *et al.*, 1992).

1.4.2 Intercisternal Structures and Stacking

The most striking feature of the Golgi apparatus is its unique morphology - the cisternal stack. The fact that the stack can be visualised in virtually all eukaryotes suggests that it serves an important function and so has been conserved throughout evolution. To date, however, the function of the stack and the mechanism by which it is generated and maintained has remained elusive.

The fact that Golgi membranes can be purified in a stacked form from various sources (Morré and Mollenhauer, 1964; Morré *et al.*, 1970; Hino *et al.*, 1978; Fleischer and Fleischer, 1970; Leelavathi *et al.*, 1970) strongly suggests that the stack is stable. Furthermore structures must exist which keep cisternal membranes apposed because stacking is sensitive to proteases (Mollenhauer *et al.*, 1973; Cluett and Brown, 1992). This protease-sensitivity is restricted to a certain subset of proteases, suggesting that the effect is specifically due to the removal of structural proteins within the intercisternal

space and not to a general effect of protease treatment of Golgi membranes. These structures must be distributed throughout the length of the Golgi cisternae since they are apposed for their entire length with a constant spacing of 10nm (Cluett and Brown, 1992).

Stacking does not seem to require components of the cellular cytoskeleton. As described above, treatment of cells with nocodazole to disrupt microtubules, converts the single copy Golgi apparatus into a multi-copy form dispersed throughout the cytoplasm. These dispersed forms, however, consist of small stacks, demonstrating that microtubules play a role in the subcellular localisation of the Golgi apparatus, but not in stacking the cisternae (Thyberg and Moskalewski, 1985). Treatment with cytochalasin D to disrupt actin-based microfilaments has no effect on Golgi morphology, and neither does the microinjection of antibodies specific for intermediate filaments (Ho *et al.*, 1989). Having said this, both microtubule (Bloom and Brashear, 1989) and actin (Weiner *et al.*, 1993) binding proteins have been localised to the Golgi apparatus (though not to the intercisternal space), but their functions are as yet unknown. Finally, an isoform of spectrin, a intermediate-type filament protein of the erythrocyte plasma membrane scaffold, has also been localised to the Golgi apparatus, but its function and location within the stack is again not known (Beck and Nelson, 1993).

Various electron microscopic studies over the past thirty years have identified electron dense structures between, and surrounding, the Golgi cisternae, and it has been suggested that these may be involved in the stacking phenomenon, though these conclusions are somewhat spurious (see Mollenhauer and Morré (1978) for a review).

These structures have generally been classified into three categories. Firstly, the intercisternal elements consist of a electron dense fibres which run parallel to and between adjacent cisternae. Such structures have been identified in plants such as maize (Mollenhauer, 1965), *Nicotiana* and *Arabidopsis* (Staehelin *et al.*, 1990), the alga *Distigma* (Mignot, 1965) and in the unicellular flagellate *Trichomonas* (Amos and Grimstone, 1968). Secondly, smaller, more discrete structures termed intercisternal cross-bridges have been observed which appear to join adjacent cisternae. These have been observed in rat-liver and various plants (Cluett and Brown, 1992; Franke *et al.*, 1972), as well as in closely apposed membranes of other organelles such as the ER and the thylakoid membranes of the chloroplast (Franke *et al.*, 1971). Thirdly, an electron dense "zone of exclusion", which excludes cytoplasmic components and organelles such as ribosomes and mitochondria, can be often visualised surrounding the Golgi stack *in vivo* (Morré and Ovtracht, 1977; Mollenhauer and Morré, 1978). These studies, however, present no direct evidence that the structures observed are involved in the stacking of the Golgi apparatus. Furthermore, electron microscopy of freeze-fractured plant Golgi

Enzyme	% Unstacking
Amylase	0
Carboxypeptidase Y	0
Collagenase	0
V-8 Protease	0
Papain	10
Trypsin	15
Dispase	60
Elastase	74
Subtilisin	75
Chymotrypsin	75
Proteinase K	90

Table 1.1: Effect of treatment of various hydrolytic enzymes on Golgi stacking *in vitro*. The table shows a summary the results obtained by Cluett and Brown (1992) on the extent of unstacking produced by treatment of isolated rat liver Golgi stacks with various proteases or with amylase.

membranes led to the observation that the membrane-proteins were not randomly distributed in the bilayer but arranged in ordered rows (Staehelin *et al.*, 1990). This suggested that these proteins were interacting with a structural matrix to yield this highly ordered arrangement, and such a matrix might also be involved in cisternal stacking, though again the matrix was not identified.

A more biochemical approach towards investigating the mechanism of Golgi stacking was that employed by Cluett and Brown (1992). By treating isolated rat-liver Golgi stacks with a large selection of proteases followed by quantitative examination by electron microscopy, they were able to show that stacking was highly sensitive to proteolysis, though not all proteases were capable of inducing unstacking. Stacking was also shown to be insensitive to incubation with, or depletion of, the divalent cations calcium, magnesium and manganese. Their data on the effect of various proteases on Golgi stacking are summarised in table 1.1. Additionally, stacking was not sensitive to amylase as was previously reported (Ovtracht *et al.*, 1973), and it was suggested that the earlier observation was due to protease contamination of the amylase used in that study. These data suggested that Golgi stacking was maintained at least, in part, by proteinaceous interactions and not by the presence of carbohydrates. Furthermore, it was shown that proteolytic treatment under conditions which unstacked cisternae also led to

the removal of the intercisternal cross-bridges which could be visualised by tannic-acid staining of isolated rat liver Golgi stacks and *in vivo* in both rat hepatocytes and bovine testicular cells. The cross-bridges observed were very similar in dimensions to those previously reported by Franke *et al.* (1972), being 11nm in height and 8.5nm in width, and were unlikely to be fixation artefacts since they could be visualised by two independent methods using either tannic acid or negative staining with phosphotungstic acid. This correlation suggested that these cross-bridges were indeed involved in the stacking mechanism, though again the evidence is indirect, and their molecular composition could not be studied because they were not purified biochemically.

The relationship between intercisternal elements and cross-bridges is unclear. They may represent two entirely unrelated structures or alternatively cross-bridges may represent discrete regions of the elements which were preferentially stained by the methods used in the studies where they were observed. Furthermore, both cross-bridges (Franke *et al.*, 1972) and elements (Mignot, 1965) have been observed within the lumen of Golgi cisterna. Such intracisternal structures may be involved in the maintenance of the classical flattened cisternal morphology. The other way in which this could be achieved would be by the presence of specific channels within the Golgi membranes which would function to reduce the volume of the cisternal lumen. However, this latter possibility seems unlikely since the morphology of cisternae in purified Golgi stacks is unaffected after treatment with solutions of high ionic strength (Cluett and Brown, 1992). Unstacking of Golgi cisternae by proteolysis releases individual cisternae whose morphologies are indistinguishable from those present in untreated stacks, arguing that stable interactions within the Golgi lumen are responsible for maintaining the flattened cisternal shape.

In summary, although the stacking of Golgi cisternae appears to be a highly conserved phenomenon, no direct evidence yet exists for the mechanism by which this is achieved. The evidence available, however, does indicate that it occurs by means of an interaction of apposed cisternal membranes with a cytoplasmic matrix, though such a matrix has not yet been identified biochemically.

1.4.3 The Function of the Stack

Although the stacked nature of the Golgi cisternae has been well preserved throughout evolution, it as yet serves no known function. The simplest explanation of the function of the stack, that is that it serves to concentrate the Golgi cisternae within the cell and thus increase the efficiency of transfer of transport vesicles from one cisterna to the next, though no evidence exists to suggest that this is so.

The budding yeast *Saccharomyces cerevisiae* does not possess a highly ordered stack, with most of its cisternae appearing to be free in the cytoplasm and yet the organism survives, suggesting that permanently stacked Golgi cisternae are not required for efficient exocytic transport. This organism, however, does appear to possess mechanisms to form a stack, since incubation of *sec7* mutant cells at the non-permissive temperature leads to the formation of stacked cisternae (Franzusoff and Schekman, 1989), though these do not appear to be *bona fide* stacks since thee-dimensional reconstruction has demonstrated that the cisternae in these structures appear to be connected (Rambourg *et al.*, 1993). The *sec7* gene has been shown to encode a protein which is a component of the coat of transport vesicles and is essential for budding to occur (Franzusoff *et al.*, 1992), though the mechanism by which its inactivation leads to cisternal stacking is still unclear.

The fission yeast *Schizosaccharomyces pombe*, in contrast, possesses a highly stacked Golgi apparatus in wild-type cells. The cisternae can, however, be easily separated by treatment with the microtubule-disassembling agent thiabendazole (Ayscough *et al.*, 1993). This effect was not due to a secondary effect of this drug since it was also observed when *nda3* cells, which bear a temperature-sensitive mutation in the β-tubulin gene which prevents microtubule assembly, were incubated at the non-permissive temperature. Unstacking did not affect growth or the rate or extent of transport to the cell surface, suggesting that the stack does not function to improve the efficiency of transfer of vesicles between cisternae. The lack of any other observable effects caused by Golgi unstacking meant that it was not possible to determine a function for the stack. Microtubules cannot serve as stacking components directly since they are too large to penetrate the intercisternal space, even in yeast cells where this distance between cisternae is larger than that in mammalian cells (13-28nm compared to 10nm). This effect also contrasts sharply with the effect of microtubule depolymerisation on the Golgi apparatus in mammalian cells, as described above. In this case, the Golgi is fragmented but these fragments still retain a stacked cisternal morphology.

This suggests that the stacking mechanism of the Golgi apparatus in higher eukaryotes is very different to that in *S. pombe*. The mammalian Golgi stack presumably contains proteins which are not present in yeast which function in maintaining the close apposition of cisternal membranes. The difference in the dynamics of the stack between yeast and higher eukaryotes was further demonstrated by a study which showed that inhibition of protein synthesis in *S. pombe*, also led to a disruption in cisternal morphology (Ayscough and Warren, 1994), with an almost complete loss of a detectable Golgi apparatus after a sufficiently long incubation. No such effects have ever been reported in mammalian cells.

Since unstacking does not seem to affect the transport of proteins in *S. pombe*, the stack does not appear to play a role in this process in this organism. Nevertheless, it is clear that the stacks in mammalian cells are very different to those in yeast, and it is still possible that cisternal stacking may play a role in increasing transport efficiency in higher eukaryotes, though this may be a subtle effect since no difference can be detected in the kinetics of protein transport between spatially separated Golgi stacks (Rothman *et al.*, 1984; Rothman *et al.*, 1984). The fact that the function of the stack in yeast is unknown has made it impossible to devise a screening procedure to produce mutants which could be used in studying this phenomenon. Furthermore if such mutants could be obtained, it is not clear how useful they would be in determining the stacking mechanism in higher eukaryotes. It seems likely that only the biochemical identification of the stacking material in higher eukaryotes will enable the elucidation of this mechanism and begin to answer questions regarding the function of stacked cisternae.

1.4.4 N-Glycan Processing

Of the many post-translational modifications that are carried out by the Golgi apparatus, none are as well characterised as those occurring to the asparagine-linked oligosaccharides of the proteins in transit through this organelle to form the biantennary complex-type oligosaccharides such as the one depicted in figure 1.6B. The N-glycans of various proteins contain a large diversity of structures. Some are incompletely processed while others contain three or four branches (see Spiro (1973) for a review). The mechanisms by which such divergence occurs are not completely understood. The synthesis of the classical biantennary structure is the best characterised, and most commonly used marker for protein transport through the Golgi and it is this structure that is discussed here.

The process is initiated in the ER by the co-translational en-block transfer of a Glc_3-Man_9-$GlcNAc_2$ precursor onto the nascent polypeptide chain (Tabas *et al.*, 1978). The precursor is synthesised in the ER and its structure immediately after transfer to a polypeptide by oligosaccharyltransferase is depicted in figure 1.6A (Li *et al.*, 1978). Synthesis occurs in several steps on both the cytoplasmic and lumenal faces of the ER membrane, though this mechanism is not yet completely clear (see Kornfeld and Kornfeld (1985) and Roth (1987) for reviews). The final precursor is present in the lumen of the ER, bound to a lipid carrier, dolichol phosphate. Transfer occurs onto the peptide at asparagine residues bearing the consensus sequence Asn-X-Ser/Thr, though not all such sequences are modified, probably due to a conformational requirement as well as the correct consensus sequence. This precursor is then subjected to enzymatic trimming before the addition of new sugar residues to form the mature oligosaccharides. This

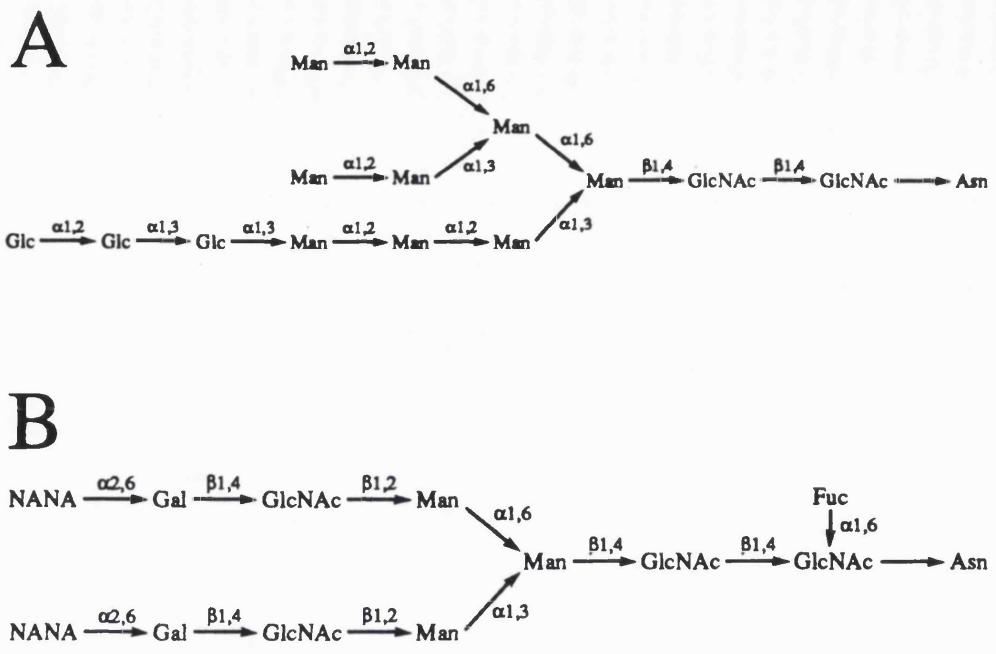

Figure 1.6: Structure of N-linked oligosaccharides. The diagram shows the structure of the precursor that is transferred to polypeptides in the ER (A), and of the mature biantennary oligosaccharide (B). Glc=glucose, Man=mannose, GlcNAc=N-acetylglucosamine, Gal=galactose and NANA=sialic acid.

trimming is initiated in the ER with the removal of the three terminal glucose residues and one mannose residue to yield a Man_8-$GlcNAc_2$ product which moves on to the Golgi for further processing.

The enzyme-catalysed processing reactions which occur to the initial structure are depicted schematically in figure 1.7, as based on the scheme described by Kornfeld and Kornfeld (1985). It is thought that the reactions are physically separated in the various compartments of the Golgi, and the evidence for their sub-cellular locations are described later. In this scheme, Golgi processing begins by the trimming of 3 mannose residues by the enzyme α1,2-Mannosidase I (Mann I). Next, addition of a single GlcNAc residue by β1,2-N-acetylglucosaminyltransferase I (NAGT I) functions as a switch to facilitate removal of two mannose residues by α1,3-α1,6-mannosidase II (Mann II) to yield the final tri-mannosyl core of the mature oligosaccharide. The next step is the addition of a second GlcNAc by β1,2-N-acetylglucosaminyltransferase II (NAGT II). Addition of fucose by α1,6-fucosyltransferase (FucT) can occur after any of the three previous stages *in vitro*, though its precise point of action *in vivo* has not been elucidated. Glycosylation terminates with the sequential additions of galactose by β1,4-galactosyltransferase (GalT)

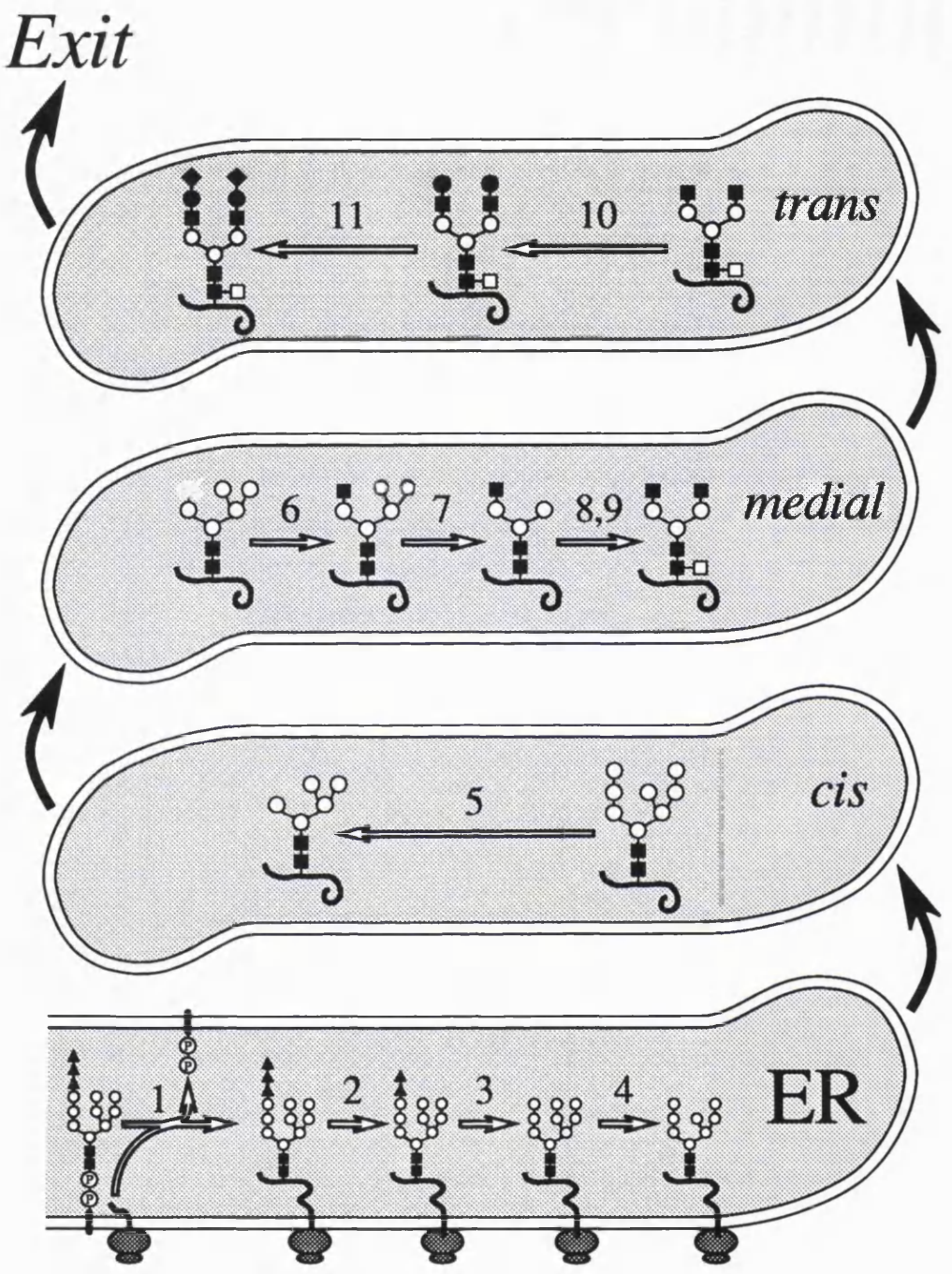

Figure 1.7: Processing of N-linked oligosaccharides. The diagram shows the steps involved in the synthesis of classical N-linked biantennary oligosaccharides after initial transfer from dolichol. 1=oligosaccharytransferase, 2=α1,2-Glucosidase I, 3=α1,3-Glucosidase II, 4=ER α1,2-Mannosidase, 5=Mann I, 6=NAGT I, 7=Mann II, 8=NAGT II, 9=FucT, 10=GalT, 11=sialylT. ○=mannose, ■=N-acetylglucosamine, ●=galactose,▲=glucose, ❑=fucose◆=sialic acid.

and sialic acid by α2,6-sialyltransferase (SialylT) to yield the final mature form of the oligosaccharide.

The sequential trimming and addition of sugar residues in the early stages of this process was established in a classic study by Kornfeld *et al.* (1978) where the oligosaccharides of the VSV G protein were analysed after being chased for various times after their synthesis. This showed that the earlier intermediates of the trimming pathway consisted of oligosaccharides whose terminal glucose residues had been sequentially removed, with complete removal occurring within 20-30min after protein synthesis. The removal of glucose was a prerequisite of mannose trimming since no mannose removal could be detected before loss of all the glucose residues. The removal of glucose appeared to be a rate-limiting step, since subsequent mannose trimming appeared to occur very rapidly after loss of the final glucose. Analysis of such intermediates in clone 15B CHO cells, which lack the enzyme NAGT I, showed an accumulation of a Man_5-$GlcNAc_2$ product, which indicated that the action of this enzyme was required before final mannose trimming could occur. The lack of trimming could not be explained due to a lack of this later mannose trimming activity in clone 15B cells because membrane extracts from these cells were capable of cleaving mannose from the product of the NAGT I reaction (Tabas and Kornfeld, 1978). A similar NAGT I-dependant mannosidase was also shown to occur in rat liver (Harpaz and Schachter, 1980c).

Thus, mannose trimming occurs in two stages, one directly before and one directly after the action of NAGT I. The first stage involves cleavage at α1,2 linkages only while the second involves trimming at α1,3 and α1,6 linkages. This was shown to be performed by two different enzymes (Mann I and Mann II respectively) when these proteins were purified. Mann I was shown to specifically remove only α1,2 linked mannose residues and had a negligible activity on the α1,3 and α1,6 linked residues (Tabas and Kornfeld, 1979). Further purification seems to indicate that Mann I activity is present in two forms, Mann IA and Mann IB. Although their kinetic properties are very similar, they can be separated chromatographically on a cellulose phosphate column (Tulsiani *et al.*, 1982b). The fact that both enzymes can be recognised by antibodies against Mann IA (Tulsiani and Touster, 1988) suggests that the proteins are related or are the same enzyme bearing different post-translational modifications (Tulsiani and Touster, 1988), and thus the *in vivo* significance of these differences is unclear. Unlike Mann I, Mann II displays a substrate specificity for only for α1,3 and α1,6 linked residues, and only when a GlcNAc residue has been added to the structure (Tulsiani *et al.*, 1982b). The enzyme has little activity towards the α1,2 linked mannoses, and can also be distinguished from Mann I

because it can cleave the artificial chromogenic substrate p-nitrophenol-α-D-mannopyranoside while Mann I cannot.

Both GlcNAc residues are added to the oligosaccharide in a β1,2 linkage, though one is added to the mannose which is joined to the central mannose of the trimannosyl core by an α1,3 linkage (NAGT I) while the other is added to the mannose which possesses the α1,6 linkage (NAGT II). These activities were shown to be carried out by two different enzymes because they could be chromatographically separated from bovine colostrum (Harpaz and Schachter, 1980b). Purification of NAGT I from rabbit liver showed that it was indeed specific for the α1-3 linked mannose (Oppenheimer and Hill, 1981) and occurred most readily on a Man_5-$GlcNAc_2$ substrate, in agreement with its predicted substrate specificity. Similarly NAGT II was purified from rat liver (Bendiak and Schachter, 1987b) and consequently shown to be specific for the catalysis of the addition of GlcNAc to the α1,6 linked mannose (Bendiak and Schachter, 1987a).

The addition of an α1,6 fucose to the asparagine-linked GlcNAc was shown only to occur to the products of NAGT I, Mann II and NAGT II, and would not occur to high mannose oligosaccharides which had not been processed by NAGT I (Longmore and Schachter, 1982). This indicated that α1,6 fucosyltransferase could act at any point in the processing pathway that was carried out by these three enzymes.

The next steps in the process are the elongation of the oligosaccharide chains, firstly by the addition of galactose residues by β1,4-galactosyltransferase. This enzyme has been purified from several sources such as pig thyroid (Bouchilloux, 1979), rat mammary gland (Navaratnam *et al.*, 1988) and liver (Bendiak *et al.*, 1993) and human milk (Khatra *et al.*, 1974). The purified rat liver enzyme has been shown to exhibit a branch preference for galactosylation (Pâquet *et al.*, 1984). This study showed that reaction with a $GlcNAc_2$-Man_3-$GlcNAc_2$ substrate formed a Gal-$GlcNAc_2$-Man_3-$GlcNAc_2$ intermediate and the galactose was added more rapidly onto the branch bearing the α1,3 linked mannose with the digalactosylated form accumulating slowly. This differential specificity was suggested as being a mechanism for the generation of some of the diversity which is observed in many N-glycans, with the glycoprotein's residence time in the GalT-containing cisternae playing a role in the extent of galactosylation in the oligosaccharide.

A similar branch specificity was found to occur with the next enzyme in the cascade, α2,6-sialyltransferase, which catalyses the final stage of the maturation pathway of the biantennary complex N-glycans. This enzyme has been purified from porcine submaxilary gland (Sadler *et al.*, 1979) and rat liver (Weinstein *et al.*, 1982a; Weinstein *et al.*, 1982b). A study of partially purified enzyme from bovine colostrum showed that, like GalT, it also displayed preferential activity for the α1,3 arm of the biantennary structure, with the α1,6 arm being sialylated more slowly (van den Eijnden *et al.*, 1980).

Other modifications also occur to N-glycans to form oligosaccharides which are different to those described above. Lack of mannosidase processing yields N-glycans of the high mannose type which lack galactose and sialic acid residues and contain only the two GlcNAc moieties closest to the asparagine. Processing by NAGT III, IV and V cause the production oligosaccharides with more than two branches. Other galactosyl, fucosyl- and sialyltransferases which catalyse the formation of different covalent bonds can cause branching in the terminal sugar groups. These alternative structures have been reviewed in detail by Kornfeld and Kornfeld (1985).

Additionally, the Golgi apparatus functions in the formation and processing of O-linked glycans which are connected to glycoproteins via serine or threonine residues. The formation of these structures have been reviewed by Kornfeld and Kornfeld (1976).

1.4.5 Compartmentalisation

It is widely accepted that the Golgi apparatus is divided into a series of sub-compartments which contain distinct sets of proteins. Although the function of such compartmentalisation is unknown, it is likely that it increases the efficiency of post-translational modification. Because the enzymes catalysing these modifications function strictly in sequence, with the product of one reaction being the substrate for the next, there is no *a priori* reason to separate these activities. However, sequestration of the enzymes into differing subcompartments would increase the local concentrations of the required enzymes at each step of the exocytic pathway and thus increase the rate and efficiency of the post-translational modifications. The sub-compartmentalisation has been demonstrated both biochemically and by immunoelectron microscopy, as described below.

Initially, studies showed that the Golgi apparatus contained several nucleoside pyrophosphatase activities, and that the organelle could be visualised by light microscopy by incubation of tissue sections in a nucleoside pyrophosphate buffer containing lead ions (Novikoff and Goldfischer, 1961). Enzymatic release of inorganic phosphate caused a precipitation of lead phosphate at the site of enzyme activity and this could be visualised by microscopy. This study was critical in demonstrating that the Golgi apparatus was a ubiquitous organelle, since this staining technique demonstrated for the first time that it was present in all eukaryotic cells. The pyrophosphatases of the Golgi apparatus are responsible for the cleavage of diphosphonucleotides to yield nucleotide monophosphates. The nucleotides are imported into the Golgi lumen via specific pumps as nucleotide sugars which act as the donors in glycosylation reactions to yield the diphosphonucleotides. These cannot exit the lumen and must be converted to nucleotide monophosphates to be pumped back out.

The first evidence for compartmentalisation within the Golgi stack came from the observation that osmium impregnation of tissue sections stained cisternae at the *cis*-face of the Golgi apparatus of several cell lines when examined by electron microscopy while the *medial*- and *cis*-cisternae were unaffected. Subsequently cytochemistry utilising the presence of thiamine pyrophosphatase (TPPase) in the Golgi apparatus, but this time coupled to examination of tissue sections by electron microscopy, showed that TPPase activity restricted to the two *trans*-most cisternae of the stack in rat liver, epididymal and pancreatic acinar cells (Cheetham *et al.*, 1971). Finally, another cytochemical study utilising phosphate release by nicotinamide adenine dinucleotide phosphatase showed that this activity was restricted to the *medial*-cisternae of rat incisor ameloblasts (Smith, 1980).

Biochemical evidence for sub-compartmentalisation was obtained by isopycnic fractionation of Golgi membranes on sucrose gradients followed by determination of the densities of the membranes containing various endogenous Golgi enzymes. Initially, the early Golgi enzyme Mann I was shown to be present in denser membranes than the late enzyme GalT in the Golgi apparatus of CHO cells (Dunphy *et al.*, 1981). A subsequent, more thorough, study using macrophage membranes showed that the GalT containing membranes were significantly less dense than those containing NAGT I, Mann II, NAGT IV and FucT (Goldberg and Kornfeld, 1983). These results were confirmed soon afterwards using CHO cell membranes and were extended to show that Mann I and NAGT II were also present in the denser membranes while SialylT containing membranes displayed the same, lighter density as those containing GalT (Dunphy and Rothman, 1983). These studies indicated that the late processing enzymes were present in membranes which were physically distinct from the ones containing the earlier ones.

These biochemical observations have been substantiated by the immuno-localisation of some of the Golgi enzymes by electron microscopy (see Roth (1987) for a comprehensive review). The first enzyme in the Golgi processing system is Mann I. This enzyme has been considered to be a *cis*-Golgi marker by several indirect criteria, due to the lack of an antibody that could be used in immunoelectron microscopic localisation. Firstly, since its activity occurs early in the processing pathway, it was considered likely that it would be present in an early Golgi compartment. Secondly, in an *in vitro* ER-Golgi transport system, Mann I activity i.e. the acquisition of Man_5-$GlcNAc_2$ N-glycans, only occurs after a vesicular transport step, showing that the enzyme is present in a post-ER compartment (Balch *et al.*, 1987). Thirdly, Mann I activity is present in denser membranes than those of the later acting enzyme GalT and SialylT (Dunphy and Rothman, 1983). More recently, however, an immunoelectron microscopic study has challenged this classical view (see below).

The next enzyme in the pathway is NAGT I, and this was localised to two *medial*-cisternae in rabbit hepatocytes by immunoelectron microscopy using monoclonal antibodies raised against the rabbit enzyme and detection by peroxidase (Dunphy *et al.*, 1985). The *cis*- and *trans*-most cisternae were not stained. This distribution was confirmed in a subsequent study (see later). Mann II, which acts just after NAGT I in the processing pathway, was shown to match the distribution of NAGT I, again by using a monoclonal antibody, but this time using colloidal gold. This antibody showed that Mann II was also localised to the *medial* cisternae of the Golgi stack in NRK cells (Burke *et al.*, 1982). A subsequent study using a polyclonal antibody and peroxidase also showed the enzyme to be present in *medial* cisternae in rat liver hepatocytes though the distribution was somewhat broader, with some staining being also visible in the *trans* cisterna but not the *cis* (Novikoff *et al.*, 1983). The next enzyme in the pathway, NAGT II, has not been immuno-localised due to the absence of a specific antibody. The fact that it acts after Mann II and that it is present in the denser membranes containing early-acting enzymes (Dunphy and Rothman, 1983), however, suggests that it too is a *medial*-Golgi protein.

GalT has been localised to the *trans* cisternae of the Golgi stack of Hela (Roth and Berger, 1982) and HepG2 (Slot and Geuze, 1983) cells, in keeping with the observation that it appears to be present in distinct membranes with a different density to those containing the earlier-acting enzymes. Furthermore, a combination of immunocytochemistry and immuno-gold labelling demonstrated that GalT was present in the same cisternae as TPPase, barring the *trans*-most cisterna (Roth and Berger, 1982). The final enzyme of the processing pathway, SialylT, has also been localised to the *trans*-Golgi and is also present in the TGN (Roth *et al.*, 1985), again in agreement with the biochemical data of the density of the compartment in which this enzyme resides.

The above studies indicated that the various processing enzymes of N-glycans were sub-compartmentalised within the Golgi and that the sub-compartmentalisation roughly reflected the order in which they acted enzymatically. More recent work, however, suggests that this generalisation is a gross oversimplification of the true situation *in vivo*. For example, one study on the immunolocalisation of Mann II using a polyclonal antibody showed that it was restricted to *medial* cisternae in CHO and NRK cells, but in a large variety of other cell lines it was also present in *trans* cisternae (Velasco *et al.*, 1993). This same study also showed that Mann I seemed to exhibit a similar distribution as Mann II and was not present in the *cis*-Golgi. In another study, SialylT was found to be present in the *trans* Golgi of rat goblet cells, but distributed throughout the stack of adjacent absorptive cells in the same tissue sections (Roth *et al.*, 1986). Quantitation of these data showed that this distribution was polar, however, with more SialylT being present on the *trans* side compared to the *cis*, suggesting that the stack was still sub-

compartmentalised, but not the as tightly as in the goblet cells. These studies demonstrate that descriptions of enzyme localisations are only broad generalisations and that large differences occur not only between species but between different cell types in the same organism. To determine the true localisation of protein in a particular cell, immunoelectron microscopy must be performed, which is not always possible due to the lack of appropriate mono- and polyclonal antibodies which can be used for immunoelectron microscopy. In such cases, the generalised model of *cis/medial/trans* compartmentalisation of glycosylation enzymes has to suffice.

The fact that the Golgi stack is so pleomorphic i.e. the number of cisternae vary greatly in number between different species and cell types, led to a problem of interpretation of the above immunolocalisations. It was not possible to state for certain whether, for example, GalT and NAGT I were truly present in different cisternae since labelling was performed on different cell types. This problem was overcome in the first study to localise two different glycosyltransferases within the Golgi stack (Nilsson *et al.*, 1993a). This was made possible by the construction of a stable Hela cell line which expressed a NAGT I molecule which bore the *myc*-epitope tag. NAGT I could then be localised using a monoclonal antibody specific to the *myc*-epitope, and GalT could be visualised using a specific polyclonal antibody. The two enzyme distributions could then be localised simultaneously in the same cells using anti-mouse antibodies coupled to 5nm colloidal gold (for NAGT I) and anti-rabbit antibodies coupled to 10nm gold particles (for GalT). GalT labelling was observed mainly in the TGN and *trans*-most cisterna, with a smaller amount present in the *medial*. In contrast, NAGT I was mostly absent from the TGN and localised to the *trans* and *medial* cisternae. Thus the distribution of NAGT I and GalT overlapped in the *trans* cisterna, but were mutually exclusive in the TGN and *medial* cisterna. Quantitation showed that the *trans* cisterna contained 53% of the total GalT and 35% of the NAGT I. This led to a model suggesting that a cisterna was not defined by the presence of a single set of proteins uniquely found in that compartment, but by a unique composition of proteins which could be present in different ratios in different cisternae (Nilsson *et al.*, 1993b). Such a model is more flexible than the traditional view of each enzyme being restricted to a single cisterna since it can accommodate the various differences in enzyme distributions in varying cell types and also for the large number of cisternae present in Golgi stacks of certain cells. In this model, each cisterna would be unique simply because it contained a unique mixture of enzymes, as in the absorptive intestinal cells where SialylT concentrations seem to increase in a *cis-trans* direction through the stack. The mechanism by which cells would control the cisternal composition is unknown, but since Golgi enzymes can be retained by their membrane-spanning domains, it is tempting to speculate that cellular regulation of

the lipid composition of the Golgi membranes may play a role. Regulation of enzyme localisation may play a role in the control of oligosaccharide trimming, since no enzymatically operated regulation mechanism has yet been reported which is independent of the transferases substrate-specificities themselves.

1.4.6 The Effect of Brefeldin A Golgi Structure

Brefeldin A (BFA) is a fungal metabolite which was initially shown to block protein secretion and cause a dilation of the endoplasmic reticulum (Misumi *et al.*, 1986). Subsequently it was shown that this was due to the loss of a recognisable Golgi apparatus due to its redistribution into the ER (Fujiwara *et al.*, 1988; Doms *et al.*, 1989; Lippencott-Schwartz *et al.*, 1989; Lippencott-Schwartz *et al.*, 1990). BFA is thought to be an inhibitor of a membrane-bound GTP exchange factor for ARF (Helms and Rothman, 1992; Donaldson *et al.*, 1992). Thus BFA prevents activation and membrane-binding of ARF which cannot therefore promote coatomer binding the formation of transport vesicles (Donaldson *et al.*, 1990). Since the COP coat uncouples budding from fusion (Elazar *et al.*, 1994), BFA induces the fusion of Golgi membranes with the ER and thus the redistribution of Golgi proteins.

Interestingly, several electron-microscopic studies have indicated that small tubulo-vesicular structures containing Golgi proteins still remain in the cytoplasm of cells treated with BFA (De Lemos-Chiarandini *et al.*, 1992; Hidalgo *et al.*, 1992; Hendricks *et al.*, 1992; Ulmer and Palade, 1991). Since there is no reason why such structures should remain after treatment because of the nature of the BFA lesion, it is possible that these Golgi remnants represent Golgi membranes which have been physically prevented from entering the ER. Such a block could be conferred by binding of these Golgi membranes to a stable cytoplasmic structure, such as that which stacks the cisternae. Although no such structures were observed, in most of the studies, it is possible that this was due to the fixation and staining techniques used, since observation of intercisternal material in the Golgi apparatus is often dependent on sample processing. One study, however, on the reassembly of the Golgi apparatus after removal of BFA showed that after BFA treatment cells contained a perinuclear area which appeared to exclude the ER but contain the MTOC (Acalde *et al.*, 1992), and it was in this area that the Golgi apparatus re-formed. It was suggested that this area represents the zone of exclusion described above, and suggests that this is a stable, structural component of the Golgi apparatus.

1.4.7 The Golgi Apparatus in Mitosis

The fact that the mammalian Golgi apparatus is a single-copy organelle leads to a problem during mitosis when the organelle is partitioned between the two daughter cells.

The simplest mechanism by which this breakdown could occur would be the induction of a mechanism which prevents vesicle fusion during transport. The Golgi would continuously form vesicles which would be unable to fuse with the next cisterna in the stack and this would quickly result in the fragmentation of the Golgi apparatus (Warren, 1989; Warren, 1985). After mitosis, this fusion block would be released and the vesicles would then fuse again to form intact Golgi stacks in the two daughter cells.

Electron-microscopic studies have shown that during mitosis, the Golgi apparatus fragments to form approximately 150 tubulo-vesicular clusters which then shed vesicles which disperse throughout the cytosol (Lucocq *et al.*, 1987; Lucocq and Warren, 1987), after which segregation of Golgi-derived vesicles can occur presumably by a stochastic process (Birky, 1983). Upon exit from mitosis, Golgi-derived vesicles re-aggregate to form the interphase stack (Lucocq *et al.*, 1989).

Interestingly, the mitotic Golgi clusters appear to be embedded in an electron-dense matrix. The nature of this structure is unclear, but it may represent a mitotic form of the zone of exclusion or the intercisternal material described earlier, which may be required for the maintenance of Golgi stacking and/or morphology. This material would also have to be partitioned between daughter cells during mitosis, presumably also by a fragmentation process. The fact that it surrounds the Golgi clusters suggested that it binds to the membranes of the fragmented Golgi structures, as would be expected of such a matrix.

Fragmentation is thought to occur by a mitotic inhibition of vesicle fusion. Under such conditions, the Golgi apparatus would continue to produce vesicles but would not consume them, leading to the production of a fragmented Golgi apparatus (Warren, 1989; Warren, 1985). Such a mechanism seems possible since several other membrane trafficking steps, such as receptor-mediated endocytosis, ER-Golgi and Golgi-cell surface transport are also inhibited in mitosis (see Warren (1993b) for a review). In support of this hypothesis, inhibition of intra-Golgi transport has been shown to occur both *in vivo* (Collins and Warren, 1992) and *in vitro* (Stuart *et al.*, 1993; Mackay *et al.*, 1993). While resumption of transport after mitosis has been shown to occur at about the same time as reassembly of the Golgi stack (Souter *et al.*, 1993).

These data correlate the inhibition of transport with the mitotic fragmentation of the Golgi apparatus. However, they do not preclude the possibility that the Golgi stack is fragmented by a different mechanism and that the loss of transport is a direct consequence of this fragmentation. This possibility has largely been eliminated by the development of an *in vitro* assay which reconstitutes mitotic Golgi fragmentation (Misteli and Warren, 1994). After incubation with mitotic cytosol, the length of Golgi stacks reduce and there is a concomitant increase in the number of uncoated, small and large

vesicular profiles similar to those described *in vivo*. Incubation of Golgi stacks with mitotic cytosol in the presence of GTPγS causes an accumulation of COP-coated vesicles and furthermore depletion of coatomer from mitotic cytosol prevents vesicle accumulation and induces the formation of tubular networks characteristic of the induction of uncoupled membrane fusion by the removal of coatomer by Brefeldin A (Orci *et al.*, 1991).

These results indicate that Golgi fragmentation does occur as a result of the production of COP-coated vesicles during vesicular transport, and therefore, that Golgi fragmentation does indeed occur as a consequence of the inhibition of vesicle fusion. The fact that the vesicles that accumulate are not coated indicates that fusion is inhibited after vesicle docking and uncoating. This may explain the morphology of the Golgi clusters, since the docked vesicles could aggregate after uncoating. Interestingly, although treatment with interphase or mitotic cytosol in the presence of GTPγS leads to an increase in the amount of COP-coated vesicles observed, the morphology of the cisternae treated with the interphase cytosol appears not to alter while those treated with mitotic cytosol greatly reduced in length. Thus the budding of vesicles under interphase conditions does not lead to a fragmentation of the Golgi cisternae, suggesting that the central regions of the cisternae were resistant to fragmentation. Under mitotic conditions, almost complete fragmentation occurs as would be expected from the *in vivo* studies described above. This suggests that the central portions of the cisternae are stabilised in some way while the cisternal rims are not. This stabilisation may be performed by proteinaceous Golgi matrices such as those observed by electron microscopy, which could also be involved in cisternal stacking.

1.5 Proposed Golgi Stacking Mechanisms

As discussed above, there are as yet no direct data that have been able to explain the mechanism by which Golgi cisternae are stacked. What has been presented has been entirely microscopic and/or circumstantial, though the evidence is highly suggestive that the stack is stably maintained by specific protein interactions. Two speculative models regarding Golgi stacking have been recently suggested, and both are based on the observation that in Hela cells, the *medial*- and *trans*-Golgi enzymes are not uniquely present in *medial*- and *trans*-Golgi cisternae (Rabouille *et al.*, 1994; Nilsson *et al.*, 1993a). In fact, the *medial*-enzymes are present in the *medial*- and *trans*-cisternae while the *trans*-enzymes are present in the *trans*-cisterna and TGN. Thus the enzyme distributions overlap in the *trans*-cisterna, and though no evidence yet exists, it has been postulated that this distribution holds true for the *cis/medial* cisternae (Nilsson *et al.*, 1993b). Furthermore, it has also been postulated that the enzymes may not be distributed

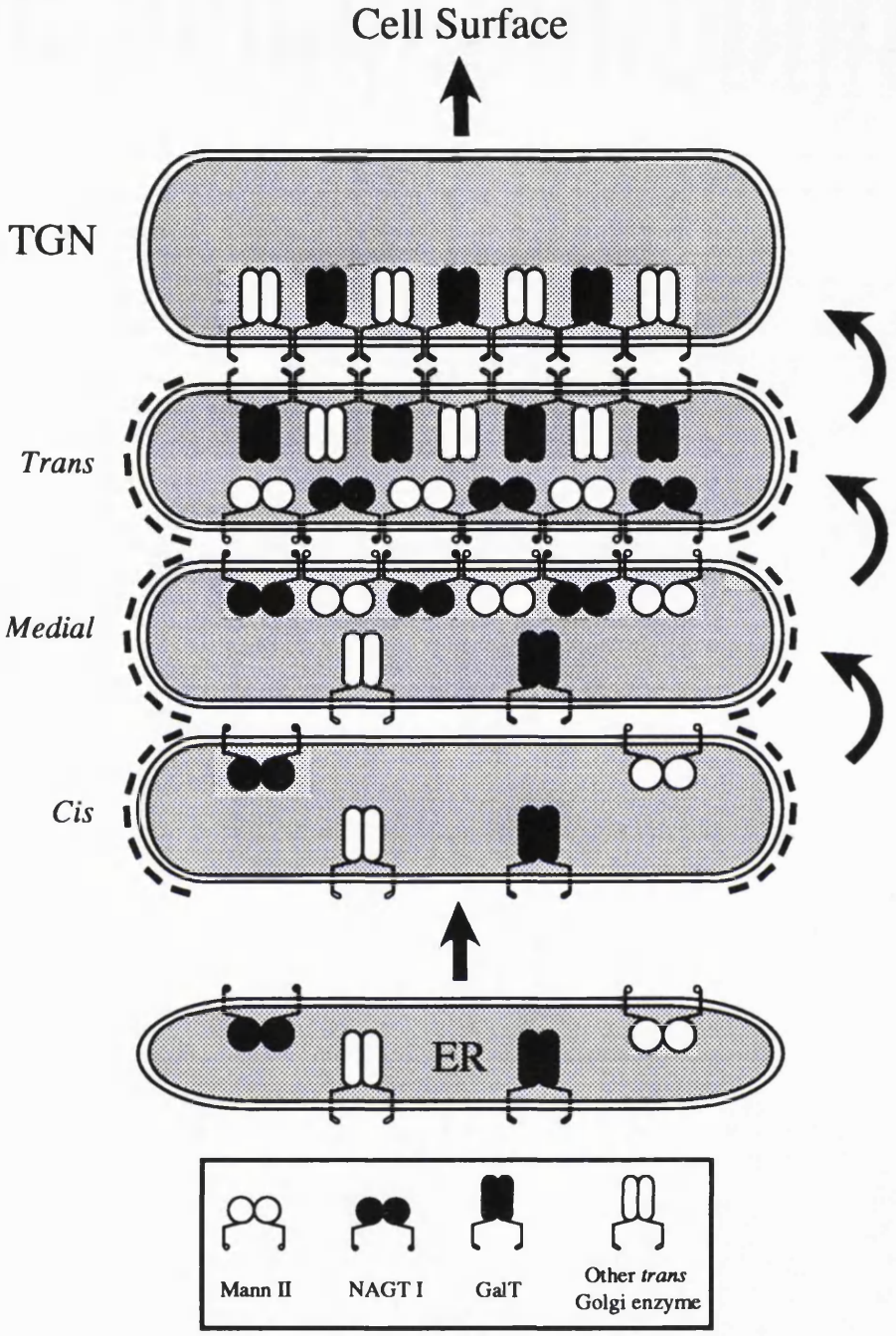

Figure 1.8: Schematic diagram of hypothesised overlapping distribution of Golgi enzyme. Golgi enzymes are not present in single Golgi cisternae but overlap between two. Enzymes are restricted to single cisternal faces of apposing cisternae. Newly synthesised enzymes progress from the ER and through the Golgi via COP coated vesicles which bud from dilated rims (- - - -) until they reach their correct location, at which point they are retained either by oligomerisation with other residents or because of the lipid composition of the bilayer in the target cisterna.

evenly within cisternae, but restricted to apposing faces of the adjacent cisternae. This is depicted schematically in figure 1.8, where newly synthesised enzymes are also shown progressing throughout the stack by vesicular transport until they arrive at their correct position of the stack where they are retained by their membrane-spanning domains either by oligomerisation or by the lipid composition of the cisternal membrane. It should be noted that the overlapping distribution need not only apply to resident Golgi enzymes but to all resident proteins, no matter what their function.

Such an arrangement of resident proteins could lead to stacking in two ways. The first hypothesis suggests that the resident proteins of the cisternae can interact with a cytoplasmic matrix (Nilsson *et al.*, 1993b). The possibility that the proteins in the apposing cisternal faces are the same simplifies such a mechanism since it minimises the number of different Golgi-protein binding sites that such a matrix would need to possess. Such a model postulates that several different matrices would exist, one for each apposed set of cisternae. The depiction of the interaction of two apposing cisternal membranes to such a matrix, as predicted by such a model, is depicted schematically in figure 1.9A. It should be noted that the Golgi proteins shown interacting with the hypothetical matrix via their cytoplasmic tails could be Golgi enzymes or other structural proteins. Interaction of the cytoplasmic domains of membrane-spanning proteins with components in the cell cytosol has precedence. For example, the spectrin-based membrane skeleton of erythrocytes interacts with the integral-membrane protein band III via ankyrin (Bennett, 1990). Membrane-spanning proteins are incorporated into endocytic vesicles by signals in their cytoplasmic tails which are recognised by cytoplasmic machinery (Glickman *et al.*, 1989). Furthermore, these signals can be very small since TGN38 can be recycled from the plasma membrane via a signal containing just four amino acids (Bos *et al.*, 1993), while endocytosis of influenza haemagglutinin can occur by the substitution of a single cysteine by a tyrosine residue (Lazarovits and Roth, 1988). Such a matrix would thus correspond to the intercisternal material observed by electron microscopy. Additionally, such a matrix could also function in maintaining the stability of the cisternae by, for example, preventing vesicle budding from regions other than the dilated rims.

The second model for Golgi stacking is also speculative and draws upon the SNARE hypothesis. According to this theory, apposing cisternae contain v-SNAREs and t-SNAREs respectively which can interact with each other. It has been proposed that interaction of the v-SNAREs of a given cisterna with the cognate t-SNAREs of the next cisterna would facilitate stacking (Rothman and Warren, 1994). Such a mechanism needs to invoke the existence of "fusion clamps" - proteins which prevent membrane fusion after SNARE coupling - to prevent uncoupled fusion of cisternal contents after the SNAREs have met by inhibiting the binding of SNAPs and NSF. Such a clamp is thought

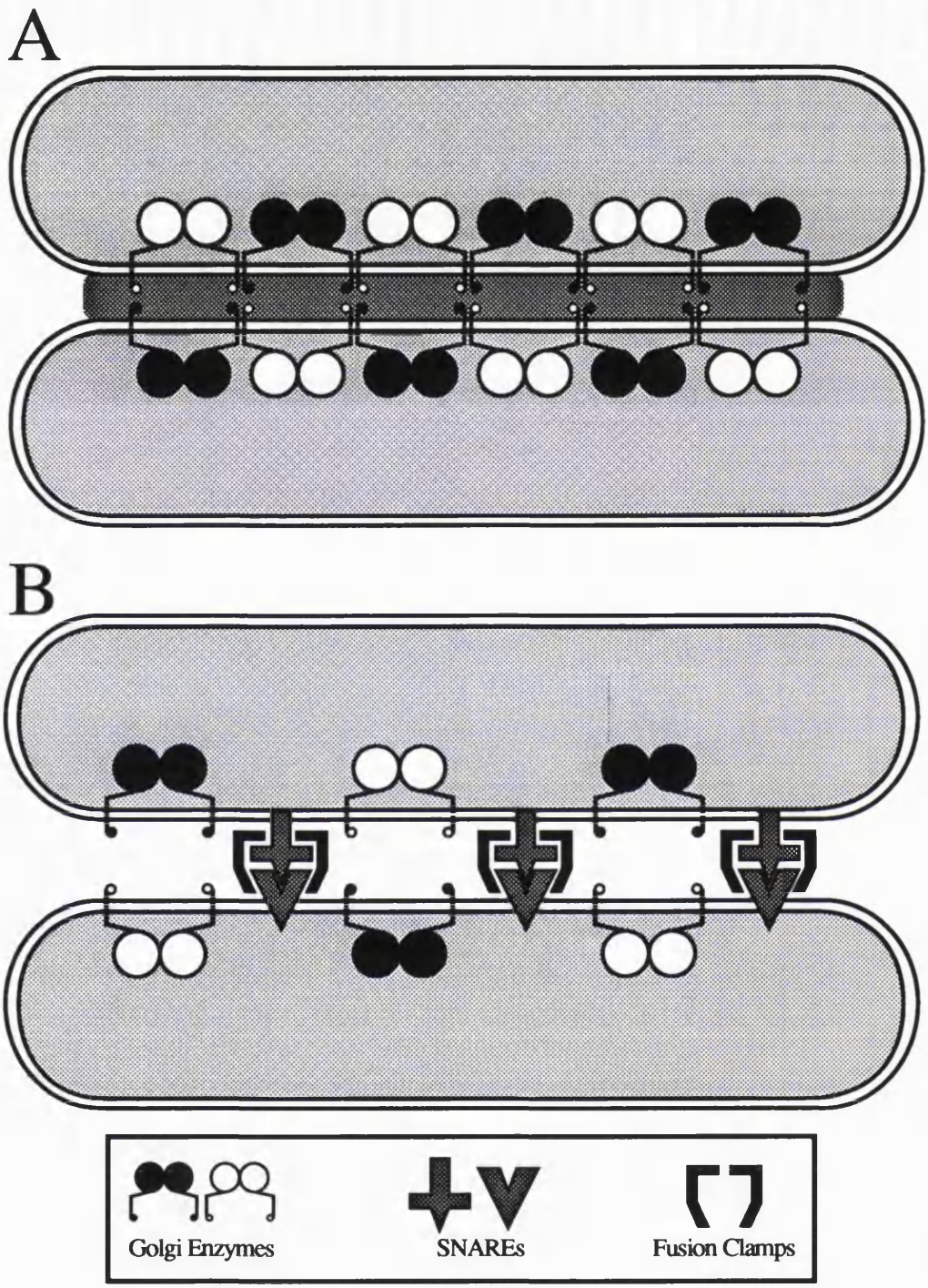

Figure 1.9: Schematic diagram of Golgi stacking mechanisms. Two proposed mechanisms of cisternal stacking are depicted. (A) Golgi resident proteins in apposing cisternae interact with an inter-cisternal matrix to facilitate stacking. (B) Cognate v-SNAREs and t-SNAREs in apposing membranes interact to cause stacking. Fusion of cisternae is prevented by the presence of fusion clamps which prevent the binding of SNAPs and NSF.

to exist at the synapse where synaptic vesicles are docked at the pre-synaptic membrane and only fuse after a calcium influx. This process is thought to be regulated by synaptotagmin, a protein with calcium-binding motifs which is postulated to be the fusion clamp (DeBello *et al.*, 1993). In this model, other Golgi-specific fusion clamps would be predicted to exist. It has been noted with interest that the 10nm gap between cisternal membranes is very similar to that between docked synaptic vesicles and synaptic membranes. The SNARE-complexes could theoretically represent the intercisternal cross-bridges observed by electron microscopy, though these structures are too large to represent single SNARE pairs, and may represent clusters of SNAREs or SNARE pairs which are associated with other structural components. This model is presented schematically in figure 1.9B, and obviates the requirement for several different structural matrices within the stack, since the specificity of cisternal apposition would be encoded simply by the residents of the different cisternae. This model, however, does not provide a mechanism for the structural stabilisation of the cisternae themselves, nor the maintenance of their striking morphology. If stacking was indeed due to the interaction of SNARE pairs, structural Golgi matrices would still be required to keep cisternae flattened. Furthermore, it is conceivable that SNAREs may simply add polarity to the stacking mechanism, while intercisternal matrices could still be required to stabilise the cisternal interactions.

At the moment, both models remain highly speculative. They do, however, provide for the first time specific models that can be tested experimentally. The lack of such intellectual frameworks has prevented biochemical analysis of the Golgi stacking mechanism in the past, but only future studies will be able to discriminate between these models or eliminate them entirely as viable possibilities.

1.6 Objective of the Thesis

The aim of this thesis was to isolate a structural matrix which was associated with the Golgi apparatus. Such matrices have been long observed by electron microscopy, and their existence has been inferred from several lines of indirect evidence, but to date none have been isolated in a biochemically functional form. Isolation of such a matrix would allow, for the first time, molecular studies regarding the mechanisms by which the Golgi cisternae are stacked and kept flattened, and also provide a foothold by which questions could begin to be addressed regarding the function of this striking morphology.

The approach taken was to draw an analogy between the previously observed Golgi intercisternal material and the major structural elements of the cell - the cytoskeleton. These consist of microtubules, actin-based microfilaments and intermediate-type filaments. All of these macro-molecular structures are resistant to extraction with non-

ionic detergents and salt. It was reasoned that a Golgi matrix which played a structural role in the maintenance of the classical Golgi morphology would also possess such properties. Thus purified stacked Golgi membranes from rat liver were extracted with the non-ionic detergent Triton X-100 and the insoluble material was examined in an attempt to identify putative Golgi matrix components. It was hoped that by identification of these proteins it would be possible to begin to study the components of the Golgi matrix and thus to begin to elucidate its structure and function.

Following identification of such candidate proteins, they could be characterised to determine whether they exhibited the properties that would be expected for Golgi matrix components before continuing with studies to elucidate their true nature and to identify molecules with which they might interact.

In this thesis, I describe the identification of such a Triton X-100 insoluble protein and studies which have utilised it in identifying a proteinaceous Golgi matrix with the above properties.

Chapter 2

Materials and Methods

2.1 Chemicals

All reagents were of analytical grade or better and were purchased from either Sigma or BDH, unless indicated otherwise. Radiochemicals were all purchased from NEN. All water was filtered by the Whatman Milli-Q system.

2.2 Working Reagents

2.2.1 Stock Solutions

Acrylamide/bis-Acrylamide (40%; 29:1): Dissolve 6.6g of N,N'-methylene bis-acrylamide in 100ml of H_2O, add 193.4g of acrylamide and make up to a final volume of 500ml. Add 20g of deionising AG 501-X8 beads (BioRad) to remove any acrylic acid and stir for 1hr. Filter the solution through Whatman No.1 paper and store at 4°C..

AMC Buffer: Dissolve 2.1g of sodium acetate in 450ml of H_20, adjust to pH5.5with HCl. Add 0.5ml of 2M $MnCl_2$ and $CaCl_2$ and make up to a final volume of 500ml. Store at room temperature.

AMP (50mM): Dissolve 17.4mg in 1ml of H_2O and store at -20°C.

Ammonium persulphate (10% (w/v)): Just before use dissolve 100mg in 1ml of H_2O.

ATP pH7.0 (0.2M): Dissolve 605 mg in 3ml of H_2O. Adjust to pH 6.5-7.0 with 1M NaOH and make up to a final volume of 5.0ml. Store at -20°C in 200µl aliquots. Do not freeze-thaw more than 5 times.

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

BSA/PBS (0.5% (w/v)): Dissolve 0.25g of BSA fraction V (Boehringer Manheim) in 50ml of ice-cold PBS. Pass through a 0.45µm nitrocellulose filter and store at 4°C. Use within one week.

$CaCl_2$ (2M): Dissolve 29.4g of $CaCl_2$ in a final volume of 100ml of H_2O. Store at room temperature.

CAPS pH11.0 (100mM): Dissolve 11.1g of CAPS in 450ml of ddH_2O. Adjust the pH to 11.0 with NaOH and make up to 500ml. Store at room temperature.

CMP-NANA (5mM): Dissolve 1mg in 325µl of H_2O and store in 50µl aliquots at -20°C.

Coomassie Blue Stain: Dissolve 5.0g in a solution composed of 100ml of methanol, 150ml of acetic acid and 850ml of H_2O. Filter through Whatman Number 1 paper and store at room temperature.

Cycloheximide (3mg/ml): Dissolve 45mg in 15ml of H_2O and use immediately.

Cytochalasin B (20mg/ml): Dissolve 20mg in 1ml of DMSO and store at 4°C.

Cytochalasin B (20.9mM): Dissolve 10mg in 1ml of DMSO and store at 4°C.

Cytochrome c (1mM): Dissolve 185.7mg of horse heart cytochrome c in 15ml of H_2O, pass through a 0.45µm filter and store at -20°C in 1ml aliquots..

DTT (1M): Dissolve 15.4mg in 1ml of H_2O and use immediately.

EDTA (200mM): Dissolve 7.4g in 70ml of H_2O and adjust pH to 7.2 with KOH. Make up to 100ml and store at room temperature.

EGTA (0.5M): Dissolve 95.1g in 450ml of H_2O and adjust pH to approximately 7.0 with KOH. Make up to 500ml and store at room temperature.

FFS Buffer (10x): Dissolve24.2g of Tris and 10g of TX-100 in 900ml of H2O. Adjust the pH to 7.5 with HCl and make up to a final volume of 1l. Store at 4°C.

GlcNAc (1M): Dissolve 221mg in 1ml of H_2O and store at -20°C..

Glycine (1M): Dissolve 7.4g of glycine in a final volume of 100ml of H_2O. Store at room temperature.

Glycine (50mM) pH10.4/5mM EDTA: Dissolve 1.88g in 450ml H_2O and increase pH to approximately 10 with NaOH. Add 12.5ml of EDTA, adjust pH to 10.4 and make up to 500ml. Store at room temperature.

HCl (10mM): Add 87.7µl of 11.4M HCl to 100ml of H_2O and store at room temperature.

HEPES pH7.0 (50mM): Dissolve 1.2g in H_2O, adjust pH to 7.0 with KOH and make up to a final volume of 100ml. Store at room temperature.

HMS Buffer: Dissolve 2.4g of HEPES, 20g of sucrose and 10µl of 2M $MgCl_2$ in 180ml of H_2O and adjust pH to 8.0 with NaOH. Store at room temperature.

Iodoacetamide (0.5M): Dissolve 9.25mg in 100µl H_2O and use immediately.

IP lysis buffer (2x): Dissolve 484mg of Tris, 1.75g of NaCl and 1g of TX-100 in 80ml of H_2O and adjust pH to 8.0 with HCl. Make up to 100ml and store at 4°C.

IP wash buffer Dissolve 1.2g of Tris, 4.4g of NaCl and 0.5g of TX-100 in 450ml of H_2O and adjust pH to 8.0 with HCl. Make up to 500ml and store at 4°C.

KCl (1M): Dissolve 7.5g in a 100ml final volume of H_2O and store at room temperature.

KCN (30mM): Dissolve 195.4mg in 100ml of H_2O and use immediately.

KEHM Buffer (5x): Dissolve 9.2g of KCl and 29.8g of HEPES in 400ml of ice-cold H_2O. Add 2.4ml of 2M $MgCl_2$ and 50ml of 0.5M EGTA. Adjust pH to 7.4 with KOH and make up to 500ml with H2O. Store at 4°C and dilute 5 times before use.

MES pH6.1 (0.5M): Dissolve 9.8g in 90ml of H_2O and adjust pH to 6.1 with NaOH. Make up to a final volume of 100ml and store at room temperature.

$MgCl_2$ (2M): Dissolve 40.7g of $MgCl_2.6H_2O$ in final volume of 100ml of H_2O and store at room temperature.

MMS Buffer: Exactly as for TMMS but lacking TX-100.

$MnCl_2$ (2M): Dissolve 9.9g of $MnCl_2.4H_2O$ in a final volume of 25ml of H_2O. and store at room temperature.

Na_2CO_3 (0.25M): Dissolve 13.3g in a final volume of 500ml of H_2O. Store at room temperature.

NaCl (1M): Dissolve 5.8g in a final volume of 100ml of H_2O and store at room temperature..

NADH (10mM): Dissolve 7.1mg (reduced form) in 1ml of 0.2M sodium phosphate pH7.4 and use immediately.

NAG (13.6mM): Dissolve 46.6mg of NAG in 10ml of DMSO and store at -20°C in 1ml aliquots.

$NaHCO_3$ (10% (w/v)): Dissolve 10g of $NaHCO_3$ in a final volume of 100ml of H_2O. Store at room temperature.

50mM NH_4HCO_3 (10x): Dissolve 39.5g of NH_4HCO_3 in 900ml of H_2O. Make up to 1l and store at 4°C.

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

Ovalbumin (200mg/ml): Dissolve 1g ovalbumin in H_2O to a final of volume of 5ml. Pass through a 0.45μm nitrocellulose filter and store at -20°C in 500μl aliquots.

Ovomucoid (175mg/ml): Dissolve 1g ovomucoid in H_2O to a final of volume of 5.7ml. Pass through a 0.45μm nitrocellulose filter and store at -20°C in 500μl aliquots.

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

PBS (Phosphate Buffered Saline): Made up by the ICRF Central Services containing 10mM sodium phosphate pH7.2, 150mM NaCl and 3mM KCl.

PNM (20mM): Dissolve 60.3mg of PNM in 10ml of DMSO. Store at -20°C in 1ml aliquots.

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

PTA (1% (w/v))/0.5M HCl: Dissolve 5g PTA in 478ml of H_2O final volume. Add 22ml of 11.4M HCl and store at 4°C.

PTA (5% (w/v))/20% TCA: Dissolve 5g PTA and 20g of TCA in a final volume of 100ml of H_2O. Store at room temperature.

PM Buffer (0.5M potassium phosphate pH6.7): Make up 500ml solutions of 0.5M anhydrous K_2HPO_4 (43.6g) and 0.5M anhydrous KH_2PO_4 (34g). To 400ml of the latter, gradually add the former until the pH reaches 6.7. Store at 4°C.

PMSF (40mg/ml): Dissolve 40mg of PMSF was in 1ml of DMSO and use immediately.

Rotenone (1.5mM): Dissolve 2.96mg in 5ml of DMSO and use immediately.

SDS (5% (w/v)): Dissolve 5g of SDS in a final volume of 100ml of H_2O and store at room temperature.

SDS (20% (w/v)): Dissolve 20g of SDS in a final volume of 100ml of H_2O and store at room temperature.

SDS-PAGE Electrode Buffer (5x Stock): Dissolve 151.5g of Tris, 713.2g of glycine and 25g SDS in a final volume of H_2O. Dilute 5-fold with water before use.

SDS-PAGE Loading Buffer: Dissolve 34.2g of sucrose and 4mg of bromophenol blue in 10ml of 2M Tris pH8.8 and 2.5ml of 200mM EDTA and make up to 100ml with H_2O. Pass through a 0.45µm nitrocellulose filter and store at 4°C.

Sodium acetate pH6.0 (0.2M): Dissolve 1.6g of sodium acetate in H_2O, adjust pH to 6.0 with acetic acid and make up to 100ml. Store at room temperature.

Sodium cacodylate pH6.6 (0.4M): Dissolve 17.12g sodium cacodylate in 180ml of H_20, adjust to pH6.6 with HCl and make up to a final volume of 200ml. Store at room temperature.

Sodium citrate pH5.0 (0.1M): Dissolve 2.94g of trisodium citrate to a final volume of 100ml of H_2O, and 2.1g of citric acid in a further 100ml H_2O. Mix 35ml of citric acid and 65ml of trisodium citrate and store.

Sodium deoxycholate (0.15% (w/v)): Dissolve 150mg in 100ml of H_2O and store in 1ml aliquots at -20°C. Can be freeze-thawed indefinitely.

Sodium phosphate pH 6.0 (0.2M): Dissolve 2.84g of Na_2HPO_4 in a final volume of 100ml of H_2O, and 2.72g of NaH_2PO_4 in a further 100ml of H_2O. Mix 87.7ml of NaH_2PO_4 and 12.3ml of Na_2HPO_4 and store at room temperature.

Sodium phosphate 7.4 (0.2M): Dissolve 2.84g of Na_2HPO_4 in a final volume of 100ml of H_2O, and 2.72g of NaH_2PO_4 in a further 100ml of H_2O. Mix 19ml of NaH_2PO_4 and 81ml of Na_2HPO_4 and store at room temperature.

Sodium tetraborate (20mM)/2mM EDTA: Dissolve 3.8g of sodium tetraborate in 400ml of H_2O. Add 5ml of 200ml EDTA and make up to a final volume of 500ml with H_2O. Store at room temperature.

Sucrose (2M): Dissolve 342.3g in H_2O by stirring at 50°C. Make up to a final volume of 500ml and store at 4°C.

Sucrose (2.3M): Dissolve 393.7g in H_2O by stirring at 50°C. Make up to a final volume of 500ml and store at 4°C.

Sucrose (60% (w/v)): Dissolve 60g in H_2O to a final volume of 100ml. Store at 4°C.

TCA (72% (w/v)): Dissolve 72g of TCA in a final volume of 100ml of H_2O and store at room temperature.

TEA pH7.4 (10mM)/150mM KCl: Dissolve 373mg of triethanolamine and 2.8g of KCl in 200ml of ice-cold H_2O. Adjust pH to 7.4 with KCl and make up to 250ml. Store at 4°C.

TG Buffer (0.2M Tris/1.5M glycine): Dissolve 12.1g of Tris and 56.3g glycine in a total of 500ml of H_2O. Store at room temperature.

TMC Buffer: Dissolve 1.2g of Tris in 450ml of H_20 and adjust to pH7.4 with HCl. Add 0.5ml of 2M $MnCl_2$ and $CaCl_2$ and make up to a final volume of 500ml. Store at room temperature.

TMC/0.5M NaCl: Dissolve 1.2g of Tris and 14.6g of NaCl in 450ml of H_20 and adjust to pH7.4 with HCl. Add 0.5ml of 2M $MnCl_2$ and $CaCl_2$ and make up to a final volume of 500ml. Store at room temperature.

TMG Buffer: Dissolve 2.4g of methyl-a-D-mannopyranoside and 2.8g of GlcNAc in a final volume of 50ml of TMC Buffer and use immediately.

TMMS Buffer: Dissolve 1.05g of MOPS, 2g of TX-100, 10g of sucrose and 5µl of 2M $MgCl_2$ in 80ml of ice-cold H_20. Adjust the pH to 7.0 with NaOH and make up to 100ml. Store at 4°C. Just before use, make up a 1M solution of DTT and dilute 1000-fold into an aliquot of buffer to create TMMDS buffer.

Tris pH6.8 (0.5M): Dissolve 15.1g of Tris in 200ml H_2O, adjust the pH to 6.8 with HCl and make up to a final volume of 250ml. Store at 4°C.

Tris pH7.0 (10mM): Dissolve 121mg of Tris in 90ml H_2O, adjust the pH to 7.0 with HCl and make up to a final volume of 100ml. Store at 4°C.

Tris pH7.0 (0.5M): Dissolve 15.1g of Tris in 200ml H_2O, adjust the pH to 7.0 with HCl and make up to a final volume of 250ml. Store at 4°C.

Tris pH7.4 (10mM)/150mM NaCl: Dissolve 1.2g of Tris and 8.8g of NaCl in 900ml H_2O, adjust the pH to 7.4 with HCl and make up to a final volume of 1l.

Tris pH9.0 (10mM): Dissolve 121.1mg of Tris in 90ml H_2O, adjust the pH to 9.0 with HCl and make up to a final volume of 100ml. Store at room temperature.

Tris pH8.8 (2M): Dissolve 121.1g of Tris in 400ml H_2O, adjust the pH to 8.8 with HCl and make up to a final volume of 500ml. Store at 4°C.

Tris unbuffered (2M): Dissolve 24.2g of Tris in a final volume of 100ml of H_2O and store at room temperature.

Tris/maleate pH6.9 (0.5M): Dissolve 6.06g Tris and 5.81g maleic acid in H_2O, adjust to pH 6.9 with NaOH and make up to a final volume of 100ml. Store at room temperature.

TTMS Buffer: Dissolve 149mg of TEA, 2g of TX-100, 10g of sucrose and 5µl of 2M $MgCl_2$ in 80ml of ice-cold H_20. Adjust the pH to 7.5 with HCl and make up to 100ml. Store at 4°C. Just before use, make up a 1M solution of DTT and dilute 1000-fold into an aliquot of buffer to create TTMDS buffer.

UDP-galactose (10mM): Dissolve 25mg in H_2O to a final volume of 4.42ml and store at -20°C in 200µl aliquots.
UDP-GlcNAc (50mM): Dissolve 60mg in a final volume of 1.84ml of H_2O store in 200µl aliquots at -20°C.
Tween-20 (20% (w/v)): Dissolve 100g in a total of 500ml of H_2O. Store at 4°C.
TX-100 (10% (w/v)): Dissolve 10g in a final volume of 100ml of H_20 and store at 4°C.
TX-114 (10% (w/v)): TX-114 was prepared as described below.

2.2.2 Tissue Culture Reagents

All cells were grown in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) containing 4.5g/l glucose and supplemented with 10% (v/v) foetal calf serum (FCS; GIBCO), 2mM glutamine, 1% (v/v) non-essential amino acids (GIBCO) and 100U/ml each of penicillin and streptomycin in an atmosphere of 5% CO_2/95% air at 37°C. For the production of mannosidase I substrate, cells were grown as above but in DMEM that contained low concentration of glucose (500mg/l ; ICRF Central Services) and 10mM sodium pyruvate. Trypsin solutions were supplied by the ICRF Central Services and consisted of 0.25% (w/v) trypsin in versene solution.

2.2.3 Assay Mixtures

β-Hexoseaminidase: 55.6mM sodium citrate pH5.0, 0.28% (w/v) TX-100, 4.2mM NAG
GalT: 50mM sodium cacodylate pH6.6, 21.9mg/ml ovomucoid, 50mM β-mercaptoethanol, 0.25mM UDP-galactose, 0.25% (w/v) TX-100, 2.5mM ATP, 50mM $MnCl_2$, 0.5µCi/ml UDP-[^{3}H]galactose
Mann I: 62.5mM sodium acetate pH6.0, 1.25mM $CaCl_2$, 1.25mM $MngCl_2$, 0.13% (w/v) TX-100, 50DPM/µl substrate
Mann II: 145mM sodium phosphate pH6.0, 0.25% (w/v) TX-100, 5mM PNM
NADH-cytochrome c reductase: 52.6mM sodium phosphate pH7.4, 1.3mg/ml cytochrome c, 0.11% (w/v) TX-100, 0.11mM NADH, 1.6µM rotenone, 0.32mM KCN
NAGT I: 62.5mM Tris/maleate pH6.9, 25mg/ml ovalbumin, 12.5mM KCl, 1.25mM UDP-GlcNAc, 0.13% (w/v) TX-100, 6.25mM $MnCl_2$, 6.25mM $MgCl_2$, 1.25µCi/ml UDP-[^{3}H]GlcNAc
NAGT II: 125mM MES pH6.1, 125mM NaCl, 25mM $MnCl_2$, 0.63mM UDP-GlcNAc, 0.13% (w/v) TX-100, 12.5mM AMP, 250mM GlcNAc, 2µCi/ml UDP-[^{3}H]GlcNAc
SialylT: 125mM Tris pH7.0, 5mg/ml asialotransferrin, 0.63% (w/v) TX-100, 62.5µMCMP-NANA, 2.09µCi/ml CMP-[^{3}H]NANA

2.3 Enzyme Assays

Several enzymes were assayed during the course of this study as markers of various intracellular compartments. All samples were assayed in duplicate and the results averaged, except for NAGT II assays due to a limited supply of the substrate.

2.3.1 β-hexoseaminidase

This glycosidase was used as a marker for the lysosome and the assay is essentially as that described previously by Landegren (1984). The assay measures the enzymatic cleavage of p-nitrophenol-N-acetyl-β-D-glucosaminidine (NAG) to release p-nitrophenol. The accumulation of this product can be observed spectrophotometrically by an increase in absorbance at 405nm.

An assay mixture was made up containing: 2.5ml of 0.1M sodium citrate pH5.0, 125µl of 10% (w/v) TX-100 and 1.375ml of 13.6mM NAG. 180µl of this mix was added to 20µl of sample or 20µl of water to act as a blank. After incubation at 37°C for 30 min the reaction was stopped by the addition of 600µl of 50mM glycine pH10.4/5mM EDTA. Samples were spun on a benchtop microfuge at 14,000rpm for 5min and their absorbances measured at 405nm on an Ultrospec Plus spectrophotometer (LKB), after using the blank to set the machine to zero.

The linearity of the assay was determined for rat liver homogenate,

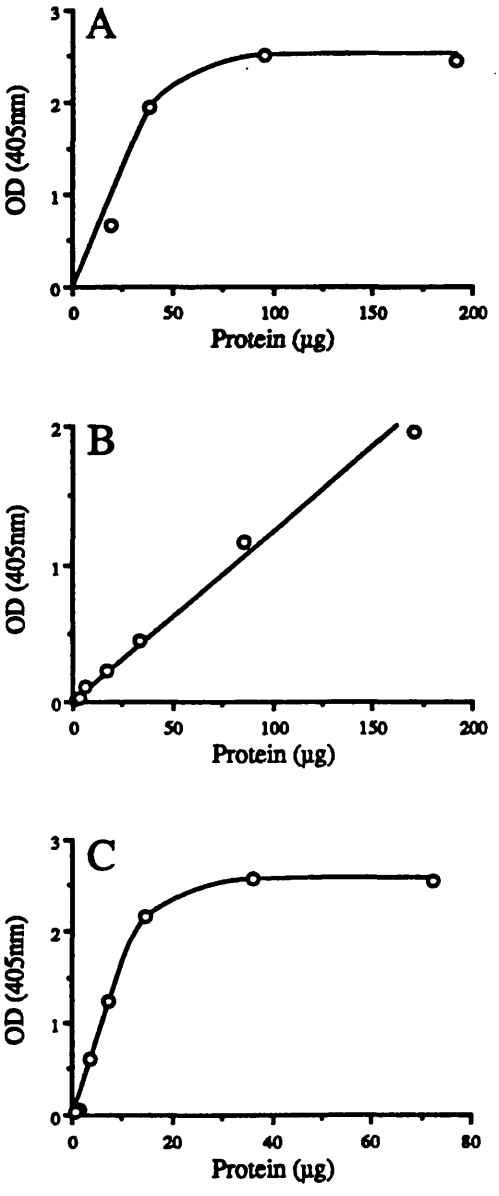

Figure 2.1: Linearity of the β-hexoseaminidase assay. Increasing amounts of rat liver homogenate (A), intermediate fractions (B) and Golgi membranes (C) were assayed for β-hexoseaminidase. The absorbance after a 30 min incubation was measured and plotted against the amount of protein in each sample.

intermediate-fraction and purified Golgi membranes. Increasing dilutions (1, 2, 5, 10, 20, 50 and 100-fold) of these samples were assayed for activity and the results are presented in figure 2.1. Due to the presence of large amounts of haemoglobin in the homogenate and intermediate fractions, it was necessary to correct for its absorbance during measurement of p-nitrophenol release. This was achieved by measuring the absorbance of parallel samples incubated in the absence of NAG. These values were subtracted from the sample absorbances to correct for the presence of haemoglobin. Additionally, a degree of turbidity was observed in the more concentrated homogenates. To assess the level of interference due to light scattering in these samples, the percentage transmission at 700nm was measured. This was found to range from 36-82% in the 1-5-fold diluted samples and thus these were not plotted. Loss of transmission for the intermediate and Golgi fractions were found to be negligible.

These data demonstrated that the assay was linear for the homogenate at 50-100-fold dilutions (up to 38μg of protein), for the intermediate fraction at 1-100-fold (up to 170μg) and for the Golgi membranes at 5-100-fold (up to 14μg).

2.3.2 NADH-Cytochrome c Reductase

NADH-cytochrome c reductase activity was used as a marker for the ER. This was measured according to the method of Sottocasa *et al.* (1967) except that assay volumes were reduced from 5ml to 1ml. This activity is present in both mitochondria and the ER. The mitochondrial activity is due to a single polypeptide and is coupled directly to the electron transport chain. In contrast, the ER activity is not due to a single protein but to several acting in concert (Fleischer and Fleischer, 1970), and is not coupled to electron transport. The ER activity can, therefore, be distinguished from the mitochondrial by the addition of the electron transport uncoupler, rotenone (Sottocasa *et al.*, 1967).

An assay mixture was made up containing: 12.5ml of 0.2M sodium phosphate pH7.4, 5.0ml of 1mM cytochrome c, 500μl of 10% (w/v) TX-100, 500μl of 10mM NADH, 50μl of 1.5mM rotenone, 500μl of 30mM KCN and 28.45ml of H_20. 950μl of this mix was added to 50μl of sample or 50μl of water as a blank. After incubation at 37°C for 30min samples were spun on a benchtop microfuge at 14,000rpm for 5min and their absorbances measured at 550nm after using the blank to set the absorbance to zero.

The linearity of this assay for rat liver homogenate, intermediate fraction and Golgi membranes was measured exactly as for β-hexoseaminidase, except that undiluted samples were omitted (fig. 2.2). Blanks for the correction for the presence of haemoglobin were produced with an assay mix lacking NADH. The 2 and 5-fold dilutions of the homogenate were again determined to be too turbid to be reliable and were omitted.

These data show that the assay is linear for the homogenate at 50-100-fold dilutions (up to 74μg of protein), for the intermediate fraction at 2-100-fold (up to 121μg) and for the Golgi membranes at the same range (up to 49μg).

2.3.3 α1,2-Mannosidase I (Mann I)

Mannosidase I catalyses the removal of mannose residues from Man_{7-8}-$GlcNAc_2$ N-glycans to yield Man_5-$GlcNAc_2$ structures. Since this is the first step in the processing of N-linked oligosaccharides that occurs outside the ER, it is thought to be a *cis*-Golgi enzyme. The Mann I assay measured the removal of tritiated mannose from glycopeptides bearing the above oligosaccharide structure which were produced from metabolically labelled cells.

2.3.3.1 Preparation of Lectin Columns

Enzymatic release of tritiated mannose from Mann I substrate was measured by the selective retention of unreacted substrate on lectin columns. The columns consisted of a 50:50 mixture of Concanavalin A-Sepharose (ConA) and wheat germ agglutinin-agarose (WGA). The former binds the unreacted substrate while the latter binds contaminating complex oligosaccharides in the substrate preparation which would otherwise be counted as released mannose.

Columns were prepared, based on the methods of Szumilo and Elbein (1985). from 1ml syringes which had been plugged with glass wool. ConA and WGA beads were resuspended in TMS buffer and the mixture

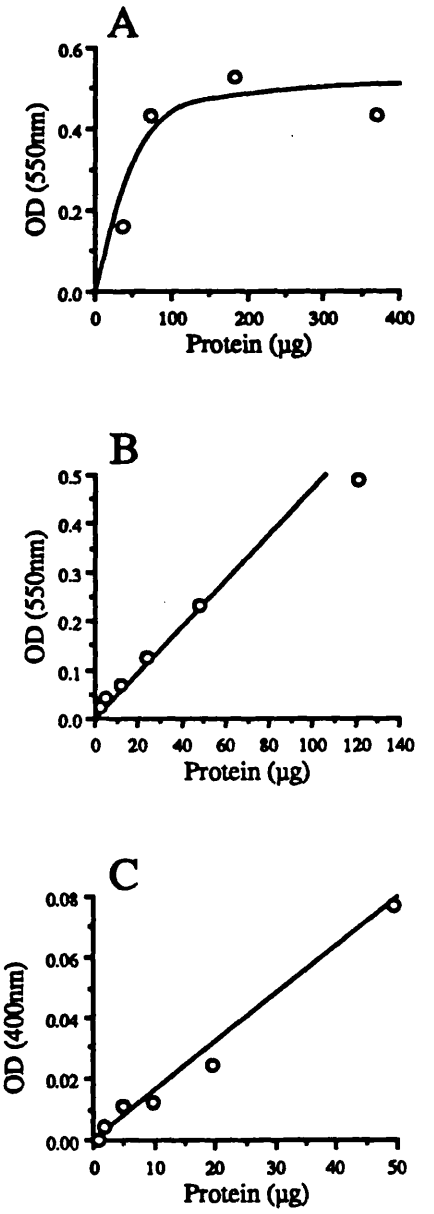

Figure 2.2: Linearity of the NADH-cytochrome c reductase assay. Increasing amounts of rat liver homogenate (A), intermediate fractions (B) and Golgi membranes (C) were assayed for NADH-cytochrome c reductase. The absorbance after a 30 min incubation was measured and plotted against the amount of protein in each sample.

poured into the syringes to give packed gel volumes of 0.5ml. The columns were washed with 5ml of TMS/0.5M NaCl followed by a further 5ml of TMS alone. Columns were stored at 4°C in TMS containing 0.02% (w/v) sodium azide.

2.3.3.2 Preparation of the Mannosidase I Substrate

Mannosidase I glycopeptide substrate (Man_{7-8}-$GlcNAc_2$- peptide) was originally prepared from processing intermediates of VSV-infected CHO cells (Kornfeld *et al.*, 1978; Tabas and Kornfeld, 1979), or from the oligosaccharide-processing mutant cell lines LPC1 (Tabas *et al.*, 1978) or clone 6 (Tabas and Kornfeld, 1978). Here an alternative approach was taken (Szumilo and Elbein, 1985), utilising the mannose analogue deoxymannojirimycin (dMM; 1,5-dideoxy-1,5-imino-D-mannitol). This has been shown to be a specific inhibitor of mannosidase I (Bischoff and Kornfeld, 1984) and to interfere with N-linked oligosaccharide processing *in vivo*. Cells treated with this drug still express their cell-surface proteins but they do not acquire Endo-H resistance, indicating that carbohydrate processing has been blocked at an early stage (Burke *et al.*, 1984; Fuhrmann *et al.*, 1984). Furthermore, glycopeptides derived from dMM treated Hela cells have been shown to bear Man_{7-8}-$GlcNAc_2$ oligosaccharides (Peat, 1993) and are, therefore, excellent substrates for Mann I.

Mann I substrate glycopeptides were prepared from integral membrane proteins of Hela S3 cells grown in dMM and [^{3}H]-mannose. Glycoproteins were isolated by TX-114 phase separation (Featherstone *et al.*, 1985) and glycopeptides produced by proteinase K digestion.

Cells were grown as described in section 2.11. Hela S3 cells were grown to 60% confluence in four 150cm^2 flasks, and for a further 2hr in 15ml of low glucose DMEM/10mM sodium pyruvate to stimulate the uptake of mannose. Sodium pyruvate was provided to allow the cells to undergo glycolysis in the absence of glucose. After this incubation, 30µl of 10mg/ml dMM (Boehringer Mannheim) was added and the cells grown for a further 2hr before the addition of 75µl of 25Ci/mmole [^{3}H]-mannose to each flask. The cells were then grown for 24hr before harvesting. Cells were washed 3 times in ice cold PBS and trypsinised. Trypsin was quenched with 10ml of low glucose DMEM and the detached cells pooled and spun in a 500E centrifuge (WIFUG Lab. Centrifuges) for 2min at 1400rpm.

TX-114 separation was carried out by resuspending the cell pellet in 5ml of 1% (w/v) TX-114/PBS and incubating on ice for 10min. The extract was divided into 1ml aliquots and spun at in a microfuge at 14,000rpm for 5min at 4°C to sediment cellular debris and the supernatants warmed to 37°C for 3min. The samples were then spun at 5000rpm for 5min at room temperature. The detergent pellets were resuspended in 1ml of ice cold

0.06% (w/v) TX-114/PBS and the above phase separation repeated 3 times. The detergent pellets were combined and resuspended in 1ml of ice cold 0.06% (w/v) TX-114/PBS followed by a final phase separation step.

The detergent pellet was resuspended in 500µl of 50mM HEPES pH7.0 containing 10mg/ml of proteinase K (Boehringer Mannheim) and incubated with rotation for 1hr at 37°C. The sample was then spun at 5000rpm for 5min in a benchtop microfuge at room temperature. 10% (w/v) TX-114 was added to the supernatant to a final concentration of 2% and the sample cooled on ice and warmed at 37°C for 3min each before being spun as before. The supernatant was removed, cooled and warmed and spun once more to remove any remaining TX-114. SDS was added to the supernatant to a final concentration of 0.1% (w/v) as were a few grains of sodium azide. The sample was incubated for 16-20hr at 57°C. Proteinase K was inactivated by heating to 95°C for 3min, cooling to room temperature and addition of 40mg/ml PMSF to a final concentration of 40µg/ml.

5µl of this final substrate was added to 4ml of Ready Micro liquid scintillant (Beckman) and counted in the tritium channel of a 6000IC scintillation counter (Beckman). The sample was diluted to 1000dpm/µl with H_2O, aliquoted and stored at -20°C. If samples contained less than 1000dpm/µl they were freeze dried and taken up in the appropriate volume of water.

2.3.3.3 Procedure

The assay is based on that described by Szulmilo and Elbein (1985), except that separation of the reaction products was achieved using columns that contained a 50:50 mixture of Concanavalin A-Sepharose and wheat germ agglutinin-agarose.

An assay mixture was made up containing 1.25ml of 0.2M sodium acetate pH6.0, 2.5µl each of 2M $CaCl_2$ and 2M $MgCl_2$, 50µl of 10% (w/v) TX-100, 250µl of substrate and 2.445ml of H_20. 80µl of this mixture was added to each 20µl sample (and to 20µl of water to act as a blank), and incubated at 37°C for 1hr. The reaction was stopped by the addition of 100µl of 5% (w/v) TCA/ 20% (w/v) TCA. Samples were spun in a microfuge at 14,000rpm for 5 min to remove precipitated protein and the supernatants removed. 125µl of 10% (w/v) $NaHCO_3$ was added to each sample and incubated at 95°C until the effervescence had stopped. Samples were microfuged at 14,000rpm for 5min after the addition of 600µl of TMC buffer and the supernatants loaded onto 0.5ml WGA/ConA columns. Eluents from each column were collected and pooled with a subsequent wash-out with 0.5ml of TMC. The samples were counted in the tritium channel of a scintillation counter after the addition of 4ml of liquid scintillant. The value of the blank was subtracted from each of the samples.

The linearity of the Mann I assay was determined for rat liver Golgi membranes. Samples were diluted 50, 100, 200, 300, 400 and 500-fold and assayed for Mann I (fig. 2.3). These data showed that the Mann I assay was linear for Golgi membranes at these dilutions (0-1.4µg of protein).

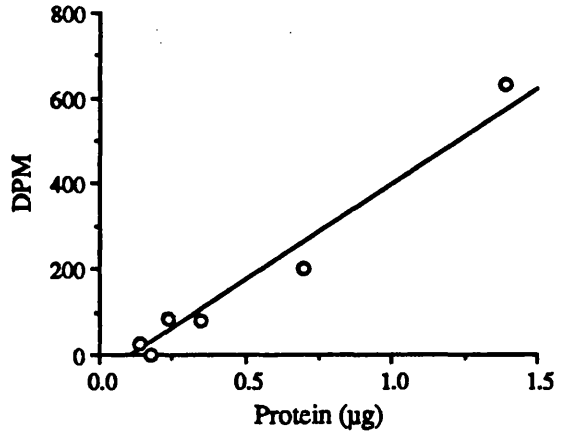

Figure 2.3: Linearity of the Mannosidase I Assay. Increasing dilutions of Golgi membranes were assayed for Mann I. After a 60min incubation he amount of tritiated mannose released was plotted against the total amount of protein in each sample.

2.3.3.4 Regeneration of Lectin Columns

Oligosaccharides were eluted using TMG elution buffer consisting of TMS buffer containing 250mM methyl-a-D-mannopyranoside and 250mM GlcNac. The elutes removes bound, unreacted Mann I substrate from ConA and the latter elutes the complex oligosaccharides from WGA.

Columns were washed with 1ml of TMG and left to stand in this buffer at 4°C overnight. Columns were then washed with 5ml of TMC/0.5M NaCl, 5ml of AMC and 5ml of TMC. Columns were then stored at 4°C in TMC containing 0.02% sodium azide.

2.3.4 β1,2-N-acetylglucosaminyltransferase I (NAGT I)

This *medial*-Golgi enzyme catalyses the addition of N-acetylglucosamine onto Man_5-$GlcNac_2$ N-glycans, the products of Mann I catalysis. This assay measures the transfer of tritiated GlcNAc from a UDP-GlcNAc donor onto ovalbumin, which contains incompletely processed N-linked oligosaccharides which can act as a substrate for NAGT I (Vischer and Hughs, 1981).

The assay mixture was made up as follows: 500µl of 0.5M Tris/maleate pH6.9, 500µl of 200mg/ml ovalbumin, 50µl of 1M KCl, 100µl of 50mM UDP-GlcNac, 50µl of 10% (w/v) TX-100, 12.5µl of 2M $MnCl_2$, 12.5µl of 2M $MgCl_2$, 50µl of 100µCi/ml UDP-[^{3}H]GlcNac and 2.72ml of H_2O. 80µl of this assay mix was added to 20µl of sample (or 20µl of H_2O as a blank) and incubated for 2.5hr at 37°C. The reaction was stopped by protein precipitation by the addition of 1ml of ice cold 1% phosphotungstic acid/0.5M HCl (PTA/HCl). The samples were centrifuged at 14,000rpm for 7sec on a benchtop microfuge and the supernatants discarded. The pellets were resuspended in a fresh 1ml of

PTA/HCl by scraping and spun as before. These pellets were resuspended in 1ml of ice cold 95% ethanol (Hayman Ltd.) and subjected to a final centrifugation step. After removal of the ethanol the pellet was dissolved in 50µl of 2M unbuffered Tris and 200µl of 5% (w/v) SDS by shaking at room temperature for 1hr. 1ml of liquid scintillant was then added, the samples vortexed and counted under the tritium channel of a scintillation counter. The DPM value of the blank was subtracted from the sample values.

The assay was tested for linearity with rat liver homogenate, intermediate fraction and Golgi membranes. Samples were diluted 2, 5, 10, 20, 50 and 100-fold and assayed (fig. 2.4).This showed that the assay was linear in the 5-100-fold range for all three samples, requiring approximately 0-65µg of protein for the homogenate and intermediate fraction and 0-12µg for Golgi membranes.

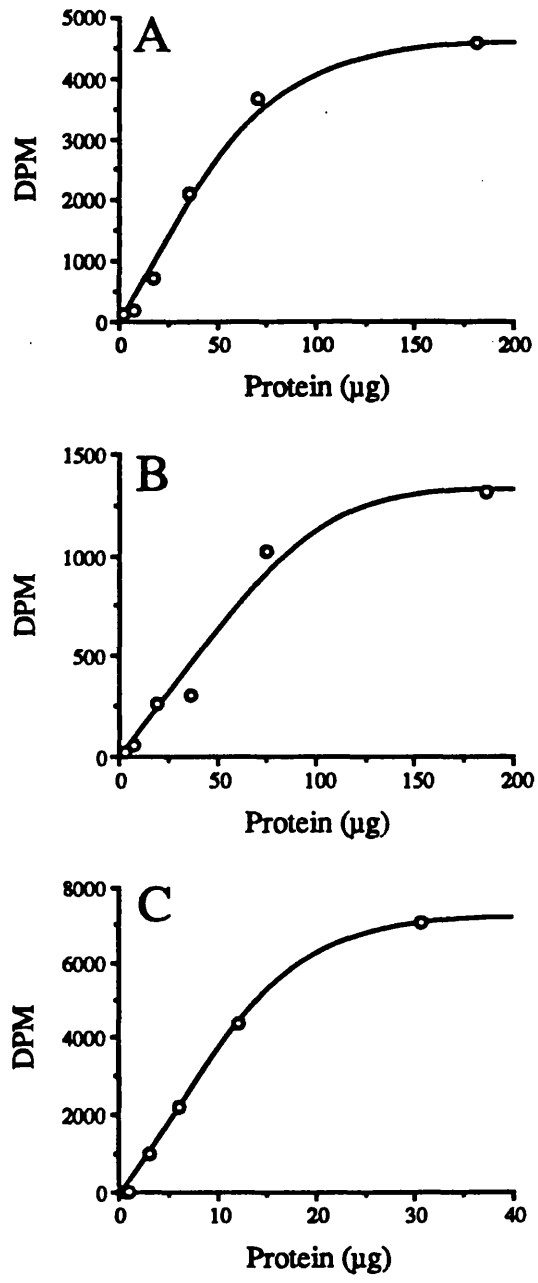

Figure 2.4: Linearity of the N-acetylglucosaminyltransferase I assay. Increasing amounts of rat liver homogenate (A), intermediate fractions (B) and Golgi membranes (C) were assayed for NAGT I. The amount of radioactivity incorporated after a 150 min incubation was measured and plotted against the amount of protein in each sample.

2.3.5 α1,3-1,6-Mannosidase II (Mann II)

Mannosidase II acts directly after NAGT I by catalysing the conversion of GlcNAc-Man_5-$GlcNAc_2$ to GlcNAc-Man_3-$GlcNAc_2$. The Mann II assay is spectrophoto metric and utilises

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

2.3.5.1 Procedure

The Mann II assay was based on the method of Bischoff and Kornfeld (1984). An assay mix was made up containing: 2.9ml of sodium phosphate pH6.0, 100µl of 10% (w/v) TX-100 and 1ml of 20mM PNM. 80µl of this mixture was added to each 20µl sample (and 20µl of water to act as a blank) and the samples incubated at 37°C for 1 hour. Reactions were stopped by the addition of 1ml of 0.25M Na_2CO_3. The blank was used to zero the spectrophotometer, and the absor-bance of the samples was measured at a wavelength of 400nm.

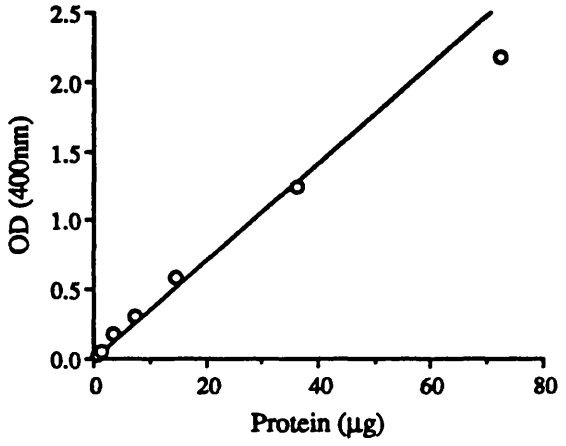

Figure 2.5: Linearity of the Mannosidase II Assay. Increasing dilutions of Golgi membranes were assayed for Mann II. Following a 60min incubation sample absorbance was measured at 400nm and plotted against protein concentration.

The assay was shown to be linear for rat liver Golgi membranes. Samples were diluted 1, 2, 5, 10, 20, 50 and 100-fold and assayed for Mann II (fig. 2.5). These data show that assay is linear for Golgi membranes which have been diluted 2-100 times (or containing up to 36µg of protein).

2.3.5.2 Assay Specificity

The problem inherent in the Mann II assay is its lack of specificity. The substrate is cleaved not only by Mann II, but by the mannosidases of the ER and by lysosomal mannosidase, though not by Mann I (Bischoff and Kornfeld, 1984). Thus, in order to be confident that the assay in this instance was only measuring Mann II activity, it was necessary to demonstrate that the Golgi preparations used in these studies were not contaminated by these other activities.

Such a contamination is unlikely because lysosomes and microsomal membranes are not large contaminants of the rat liver Golgi membranes (table 2.3). This was confirmed by the use of the mannosidase inhibitor swainsonine (8αβ-indolizidine-1α,2α,8β-triol)

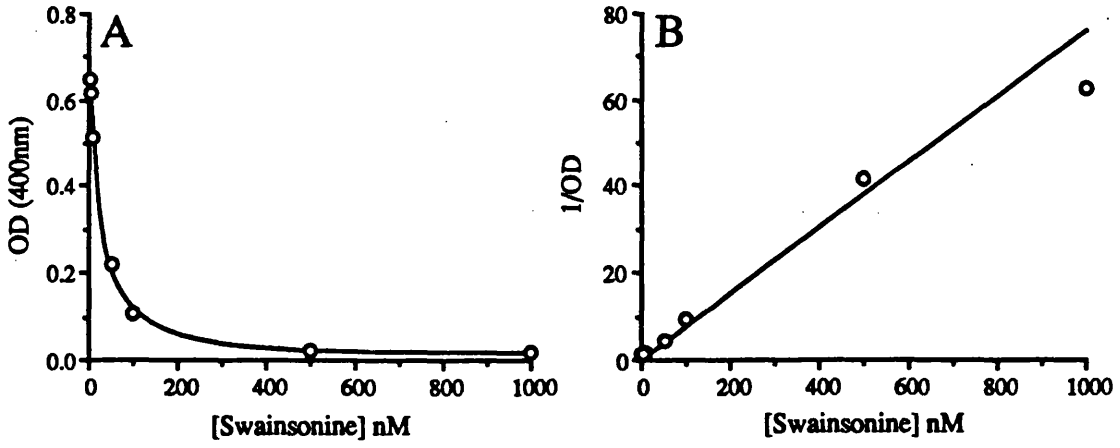

Figure 2.6: Swainsonine inhibition of the Mannosidase II assay.; 15µg aliquots of Golgi membranes were assayed for Mann II in the presence of increasing concentrations of swainsonine. After 60min of incubation, reactions were stopped and the absorbance of each sample at 400nm determined. The data was plotted as the absorbance (A) or the reciprocal of the absorbance (B) against the swainsonine concentration.

which inhibits all mannosidases except Mann I and ER mannosidases (Tulsiani *et al.*, 1982a; Dorling *et al.*, 1980). Thus, in the presence of swainsonine, this assay should only measure ER mannosidase activity (since PNM is not a substrate for Mann I). When the assay was carried out in the presence of increasing concentrations of swainsonine, virtually no activity was observed beyond an inhibitor concentration of 500nM (fig. 2.6 A). This confirmed the lack of contaminating ER activity and the inability of Mann I to hydrolyse the substrate. When these data were graphed as a Dixon Plot (the reciprocal of the absorbance versus the swainsonine concentration), a straight line was obtained (fig. 2.6 B). This is characteristic of the inhibition of a single enzyme, not a mixture, and thus confirms the lack of lysosomal mannosidase activity in the Golgi membranes, as suggested by the organelle's depletion in these preparations.

2.3.6 β1,2-N-acetylglucosaminyltransferase II (NAGT II)

NAGT II catalyses the step directly downstream of the action of Mann II. The enzyme adds an extra GlcNAc residue onto N-glycans bearing the structure GlcNAc-Man_3-$GlcNAc_2$. The acceptor for in this assay was kindly donated by Dr. Harry Schachter and is an artificial substrate with the structure Manα1→6(GlcNAcβ1→2 Manα1→3)Manβ-$(CH_2)_8COOCH_3$ (GnM_3-octyl). This oligosaccharide moiety acts as the GlcNAc acceptor while the hydrophobic octyl group is utilised in the separation of the substrate from unreacted UDP-^{3}[H]GlcNAc donor by reverse-phase chromatography (Palcic *et al.*,

1988). The assay is based on that used by Bendiak and Schachter (1987b), the major difference being the use of this substrate.

An assay mixture was made up containing: 500ng of GnM_3-octyl, 200µl of 0.5M MES pH6.1, 100µl of 1M NaCl, 10µl of 2M MnCl2, 10µl of 50mM UDP-GlcNAc, 10µl of 10% (w/v) TX-100, 200µl of 50mM AMP, 200µl of 1M GlcNAc, 20µl of UDP-^{3}[H]GlcNAc and 50µl of H_2O. 20µl of this mixture was added to each 5µl sample. The samples were pulsed in a centrifuge and vortexed prior to incubation at 37°C for 60min. Reactions were stopped by the addition of 400µl of 20mM sodium tetraborate/2mM EDTA.

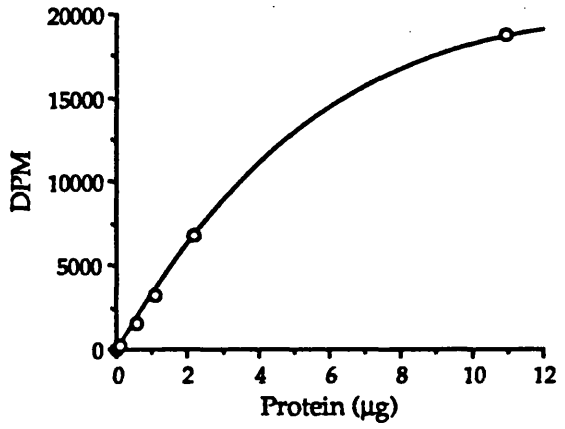

Figure 2.7: Linearity of the NAGT II Assay. Increasing concentrations of Golgi membranes were assayed for NAGT II activity. After a 60min incubation, the amount of radioactivity incorporated into the substrate was measured and plotted against the amount of protein in the sample.

The substrate was separated from unreacted UDP-GlcNAc using Sep-Pak C18 reverse-phase cartridges (Waters Ltd.). Columns were pre-wetted with 10ml of methanol and washed with 20ml of water using a 20ml syringe. The samples were then diluted with 4.6ml of water and loaded onto the cartridges and the cartridges washed with 10ml of water to remove the UDP-GlcNAc. GnM_3-octyl was then eluted with 5ml of methanol into a scintillation vial. 10ml of scintillation cocktail was added and the samples counted in the tritium channel of a scintillation counter.

Typically, each cartridge was reused 10 times. After elution of the GnM_3-octyl, the cartridge was washed with 10ml of methanol followed by 10ml of water before loading of the next sample.

The assay was tested for linearity in reactions with Golgi membranes. Dilutions of 1, 5, 10, 20, 100 and 500 were produced and these samples assayed for NAGT II activity (fig. 2.7). These data showed that the assay is linear for dilutions of 5-fold or greater of Golgi membranes (or up to 6µg of protein).

2.3.7 β1,4-galactosyltransferase (GalT)

This *trans*-Golgi marker was measured by a technique developed by Bretz and Staubli (1977). This enzyme catalyses the addition of galactose onto N-linked oligosaccharides

bearing the $GlcNAc_2$-Man_3-$GlcNAc_2$ structure, Such an incompletely processed structure is carried by the glycoprotein ovomucoid (see Spiro (1973) for a review of glycoprotein structure). This assay is very similar to that for NAGT I except that it measures the incorporation of tritiated galactose, from a UDP-[^{3}H]Galactose donor, into a different acceptor, ovomucoid.

An assay mixture was made up containing: 500µl of 0.4M-cacodylate pH6.6, 500µl of 175mg/ml ovomucoid, 15µl of 2-mercaptoethanol, 100µl of 10mM UDP-galactose, 100µl of 10% (w/v) TX-100, 50µl of 0.2M ATP, 100µl of 2M $MnCl_2$, 25µl of 100mCi/ml UDP-[^{3}H] galactose and 2.6ml of H_2O. 80µl of assay mix was added to each 20µl sample and to 20µl of H_2O to serve as a blank and the samples incubated at 37°C for 30min. The reactions were stopped, processed and counted exactly as for the NAGT I assay (see section 2.3.4, above). The only difference occurred in the SDS-solubilisation of the ethanol-washed pellet which occurred much more readily, in the space of a few seconds.

The linearity of this assay for rat liver homogenate, intermediate fraction and Golgi membranes was tested in much the same way as that for NAGT I (fig 2.8). Although the

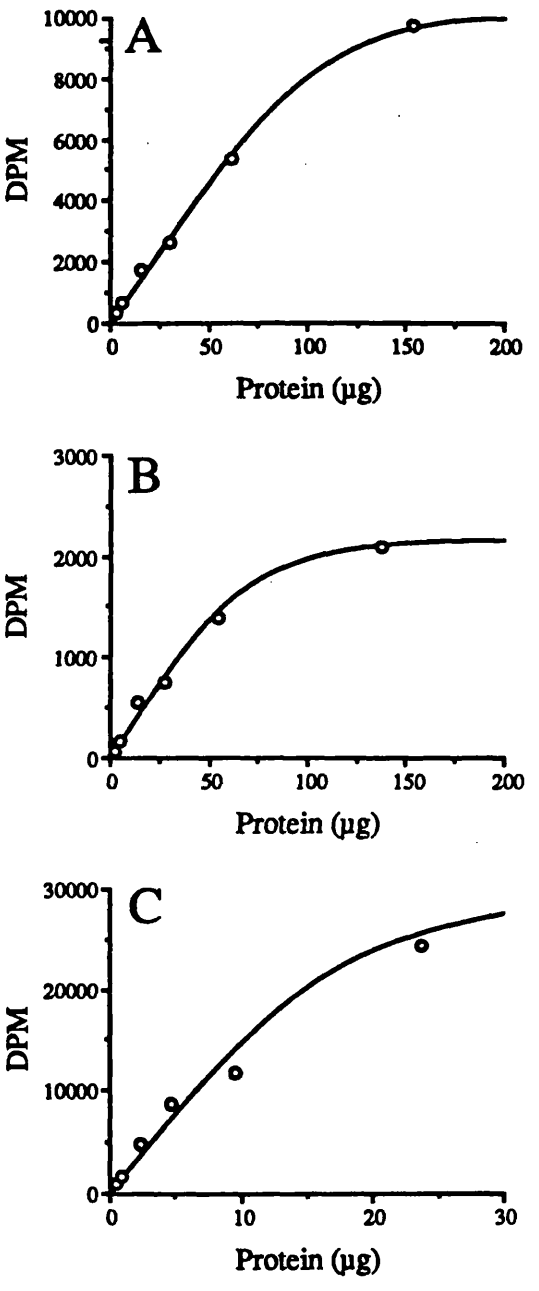

Figure 2.8: Linearity of the galactosyl-transferase assay. Increasing amounts of rat liver homogenate (A), intermediate fractions (B) and Golgi membranes (C) were assayed for galactosyltransferase. The amount of radioactivity incorporated after a 30 min incubation was measured and plotted against the amount of protein in each sample.

GalT assay is significantly more sensitive than that for NAGT I (c.f. y-axes in figs. 2.4 and 2.8), its linearity in response to increasing sample concentrations is very similar.

2.3.8 α2,6-sialyltransferase (SialylT)

This enzyme catalyses the final step in the maturation of complex N-glycans and acts directly after GalT. The assay measures the transfer of tritiated sialic acid (N-acetyl neuraminic acid; NANA) from the donor (CMP-^{3}[H]NANA) to the acceptor, asialotransferrin, which bears the oligosaccharide structure (Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$. The assay is a modification of that described by Dunphy and Rothman (1983), the major differences being in the sample processing after the assay and in the choice of the acceptor.

2.3.8.1 Production of Substrate

The substrate chosen was not asialofetuin as described by Dunphy and Rothman (1983) but asialotransferrin. This was chosen because fetuin contains both N- and O-glycans (Spiro, 1960). Since transferrin contains only N-linked oligosaccharides (see Spiro (1973) for a review), the possibility of interference of the O-glycan sialyltransferase in this assay was eliminated.

Asialotransferrin was prepared by the removal of NANA from holotransferrin by acid hydrolysis essentially as described by Hatton *et al.* (1979). Briefly, 50mg of holotransferrin was dissolved in 5.0ml of 50mM H_2SO_4 (272µl of concentrated H_2SO_4 in 100ml of H_2O). This was incubated at 80°C for 1hr and cooled. The solution was then dialysed overnight at 4°C against 1l of 10mM Tris pH9.0. This neutralised the acid and removed hydrolysed NANA which is a competitive inhibitor of the enzymes. The substrate was divided into 1ml aliquots and stored at -20°C.

2.3.8.2 Procedure

An assay mixture was made consisting of: 300µl of 0.5M Tris pH7.0, 600µl of 10mg/ml of asialotransferrin, 75µl of 10% (w/v) TX-100, 15µl of 5mM CMP-NANA, 25µl of 100mCi/ml CMP-^{3}[H]NANA and 185µl of H_2O. 24µl of this mixture was added to each 6µl sample and incubated at 37°C for 30min. Reactions were stopped and samples processed exactly as for the GalT and NAGT I assays.

The linearity of the assay was tested for Golgi membranes using dilutions of 20, 50, 100, 250 and 500-fold. The assay was also carried out using transferrin to demonstrate that de-sialylation had been successful (fig. 2.9). These data showed that the assay was linear at 100-fold or greater dilutions of Golgi membranes, or at up to 180ng of protein.

Additionally, these data showed that very little activity is observed in the presence of transferrin. Thus, the assay is strictly dependent on the presence of asialotransferrin.

2.3.9 Catalase

Catalase was assayed as described in the Sigma catalogue. An assay mixture was made up consisting of: 5.0ml of 0.5M sodium phosphate pH6.7, 100µl of 30% (w/v) H_2O_2 and 44.9ml of H_2O. 10µl of sample was added to 1ml of assay mix in a silica cuvette (Pharmacia) and mixed. The drop in absorbance of the solution at 240nm, corresponding to the hydrolysis of hydrogen peroxide to oxygen and water, was measured over the first 10sec of the reaction and recorded.

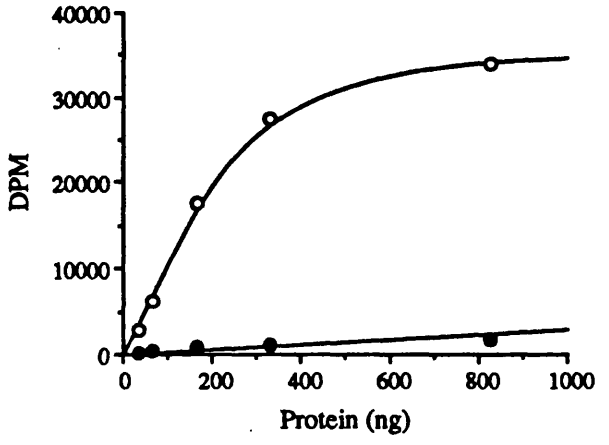

Figure 2.9: Linearity of the sialyltransferase assay. Increasing concentrations of Golgi membranes were assayed using either transferrin (●) or asialotransferrin (○) as an acceptor. The amount of radioactivity incorporated after a 30min incubation was plotted against the total amount of Golgi protein present.

2.3.10 Protein

Sample protein concentrations were determined using the BCA Protein Assay Reagent (Pierce Chemicals) as described in the handbook. Standard curves were constructed containing 0, 10, 20, 30 and 40µg of protein (using 0, 5, 10, 15 and 20µl of 2mg/ml Bovine Serum Albumin diluted to 50µl with water), and sample volumes were 50µl at the appropriate dilution. The spectrophotometer was set to zero by a blank sample containing 50µl of water.

2.4 Rat Liver Golgi Preparation

Golgi membranes were routinely prepared and characterised as described below.

2.4.1 Isolation

Rat liver Golgi were prepared using extensive modifications of the procedure of Leevlavathi *et al.* (1970), as described by Slusarewicz *et al.* (1994a). This was performed using discontinuous sucrose-gradient centrifugation using the buffers A-E as outlined in Table 2.1. Six discontinuous sucrose-gradients, consisting of 13ml of buffer D

Buffer	A	B	C	D	E
Sucrose Concentration	0M	0.25M	0.5M	0.86M	1.3M
PM Buffer	20ml	40ml	80ml	20ml	12ml
2M Sucrose	0ml	25ml	100ml	43ml	39ml
2M $MgCl_2$	0.25ml	0.5ml	1ml	0.25ml	0.15ml
Water	79.8ml	135ml	219ml	36.8ml	8.9ml

Table 2.1: Sucrose buffers used for purification of rat liver Golgi membranes.

underlain with 7.5ml of buffer E were poured into Beckman Ultraclear SW-28 rotor tubes and kept on ice. Underlaying of buffer E was performed with a 10ml syringe connected to a plastic tube.

Four to six rats were starved for 24 hours prior to sacrifice. Livers were quickly immersed in 200ml of ice-cold buffer C and swirled and squeezed occasionally to expel blood and to speed cooling. 36g of liver was placed into fresh buffer C and cut into several pieces to release as much blood as possible. Excess buffer was decanted to leave a volume of less than 80ml, and the livers minced into small pieces (4-5mm diameter, approximately) with a pair of fine scissors.

This material was homogenised by gently pressing through a 150µm-mesh steel laboratory test sieve (Endecotts Ltd.) with the bottom of a conical flask and the homogenate collected in a plastic tray. This is a relatively gentle method of homogenisation and reduces the possibility of cisternal unstacking by mechanical shear (Hino *et al.*, 1978). Homogenate was poured into a 100ml measuring cylinder and buffer C added to a final volume of 80ml, followed by thorough mixing.

13ml of this homogenate was overlaid onto each of the gradients, and was topped up with buffer B. These gradients were centrifuged in a L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) using a SW-28 rotor at 28,000rpm for 1 hour at 4°C. A 100µl aliquot of the homogenate was kept on ice for later assay.

After centrifugation, the lipid layer on the surface of the gradient was removed by aspiration. The Golgi fraction from the interface between buffers C and D was collected using a Pasteur pipette (approximately 2-3ml from each gradient). These membranes were pooled and diluted to 8-9% (w/w) sucrose using buffer A. The concentration was checked using a 0-50% Delta refractometer (Bellingham and Stanley Ltd., Tunbridge Wells, U.K.).

This intermediate fraction was poured into two fresh SW28 centrifuge tubes and 100µl was kept for enzyme assay. These were topped up with buffer B and 100µl of

buffer E was slowly added to both tubes to form a sucrose cushion. The samples were then centrifuged at 7000rpm for 30 minutes at 4°C.

The supernatant was removed by aspiration, and the pellets resuspended in 2ml of buffer B each and pooled. This suspension was made up to 38ml with buffer B, placed into a fresh centrifuge tube, and 100µl of buffer E added to form another sucrose cushion. This sample was centrifuged at 7000rpm for 30 minutes at 4°C.

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

2.4.2 Biochemical Characterisation

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

Golgi preparations were assayed routinely for GalT activity. The preparations were typically purified 80-fold over the homogenate for this enzyme (table 2.2 A). Occasionally, the preparations were assayed for NAGT I activity (table 2.2 B). In this case, the purification was in excess of twice that for GalT.

This difference is probably due to some loss of the *trans*-Golgi network during purification since this organelle contains a significant amount of GalT but no NAGT I (Nilsson *et al.*, 1993a).

The GalT, NAGT I and protein assays were carried out as detailed in section 2.3. Homogenates, intermediate-fractions and Golgi membranes were diluted 200, 20 and 5 times respectively before assaying for protein and all samples were diluted 20 times for the GalT and NAGT I assay. These dilutions ensured that sample concentrations were in the linear range for these assays (see section 2.3).

To determine the concentration of GalT in any given sample, a 10µl aliquot of the assay mixture, mixed with 40µl of 2M unbuffered Tris and 200µl of 5% SDS, was counted with the samples to allow calculation of the specific activity of the UDP-Galactose in the reaction. The specific activity of the UDP-Galactose in DPM/nmole) was calculated as shown below:

S.A. UDP-Galactose = (DPM of standard - DPM of blank)/2.5 *(i)*

A

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

B

Fraction	Volume	[Protein] (mg/ml)	[NAGT I] (nmol/h/ml)	Specific Activity	Yield (%)	Purification Fold
Homogenate	78	78.9	45.2	0.6	100	1x
Intermediate	74.8	5.2	49.6	9.6	88.3	16.6x
Golgi	4.9	2.7	370	144	51.0	192x

Table 2.2: Purification of Golgi markers in Golgi preparations. These tables show the enrichment of a *trans*-Golgi marker, GalT (A); and a *medial*-Golgi marker, NAGT I (B); over the homogenate in both the intermediate fraction and the final Golgi preparation. The GalT table was compiled using the results from 24 separate fractionations and are presented as the mean ±SEM. for each parameter. Note that the specific activity, yield and purification-fold are not, therefore, arithmetically related to the GalT and protein concentrations. The NAGT I table shows the average results from two fractionations.

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

$$[Protein] = ((Absorbance\ of\ sample - c) \times Dilution\ Factor \times 0.02)/m \qquad (ii)$$

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

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

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

Determination of NAGT I concentrations was very similar. Again a 10µl standard of assay mixture was counted. The calculation the specific activity of UDP-GlcNAc was exactly as in equation (i), above, except that DPMs were divided by 1.25×10^{-5} instead of

A

Fraction	(Volume) ml	[Protein] (mg/ml)	OD (405nm)	Spec. Activity (Abs./mg)	Yield (%)	Purification Fold
Homogenate	78.0	95.89	1.90	50.8	100.0	1.0x
Intermediate	77.6	8.0	0.03	9.1	1.6	0.2x
Golgi	4.8	3.58	0.06	38.0	0.2	0.8x

B

Fraction	(Volume) ml	[Protein] (mg/ml)	OD (550nm)	Spec. Activity (OD/mg)	Yield (%)	Purification Fold
Homogenate	78.0	74.0	0.2	4.2	100.0	1.0x
Intermediate	74.7	4.8	0.2	4.8	6.3	1.1x
Golgi	4.7	2.0	0.1	1.6	0.2	0.4x

Table 2.3: Purification of lysosomal and ER markers in Golgi preparations.; These tables show the depletion of the lysosomal marker, β-hexoseaminidase (A); and the ER marker, NADH-cytochrome c reductase (B); over the homogenate in both the intermediate fraction and the final Golgi preparation. Both tables show the average results from two experiments.

2.5 to give a value in DPM/nmole. The specific activity of NAGT I was also essentially the same as for GalT except that 2000 in equation (iii) was replaced by 400 to also yield a value in nmoles/hr/ml.

Coupled to this large enrichment of Golgi markers in these Golgi preparations, there was also a depletion of enzymatic markers of the lysosomes (table 2.3 A) and ER (table 2.3 B). The specific activity of these enzymes is presented as OD/mg as conversion to moles/min is not necessary to determine the degree of purification.

2.5 Isopycnic Centrifugation

Linear isopycnic sucrose gradients were poured into Beckman Ultraclear SW-40 rotor tubes, from bottom to top, using an Auto Densi-Flow automated fractionator (Buchler Instruments) at a flow rate of 1.5ml/min. The chambers of the device were filled with 6ml of the high- and low-concentration sucrose buffer respectively.

After pouring, 0.5ml aliquots of sample were loaded onto each gradient and the tubes balanced with sucrose-free buffer. Samples were spun at 40,000rpm and 4°C for 16hr in an L8-70M ultracentrifuge and the fractions were collected with the same apparatus in 1ml aliquots from top to bottom and at the same flow rate.

The two types of gradient used in this study are described below.

2.5.1 TMMDS Gradients

These gradients contained TX-100 and were used in studies on detergent-extracted Golgi membranes. The range of the gradients used was either 15-75% or 60-75% (w/v) of sucrose. The gradient buffers were made up as TMMS buffer containing higher than normal sucrose concentrations i.e. 15g/100ml for 15%, 60g/100ml for 60% and 75g/100ml for 75%. 1M DTT was added to each gradient buffer to a final concentration of 1mM just before use.

2.5.2 KEHM Gradients

These gradients contained no detergent and were used for the subcellular fractionation of post-nuclear supernatants from cells grown in culture. Because of the presence of cytosolic proteases in these samples, the gradients also contained a cocktail of protease inhibitors (PIC). Cycloheximide was also added to prevent detachment of ribosomes from the RER in order to maximise the density of this compartment. The range of the gradients was 20.5-63% (w/v) sucrose (0.6-1.84M) and the gradient solutions were made up as follows using stocks of 5xKEHM buffer and 2.3M sucrose. The 20.5% buffer contained 4ml of 5xKEHM, 5.1ml of 2.3M sucrose, 20µl of PIC, 60µl of 3mg/ml cycloheximide and 10.8ml of H_2O. The 63% buffer was made up in exactly the same way except that 16ml of 2.3M sucrose and no H_2O were used.

2.6 TX-114 Phase Separation

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

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

This low cloud point has been exploited as a method for isolation of membrane spanning proteins (Moremen *et al.*, 1991; Featherstone *et al.*, 1985; Pryde, 1986; Bordier, 1981). During phase separation, amphipathic proteins (such as those that span the lipid bilayer) preferentially enter the detergent phase while hydrophilic proteins remain in the

aqueous phase. Although the behaviour of individual proteins varies with regards to their phase partitioning, this provides a qualitative method of isolating membrane spanning proteins and of determining whether a novel protein spans the lipid bilayer.

2.6.1 Precondensation

Each member Triton X series of detergents consists of a hydrophobic octylphenyl residue onto which several oxyethylene moieties have been condensed. The oxyethylene chain length distinguishes the different members of the family. Due to the method of synthesis, each member of the series does not have a fixed chain length but its own unique length distribution. Thus although the majority of molecules in a commercial batch of TX-114 will contain 7-8 residues per chain, significant amounts of molecules of other chain lengths will be present. Contamination by more hydrophilic molecules will increase both the cloud point and the detergent concentration in the aqueous phase after phase separation, and may therefore interfere with the correct partitioning of protein (Bordier, 1981). Such hydrophilic molecules were removed by repeated cycles of phase separation as described below.

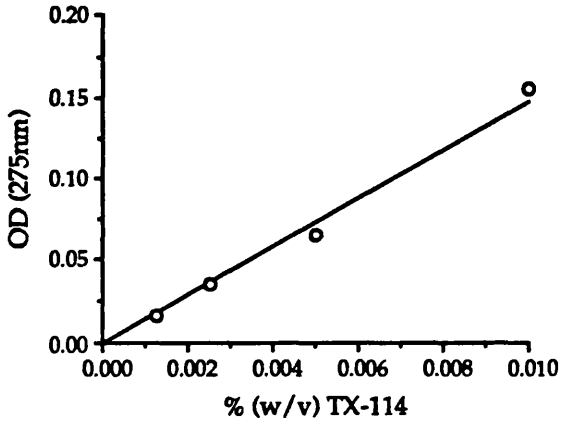

Figure 2.10: TX-114 Standard Curve. Varying concentrations of TX-114 were measured for absorbance at 275nm in a silica cuvette.

20g of TX-114 and 16mg of butylated hydroxytoluene were dissolved in 980ml of 10mM Tris pH7.4/150mM NaCl. The solution was cooled at 4°C until the solution cleared and then left at room temperature for phase partitioning to occur. The solution was left at room temperature overnight for the phases to separate and the upper aqueous phase was decanted and discarded. The detergent phase was made up to 1l with fresh 10mM Tris.HCl pH7.4/150mM NaCl, cooled, warmed and decanted as before. This process was repeated once more to yield the final stock detergent. The TX-114 concentration in this stock was determined by reading the absorbance of a 4000-fold diluted sample at 275nm using a crystal cuvette. This was compared to a standard curve (fig. 2.10) constructed from uncondensed TX-114 at concentrations of 1.25×10^{-3}%-0.01% (w/v). This showed that the precondensed TX-114 was a 10.8% (w/v) solution. The detergent was divided into 50ml aliquots which were stored at -20°C while the working solution was stored at 4°C.

	10%	15%	Stack
40% Acrylamide/bis (29:1)	5ml	7.5ml	2.5ml
60% Sucrose	0ml	4ml	5ml
2M Tris.HCl pH8.8	4ml	4ml	0ml
0.5M Tris.HCl pH6.8	-	-	2.75ml
20% SDS	0.1ml	0.1ml	0.1ml
H_2O	10.9ml	4.4ml	9.6ml
TEMED	10µl	10µl	10µl

Table 2.4: Solutions used for SDS-PAGE.

2.6.2 Procedure

Unless specified, TX-114 extractions were carried out as follows. The sample was taken up in 200µl of TX-114 Extraction Buffer (200µl of PM buffer, 2.5µl of 2M $MgCl_2$, pre-condensed TX-114 to give a final concentration of 1% (w/v) and H_2O to a final volume of 1ml). This was incubated on ice for 10min followed by a 3min incubation at 37°C and a further 10min on ice. The sample was then spun at 14,000rpm at 4°C for 5min in a benchtop microfuge to remove unsolubilised material The supernatant was then incubated at 37°C for 3min and overlaid onto a 50µl Cushion Buffer (as for extraction buffer but containing 0.06% (w/v) TX-114 and 10%(w/v) sucrose). This was spun at room temperature for 5min at 5000rpm to yield a detergent pellet and an aqueous supernatant.

2.7 SDS-PAGE

Electrophoresis was carried according to the protocol of Blobel and Dobberstein (1975). The methods used are described below.

2.7.1 Gel Casting

Gels consisted of linear gradients of 10-15% acrylamide. The separating gel and the stacking gel solutions were poured as described in table 2.4. Polymerisation was initiated by the addition of 100µl of 10% (w/v) ammonium persulphate to each solution just before use.

The gels were poured at a thickness of 0.75mm using a simple two-chambered gradient maker. The separating gels were overlaid with distilled water to exclude oxygen and to ensure a smooth interface and allowed to set. Once set, the water was removed and the gel washed once with stacking solution, to which 100µl of ammonium persulphate solution had been added, before pouring the stacking gel.

2.7.2 Protein Precipitation

If necessary, protein was precipitated prior to electrophoresis to either reduce the sample volume or remove reagents which would interfere with the running of the gels. Two methods were employed. For samples of up to 100µl, the methanol/chloroform method of Wessel and Flugge (1984) was used. Samples of 0.1-1ml volume were precipitated using trichloroacetic acid (TCA).

2.7.2.1 Chloroform/Methanol Method

Samples were made up to 100µl with H_2O. 0.4ml of methanol was added, and the sample vortexed and spun down in a microfuge at 12,000 rpm for 10 sec. Then 0.2ml of chloroform was added and the sample vortexed and spun as before. Next, 0.3ml of water was added followed by vortexing and spinning at 12,000rpm for 1min. The upper phase was discarded, to leave the interface and the lower phase. Finally, 0.3ml of methanol was added and the precipitate spun down at 12,000rpm for 2min to pellet the precipitate. The supernatant was removed and samples incubated at 37°C for 10min to evaporate excess solvent. Pellets were then dissolved in SDS-PAGE loading buffer and processed for electrophoresis.

2.7.2.2 TCA Method

Samples were made up to 1ml with H_2O and 100µl of 0.15% sodium deoxycholate added. After vortexing and a 5min incubation at 4°C, 200µl of 72% (w/v) TCA was added and the sample left on ice for 1hr. Samples were spun at 14,000rpm for 5min in a benchtop microfuge and the supernatants discarded. The pellets were washed and resuspended (by scraping) in 1ml of acetone that had been pre-cooled on dry-ice. Following a second spin, samples were washed once more in acetone and spun followed by evaporation of excess solvent for 10min at room temperature. The resulting pellets were dissolved in SDS-PAGE loading buffer and processed for electrophoresis.

2.7.3 Sample Preparation

A 1ml aliquot of SDS-PAGE loading buffer was diluted with 0.3ml of 20% (w/v) SDS and 10µl of 1M DTT added. Samples were dissolved in 40µl of this solution by shaking for 30min. Samples were then heated at 95°C for 3 min and cooled to room temperature. Once cooled, 5µl of 0.5M iodoacetamide was added and the samples shaken for 20min. The samples were then spun for 14,000rpm for 5min in a benchtop microfuge before being loaded onto the gel with a Hamilton syringe. In addition, 0.75µl of BioRad high and/or low molecular weight markers were processed as above and loaded in one lane.

2.7.4 Electrophoresis

Gels were placed into the apparatus and the upper and lower tanks filled with SDS-PAGE running buffer. Gels were electrophoresed at a constant current of 45mA for 3-4hr with the water cooling system operating.

2.7.5 Fixing and Staining

Gels were stained by a 10min incubation in a solution containing 0.25% (w/v) Coomassie Brilliant Blue R. This solution also contained 7.5% (v/v) acetic acid and 50% (v/v) methanol which served to fix the gel by protein precipitation. Gels were then destained by numerous washes in 7% (v/v) acetate/40% (v/v) methanol. Gels were then photographed or dried under vacuum using a Model 453 gel drier (BioRad).

2.8 Western Blotting

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

2.8.1 Immobilon-C

Immobilon-C is a nitrocellulose membrane with a nylon backing which confers mechanical strength. Nitrocellulose is a matrix which binds proteins by hydrophobic interaction. Transfer buffer was made up with 20ml of TG Buffer (0.2M Tris/1.5M glycine), 40ml of methanol and 1.0ml of 20% (w/v) SDS made up to 200ml of H_2O. Transfer was carried out at a constant current of 100mA for 1 hr. Blots were stained with Ponceau S solution (Sigma) to determine the position of molecular weight markers which were marked with a pencil. Blots were then probed with antibodies as described below.

2.8.2 PVDF

PVDF is a strong matrix which can withstand the conditions encountered in a solid-phase peptide sequencing (Matsudaira, 1987). The matrix binds proteins by ionic interaction and, therefore, blotting in SDS must be avoided since this anionic detergent will block the

binding sites on the membrane. Blotting was, therefore, carried out in a highly alkaline pH in order to ensure that all acidic residues on the proteins being blotted were ionised. Transfer buffer was made up using 25ml of 100mM CAPS pH11 and 5ml of methanol made up to 250ml with H_2O and transferred as above but at 250mA for 2hr. Note that these conditions were determined specifically for the blotting of the protein of interest. Methanol is required to strip off any SDS remaining in gel from the filter. However, small amounts of SDS will help the transfer of proteins onto the membrane without blocking the filter. The SDS and methanol concentrations, current and transfer-time required for the transfer of any given protein must be determined for each individual polypeptide.

Proteins were visualised on the membranes using sulpho-rhodamine B as described by Coull and Pappin (1990). Sequencing was carried out on a MilliGen 6000 solid phase protein sequencer in the ICRF Protein Sequencing Laboratory by Dr. Darryl Pappin and Dinah Rahman.

2.9 Immunodetection

Western blots were probed using the ECL (Enhanced Chemi-Luminescence) kit supplied by Amersham. Blots were blocked by overnight incubation in blocking buffer (5% low fat milk powder, 0.2% (w/v) Tween-20/PBS) at 4°C. Milk powder was purchased from Boots The Chemist. Blots were then sealed into plastic bags containing 3ml of blocking buffer containing the appropriate antibody (spun at 14,000rpm for 5min in a benchtop microfuge to sediment any aggregates) with care being taken to exclude any air bubbles. Blots were then placed on a rocker and incubated at room temperature for 1hr under a bag of water. The blot was then washed twice briefly with blocking buffer followed by two 30min washes and two more brief ones. The blot was then sealed into a fresh bag with 3ml of blocking buffer containing 3µl of horse-radish peroxidase-conjugated anti-rabbit IgG (TAGO) and incubated for 1hr as before. The blot was washed again as above. The blot was then washed briefly in 0.5% (w/v) Tween-20/PBS and once with PBS alone. The blot was then placed onto a plastic dish and 6ml of a 50:50 mixture of the ECL reagents poured over it. After 1min, the excess was drained off and the blot placed onto the shiny side of a piece of benchcote. The blot was covered with cling-film and exposed to X-ray film (X-OMAT AR, Kodak) for periods of 1sec-5min.

2.10 Immunoprecipitation

Immunoprecipitation was carried out using either magnetic beads (Dynal) covalently coupled to anti-mouse IgG, or with Protein A-Sepharose.

2.10.1 Magnetic Beads

This was based on the methods described by Howell *et al.* (1989). These beads are synthesised in the presence of iron and are, therefore, magnetic. The beads can easily be recovered by placing them in a strong magnetic field and this avoids the need for centrifugation for recovery.

The beads used were purchased pre-coated with anti-mouse IgG which had covalently cross-linked by cyanogen bromide activation (Dynal). 20µl aliquots of beads were resuspended in 1ml of 0.5% (w/v) BSA/PBS, recovered and resuspended in a further 1ml of BSA/PBS. 20µl of secondary antibody was added and this mixture was incubated overnight at 4°C with end-over-end rotation at 6rpm. After this incubation, unbound secondary antibody was removed by 5 washes of 1ml BSA/PBS, with 15min incubations before each wash. The samples were then added to the washed beads, the volumes made up to 1ml with BSA/PBS, and the samples rotated at 4°C for 2hr and washed as before to yield the final pellet of beads.

2.10.2 Protein A-Sepharose

The samples were made up to 0.4ml with H_2O and 0.4ml of 2x IP lysis buffer (40mM Tris pH8.0, 300mM NaCl, 1% (w/v) TX-100) added. 80µl of 10% (w/v) protein A-sepharose (PAS) was added along with 4µl of polyclonal serum which had been prespun at 14,000rpm for 5min. The samples were then rotated at 4°C for 2hr and spun at 14,000rpm for 5sec on a benchtop microfuge. The supernatant was removed and the beads resuspended in 1ml of IP wash buffer (20mM Tris pH8.0, 150mM NaCl, 0.1% TX-100). Samples were vortexed briefly and spun again to the recover the beads. The above process was carried out two more times to yield the final pellet of beads.

2.11 Tissue Culture

Two stably-transfected Hela cell-lines produced by Dr. Tommy Nilsson in this laboratory were grown in tissue culture. The cell-line 4:12 (Nilsson *et al.*, 1993a) expresses NAGT I which is tagged with the *myc*-epitope (Evan *et al.*, 1985), while 4:48 (Nilsson *et al.*, 1994) expresses *myc*-tagged NAGT I carrying the cytoplasmic domain of the ER resident form of human invariant chain, p33 (Lotteau *et al.*, 1990).

2.11.1 Passaging

Cells were grown on 150cm^2 flasks (Falcon) in DMEM containing 0.5mg/ml Geneticin-418 (G-418 sulphate; GIBCO). and passaged every three days, by which time they had reached 60-70% confluence. The medium was removed and replaced with 5ml of

trypsin/versene solution which was itself removed after 10sec. Cells were incubated at 37°C until they had been dislodged and were resuspended in 5ml of fresh DMEM/G-418. After 10 passages in a glass pipette, 1ml of 4:48 or and 0.5ml of 4:12 suspension was added to new flasks containing 12ml each of fresh medium. Hela S3 cells were grown in exactly the same way as 4:12 except that the medium did not contain G-418.

2.11.2 Expansion

For the production of post-nuclear supernatant (PNS), each cell line was grown in five 850cm^2 roller-bottles (Falcon). Firstly, the cells were expanded into five 150cm^2 flasks by the method described above. Once they had reached 60-70% confluence, the cells were trypsinised and each aliquot added to a roller-bottle containing 100ml of DMEM without G-418. These cells were grown until 70-80% confluent (approximately 3 days) whilst rotating at 0.25rpm.

2.11.3 Production of Post-Nuclear Supernatants

Once cells had reached 70-80% confluency, 1ml of 3mg/ml cycloheximide (Boehringer Manheim) was added and the cells incubated for a further 10min. At this point, sterile conditions were no longer maintained. The medium was poured off and kept and the cells were then trypsinised with 20ml of trypsin per bottle and rotated until the cells had been dislodged (note that trypsin was not removed). Trypsin was then quenched by the addition of 30ml of the used medium and the cells collected into 50ml centrifuge tubes (Falcon). Cells were recovered by centrifugation at 1400rpm for 3min in a 500E benchtop centrifuge, The cells were resuspended and pooled in 50ml of used medium containing 50µl of 20mg/ml cytochalasin B and 1ml of 1M HEPES pH7.4. The cells were then rotated at 4°C for 45min to depolymerise intracellular actin. and recovered as before. The cells were then subjected to a swelling step by resuspending in 25ml of 10mM TEA pH7.4/150mM KCl buffer containing 0.25ml of 3mg/ml cycloheximide and incubating at 4°C for 10min. Cells were then recovered, resuspended in 25ml of KEHM buffer (50mM HEPES pH7.4, 50mM KCl, 10mM EGTA, 1.92mM $MgCl_2$) containing 25µl of 1M DTT and 0.25ml of 3mg/ml cycloheximide and recovered again. This final cell pellet was taken up in an equal volume of KEHM containing 1mM DTT, 30mg/ml cycloheximide and 1x Protease Inhibitor Cocktail (PIC). The cell suspension was homogenised with 10 passes in a ball-bearing homogeniser with a 10µm clearance (Balch *et al.*, 1984a). Nuclei were removed by a 5min spin at 3000rpm at 4°C in a microfuge. The PNS was removed with care being taken not to disturb the nuclear pellet. The PNS was divided into aliquots of 0.5ml, frozen in liquid nitrogen and stored at -70°C.

2.11.4 Long Term Storage

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

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

2.12 Digestion with Proteases

Golgi membranes were digested with three different proteases; Trypsin, chymotrypsin and proteinase K. These methods are described below.

2.12.1 Trypsin

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

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

2.12.2 Chymotrypsin

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

2.12.3 Proteinase K

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

2.13 Treatment with SPITC

SPITC (4-sulphophenyl-isothiocyanate) is a derivative of Edman's reagent. The difference lies in the substitution of a sulphate group in the *para* position of the benzene ring. This charged moiety renders the reagent impermeable to lipid bilayers. The reagent still retains the ability to electrophilically attack primary amines. Thus, if added to intact membranes, the reagent will modify the N-termini and lysine residues of all proteins that are exposed on the cytoplasmic face.

Golgi membranes were first digested with 0.5mg/ml proteinase K and proteolysis terminated by the addition of PMSF. Samples were then spun at 50,000rpm and 4°C for 5min in a TLA-100 ultracentrifuge (Beckman). The pellets were washed with two volumes of HMS buffer (50mM HEPES pH8.0, 0.1mM $MgCl_2$ and 10% (w/v) sucrose). The pellets were then resuspended in HMS buffer supplemented with the appropriate concentration of SPITC, to a final concentration of 1mg/ml of original Golgi protein. Samples were incubated with shaking at room temperature for 30min. Excess SPITC was then quenched by the addition of 1M glycine to a final concentration of 100mM.

2.14 Extraction of Golgi Membranes

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

2.14.1 Using TX-100 Alone

Rat liver Golgi membranes were spun in at 50,000rpm for 5min at 4°C in a TL-100 ultracentrifuge (Beckmann) using either TLA-100.1, TLA-100.2 or TLA-100.3 rotors. The membranes were then resuspended in TMMDS buffer (2% (w/v) TX-100, 50mM MOPS pH7.0, 0.1mM $MgCl_2$, 1mM DTT, 10% (w/v) sucrose), using a P200 Gilson pipette until the suspension was homogenous (10-40 passages) and to a final protein concentration of 1mg/ml. The samples were incubated on ice for 30min and spun at 20,000rpm and 4°C for a further 30min. The supernatant was removed and the tube containing the pellet rinsed gently in two volumes of TMMDS before resuspension in one volume of TMMDS until the suspension was homogenous.

2.14.2 Using TX-100 and NaCl

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

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

2.15 Rebinding Studies

Rebinding of solubilised *medial*-Golgi enzymes to the 150mM NaCl/TMMDS pellet (or matrix) was carried out in two ways. The first method used enzyme solutions in which the enzymes had been solubilised by salt. Removal of the salt by dialysis allowed the enzymes to rebind. The second method used enzymes solutions lacking salt. The enzymes were solubilised in TX-100 alone, from Golgi membranes that had been digested with proteinase K. In this case, dialysis was not required for rebinding to occur.

2.15.1 With Dialysis

Golgi membranes were extracted in 100µl aliquots of TMMDS buffer. The Triton insoluble pellets were then further extracted with equal volumes of either 150mM NaCl/TMMDS to yield the matrix pellet, or with TMMDS containing a lower salt concentration (as described in the text) to yield supernatants containing the solubilised enzymes. Each of the matrix pellets were then resuspended in a salt supernatant and dialysed for 16hr against TMMS buffer at a flow rate of 0.3ml/min, using a System 100 microdialyser (Pierce) fitted with a 12-14kD cut-off membrane (Spectrum Medical Industries). Dialysates were then removed and spun at 20,000rpm for 30min at 4°C in a TL-100 ultracentrifuge. Supernatants were removed and the pellets were then rinsed in two volumes of TMMS before resuspension in one volume of TMMS.

Alternatively, 100µg aliquots of Golgi membranes were extracted in 150mM NaCl/TMMDS. These extracts were then dialysed and spun as above before separation of the supernatants and pellets.

2.15.2 Without Dialysis

150mM salt pellets were prepared from 100µg aliquots of Golgi membranes. Additionally, 100µg aliquots were digested with proteinase K as described above and extracted in TMMDS buffer, followed by centrifugation. The supernatants from these extracts were used to resuspend the 150mM salt pellets and these suspensions incubated

at 4°C for 1hr before recentrifugation. The supernatants and pellets were then separated as before.

2.16 Scatchard Analysis

Assuming that a ligand binds to a receptor via a single binding site (or several binding sites with the same affinity), the affinity of the interaction can be determined by utilising a Scatchard plot. Increasing concentrations of ligand are bound to a constant amount of receptor and the amounts of free and bound ligand determined for each sample. If the above assumption holds true, the data will form a straight line when plotted as [Bound Ligand] on the y-axis and [Bound Ligand]/[Free Ligand] on the x-axis. The slope of this line represents the affinity of the ligand-receptor interaction in units equal to those of the y-axis.

2.16.1 Procedure

350µg of Golgi membranes were extracted first in TMMDS buffer followed by a further extraction with 25mM NaCl/TMMDS. These supernatants were divided into 20, 40, 60, 80 and 100µl aliquots and made up to 100µl with 25mM NaCl/TMMDS. These supernatants were used to resuspend matrix (150mM NaCl) pellets produced from 25µg of Golgi membranes each, and the remainder was stored at 4°C. The samples were dialysed and centrifuged as described above and the supernatants removed. Pellets were rinsed with two volumes of TMMDS and resuspended in one volume of the same buffer. The supernatants and pellets were then assayed for Mann II and NAGT I activity, along with a sample of undialysed 25mM salt supernatant.

2.16.2 Mannosidase II

In order to be able to produce Scatchard plots from these data, it is necessary to determine enzyme (ligand) concentrations in the bound and unbound samples. This can be achieved if both the specific activity and molecular weight of the enzyme are known. This allows the conversion the enzymatic activity measured in an assay to a protein concentration. Fortunately, the specific activity of rat liver Mann II was determined when the enzyme was purified (Tulsiani and Touster, 1986; Moremen *et al.*, 1991) to be 6.75 µmoles/min/mg. Additionally, the molecular weight of the protein is known, both by SDS-PAGE analysis and gel filtration (Moremen *et al.*, 1991) and by translation of the cDNA (Moremen and Robbins, 1991) and is 125kD.

In order to determine the amount of Mann II in a given assay, it was necessary to determine the concentration of the p-nitrophenol released. A standard curve was constructed by the addition of 1ml of 0.25M Na_2CO_3 to duplicate 100µl aliquots of Mann

II assay mixture which contained 50, 100, 250, 500 and 1000μM of p-nitrophenol (but lacked PNM). This showed that an absorbance of 1OD after addition of the Na_2CO_3 corresponded to 577.5μM of p-nitrophenol in the original assay mixture (fig. 2.11).

20μl of each dialysed pellet fraction (containing bound Mann II ligand) was assayed for Mann II as was 20μl of undialysed 25mM salt supernatant. Dialysed supernatants were also assayed to determine the enzyme recoveries in order to exclude the possibility of denaturation during dialysis. The amount bound was considered to be that in the pellet while the amount remaining free was considered to be the amount loaded minus the amount bound. The amount loaded was calculated from the assay of the 25mM salt supernatant i.e. 1, 0.8. 0.6, 0.4, and 0.2x the 25mM supernatant value. The concen-tration of Mann II in each sample was calculated as follows.

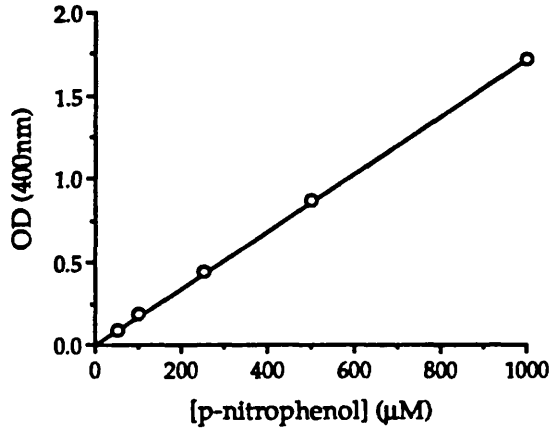

Figure 2.11: Standard curve for p-nitrophenol.; Increasing concentrations of p-nitrophenol were made up in 100μl of Mann II assay mixture. 1ml of 0.25M Na_2CO_3 was added and the absorbance for each sample at 400nm recorded.

The amount p-nitrophenol released by the entire 100μl sample was calculated by multiplying by 5 and the μM concentration of Mann II in a 100μl sample was obtained simply by multiplying by 577.5. The number of μmoles released by this 100μl was then calculated by dividing by 10,000, and the rate of release (in μmoles/min) by the dividing by the time of incubation (60min). Since the specific activity of Mann II is 6.75 μmoles/min/mg, division of the rate of p-nitrophenol release by 6.75 gave the amount of Mann II present in the 100μl sample in milligrams and dividing by 1000 gave the value in grams. Next, division of this value by the molecular weight of Mann II (125,000D) yielded the number of moles of Mann II present in the 100μl sample, and a further multiplication by 10^{13} yielded the final Mann II concentration as a nM value. These calculations resulted in a final factor of 48.9 i.e. multiplication of the p-nitrophenol absorbance of these samples by 11.4 yielded their Mann II concentrations in nM units.

2.16.3 NAGT I

Unfortunately, the determination of NAGT I affinity was not as accurate as for Mann II because only the specific activity of the purified rabbit (not rat) enzyme has been reported (Oppenheimer and Hill, 1981). Additionally, the substrate used in this determination was not ovalbumin as used in those previous studies, but an oligosaccharide purified from this substrate. Past work had shown the kinetics of reaction of glycosyltransferases and glycosidases for glycoprotein and oligosaccharide substrates varies (Tabas and Kornfeld, 1979; Harpaz and Schachter, 1980a). Nonetheless, the specific activity of the rabbit enzyme could be used to obtain a crude estimate of the NAGT I binding affinity. The calculation was exactly as that for Mann II except that the NAGT I concentrations of the samples were calculated as described below.

To determine the specific activity of UDP-GlcNAc in the assay mixture, 10µl was counted in the scintillation counter and the DPM value divided by 60 to give the specific activity in DPM/nmole. Next the DPM of the blank was subtracted from that of the sample and this value divided by the specific activity of UDP-GlcNAc to determine the total number of nmoles of GlcNAc incorporated in each assay. This was then divided by 100 and by the time of incubation (150min) to give the number of µmoles released per minute by the 100µl sample. This value was divided by the specific activity of NAGT I (2.51µmoles/min/mg) to give the number of milligrams present and by 1000 to give the number of grams. Next, the value was divided by the molecular weight of NAGT I (55000D; (Kumar and Stanley, 1989)). This gave the number of moles of NAGT I in the 100µl sample and multiplication by 10^{13} yielded the nM concentration of the enzymes. This meant that the conversion could be achieved simply by dividing the corrected sample DPM by 4.8 and the specific activity of UDP-GlcNAc in the assay mixture.

2.17 Fixation for Electron Microscopy

Golgi membranes were fixed for electron microscopy in a solution containing 60µl of 50% (w/v) glutaraldehyde (Fluka), 125µl of 2M sucrose, 2.5µl of 2M $MgCl_2$, 200µl of PM buffer pH6.7 and 612.5µl of H_2O. 100µg of Golgi membranes were fixed in suspension overnight and dehydrated and embedded as described by Pypaert *et al.* (1991). Samples were examined on a Phillips CM10 transmission electron microscope.

2.18 Density Alteration with Colloidal Gold

In this procedure, TX-100 extracted Golgi membranes were labelled antibodies specific for Mann II followed by secondary antibodies conjugated to 10nm colloidal gold. The change in density of the Mann II containing material was assessed by determining

whether the enzyme could be centrifuged through a 75% (w/v) sucrose cushion after this treatment, since the untreated Triton pellet could not.

Aliquots of 400µg of Golgi membranes were extracted in TMMDS buffer and the pellets resuspended in 400µl of fresh TMMDS buffer including 40µl of anti-Mann II ascitic fluid, or of control ascites which contained an equal concentration of immunoglobulins (P5D4). After a 40min incubation at 4°C, the samples were spun again under the same conditions as after the extraction, and the pellets rinsed thoroughly before resuspension in a fresh 400µl of TMMDS containing 20µl of anti-mouse IgG coupled to 10nm colloidal gold (Biocell). After a further 30min incubation, samples were overlaid onto discontinuous sucrose gradients. These consisted of 6.5ml of 15% (w/v) sucrose in TMMDS buffer overlaid onto 6ml 75% (w/v) sucrose in the same buffer in a SW40 centrifuge tube.

The samples were centrifuged at 40,000rpm for 1hr at 4°C and 1ml fractions collected from the top downward before assaying for the appropriate enzymes. The position of the step was determined by using a 0-50% Delta refractometer (Bellingham and Stanley) to determine the sucrose concentration.

2.19 Mann II Chymotryptic Fragment Purification

The chymotryptic fragment of Mann II (CT-Mann II) was purified for the production of polyclonal antibodies against the enzyme and for use in binding studies. The purification method used was similar to that described by Moremen *et al.* (1991), but two changes were introduced. Firstly, solubilisation of Mann II from Golgi membranes was not carried out using multiple TX-114 extractions. Instead, Mann II derived from a 150mM salt supernatant was used as the source of the enzyme. This circumvented the need for the laborious process of performing several bulk TX-114 phase separations and yielded a Mann II source from which many other Golgi proteins had been removed. The second difference was that a Fast Flow SP Sepharose (FFS) column was used in the ion-exchange chromatography, not a Mono-S column as was used by Moremen and colleagues, since such a column was not available. This column was packed manually and because of the slightly greater size-distribution of the beads compared to the pre-packed Mono-S, a slight loss in resolution would occur, but this did not prevent the purification of CT-Mann II to homogeneity.

Two or more Golgi membrane preparations were pooled to yield at least 22mg of membranes. Of this, 21mg were extracted in TMMDS buffer and the subsequent Triton pellet further extracted in TMMDS supplemented with 150mM NaCl. A 200µl aliquot of this supernatant was kept for enzyme assay and SDS-PAGE analysis and 52µl of a 10mg/ml solution of chymotrypsin added to the remainder. The solution was incubated at

15°C for 50min and the protease was quenched by the addition of 21µl of a 40mg/ml solution of PMSF in DMSO and incubation at 4°C for 10min. A 300µl aliquot of this solution was kept for future assay and the remainder was concentrated to 5ml by ultrafiltration using an Amicon 8010 pressure concentrator fitted with a 30kD cut-off membrane (YM30; Amicon) at a nitrogen pressure of 45 p.s.i. The filter had been previously incubated in PBS containing 5mg/ml rabbit serum albumin to minimise non-specific binding to the membrane, Rabbit serum albumin was chosen so that any trace contamination in the final CT-Mann II preparation would not raise an immune response if it was used for antibody production. Between uses, the membrane was stored in 50% (v/v) ethanol.

The concentrated sample was then diluted by the addition of 45ml of FFS buffer (20mM Tris pH7.5, 0.1% (w/v) TX-100) which itself was obtained by dilution of a 10x concentrated stock solution. This 50ml sample was loaded onto a 3cm x 1cm FFS column, which had previously been equilibrated with 20ml of FFS buffer, using a Pharmacia FPLC System. After a 10ml wash with FFS buffer, the bound CT-Mann II was eluted with an 80ml linear gradient of 0-150mM NaCl in FFS buffer at a flow rate of 0.1ml/min, and 1ml fractions of this elution gradient were collected. After use, the FFS column was washed with 20ml of 0.5M NaCl in FFS buffer followed by 20ml of FFS buffer alone, sealed and stored at 4°C.

20µl aliquots of each fraction were then assayed for Mann II activity and the fractions which contained 80-90% of the enzyme activity were pooled, with 300µl being kept for later assay. This pooled fractions were then concentrated to 200µl as described below and loaded onto a pre-packed Superose-6 column (Pharmacia) which had been equilibrated with 50mM NH_4HCO_3 (which had been diluted from a 10x stock). The column was then eluted with 50ml of the same buffer at a flow rate of 0.1ml/min and 1ml fractions were collected. 20µl aliquots were then assayed for Mann II activity and the fractions that contained the enzyme were pooled to yield the purified CT-Mann II preparation. If desired, this could be further concentrated by ultrafiltration but this would yield some loss of enzyme due to non-specific binding to the membrane, or by lyophilisation which would also lead to some loss of activity (Moremen *et al.*, 1991).The preparation was stored at 4°C at which temperature it is stable, since freeze-thawing seems to result in some fragmentation of the protein (Moremen *et al.*, 1991),

Chapter 3

Extraction of Golgi Membranes with Triton X-100

3.1 Introduction

Intercisternal material had long been observed between the cisternae of the Golgi stack as described in chapter 1, and this matrix had been implicated in the mechanism which stacks the Golgi cisternae. This material had, however, never been isolated biochemically in a functional form. Since these structures can be detected in a wide variety of cell lines, we postulated that they would be present in the Golgi stacks of rat liver. Furthermore, since Golgi stacks can be purified from both plants and animals in a stacked form (Morré and Mollenhauer, 1964; Morré *et al.*, 1970), the stacking material must be stable.

We drew an analogy between these highly ordered and stable structures and the nuclear lamina (see Gerace (1988) for a review). This structure is a meshwork of highly ordered filaments which are closely apposed to, and line the inside of the nuclear envelope (Aebi *et al.*, 1986). The lamina is formed by hetero-oligomerisation of its protein subunits, lamins A, B and C, which are highly homologous in both primary and secondary structure to the intermediate-type filament class of proteins (Fisher *et al.*, 1986; McKeon *et al.*, 1986). The lamina was isolated (as were many other intermediate-type filaments) by virtue of its insolubility in detergent, salt and urea (Dwyer and Blobel, 1976). After such extractions, the lamina could be sedimented at low speed and thus separated from more soluble contaminants.

We postulated that extraction of highly purified rat liver Golgi stacks under similar conditions would be a reasonable approach in attempting to isolate the intercisternal matrix. In this chapter, I describe the identification of the major insoluble protein after extraction of Golgi membranes with the non-ionic detergent Triton X-100 (TX-100), and the optimisation of the extraction conditions to maximise the insolubility of this component.

3.2 Initial Extraction Experiments

3.2.1 Extraction with TX-100

Extractions were performed on highly purified Golgi membranes. These membranes were biochemically pure (see section 2.4), and contained large numbers of stacked cisternae (fig. 3.1). Stereological analysis by Norman Hui in this laboratory, showed that 65%±4 (± SEM, n=3) of Golgi stacks in these preparations contained two or more cisternae (Slusarewicz *et al.*, 1994b). These membranes were extracted by a protocol based on that of Dwyer and Blobel (1976), at a protein concentration of 1mg/ml in TTMDS buffer (0.25% (w/v) TX-100, 10mM TEA pH7.5, 0.1mM $MgCl_2$, 1mM DTT and 10% (w/v) sucrose). After incubation on ice and centrifugation, the supernatants and pellets were separated. These samples, and an aliquot of an equivalent amount of untreated Golgi

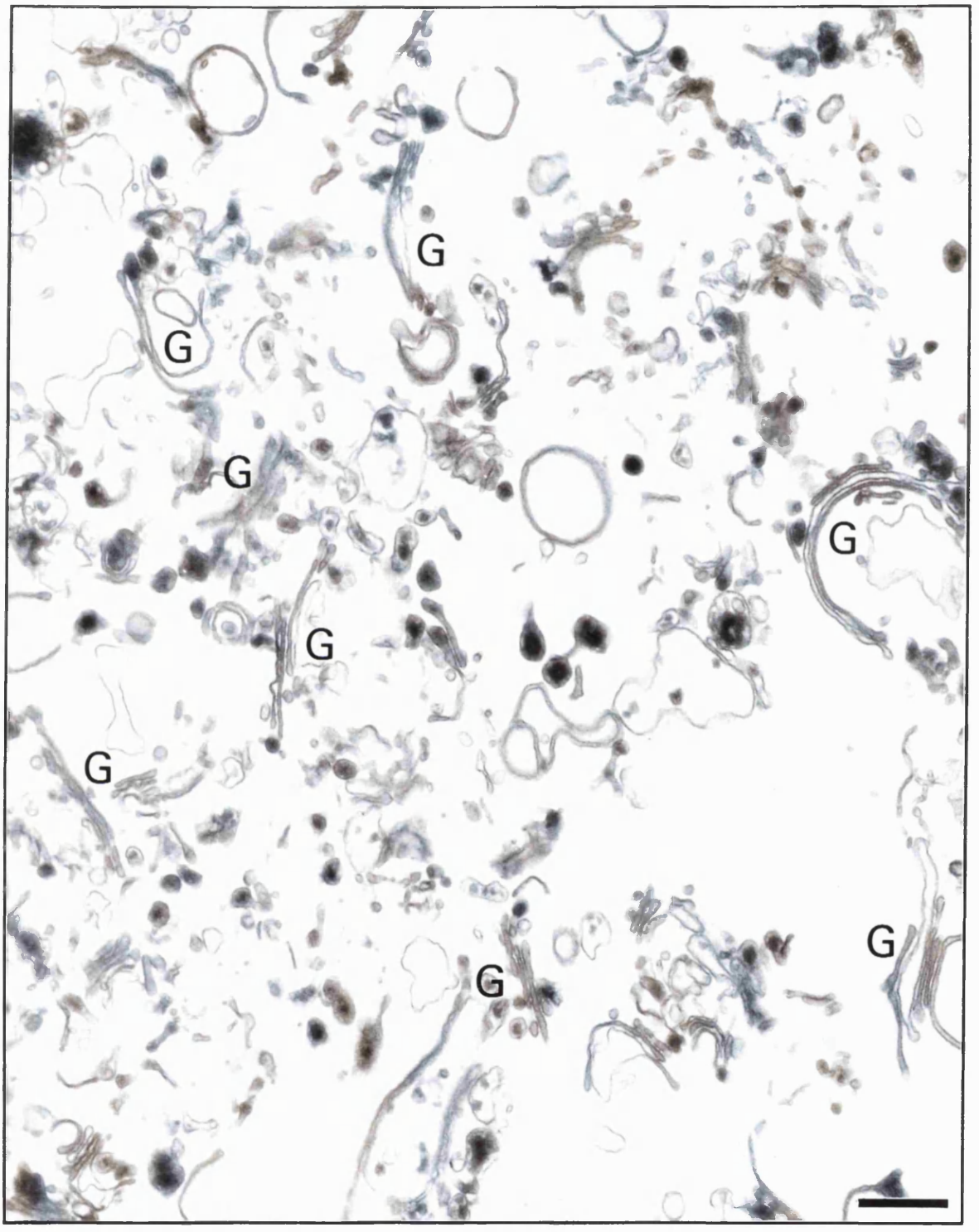

Figure 3.1: Morphology of Golgi Preparations. A Golgi membrane preparation was fixed in glutaraldehyde, embedded in Epon resin, and impregnated with osmium tetroxide and stained with uranyl acetate/lead citrate before sectioning and examination by transmission-electron microscopy. The picture shows several Golgi stacks (G). Magnification 28,750, Bar= 0.5μm.

membranes, were precipitated and analysed by SDS-PAGE. The gel was stained using Coomassie Brilliant Blue R and examined to determine whether any abundant proteins were found preferentially in the Triton pellet (fig. 3.2).

This clearly showed that a major component of the Triton pellet was a protein with a molecular weight of 125kD. This was termed GERP125 (**G**olgi **E**xtraction **R**esistant **P**rotein **125**kD). This protein was very abundant and was present in amounts comparable to rat serum albumin (RSA; fig. 3.2, lane 1), a major secretory protein and the most abundant protein in these preparations. Additionally, while the RSA, an entirely lumenal protein, was completely solubilised, GERP125 remained almost completely insoluble (c.f. lanes 2 and 3).

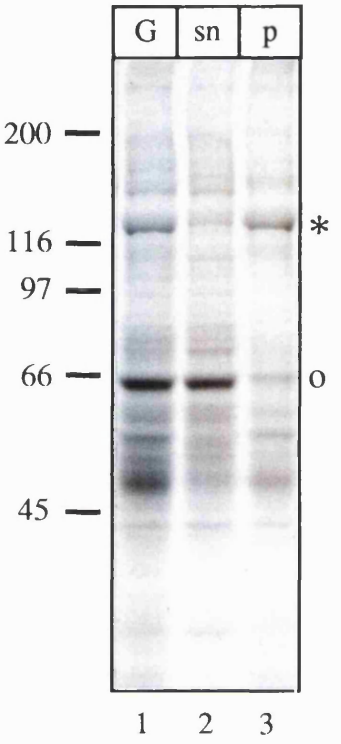

Figure 3.2: SDS-PAGE of solubilised Golgi membranes. 20µg of Golgi membranes (G), was extracted in TTMDS buffer. After centrifugation, the supernatant (sn) and pellet (p) were analysed by SDS-PAGE. The positions of the major insoluble protein, GERP125 (*), and of rat-serum albumin (o) are shown on the right, and the molecular weight standards (in kD) are indicated on the left.

To investigate the solubilisation of membrane-spanning proteins during this extraction procedure, the extracted supernatants and pellets, and an aliquot of untreated Golgi membranes, were assayed for GalT activity. This showed that GalT was almost completely solubilised after this extraction (fig. 3.3). The recovery of GalT compared to untreated membranes was 97.5%.

Thus GalT was, like RSA, almost completely solubilised by this treatment, and because the recovery of enzyme activity was very close to the amount in the original Golgi membranes little enzyme activation or inactivation had occurred during this procedure.

The fact that these typical Golgi lumenal and membrane-spanning proteins were soluble after this extraction but GERP125 was not, coupled with the fact that GERP125 was a major component of the Golgi membrane preparation suggested that it may be a component of the Golgi matrix. The nature of this polypeptide was, therefore, further investigated to determine whether it had the properties one would expect of such a protein.

3.2.2 Topology of GERP125

The topology of GERP125 was determined by protease protection experiments. If the protein was cytoplasmic, it should be sensitive to the addition of exogenous protease while if it was lumenal, it should not be sensitive without the addition of TX-100 to disrupt the lipid bilayer. Such experiments are usually performed only if an antibody is available to the unknown protein in question in order to be able unequivocally visualise it after SDS-PAGE and Western blotting. However, since GERP125 is such an abundant protein which can easily be seen on a Coomassie-stained SDS-PAGE gel, it was possible to perform these experiments without the need for a specific antibody. Protease protection was carried out using two proteases, trypsin and chymotrypsin, as detailed below.

3.2.2.1 Digestion with Trypsin

Golgi membranes were diluted to a protein concentration of 1mg/ml and digested with trypsin at concentrations of either 10 or 100μg/ml in the presence or absence of 0.1% (w/v) TX-100. After quenching of the protease with PMSF, the protein was precipitated and analysed by SDS-PAGE (fig. 3.4).

GERP125 appeared to be partially sensitive to trypsin at a concentration of 10μg/ml (c.f. fig. 3.4 lanes 1 and 3) and completely sensitive at 100μg/ml (c.f. lanes 1 and 5). This sensitivity, however, was only observed in the presence of TX-100 (c.f. lanes 2 and 3, and lanes 4 and 5). This indicated that GERP125 was either completely lumenal or was a membrane spanning protein with either a very short cytoplasmic domain or a larger, trypsin insensitive one (since no mass decrease was detectable after proteolysis in the absence of detergent). In order to confirm this result, and to attempt to determine

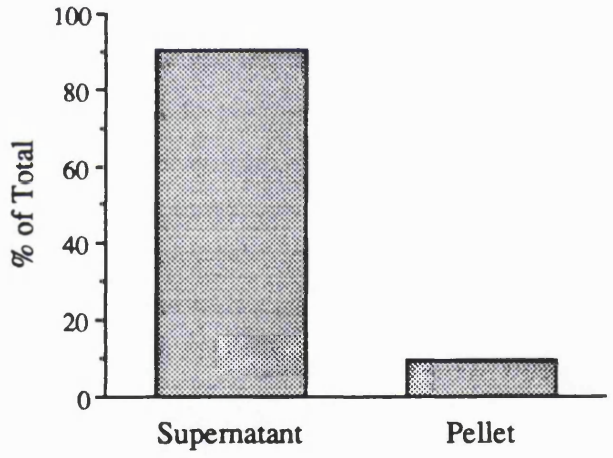

Figure 3.3: Solubilisation of GalT in TX-100. 50μg of Golgi membranes was extracted in TTMDS buffer for 30min. After a 30min spin at 20,000rpm and 4°C, the supernatant and pellet were diluted 20 times and assayed for GalT activity. The data are plotted as the percentage of the total activity found in the supernatant and pellet.

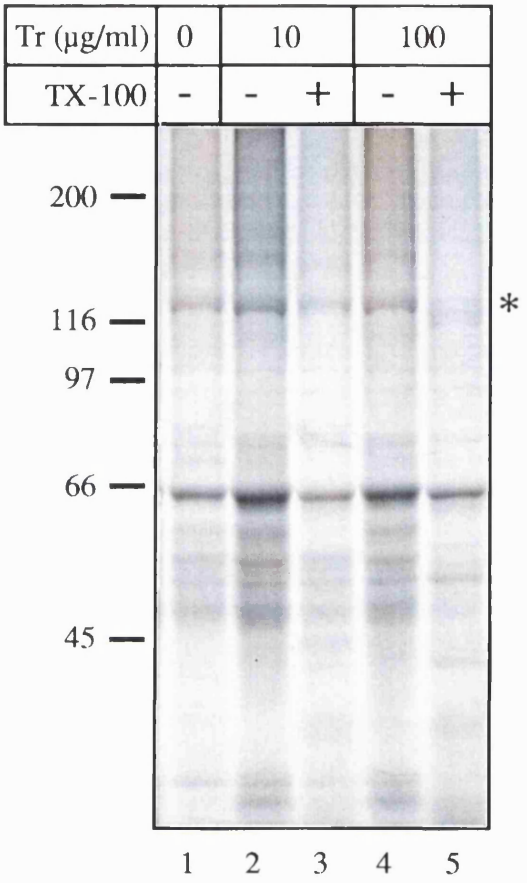

Figure 3.4: Protease protection experiment of GERP125 using trypsin. 50µg of Golgi membranes (lane 1) were digested with trypsin (Tr) at concentrations of 10µg/ml (lanes 2 and 3) or 100µg/ml (lanes 4 and 5) in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 0.1% (w/v) TX-100. Positions of molecular weight markers (in kD) are shown on the left and of GERP125 (*) on the right.

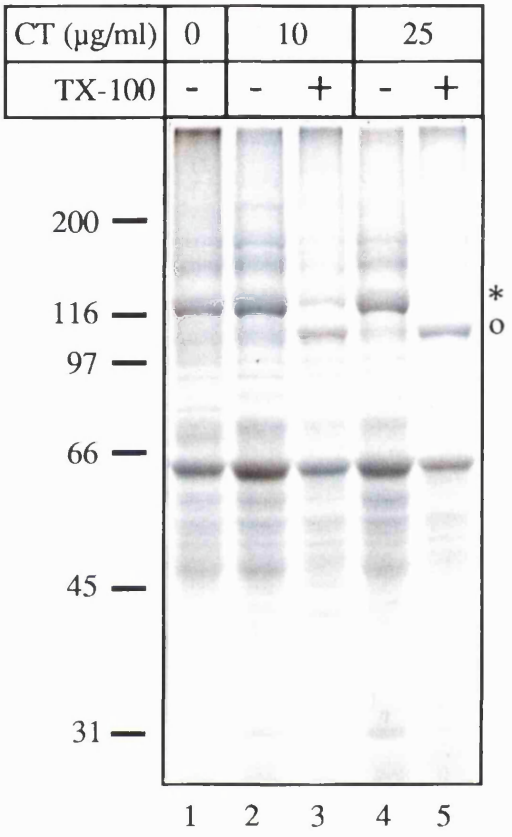

Figure 3.5: Protease protection experiment of GERP125 using chymotrypsin. 50µg of Golgi membranes (lane 1) were digested with chymotrypsin (CT) at concentrations of 10µg/ml (lanes 2 and 3) or 25µg/ml (lanes 4 and 5) in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 0.1% (w/v) TX-100. Positions of molecular weight markers (in kD) are shown on the left and GERP125 (*) and GC110 (o) on the right..

whether GERP125 had a significant cytoplasmic domain, this experiment was repeated with a different protease.

3.2.2.2 Digestion with Chymotrypsin

Chymotrypsin digestion was carried out exactly as with trypsin except that 10 and 25µg/ml concentrations of chymotrypsin were used instead. After SDS-PAGE it was apparent that GERP125 was behaving exactly as it did with trypsin digestion (fig. 3.5). At 10µg/ml over 50% of GERP125 was digested, but only in the presence of detergent (c.f. lanes 2 and 3), while at 25µg/ml no intact protein remained (lane 5).

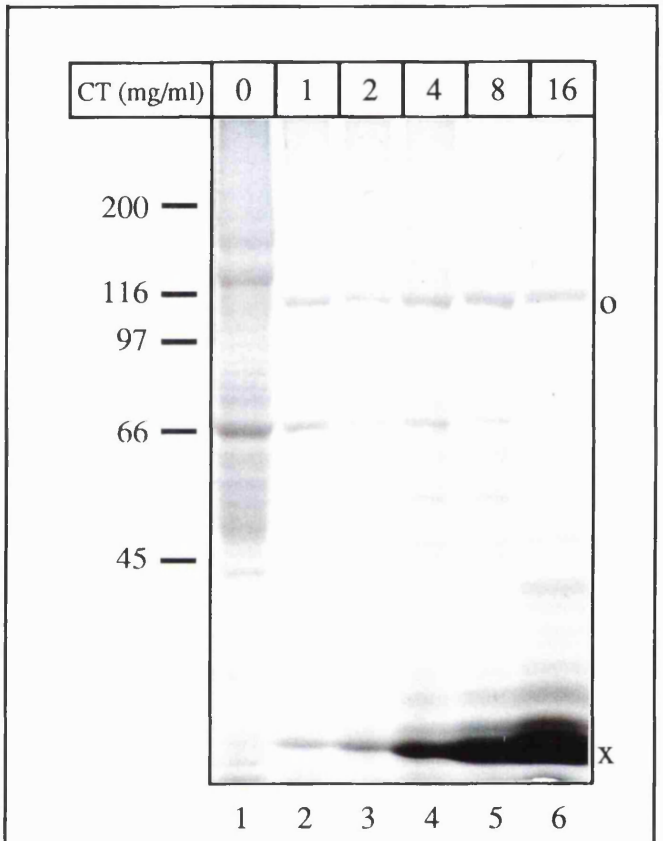

Figure 3.6: Digestion of Golgi membranes at high chymotrypsin concentrations. 30µg of Golgi membranes (0) was digested with increasing concentrations of chymotrypsin (CT; lanes 2 to 6) in the presence of 0.1% (w/v) TX-100. Positions of molecular weight markers (in kD) are shown on the left and GC110 (o) and chymotrypsin (x) on the right.

Interestingly, as GERP125 was digested in the presence of TX-100, there was a concomitant appearance of a new band with a molecular weight of 110kD (lanes 3 and 6). This had to be a chymotrypsin-resistant fragment of GERP125 since it was equally abundant and there were no proteins of higher molecular weight that were present in sufficient amounts to produce this band. An alternative explanation was that several of the higher molecular weight bands in the Golgi preparation (fig 3.5, lane 1) produced 110kD chymotrypsin-resistant fragments but this seemed highly unlikely. This fragment was therefore termed GC110 (**G**ERP125 **C**hymotryptic fragment of **110**kD).

These data corroborated the results from trypsin digestion in that they suggested that GERP125 was a lumenal protein. Additionally the lack of a detectable mass decrease in the absence of TX-100 showed that if this protein spanned the lipid bilayer, its

cytoplasmic domain was very short. It was unlikely that this would be a large domain because it would have to be resistant to both trypsin and chymotrypsin which catalyse proteolysis at completely different residues (chymotrypsin attacks aromatic and bulky aliphatic residues while trypsin attacks only at lysines and arginines).

The chymotrypsin resistance of GERP125 was further investigated by digesting Golgi membranes in the presence of TX-100 and increasing concentrations of protease. Aliquots of 30µg of Golgi membranes were digested at a protein concentration of 1mg/ml and chymotrypsin concentrations of 1, 2, 4, 8, and 16mg/ml in the presence of 0.1% (w/v) TX-100. After quenching with PMSF, protein was precipitated and analysed by SDS-PAGE (fig.3.6). This showed that GC110, produced by chymotryptic digestion of GERP125, was extremely resistant to further proteolysis by chymotrypsin. Even at a protease concentration of 16mg/ml, GC110 appeared to be completely resistant to further digestion by this enzyme.

3.2.3 TX-114 Phase Separation

In order to ascertain whether GERP125 was a integral membrane protein, its behaviour upon TX-114 solubilisation and phase separation was examined. Membrane proteins have been shown to partition preferentially into the detergent phase of TX-114 after cloud-point separation followed by centrifugation (Bordier, 1981), due to the presence of the hydrophobic membrane-spanning domain. This is a qualitative procedure and separation into the detergent phase cannot be considered proof that a protein spans the lipid bilayer, but is highly indicative. The phase partitioning behaviour of both GERP120 and GC110 are described below.

3.2.3.1 GERP125

Golgi membranes were solubilised in TX-114. The extract was prespun at 4°C to remove any unsolubilised material and then incubated at 37°C to induce phase separation. After centrifugation at room temperature, the phases were separated and the protein precipitated and analysed by SDS-PAGE (fig. 3.7).

This showed that GERP125 partitions preferentially into the detergent phase of TX-114. Although this separation was not complete (approximately 40% remains in the aqueous phase; c.f. lanes 2 and 3), it still suggested that GERP-125 may be an integral membrane protein.

3.2.3.2 GC110

A 50µl aliquot of Golgi membranes was first digested with chymotrypsin at a concentration of 100µg/ml in the presence of 0.1% (w/v) TX-100 as described above.

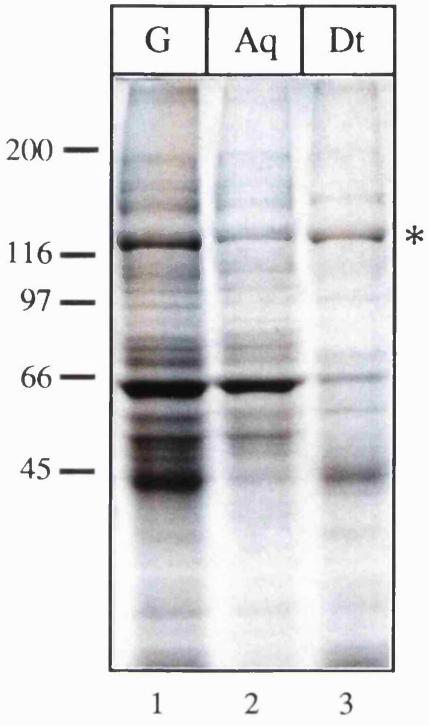

Figure 3.7: TX-114 phase separation of GERP125. A 50µg aliquot of Golgi membranes (G) was subjected to TX-114 phase partitioning and the aqueous phase (Aq) separated from the detergent phase (Dt). Positions of molecular weight markers (in kD) are shown on the left and GERP125 (*) on the right.

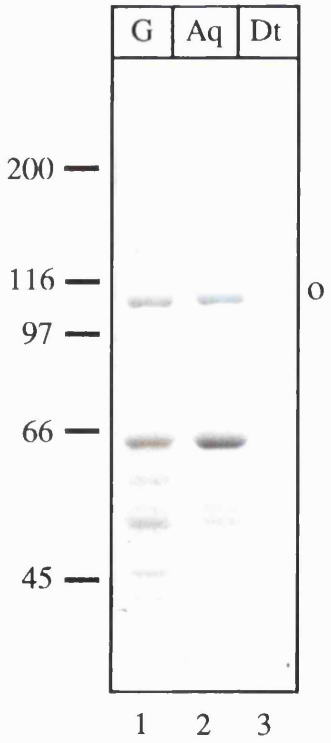

Figure 3.8: TX-114 phase separation of GC110. A 50µg aliquot of Golgi membranes was digested with chymotrypsin in the presence of TX-100 (lane 1). This sample was subjected to TX-114 phase partitioning and the aqueous phase (lane 2) separated from the detergent phase (lane 3). Positions of molecular weight markers (in kD) are shown on the left and GC110 (o) on the right.

After quenching of the protease, the membranes were extracted with TX-114 and the phases separated. The phases and an aliquot of digested but extracted membranes were precipitated and analysed by SDS-PAGE (fig. 3.8).

This showed clearly that GC110 separates entirely into the aqueous phase (lane 2), and therefore behaves like a soluble protein. No proteins were detected in the detergent phase (lane 3), presumably because digestion with chymotrypsin had fragmented them into peptides which were hydrophilic and/or too small to be resolved by the gel.

Taken together, these data suggest that GERP125 is an integral membrane protein with a very small cytoplasmic tail (less that 2kD, since a mass shift of this magnitude would be difficult to resolve for a protein of this molecular weight). Because it does not completely partition into the detergent phase of TX-114, the major part of the protein is probably hydrophilic. The majority of GERP125 is resistant to chymotrypsin and is converted into a 110kD form (GC110) upon digestion with this protease. Since this cleavage can only occur in the presence of TX-100, the cleavage site must be lumenal and since GC110 partitions completely into the TX-114 aqueous phase, the lost 15kD fragment presumably contains a region which interacts with the lipid bilayer. The complete aqueous phase solubility of GC110 also confirms the highly hydrophilic nature of this domain as does the fact that the protein is completely digested by trypsin which recognises only arginyl and lysyl residues.

3.2.4 Identification of GERP125

In order to gain more information on GER125, both it and GC110 were subjected to N-terminal microsequencing. This would identify the molecule if it had already been cloned, or provide information for the synthesis of degenerate oligonucleotides which could be used in cloning of its cDNA. It was, of course, possible that the loss of the 15kD fragment from GERP125 upon chymotrypsin digestion was from the C-terminus of the protein and that both GERP125 and GC110 would have identical N-terminal sequences. This would easily be determined upon microsequencing.

Aliquots of 2mg of Golgi membranes were either digested with chymotrypsin at 25µg/ml in the presence of 0.1% (w/v) TX-100 to provide samples of GC110, or left untreated to provide samples of GERP125. After TCA precipitation and separation by SDS-PAGE, the samples were blotted onto PVDF membrane for peptide microsequencing. GERP125 and GC110 were visualised on the membrane by staining with sulpho-rhodamine B (Coull and Pappin, 1990) and sequenced by Dr. Darryl Pappin and Dinah Rahman in the ICRF Protein Sequencing Laboratory. Because of its large molecular weight, GERP125 proved difficult to transfer onto the membrane. The high

GERP-125	X	X	X	S	X	Q	F	T	V	F	X	S	A	X	F	X	V	V	I	F	X	L
Man II	M	K	L	S	R	Q	F	T	V	F	G	S	A	I	F	C	V	V	I	F	S	L

GC-110	X	X	Q	A	D	P	X	D	X	L	F	A	S	Q	X	G	X	Q
Man II 110kd	A	L	Q	A	D	P	R	D	C	L	F	A	S	Q	S	G	S	Q

Figure 3.9: Sequence of GERP125 and GC110. GERP125 and GC110 were blotted onto PVDF membrane and subjected to N-terminal microsequencing. The data obtained are compared to the sequence of the translated cDNA of mannosidase II (Mann II). Amino acids are given in one-letter code (X=unassigned residue).

molecular weight also meant that there was a relatively low number of N-termini per μg of protein. The combination of these two factors made it difficult to obtain long stretches of sequence information from both GERP125 and GC110. However, enough residues were assigned to identify GERP125 as being the *medial*-Golgi glycosidase, mannosidase II (fig 3.9) by comparison with the sequence of the murine cDNA which had just been cloned (Moremen and Robbins, 1991).

Mann II is a resident of the *medial*-Golgi (Baron and Garoff, 1990; Burke *et al.*, 1982). It has a molecular weight of 125kD and produces a 110kD chymotryptic fragment after digestion with this protease (Moremen *et al.*, 1991). It is a type II membrane glycoprotein with a very short (5 amino acid) cytoplasmic tail. This, in combination with the sequence data showed conclusively that GERP125 and Mann II were the same protein.

This result was initially surprising but two properties of Mann II were intriguing. Firstly, it was almost entirely insoluble in TX-100 while in direct contrast, the *trans*-Golgi enzyme GalT was completely solubilised. This TX-100 insolubility of Mann II was reported previously when the enzyme was first purified (Tulsiani *et al.*, 1977). Secondly, Mann II was an extremely abundant protein and could be easily distinguished after SDS-PAGE of whole Golgi membranes. Calculations from published purification tables of other N-glycan processing enzymes show that they constitute significantly smaller amounts of Golgi membranes then Mann II. For example, NAGT II constitutes approximately 0.2% of total Golgi protein (Bendiak and Schachter, 1987b), GalT approximately 0.1% (Bendiak *et al.*, 1993) and SialylT approximately 0.4% (Weinstein *et al.*, 1982a). Values for NAGT I are less precise because the specific activities of the enzymes in crude homogenates or Golgi membranes are not published. However, rough

estimates suggest that NAGT I constitutes less than 0.5% of total Golgi protein (Oppenheimer and Hill, 1981). In contrast, Mann II constitutes approximately 1% of the total Golgi proteins (Moremen *et al.*, 1991; Tulsiani *et al.*, 1977).

These two observations suggested that Mann II may either be bound to or constitute part of a cytoplasmic or lumenal Golgi matrix while GalT is not.

3.3 Optimisation of TX-100 Extraction

In order to investigate the hypothesis of Mann II being a component of a Golgi matrix, it was necessary to optimise the extraction protocol in order to maximise the solubility of GalT whilst maintaining minimum Mann II solubilisation. If the above hypothesis held true, Mann II would act as a marker for the Golgi matrix while GalT would act as the marker for non-matrix Golgi proteins. Such optimisation would give a maximal yield of matrix for future study, whilst removing as many non-matrix proteins as possible.

To obtain quantitative data, solubilisation of Mann II and GalT was monitored by the solubilisation of their enzymatic activities. The reason for this choice was two-fold. Firstly there was a shortage of good quality antibodies against these two rat-liver enzymes which could be used for quantitative Western blotting. Secondly, enzyme assay proved a be a much swifter method for obtaining quantitative data of enzyme solubilisation. In such experiments, however, care had to be taken that solubilisation of enzymatic activity truly reflected the solubilisation of the protein. For example, if a small amount of GalT was solubilised but this free enzyme was significantly activated, the enzyme assay would still indicate that large amounts had been solubilised. To exclude this possibility, it was necessary to show that no activation or inactivation of enzyme activity had occurred after extraction. Thus in each experiment, the total amount of Mann II or GalT in the supernatant and pellet was compared to an equivalent amount of untreated Golgi membranes to gauge whether activation or inactivation had occurred. If the recoveries were close to 100%, it would be reasonable to assume that no changes in enzyme activity had occurred.

I will now describe the optimisation of the extraction protocol for several parameters. These are; the buffer used, its pH, the TX-100 concentration, the concentration of Golgi membranes during extraction, the centrifugation velocity after extraction, and effect of the increased ionic strength caused by the presence of both mono- and divalent cations.

3.3.1 Effect of Different Buffers

The extraction protocol described earlier in this chapter was based on that used to isolate the nuclear lamina (Dwyer and Blobel, 1976). This proved to be a useful starting point in the initial extraction experiments. However, after identification of Mann II as the major

unextracted protein, the extraction procedure was modified to optimise the insolubility of Mann II and maximise the solubility of GalT.

The first parameter examined was the concentration of TEA in the extraction buffer. Initially a concentration of 10mM was used, but this was considered to be quite low. In order to eliminate the possibility of the buffering capacity being exceeded during extraction, the concentration of TEA in the extraction buffer was increased to 50mM. The solubility of Mann II in extraction buffer containing either the usual 10mM, or an increased concentration of 50mM, of TEA was examined to determine if the increase in ionic strength caused by the increase in buffer concentration caused a greater solubilisation of this enzyme. Golgi stacks (100µg aliquots) were extracted in TTMDS buffer which contained either the usual 10mM or an increased concentration of 50mM TEA at pH7.5. After centrifugation the supernatants and pellets, and an aliquot of untreated Golgi-membranes, were assayed for Mann II activity. The percentage of total Mann II activity found in the pellet was calculated and plotted (fig. 3.10). The enzyme recoveries for these extractions were 97.3% (10mM) and 94.7% (50mM). This showed that the distribution of the enzyme activity was a true reflection of the protein solubilisation and that the increase in buffer TEA concentration had virtually no effect of the insolubility of Mann II.

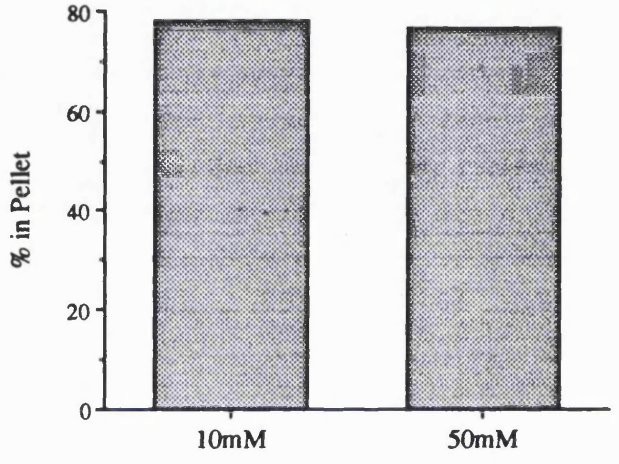

Figure 3.10: Effect of increased TEA concentration on Mann II extraction. 100µg of Golgi membranes were extracted in TTMDS containing either 10mM of 50mM TEA. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II. The data are presented as the percentage of the total Mann II activity present in the pellet.

Once the fact that increasing the TEA buffer concentration to 50mM had no effect on Mann II insolubility had been established, the effect of different buffers on this enzyme's extraction properties was examined.

The buffers tested were MES, HEPES, MOPS and PIPES. Since the pKa of MES is 6.1, and is close to the limit of its useful buffering range at pH7.0, the extractions were performed at pH 6.75 so that all the buffers would be within their pH ranges. As a control, extractions were also performed in TEA buffers at pH 7.5 and 6.75. 100µg

aliquots of Golgi membranes were extracted in TTMDS buffer containing either 50mM TEA at pH7.5 or pH 6.75, or where the TEA was replaced by 50mM MES, HEPES, MOPS or PIPES at pH 6.75. After extraction, the supernatants and pellets were diluted 20-fold and assayed for Mann II activity as was an appropriate amount of untreated Golgi membranes (fig. 3.11). Enzyme recoveries were 94.7%±8.9 (±SD) compared to untreated Golgi membranes.

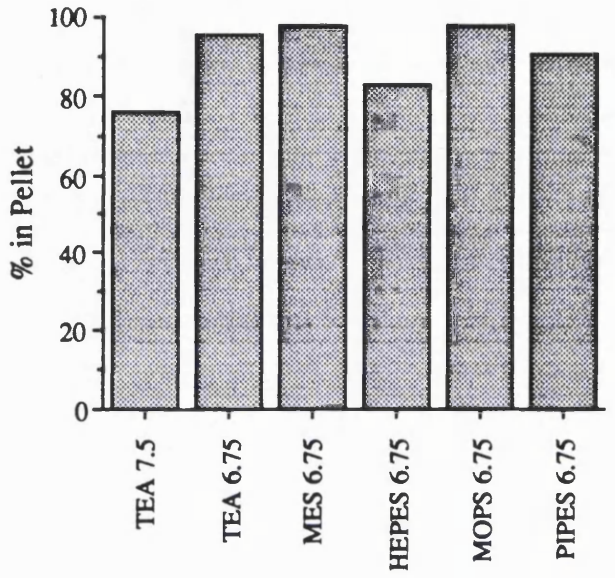

Figure 3.11: Effect of different buffers on Mann II insolubility. 100µg of Golgi membranes were extracted in TTMDS containing either 50mM TEA at pH7.5 or 6.75, or MES, HEPES, MOPS or PIPES at 50mM pH6.75. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II. The data are presented as the percentage of the total Mann II activity present in the pellet.

These data showed that Mann II was significantly insoluble in all buffers. However both MES and MOPS buffers were slightly more effective at maintaining the Mann II insolubility, with TEA being close behind. PIPES and HEPES were least efficient (albeit slightly) in maintaining this insolubility. Interestingly, TEA buffer at pH6.75 seemed to sustain Mann II insolubility more effectively than at pH 7.5, indicating that Mann II insolubility might be pH dependent.

3.3.2 Effect of pH

Since extraction of Golgi membranes at pH7.5 solubilised more Mann II than at pH6.75 (fig. 3.11), the effect of pH on Mann II solubility was further investigated. In addition, since Mann II solubility appeared to be affected, the effect of pH on GalT solubility was also investigated. The buffer chosen was MOPS since this showed to be most effective in maintaining Mann II insolubility and also had a pKa of 7.2. This meant that it could be used to produce buffers in the 6.0-8.0 pH range whereas the other buffers could not.

100µg aliquots of Golgi membranes were extracted with TTMDS buffers in which TEA had been replaced by 50mM MOPS, and whose pHs had been adjusted to 6.0, 6.5,

7.0, 7.5 and 8.0 respectively. Supernatants and pellets were diluted 20-fold and assayed for Mann II and GalT as was a suitably diluted sample of untreated Golgi membranes. Enzyme recoveries were found to be 95.4%±12.1 (±SD) for Mann II and 103.2%±5.3 (±SD) for GalT.

This showed that both Golgi enzymes displayed maximal insolubility at pH6.0 with GalT insolubility showing a particularly marked increase (fig. 3.12). Mann II solubility increased gradually with increasing pH while GalT solubility increased to a maximum at pH7.0 and decreased again more alkaline pH. This clearly showed that the separation of Mann II and GalT was greatest at pH7.0 using MOPS as a buffer. All further experiments were therefore performed using TMMDS buffer (TX-100, 50mM MOPS pH7.0, 0.1mM MgCl2, 1mM DTT and 10% (w/v) sucrose). The Triton concentration used in this buffer was next determined, and this experiment is described below.

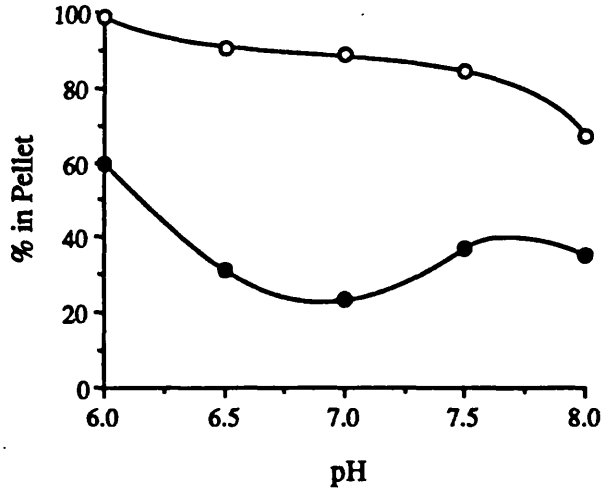

Figure 3.12: Effect of pH on Mann II and GalT insolubility. 100µg aliquots of Golgi membranes were extracted in TTMDS buffer in which TEA had been substituted by 50mM MOPS and at varying pH. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II (O) and GalT (●). The data are presented as the percentage of total enzyme activity present in the pellet.

3.3.3 Effect of TX-100 Concentration

After optimisation of buffer and pH conditions for Mann II, the insolubility of GalT had increased from 10% to 25% (c.f. fig.3.12, pH7.0 and fig. 3.3). In order to reverse this, the Triton concentration during the extraction was titrated in the hope of maintaining the Mann II insolubility and decreasing that for GalT.

Golgi membranes were extracted with TMMDS buffer containing varying TX-100 concentrations of 0, 0.25, 0.5, 1.0 and 2.0% (w/v). The supernatants and pellets were diluted 20-fold and assayed for Mann II and GalT as was an untreated aliquot of Golgi membranes which had been diluted to a protein concentration of 1mg/ml. Enzyme

recoveries were 92.7%±5.7 (±SD) for Mann II and 95.4%±7.3 (±SD) for GalT, indicating that the results were a true reflection of the protein solubilities.

Unsurprisingly, both Mann II and GalT solubility increased with increasing detergent concentration (fig. 3.13). At 2% (w/v) TX-100, however, GalT was almost completely solubilised whilst Mann II solubilisation seemed to have reached a plateau with approximately 80% of the Mann II remaining in the insoluble pellet.

This showed that extraction in 2% (w/v) TX-100 under these conditions allowed a significant separation of Mann II and GalT with less than 10% of GalT and approximately 80% of Mann II remaining in the pellet. Thus, all future extractions were performed in TMMDS buffer where the Triton concentration was 2% (w/v).

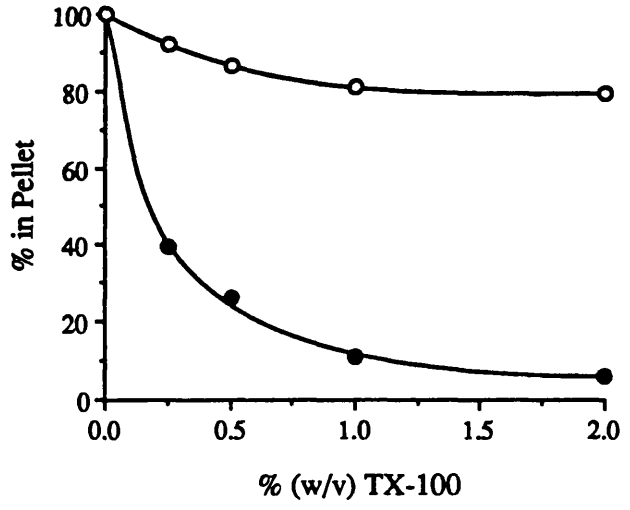

Figure 3.13: Effect of TX-100 concentration on Mann II and GalT solubility. 100µg aliquots of Golgi membranes were extracted in TMMDS buffer containing varying concentrations of TX-100. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II (○) and GalT (●). The data are presented as the percentage of the total enzyme activity present in the pellet.

3.3.4 Effect of Golgi Concentration

Having established the above extraction buffer conditions, the effect of the concentration of Golgi membranes during extraction was examined. The concentration of Golgi membranes was assumed to be directly proportional to the protein concentration in the preparation. Initially, the concentration of Golgi membranes during extraction was arbitrarily chosen to be 1mg/ml. Now that the extraction buffer had been properly optimised, the concentration of the Golgi membranes during the extraction was also investigated with respect to Mann II solubilisation.

Golgi membranes in 25, 50, 100, 150 and 200µg aliquots were recovered by centrifugation and resuspended in 100µl of TMMDS buffer to yield final protein concentrations of 0.25, 0.5, 1, 1.5 and 2mg/ml. After incubation and centrifugation, supernatants and pellets were diluted 20-fold and assayed for Mann II. Enzyme recoveries compared to untreated Golgi membranes were found to be 94.5%±8.2 (±SD).

This showed that Mann II exhibited its maximal insolubility at a protein concentration of 1mg/ml (fig. 3.14). This was not particularly surprising since the extraction had already been optimised at this protein concentration. At lower protein concentrations, more Mann II was solubilised, with complete solubilisation occurring by 0.25mg/ml of protein. This was also not surprising because an increase in the detergent:protein and thus in the detergent:lipid ratio would be expected to cause greater solubilisation of both the lipid bilayer and the membrane-spanning proteins. At protein concentrations above 1mg/ml, the Mann II appeared to become slightly more soluble, though this effect was slight and did not appear to be significant.

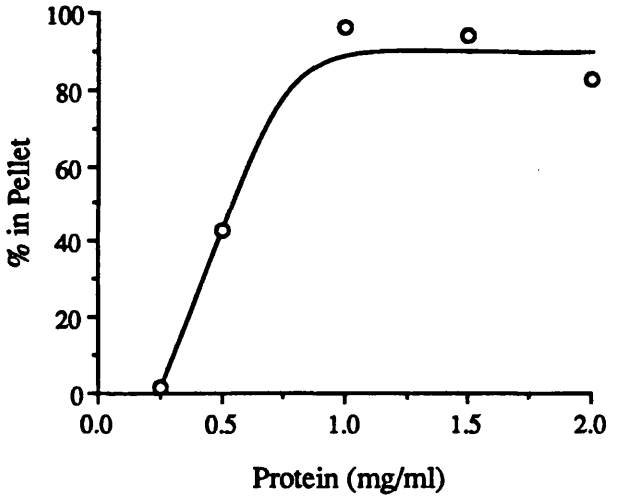

Figure 3.14: Effect of Golgi protein concentration on Mann II solubility. 25, 50, 100, 150 and 200µg aliquots of Golgi membranes were extracted in 100µl of TMMDS buffer. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II. The data are presented as the percentage of the total enzyme activity present in the pellet at each Golgi protein concentration.

Since Mann II insolubility was maximal at 1mg/ml of Golgi protein, all future extractions were carried out at this protein concentration.

3.3.5 Effect of Centrifugation Velocity

The next parameter tested was that of the centrifugation velocity. The speed of 20,000rpm for 30min in the TLA-100 ultracentrifuge had been chosen purely arbitrarily, based on the sedimentation velocity used to isolate the nuclear lamina (Dwyer and Blobel, 1976). Now that the extraction conditions had been properly characterised, the speed of the centrifugation required to separate the soluble from the insoluble material after extraction. The spin speed was kept at 30min because the centrifuge took about 5min to accelerate and brake. Thus if the duration of the centrifugation were less, the proportion of the time taken in the acceleration would have been too great compared to the total spin time. Since 80-90% of the Mann II appeared to be insoluble at 20,000rpm (g_{max}= 17,400), its sedimentation was investigated only at lower spin speeds.

Golgi membranes were extracted in TMMDS buffer and incubated at 4°C for 30min. Membranes were then centrifuged for 30min at 4°C in a TLA-100 ultracentrifuge at spin speeds of 5, 10, 15, 18 and 20 thousand rpm (or g_{max} of 1.1, 4.3, 9.8, 14.1 and 17.4 thousand). Supernatants and pellets were diluted 20-fold and assayed for Mann II and GalT. Recoveries were 89.9%±2.8 (±SD) for Mann II and 92.7%±5.6 (±SD) for GalT.

This showed that Mann II sedimentation was maximal at 17,000-20,000rpm, while GalT remained unsedimentable under all spin speeds (fig. 3.15). Thus, the centrifugation velocity used after extraction was kept at 20,000rpm for future experiments since this seemed to be sufficient to pellet all the insoluble Mann II.

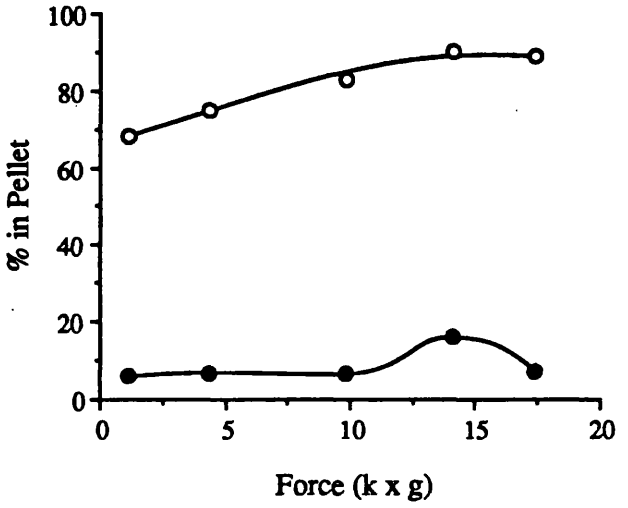

Figure 3.15: Sedimentation characteristics of Mann II and GalT. 100µg aliquots of Golgi membranes were extracted in 100µl of TMMDS buffer. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II (○) or GalT (●). The data are presented as the percentage of the total enzyme activity present in the pellet.

3.3.6 Effect of Cations

All the extractions that had been carried out so far had been in buffers of low ionic strength, with no ions being present except those of the buffer and the acid or alkali used to adjust the pH. In order to determine the consequence of changes in the ionic strength of the extraction buffer, the effect of the addition of varying concentrations of both mono- and divalent cations during extraction on the solubility of both Mann II and GalT was examined. The cations titrated were calcium, sodium and potassium.

3.3.6.1 Calcium

To ascertain whether the concentration of calcium during extraction of Golgi membranes had any effect of the solubilities of Mann II or GalT, Golgi membranes were extracted in TMMDS buffer in presence of increasing concentrations of $CaCl_2$. Calcium concentrations in biological systems range from under 1µM intracellularly to 2mM extracellularly. In order to encompass this physiologically relevant range, the extractions were performed using calcium concentrations of 0, 1, 10, 100, 1000 and 2000µM.

Since Millipore-filtered water contains low concentrations of calcium and because very low concentrations were used some of the samples in this experiment, it was necessary to utilise a calcium buffering system, in which the concentration of free calcium was regulated by the presence of the chelating agent EGTA, to ensure that the calcium ion concentrations in the samples which contained very low amounts of calcium were correct. Such a buffering system was incorporated into the 0 and 1μM samples and consisted of the appropriate concentration of calcium and EGTA as calculated by the method of Zucker and Steinhardt (1978). These two samples were supplemented with 1mM EGTA and contained 0 and 708μM concentrations of calcium respectively, which yielded the required final concentrations of 0 and 1μM of calcium in the extraction buffer.

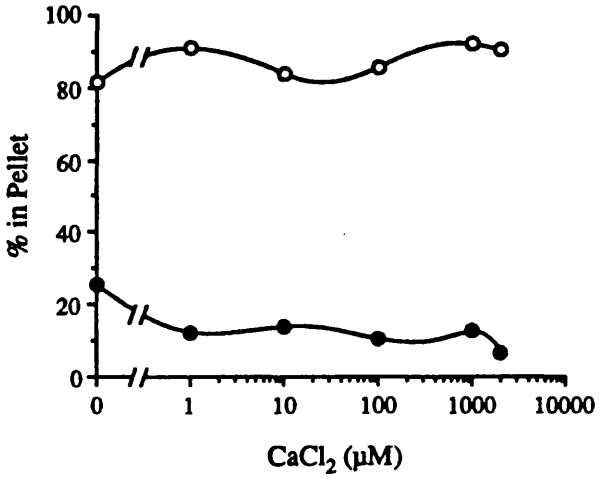

Figure 3.16: The effect of calcium on the solubility of Mann II and GalT. 100μg aliquots of Golgi membranes were extracted in 100μl of TMMDS buffer containing increasing concentrations of calcium. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II (O) or GalT (●). The data are presented as the percentage of the total enzyme activity present in the pellet.

Golgi membranes were divided into aliquots of 100μg and were extracted with TMMDS supplemented with the appropriate concentrations of calcium or of the calcium buffering system. After centrifugation, the supernatants and pellets were separated and assayed for both Mann II and GalT activities as was an aliquot of untreated Golgi membranes. The enzyme recoveries following the extraction were found to be 94.8±5.3 (±SD) for Mann II and 89.7±9.7 (±SD) for GalT.

These data clearly showed that the solubilities of both Mann II and GalT were unaffected by the concentration of calcium in the extraction buffer (fig 3.16) or by its absence.

3.3.6.2 Sodium

The effect of the presence NaCl during extraction on the solubility of both Mann II and GalT was next examined by performing extractions in TMMDS buffer supplemented

with increasing concentrations of NaCl. Extra-cellular sodium concentrations in mammalian cells are approximately 150mM, and are much lower intracellularly. Thus the concentrations chosen encompassed this physiological range and also included higher concentrations to ascertain the effects of high ionic strengths. Golgi membranes were therefore divided into aliquots of 100µg and extracted in the presence of 0, 50, 150, 250 and 500mM NaCl. After extraction, the supernatants and pellets were separated and assayed for both Mann II and GalT activities as was an aliquots of untreated Golgi membranes. The enzyme recoveries after extraction were found to be 103.4±4.1 (±SD) for Mann II and 95.8±3.6 (±SD) for GalT.

This showed that Mann II insolubility was extremely sensitive to the presence of NaCl within the physiological range (fig. 3.17). In the absence sodium, only 17% of the Mann II was solubilised after extraction, while in the presence of 50mM NaCl this increased to 52%. Once the NaCl concentration reached 150mM and beyond, almost all the Mann II had become soluble (>95%). This effect was exactly mimicked by GalT with the 10% which was insoluble in the absence of NaCl also being completely solubilised at a concentration of150mM.

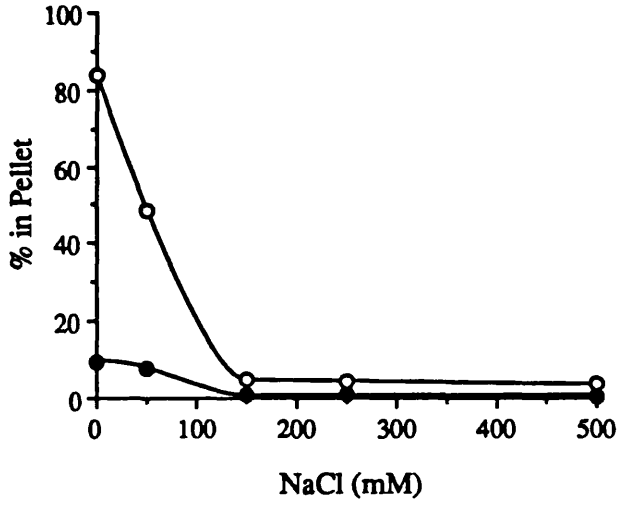

Figure 3.17: The effect of sodium on the solubility of Mann II and GalT. 100µg aliquots of Golgi membranes were extracted in 100µl of TMMDS buffer containing increasing concentrations of sodium. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II (O) or GalT (●). The data are presented as the percentage of the total enzyme activity present in the pellet.

3.3.6.3 Potassium

Since the presence of NaCl during extraction had such a dramatic effect on the solubility of Mann II (and, to a lesser extent, of GalT), it was necessary to determine whether this effect was due to the action of the sodium cations or chloride anions present in these extractions. To achieve this, a further titration was carried out using increasing concentrations of KCl in the extraction buffer. If the chloride ions were responsible for

the enzyme solubilisation, the solubilisation curve of KCl would be expected to be identical to that of NaCl. A difference in the curves would, however, have indicated that solubilisation would be due to the differing cations.

This experiment was carried out in much the same way as for NaCl. Since the concentration of intracellular potassium in mammalian cells is approximately 150mM (and much lower extracellularly), the concentrations used in the experiment were the same as for the sodium titration i.e. 0, 50, 150, 250 and 500mM. This would also allow a more direct comparison of the solubilisation curves.

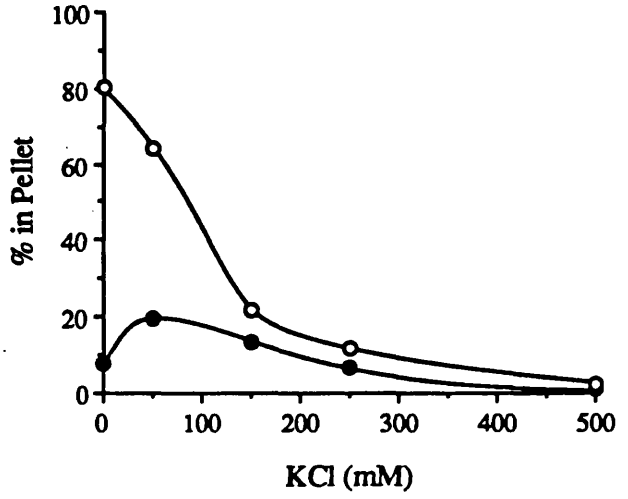

Figure 3.18: The effect of potassium on the solubility of Mann II and GalT. 100µg aliquots of Golgi membranes were extracted in 100µl of TMMDS buffer containing increasing concentrations of potassium. After extraction, supernatants and pellets were diluted 20-fold before assaying for Mann II (○) or GalT (●). The data are presented as the percentage of the total enzyme activity present in the pellet.

Golgi membranes were divided into 100µg aliquots and extracted in TMMDS buffer supplemented with increasing concentrations of KCl. The supernatants and pellets were separated and assayed for Mann II and GalT activity as was an aliquot of untreated Golgi membranes. The enzyme recoveries were 98.5±2.3 (±SD) for Mann II and 104.7±7.3 (±SD) for GalT.

This showed that Mann II was also solubilised by the presence of potassium (fig. 3.18). However, although increasing concentrations of KCl caused an increase in solubilisation of Mann II, this effect was not as pronounced as for NaCl. In contrast, GalT showed a slightly different behaviour. At a potassium concentration of 50mM, the solubility of GalT seemed to decrease slightly but began to increase again at 150mM, with complete solubilisation occurring at 500mM.

The solubilisation of Mann II by NaCl and KCl in TMMDS buffer is compared in figure 3.19A. This showed that while both salts caused complete solubilisation of Mann II at concentrations of 500mM, NaCl was more effective in solubilisation at lower concentrations. This difference also clearly indicated that the solubilisation of Mann II was being caused by the sodium and potassium cations and not by the chloride anions

present during these extractions. This was also confirmed by the difference in solubilisation of GalT by these salts (fig. 3.19B).

3.3.7 SDS-PAGE

Once these procedures had been optimised, the composition of the insoluble Triton pellet was analysed by SDS-PAGE. A 20µg aliquot of Golgi membranes were extracted using the new modified procedure and the protein in the supernatant and pellet, as well as in a 20µg aliquot of untreated membranes, were precipitated and subjected to SDS-PAGE (fig. 3.20). After staining with Coomassie Brilliant Blue R it was apparent that Mann II was still the major insoluble protein after extraction (c.f. lane 3 in figs. 3.2 and 3.20), but that more of the total protein had been solubilised. Protein assays of the extracted supernatants and pellets indicated that the Triton pellet contained only 40% of the original Golgi protein.

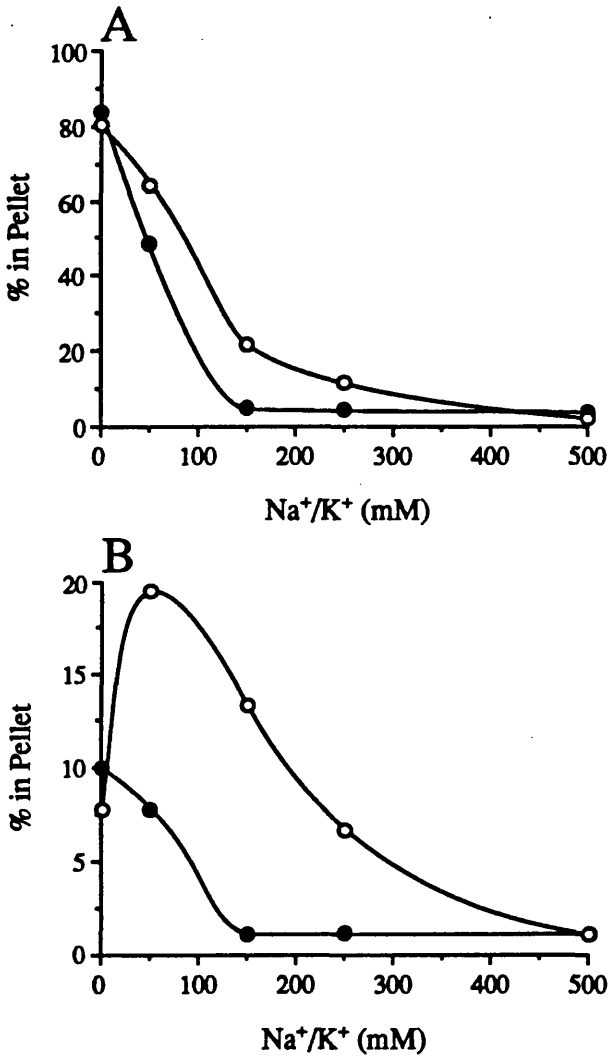

Figure 3.19: Comparison of the effect of NaCl and KCl on Mann II and GalT solubility. The graphs show the combined results of the extraction of Golgi membranes in varying concentrations off NaCl (●) and KCl (○) and their effect on the solubility of Mann II (A) and GalT (B).

Additionally this showed that the 80% insolubility of Mann II activity was a true representation of the protein solubility (fig. 3.20 c.f. lanes 2 and 3), and thus demonstrated that using enzyme activity as a marker for protein solubilisation was a valid approach.

3.4 Summary

Purified Golgi membranes were extracted with the non-ionic detergent Triton X-100 in order to identify candidate proteins of a Golgi matrix. Such molecules would be expected to be insoluble after such treatment as are the constituents of other structural cellular components such as the nuclear lamina (Dwyer and Blobel, 1976; Aebi *et al.*, 1986), the as yet uncharacterised ER matrix (Hortsch *et al.*, 1987) and many intermediate-type filaments (Tezuka and Freedberg, 1972; Geisler and Weber, 1980; Starger and Goldman, 1977).

After such treatment, a major insoluble protein with a molecular weight of 125kD was observed. This protein was named GERP125 and was the second most abundant constituent of the Golgi preparation as assessed by SDS-PAGE. Protease protection and TX-114 phase-separation experiments indicated that GERP125 was an integral membrane protein, the majority of which was probably in the lumen of the Golgi cisternae and which had a very short cytoplasmic domain. Peptide microsequencing identified GERP125 as being the *medial*-Golgi enzyme, mannosidase II.

Since Mann II was an unusually abundant protein and exhibited such Triton-insolubility, it was hypothesised that it may constitute part of a Golgi matrix. In order to investigate this possibility, the extraction procedure was optimised to maximise both the insolubility of Mann II and the solubility of another Golgi enzyme, GalT. Such optimisation would yield an insoluble pellet which would contain a purer sample of putative Golgi matrix components which could then be studied in more detail.

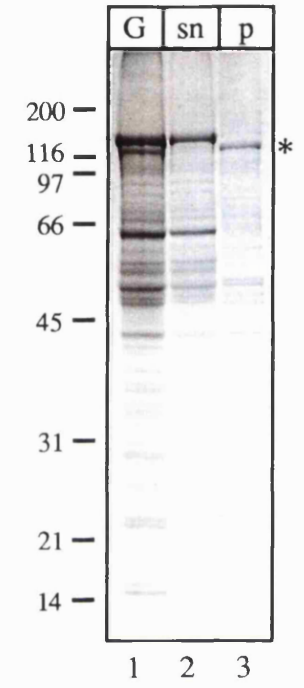

Figure 3.20: SDS-PAGE analysis of Golgi membranes solubilised in TMMDS buffer. A 20µg aliquot of Golgi membranes (G), was extracted in TMMDS buffer. After centrifugation, the supernatant (sn) and pellet (p) were analysed by SDS-PAGE. The position of Mann II (*) is shown on the right and the molecular weight standards (in kD) are indicated on the left.

Initial experiments to optimise the insolubility of Mann II showed that it was slightly dependent upon the buffer used during extraction with MES and MOPS yielding slightly greater insolubilities than TEA, HEPES and PIPES. Whether the fact that both MES and MOPS

gave slightly better results was coincidental, or due to their similar structure is unknown (both molecules contain a morpholine group conjugated to either ethanoic or propanoic acid via the nitrogen atom in the morpholine ring). This experiment also suggested that Mann II insolubility was pH dependent and MOPS was chosen as the buffer for future use since it had a slightly larger pH range which would prove useful during a titration of the effect of pH on the extraction of Golgi membranes.

A subsequent titration of the pH of the MOPS-containing extraction buffer confirmed that the solubilities of both Mann II and GalT were pH-dependent. Both enzymes were more insoluble at pH6.0, but while Mann II displayed increasing solubility up to pH8.0, GalT solubility was maximal at pH7.0 and decreased again at more alkaline pH. Because GalT solubility was maximal at pH7.0, this was chosen as the pH for future extractions.

Once the optimal choice of buffer and pH had been determined, the concentration of TX-100 during extraction was titrated. This showed that optimal separation of Mann II and GalT occurred at a TX-100 concentration of 2%(w/v). Furthermore, it was also shown that this separation was best obtained at a Golgi protein concentration of 1mg/ml, the same concentration at which all previous experiments had been performed. This was not surprising, since all the optimisations had also been carried out at this concentration of Golgi membranes. This was also found to be the case for the velocity of centrifugation after extraction which was also, therefore, kept at 20,000rpm.

Finally, the effect of the presence of mono- and divalent cations during extraction was examined. The solubility of Mann II and GalT was independent of the presence of physiologically relevant concentrations of calcium in the extraction buffer, but in contrast Mann II was efficiently solubilised in the presence of sodium as was the small amount of GalT that was normally insoluble in TX-100 alone. Potassium had a similar effect to sodium though this was not as pronounced, with more cations being required for the complete solubilisation of Mann II. In contrast, however, GalT appeared to be slightly more insoluble at physiologically relevant levels of potassium but this too was completely solubilised by 500mM. The reason for this slight elevation of GalT insolubility is unknown.

Thus, taken together, these experiments indicated that separation of Mann II and GalT could be best achieved by the extraction of Golgi membranes at a protein concentration of 1mg/ml in TMMDS buffer (2%(w/v) TX-100, 50mM MOPS pH7.0, 0.1mM $MgCl_2$, 1mM DTT, 10%(w/v) sucrose) at 4°C for 30min, followed by centrifugation at 20,000rpm and 4°C for 30min in a TL-100 ultracentrifuge. This yielded an insoluble pellet which contained over 80% of the original Mann II but less than 10% of the GalT. Protein assays also showed that 60% of the total Golgi protein was extracted by this

procedure, indicating that a 2-fold increase in the purity of Mann II (and therefore potential Golgi matrix components) had been obtained by this simple procedure.

Thus the above conditions were chosen for future experiments in which the nature of Mann II insolubility was studied in greater detail.

Chapter 4

Characterisation of the Triton Pellet

4.1 Introduction

Having optimised the Triton extraction procedure to the extent that over 80% of Mann II remained insoluble while over 90% of GalT was released into the supernatant, the nature of this Triton pellet was examined in more detail.

As mentioned previously in chapter 3, the fact that Mann II was the major unextracted protein after TX-100 extraction was a surprising result. It was, therefore of interest to ascertain whether any other Golgi resident proteins exhibited this insolubility or whether they too were solubilised like GalT. In this chapter, I describe the solubility properties of a range of Golgi marker proteins which are localised to different regions of the Golgi stack, and to the TGN or CGN respectively.

Additionally, I describe experiments which provide a more detailed characterisation of the Triton pellet, for example its morphology, its density, its isolation using antibodies against Mann II and a method for the solubilisation of Mann II from the Triton pellet.

4.2 Results

4.2.1 Solubility of Other Golgi Markers

In this section, I describe experiments carried out to determine the solubility of other Golgi proteins which are residents of subcompartments throughout the Golgi stack, as well as determining the reproducibility of the solubilities of Mann II and GalT. The markers chosen were: p58 as a marker for the CGN; Mannosidase I for the *cis*-cisternae; NAGT I, Mann II and NAGT II for the *medial*-cisternae, GalT and SialylT for the *trans*-cisternae/TGN and TGN38 for the TGN. It should be noted that the above assignments of *cis- medial-* and *trans*-Golgi are the compartments in which these molecules are classically thought to reside based on their positions in the catalytic pathway of N-glycan processing and/or on immuno-electron microscopic data. As discussed in detail in chapter 1, the actually distribution of these molecules within the Golgi stack varies greatly from species to species and even with differing cell types within the same species.

Again due to the lack of specific antibodies to many of these proteins, the solubilisation of these proteins was monitored by means of their enzymatic activities. Since p58 and TGN38 have no known function or enzymatic activity, their solubilisation was monitored by quantitative Western blotting followed by immunodetection and laser densitometry. This was made possible by the kind donation of specific antibodies to p58 and TGN38 by Drs. Jakko Saraste (University of Bergen) and George Banting (Bristol University) respectfully.

The results for each marker are described in turn below and summarised at the end of this section.

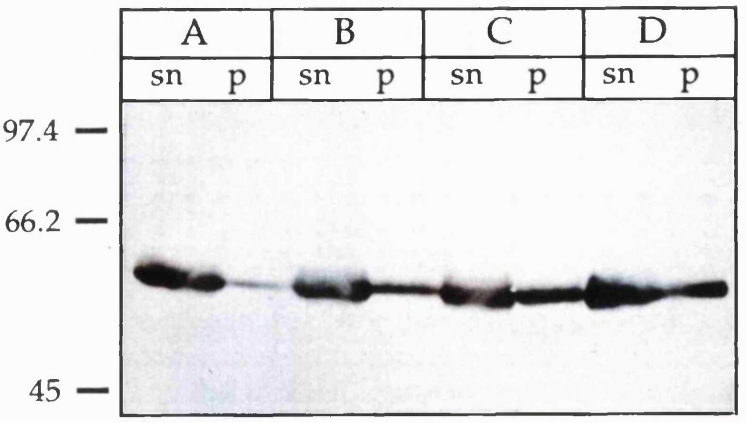

Figure 4.1: Western blot of the solubilisation of p58. 50μg aliquots of four different Golgi membrane preparations (A-D) were extracted with TTMDS buffer and the supernatants (sn) and pellets (p) precipitated and subjected to SDS-PAGE and analysed by Western blotting. The blot was probed using a polyclonal antibody against p58. Positions of molecular weight markers (in kD) are indicated on the left.

4.2.1.1 p58

This molecule, as suggested by its name, is a protein with molecular weight of 58kD. As yet, its function is unknown, but it has been shown to be a marker of the CGN and *cis*-cisterna by immuno-electron microscopy (Saraste *et al.*, 1987; Saraste and Svensson, 1991).

In order to determine this protein's solubility, 50μg aliquots of four different Golgi membrane preparations were extracted in TMMDS buffer and the supernatants and pellets separated. The protein in these samples was precipitated and subjected to SDS-PAGE followed by Western blotting. The p58 was detected by probing with a polyclonal p58-anti-serum followed by a HRP-conjugated anti-rabbit antibody (TAGO) and visualisation with the ECL kit (Amersham). This showed that a significant amount of p58 was solubilised by this procedure in all four membrane preparations (fig. 4.1).

The percentage solubilisation was calculated by quantitation of the Western blot by laser densitometry. Each band in figure 4.1 was scanned at 3 different positions and the results averaged to eliminate errors which may have arisen due to variations in the shape and density of the bands in different areas. Initially integration was carried out by cutting out and weighing the density curves from computer printouts. The values obtained were almost identical to those using automatic integration showing that the calculations performed by the machine were valid. Finally, to ensure that the exposure times for the Western blots were in the linear range for the film, two blots of 5 and 10sec exposure time were scanned and the results shown to be identical.

The percentage of p58 remaining in the pellet was calculated as being the ratio of amount in the pellet to the total amount in the supernatant and pellet. This quantitation revealed that p58 was significantly solubilised, the average amount of p58 remaining in the insoluble pellet after extraction being 34.9%±6.2 (±SEM, n=4).

4.2.1.2 Mannosidase I

Mann I was chosen as a marker for the *cis*-Golgi and its solubilisation was monitored by assaying its enzymatic activity. Mann I is classically thought to be a *cis*-Golgi marker because it is present in a post-ER compartment, by virtue of the requirement of a vesicular-mediated transport step from the ER before its substrate can gain access to it (Balch *et al.*, 1987) coupled with the fact that it catalyses a reaction prior to any of the other Golgi enzymes (Tabas and Kornfeld, 1979). More recent electron-microscopic data, however, suggest that this enzyme's distribution varies greatly within Golgi stacks in differing cell types and species (Velasco *et al.*, 1993). Due to the lack of any other more reliable, however, *cis*-Golgi markers, Mann I was utilised.

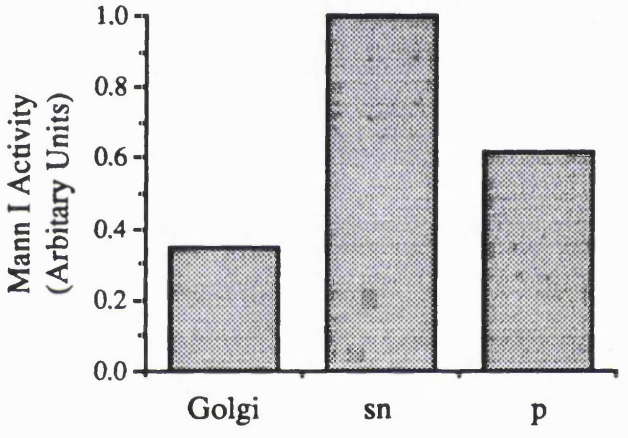

Figure 4.2: Activation of Mann I after extraction in TMMDS. A 100µg aliquot of Golgi membranes was extracted with TTMDS buffer and the supernatant (sn) and pellet (p), as well as an aliquot of untreated Golgi membranes, assayed for Mann I activity. The graph shows the relative amount of Mann I activity in each sample

Four aliquots of 100µg of different Golgi membrane preparations were extracted and the supernatants and pellets separated. These were assayed for Mann I activity as was an untreated aliquot of each membrane preparation.

The percentage of Mann I remaining insoluble after extraction was found to be 19.5±1.6 (±SEM, n=4). The recovery of enzymatic activity after extraction compared to untreated membranes was found to be 206.4%±36.8 (±SEM, n=4).

This large amount of activation after detergent extraction was further examined by determining whether the activation had predominantly occurred in the solubilised Mann I in the supernatant or in the insoluble enzyme in the pellet. To this effect, 100µg of Golgi membranes were extracted with TMMDS buffer and the supernatants and pellets assayed

for Mann I activity, as was an aliquot of untreated Golgi membranes. This clearly showed that there was more Mann I activity in both the extracted supernatant and pellet than in the untreated membranes (fig. 4.2) and thus the solubilised and insoluble Mann I had been activated after the extraction procedure. This meant that the enzyme activity was not a very reliable marker for the solubilisation of Mann I since the actual degree of activation in the supernatant and pellet could not be determined.

4.2.1.3 N-acetylglucosaminyltransferase I

NAGT I was one of the three classical *medial*-Golgi markers whose solubility was assessed after extraction. It has been localised to the *medial*-Golgi region by immunoelectron microscopy (Dunphy *et al.*, 1985; Nilsson *et al.*, 1993a).

Five 100µg aliquots of different Golgi preparations were extracted with TMMDS buffer and the supernatants and pellets assayed for NAGT I activity as were aliquots of untreated membranes. This showed that a large amount of NAGT I remained insoluble after this procedure, with 77.2%±1.1 (±SEM, n=5) remaining in the pellet after extraction. Furthermore, there seemed to be little activation or inactivation of enzymatic activity during this procedure since the average recovery was 107.9±6.1 (±SEM, n=5).

4.2.1.4 Mannosidase II

Mann II has been localised to the *medial*-Golgi in Hela cells by immunoelectron microscopy (Nilsson *et al.*, 1993a), though it does seem to exhibit cell-type and species variability (Velasco *et al.*, 1993; Novikoff *et al.*, 1983).

Although the insolubility of the *medial*-Golgi marker Mann II has been described in chapters 3 and 4, the reproducibility of this phenomenon was examined further. Five different Golgi membrane preparations were extracted in TMMDS buffer and the supernatants and pellets, as well as aliquots of untreated membranes were assayed for Mann II activity. This confirmed the insolubility of Mann II and demonstrated that the effect was extremely reproducible, with 85.8%±1.7 (±SEM, n=5) of the enzyme remaining in the insoluble pellet. Additionally, very little change in enzyme activity was noted, with the recovery averaging 91.2%±6.5 (±SEM, n=5).

4.2.1.5 N-acetylglucosaminyltransferase II

The final *medial*-Golgi marker used was NAGT II. This enzyme has not been localised by immunoelectron microscopy, however, the fact that it acts directly after Mann II suggests that it is either a *medial*- or *trans*-Golgi marker. Furthermore NAGT II is not found in the same compartment that GalT (Dunphy and Rothman, 1983), which has been

localised to the *trans*-Golgi/TGN by immuno-electron microscopy (Roth and Berger, 1982; Lucocq *et al.*, 1989), suggesting that it is not a *trans*-Golgi resident.

The assay of this enzyme was made possible by the generous donation of substrate by Dr. Harry Schachter. Five aliquots of 100µg of different Golgi preparations were extracted in TMMDS buffer and the supernatants and pellets assayed for NAGT II as were aliquots of untreated Golgi membranes. This showed that, like the other *medial*-Golgi markers, NAGT II was more than 50% insoluble after extraction, with 59.7%±2.8 (±SEM, n=5) remaining in the pellet while the enzyme recovery of 118.8%±4.7 (±SEM, n=5), indicated that little change in enzymatic activity had occurred.

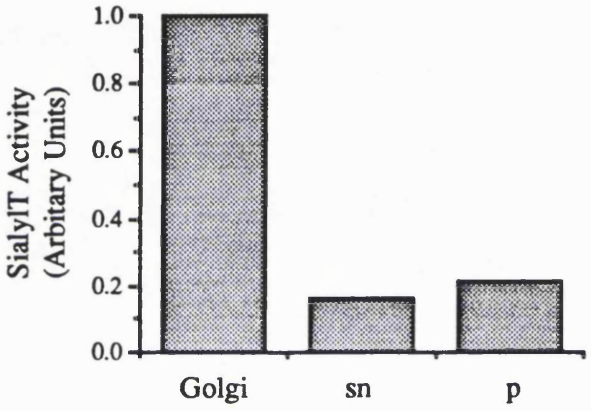

Figure 4.3: Inactivation of SialylT after extraction in TMMDS. A 100µg aliquot of Golgi membranes was extracted with TTMDS buffer and the supernatant (sn) and pellet (p), as well as an aliquot of untreated Golgi membranes, assayed for SialylT activity. The graph shows the relative amount of SialylT activity in each sample

4.2.1.6 Galactosyltransferase

GalT was chosen as a marker of the *trans*-Golgi region. It had been localised by immunoelectron microscopy to the *trans*-Golgi (Roth and Berger, 1982; Lucocq *et al.*, 1989) and to the *trans*-Golgi and TGN (Nilsson *et al.*, 1993a).

Five 100µg aliquots of different Golgi preparations were extracted in TMMDS buffer and the supernatants and pellets, as well as aliquots of untreated membranes, assayed for GalT activity. As described in chapter 3, GalT was highly soluble after such extraction with the percentage remaining in the pellet being 15.6±1.7 (±SEM, n=5) and the enzymatic recovery being 113.0%±3.7 (±SEM, n=5).

4.2.1.7 Sialyltransferase

SialylT was also chosen as a marker of the *trans*-Golgi/TGN region and has been localised to this area by immunoelectron microscopy (Roth *et al.*, 1985).

Five 100µg aliquots of different Golgi membrane preparations were extracted in TMMDS buffer and the supernatants and pellets, as well as aliquots of untreated Golgi membranes, were assayed for SialylT activity. This showed that SialylT was highly

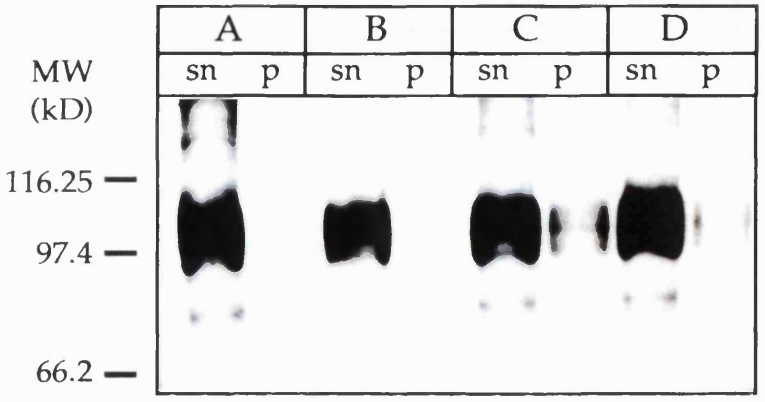

Figure 4.4: Western blot of the solubilisation of TGN38. 50µg aliquots of four different Golgi membrane preparations (A-D) were extracted with TTMDS buffer and the supernatants (sn) and pellets (p) precipitated and subjected to SDS-PAGE followed by Western blotting. The blot was probed using a polyclonal antibody against TGN38. Positions of molecular weight markers (in kD) are indicated on the left.

insoluble after this treatment, with 63.3%±4.2 (±SEM, n=5) remaining in the pellet after extraction. The enzyme recovery, however, was very low when compared to the amount present in intact Golgi membranes, with only 34.1%±5.2 (±SEM, n=5) of the original activity being recovered from the supernatant and pellet. This could have been due to an inactivation of the enzyme activity or loss of the enzyme by non-specific binding to the sides of the centrifuge tubes after extraction.

There was no visible activation of SialylT in the supernatant or pellet as both fractions contained much less activity than the original Golgi membranes (fig. 4.3). It was not possible, therefore, to determine the extent of inactivation or loss of this enzyme in the supernatant and pellet, and so it was not possible to use these data to determine the solubility of the actual protein.

4.2.1.8 TGN38

TGN38 was the chosen marker for the *trans*-Golgi network. Like p58, this is a protein with an as yet unknown function, but has been shown to reside in the TGN (Luzio *et al.*, 1990). It was possible to determine its solubility, as for p58, because of the availability of a polyclonal anti-serum against it.

Four 50µg aliquots of different Golgi membrane preparations were extracted and subjected to Western blotting as for p58, except that the anti-TGN38 antibody was used.
TGN38 appeared as a smeared band with an apparent molecular weight in the range of approximately 95-110kD (fig. 4.4). This high molecular weight is thought to be due to the

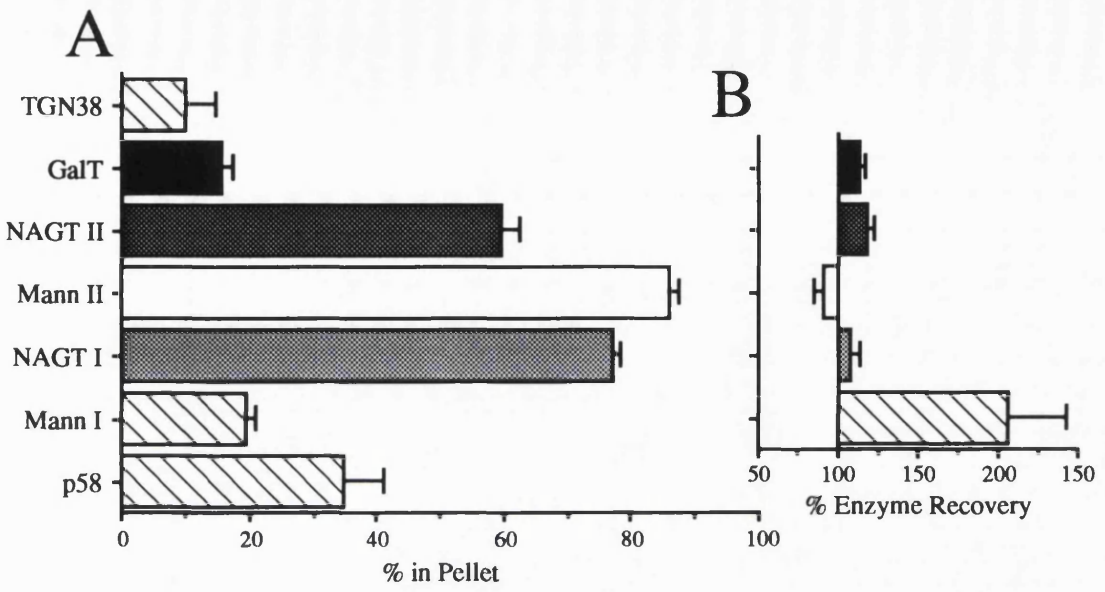

Figure 4.5: Summary of the solubilisation of Golgi markers. The data present a summary of the solubilisation of markers from through the various regions of the Golgi stack, from the *cis*-side (p58) to the *trans*-side (TGN38). Data are presented as the percentage of each marker in the pellet after extraction ±SEM (A), and the percentage recovery of enzymatic activity compared to untreated membranes ±SEM (B).

presence of a large number of post-translationally added oligosaccharides, the actual weight of the polypeptide backbone being 38kD (Luzio *et al.*, 1990), though a slightly higher molecular weight variant of 41kD has been identified (Reaves *et al.*, 1992).

The Western blot was next quantitated as for p58. The exposure of the film scanned was also shown to be in the linear range because a film that had been exposed for twice as long gave identical results. This quantitation showed that the majority of the TGN38 was solubilised by this treatment, with only 10.0%±2.3 (±SEM, n=4) remaining in the pellet.

4.2.1.9 Summary

The above experiments gave a very striking result in that it became apparent that only the *medial*-Golgi markers, NAGT I, Mann II and NAGT II were highly insoluble after this treatment (fig 4.5 A). The markers from the other regions of the Golgi stack were solubilised to a much greater extent.

The only exception was the *trans*-Golgi marker SialylT which was over 63% insoluble. This enzyme was, however, either highly inactivated during the extraction or lost by non-specific binding to the centrifuge tubes. Since it was not possible to determine whether this inactivation or loss had occurred in the supernatant or the pellet, or to what

extent, the solubilisation of the enzyme activity could not be related to the solubilisation of the protein and was therefore not considered. Thus, excluding SialylT, p58 was the most insoluble of the non-*medial* markers with 35% remaining in the pellet. All other non-*medial* markers were less than 20% insoluble compared with the *medial* markers which were 60-85% insoluble.

All the other enzyme markers displayed very low levels of activation (fig. 4.5 B) and inactivation, demonstrating that their activities were reliable markers of their actual distributions. The only exception was Mann I which showed a 2-fold activation. Enzyme activation , however, occurred in the both the supernatant and pellet. Although it was not possible to quantitate the extent of activation in the supernatants and pellets, the fact that the enzyme in each was to some extent activated, meant that the distortion of these data of the actual protein solubility would not be as great if, for example, the enzyme in the supernatant alone had been activated. Although the exact value of Mann I solubility was not certain, the data did show that it was significantly solubilised.

Since the solubilisation of p58 and TGN38 was monitored by the actual protein distribution, determination of the recoveries were not necessary.

The fact that only the *medial*-Golgi markers were significantly insoluble after this detergent extraction procedure was intriguing. This could have been coincidental, but this seemed highly unlikely. It was more probable that this shared insolubility reflected an *in vivo* phenomenon unique to these *medial*-Golgi proteins. The nature of the insolubility and of the Triton pellet was, therefore, further investigated.

4.2.2 Density of the Triton Pellet

It had been shown that after extraction Mann II was present within structures that could be sedimented at very low centrifugation speeds (see fig. 3.13). These speeds were comparable to those required to sediment intact Golgi stacks (Tom Misteli, pers. comm.).

Since these structures sedimented so rapidly, it was possible to centrifuge them to equilibrium in high concentration sucrose gradients in order to determine their density. A 500µg aliquot of Golgi membranes was extracted in TMMDS buffer and the extract was then applied to a continuous sucrose gradient consisting of TMMDS buffer whose sucrose concentration was adjusted to 15% (w/v) and 75% (w/v) respectively.

After centrifugation to equilibrium, the gradients were fractionated into 1ml aliquots and undiluted samples assayed for Mann II and GalT activity. The sucrose concentration were determined by analysing 2-fold diluted samples with a refractometer. Since the solutions rapidly became viscous at higher sucrose concentrations, a Hamilton syringe was used to sample aliquots for assays, to ensure the accuracy of the pipetting.

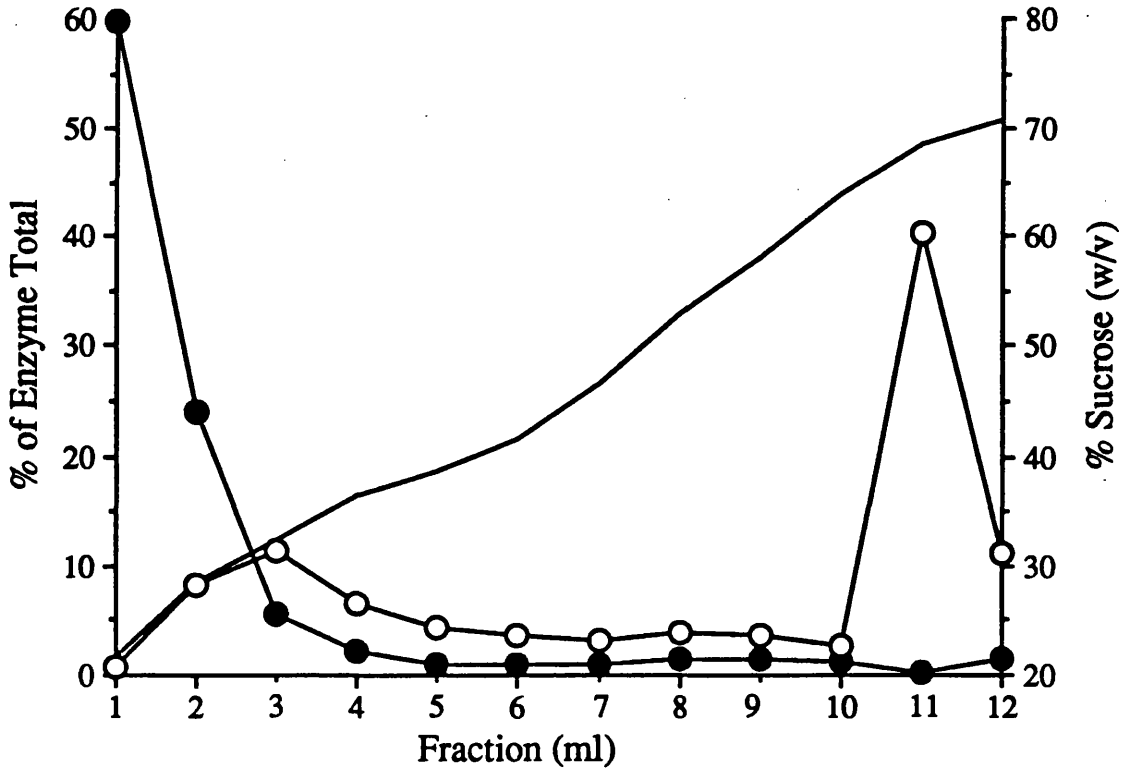

Figure 4.6: Density of Mann II in the Triton pellet. 500µg of Golgi membranes were resuspended in TMMDS buffer and overlaid onto a continuous 15-75% sucrose gradient (——) and centrifuged using a SW40 rotor at 40,000rpm for 16hr at 4°C. The gradient was fractionated into aliquots 1ml which were assayed for Mann II (○) and GalT (●) activity.

This showed that the Mann II within the Triton pellet had a very high density in sucrose (fig. 4.6) and reached equilibrium at a sucrose concentration of 68% (w/v). Furthermore, the difference in the sedimentability of Mann II and GalT was dramatically demonstrated by the fact that GalT had barely begun to enter the gradient. There was also a small peak of Mann II activity at the top of the gradient which presumably corresponded to the 10-20% of enzyme solubilised during the extraction. This small amount of Mann II had, in contrast to GalT, completely entered the gradient by virtue of its higher molecular weight (125kD compared to 51kD (Tulsiani *et al.*, 1977; Bendiak *et al.*, 1993)).

4.2.3 Electron Microscopy of the Triton Pellet

The fact that the insoluble *medial* enzymes could be sedimented so readily showed that Mann II and presumably NAGT I and NAGT II were present in large structures. The

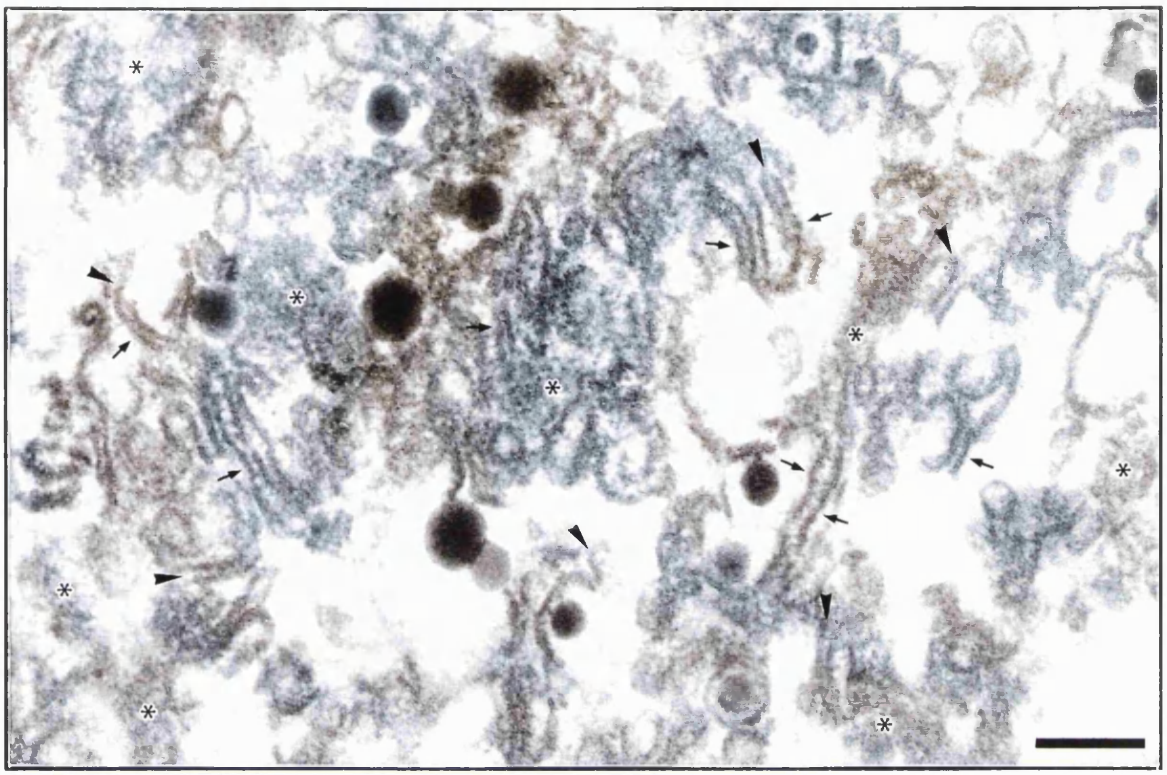

Figure 4.7: Electron micrograph of the Triton pellet. A 200µg aliquot of Golgi membranes was extracted in TMMDS buffer and the pellet fixed and processed for electron microscopy. The picture shows the partially stacked, tri-laminar membrane remnants (arrows) and the amorphous material (*) which often emanates from the membrane remnants (arrowheads). Magnification = 71,250, Bar = 0.2µm.

Triton pellet was, therefore, examined by electron microscopy in an attempt to visualise these structures.

A 200µg aliquot of Golgi membranes was extracted in TMMDS buffer and after centrifugation, the pellet was fixed with glutaraldehyde, stained with osmium tetroxide and embedded in Epon resin. After sectioning, the sample was further stained with lead citrate and uranyl acetate before examination in a transmission electron microscope.

This clearly showed that the Triton pellet contained structures with a highly organised morphology (fig. 4.7). These structures resembled the tri-lamellar appearance of lipid bilayers, consisting of two electron dense, parallel lines which sandwiched an area of less-dense material (see fig. 4.7, arrows). These structures appeared partially stacked, suggesting that they were derived from Golgi membranes. Additionally, these structures had a width of 10nm, identical to the lipid bilayers of intact Golgi membranes (Cluett and Brown, 1992). As well as the tri-lamellar material, a more amorphous material was

also observed (fig. 4.7, asterisks), and this was often seen to emanate from the tri-lamellar structures (arrowheads).

The observation of these highly ordered structures posed the question as to their composition. It was of interest to know whether the tri-lamellar or amorphous material contained the insoluble *medial* enzymes, and whether they were colocalised to the same structures or present in two discreet populations. This was determined only for Mann II and NAGT I since the substrate for NAGT II was not available when these experiments were performed.

4.2.4 Colocalisation of Mann II and NAGT I

The colocalisation of Mann II and NAGT I was achieved in two ways, both utilising a monoclonal antibody (53FC3) which recognises rat Mann II (Baron and Garoff, 1990; Burke *et al.*, 1982). These two studies are detailed below.

4.2.4.1 By Isopycnic Sedimentation

This method utilised the fact that the density of a bio-molecular structure can be increased by binding colloidal gold to it, and was used by Beardmore *et al.* (1987) to partially purify endocytic organelles. Based on this observation, 53FC3 was bound to a suspension of the Triton pellet followed by incubation with anti-mouse antibodies coupled to 10nm colloidal gold. Such samples would then be centrifuged in a continuous sucrose gradient to determine whether an increase in Mann II density had occurred. Furthermore, if NAGT I demonstrated a similar density increase, this would show that both enzymes were contained within the same structures.

Centrifugation was carried out in shallow gradients of 60-75% (w/v) sucrose. Such shallow gradients were chosen because they would allow a greater resolution of any density shifts than the steeper 15-75% gradient described above. Two 400µg aliquots of Golgi membranes were extracted in TMMDS buffer containing 40µl of 53FC3 ascitic fluid or a control ascites (P5D4 which recognises the G protein of Vesicular Stomatitis Virus). After extraction, the pellets were rinsed and resuspended in 400µl of TMMDS buffer containing 20µl of anti-mouse IgG conjugated to 10nm colloidal gold and incubated at 4°C for 30min. These mixtures were then overlaid onto continuous gradients of 60-75% (w/v) sucrose in TMMDS buffer in SW40 rotor tubes. These samples were centrifuged for 16h at 40,000rpm and 4°C and divided into 1ml fractions from top to bottom. Each fraction was assayed for Mann II and NAGT I activity as well as having its sucrose concentration determined.

After incubation of the Triton pellet with control antibodies and 10nm colloidal gold, followed by centrifugation to equilibrium, the density of Mann II remained at 68% (w/v)

of sucrose as had been determined previously (c.f. fig. 4.8 A with fig. 4.6). Additionally this showed that NAGT I equilibrated at the same density as Mann II under these conditions (fig. 4.8 A). However, after incubation with 53FC3 and 10nm gold, the densities of both Mann II and NAGT I increased and both proteins migrated as identical smears at sucrose concentrations of 68-70% (w/v). The fact that both Mann II and NAGT I densities increased, but only in the presence of anti-Mann II antibodies strongly suggested that both enzymes were present within the same structures.

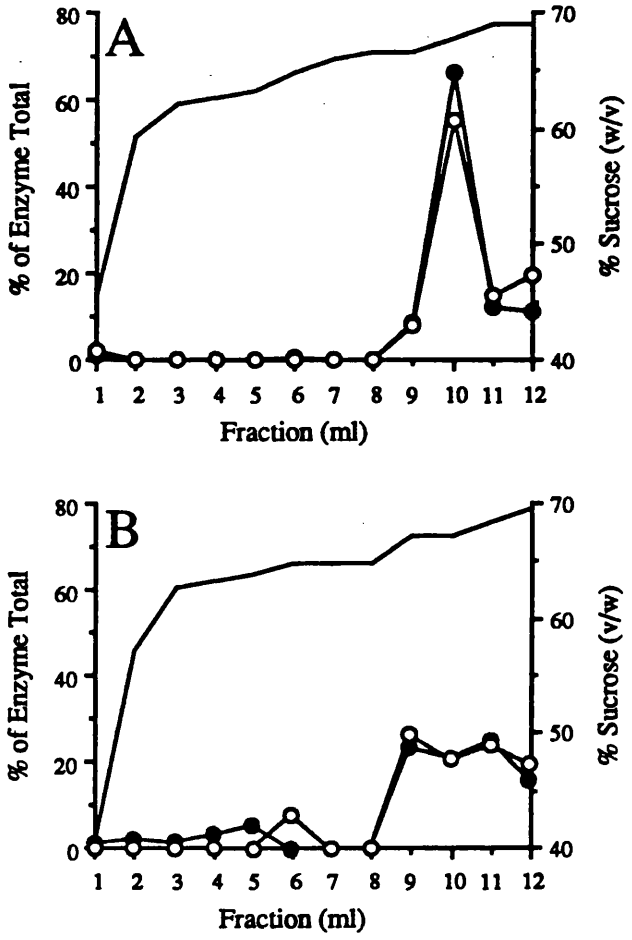

Figure 4.8: Density shift of Mann II and NAGT I using colloidal gold on a continuous gradient. 400µg aliquots of Golgi membranes were extracted in TMMDS buffer containing P5D4 (A) or 53FC3 antibodies (B). The Triton pellets were rinsed and incubated with anti-mouse antibodies conjugated to 10nm colloidal gold before being overlaid onto continuous 60-75% sucrose gradients (——) and centrifuged at 40,000rpm for 16hr at 4°C. The gradient was fractionated into 1ml aliquots which were assayed for Mann II (O) and NAGT I (●) activity

It was, however, not possible to recover enough material from these gradients for examination by electron microscopy in order to visualise the structures containing these enzymes. To attempt to recover enough gold-labelled material, labelled Triton pellets were centrifuged in a discontinuous sucrose gradient consisting of 15% (w/v) sucrose in TMMDS buffer overlaid onto 70% (w/v) sucrose. Two 400µg aliquots of Golgi membranes were extracted in TMMDS buffer containing 40µl of 53FC3 or a P5D4 ascitic fluid. The Triton pellets were rinsed and resuspended in 400µl of TMMDS containing 20µl of anti-mouse antibody conjugated to 10nm colloidal gold. These

samples were overlaid onto the gradients and centrifuged at 40,000rpm for 1hr at 4°C. Gradients were then fractionated into 1ml aliquots and undiluted samples assayed for Mann II and NAGT I activity.

In the presence of the control antibody, most of the Mann II and NAGT I in the Triton pellet accumulated at the 15/70% sucrose interface (fig. 4.9 A). However, in the presence of the Mann II specific antibody, a higher proportion (on average 57% more) of both the Mann II and NAGT I migrated past this interface and into the 70% sucrose buffer (fig. 4.9 B). This again indicated that Mann II and NAGT I were colocalised to the same structures. The fact the higher density Mann II and NAGT I seemed to migrate as a smear in the 70% sucrose was probably due to the short centrifugation time which did not allow all the material to reach the bottom of the tube, while the small amount of Mann II and NAGT I in the 70% sucrose in the controls was probably due to non-specific binding the antibody/gold conjugate. The

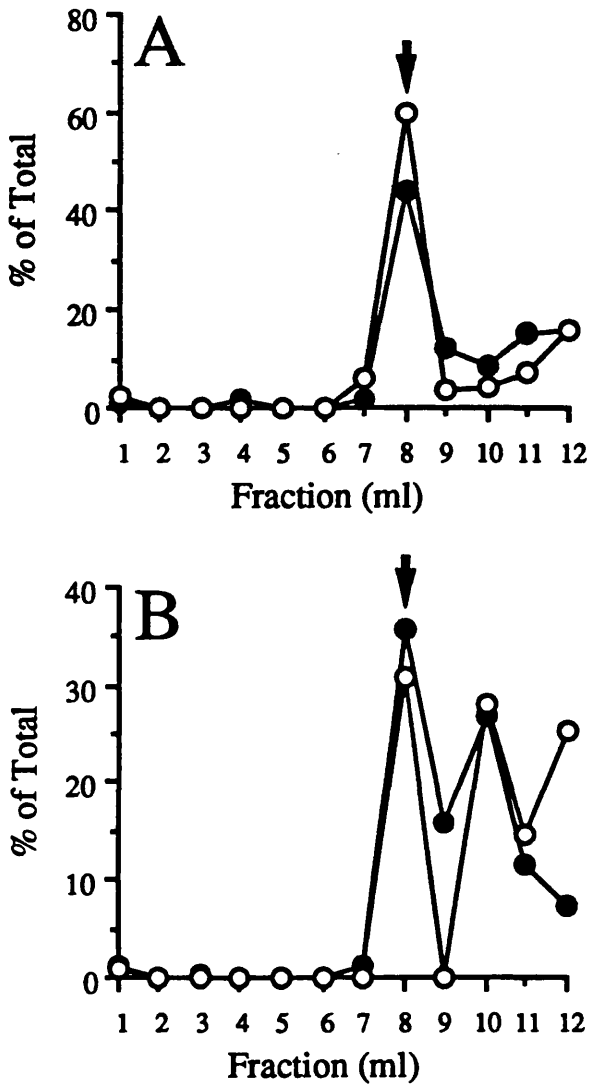

Figure 4.9: Density shift of Mann II and NAGT I using colloidal gold on a discontinuous gradient. 400µg of Golgi membranes were extracted and incubated with a control antibody (P5D4; A) or with an antibody specific to Mann II (53FC3; B) followed by anti-mouse antibodies conjugated to 10nm colloidal gold. Samples were overlaid onto a 15%/70% (w/v) discontinuous sucrose gradient and centrifuged at 40,000rpm for 1hr at 4°C. The gradients were divided into fraction of 1ml which were assayed for Mann II (○) and NAGT I (●). Sucrose concentrations were also determined to ascertain the position of the interface (arrows).

amount of Mann II and NAGT I displaying the density shift was still not sufficient to obtain enough material to be processed for electron microscopy in order to visualise the enzyme-containing structures. Thus a different approach was taken to isolate these structures, and is detailed below.

4.2.4.2 Using Immunoprecipitation

As an approach to confirming the colocalisation of Mann II and NAGT I, and for obtaining samples which could be visualised by electron microscopy, the co-immunoprecipitation of NAGT I with Mann II was investigated using 53FC3 bound to magnetic beads. Magnetic beads were chosen since they had been shown to be effective in the isolation of large intracellular structures (Howell *et al.*, 1989), and because of the ease with which these beads can be re-isolated after antigen binding and washing.

Initially, the Triton pellet was bound to the 53FC3 antibody coated beads in the presence of TMMDS buffer with subsequent washes being carried out in TMMS buffer. This led to a large amount of non-specific binding of both Mann II and NAGT I to the beads, presumably due to the presence of TX-100 or absence of NaCl in the buffer, since the protocol of Howell *et al.* (1989) used phosphate-buffered saline (PBS).

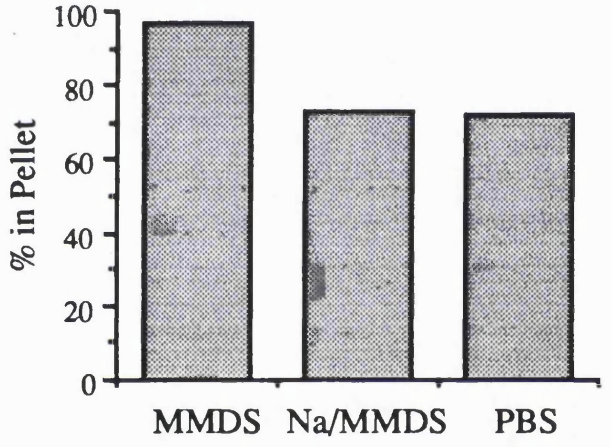

Figure 4.10: Solubilisation of Mann II from the Triton pellet by PBS. Three 100µg aliquots of Golgi membranes were extracted with TMMDS buffer. The Triton pellets were rinsed with 100µl of MMDS and resuspended in 100µl of MMDS, MMDS supplemented with 150mM NaCl or in PBS. After incubation as centrifugation under the same conditions as during extraction, the supernatants and pellets were separated, assayed for Mann II activity and the percentage of the enzyme remaining insoluble calculated.

Experiments were therefore carried out in the presence of PBS to minimise non-specific binding. Because it had been previously shown that NaCl will solubilise Mann II from intact Golgi membranes in the presence of TX-100, it was necessary to show that this did not occur in the absence of detergent. Thus 100µg aliquots of Golgi membranes were extracted in TMMDS buffer and the pellets rinsed carefully twice with 100µl of MMDS buffer. These pellets

were then resuspended in 100µl of MMDS, MMDS supplemented with 150mM NaCl or in PBS and incubated for a further 30min at 4°C. These suspensions were then re-centrifuged at 20,000rpm for 30min at 4°C as before. The supernatants and pellets were assayed for Mann II activity as was an aliquot of Triton pellet which had been resuspended in MMDS buffer but not been subjected to the second centrifugation step.

This showed that the Mann II in the Triton pellet remained almost completely insoluble after resuspension in MMDS buffer, and only 27% was solubilised in the salt buffers which lacked TX-100 (fig. 4.10). There seemed to be little difference in solubilisation using either PBS or MMDS buffers. The recovery of Mann II was 103.5%±6.8 (±SD), compared to the untreated Triton pellet.

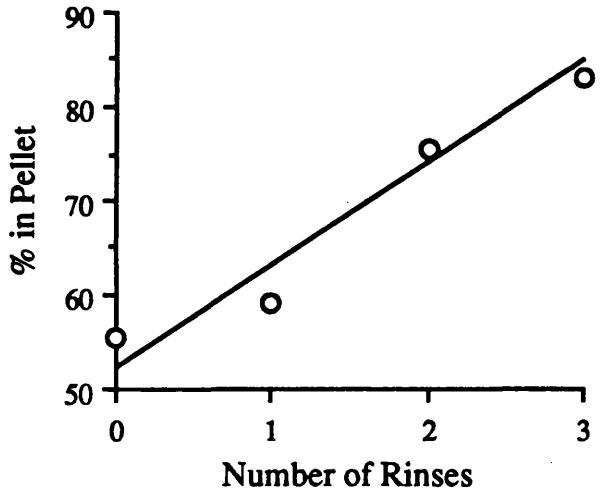

Figure 4.11: Effect of removal of TX-100 from Triton pellets on Mann II insolubility in PBS. Four 100µg aliquots of Golgi membranes were extracted with TMMDS buffer. The Triton pellets were resuspended in 100µl of PBS after rinsing 0, 1, 2 or 3 times with 100µl of MMDS buffer. After incubation as centrifugation under the same conditions as during extraction, the supernatants and pellets were separated, assayed for Mann II activity and the percentage of the enzyme remaining insoluble calculated.

The small amount of solubilisation that had occurred was probably due to the presence of small amounts of TX-100 which had not been removed by rinsing. This was tested by extracting 100µg aliquots of Golgi membranes in TMMDS and subjecting the pellets to increasing numbers of rinses in MMDS, in which Mann II remained completely insoluble (see fig. 4.10), before resuspension in 100µl of PBS, incubation at 4°C for 30min and re-centrifugation. Assaying the supernatants and pellets for Mann II activity showed that the amount of Mann II remaining in the pellet increased with increasing numbers of rinses in MMDS buffer (fig. 4.11), indicating that TX-100 was most probably responsible for the small amounts of solubilisation observed previously (fig. 4.10). As more TX-100 was removed by the MMDS rinses, the insolubility of Mann II in the Triton pellet in PBS increased, with only 17% being solubilised after three rinses with MMDS

This meant that the Triton pellet could be resuspended in PBS without significant loss of Mann II providing that as much TX-100 as possible had been removed. This therefore opened up the possibility of immunoprecipitating this material in PBS, which would lower non-specific binding, since the protocol described by Howell *et al.* (1989) utilised this buffer.

To test out whether PBS did indeed reduce the amount of non-specific binding, 20µl of magnetic beads conjugated to anti-mouse IgG suspended in 1ml of 5mg/ml BSA in PBS (BSA/PBS) were incubated with rotation for 16hr in the presence of 10µl of 53FC3 ascitic fluid or with a control ascites (OKT8; against the human CD8 antigen). The beads were washed with BSA/PBS to remove unbound antibodies and resuspended in 1ml of BSA/PBS containing Triton pellet suspensions derived from 100µg of Golgi membranes and which had been washed three times with MMDS buffer prior to resuspension in BSA/PBS. This mixture was incubated with rotation for a further 2hr before washing with BSA/PBS to remove unbound material. The beads were resuspended in 100µl of BSA/PBS and aliquots of this suspension assayed for Mann II and NAGT I activity, as well as an aliquot of the original Triton pellet. Samples were shaken gently by hand every 15min to resuspend the beads during the assay, and the beads were recovered by centrifugation at 14,000rpm for 5min at room temperature in a benchtop microfuge before measuring the absorbance for the Mann II assay to prevent them from interfering with the spectrophotometric readings. The amount bound was calculated as being the percentage of the amount of original Triton pellet loaded, and this showed that just over 25% of both Mann II and NAGT I had bound to the beads coated with 53FC3, while much less had bound to the control beads (fig 4.12).

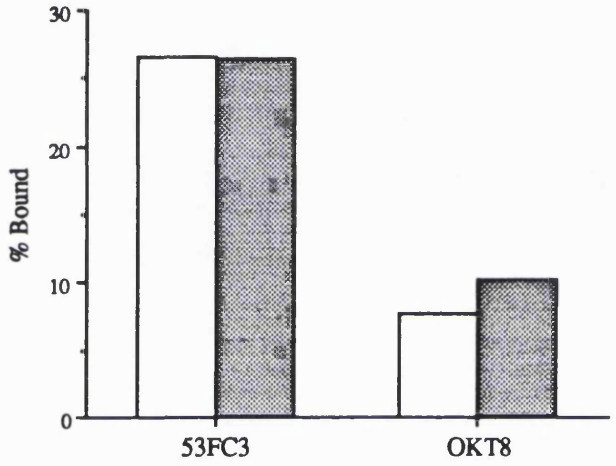

Figure 4.12: Co-immunoprecipitation of Mann II and NAGT I in the Triton pellet. Two 100µg aliquots of Golgi membranes were extracted with TMMDS buffer and bound to magnetic beads that had been pre-coated with an anti-Mann II (53FC3) or a control (OKT8) antibody. After removing unbound material, the beads were assayed for bound Mann II (❑) and NAGT I (■) activity and the percentage of the total enzyme loaded that had bound to the beads calculated.

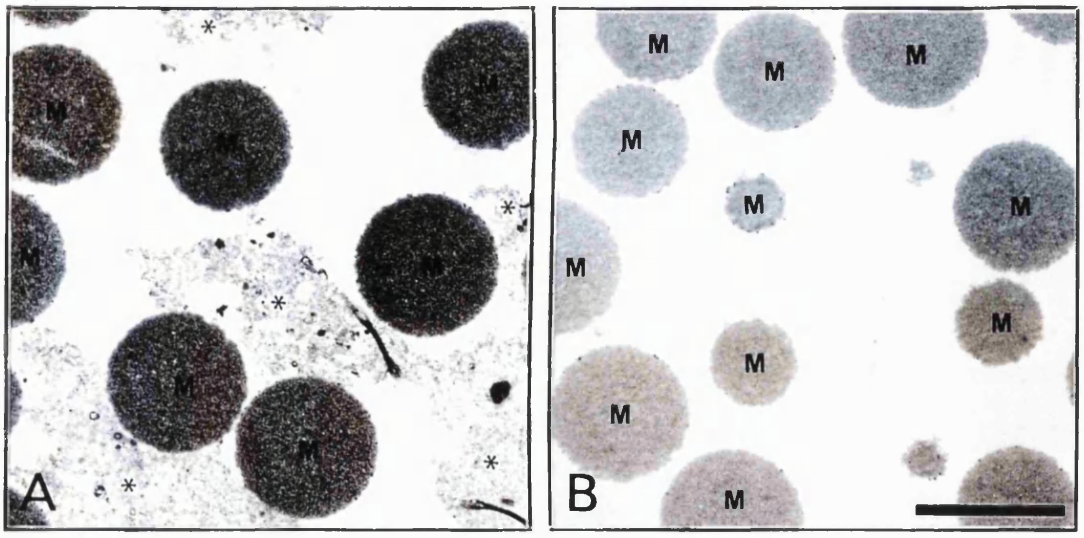

Figure 4.13: Electron micrograph of an unsonicated Triton pellet bound to magnetic beads. Two 100µg aliquots of Golgi membranes were extracted with TMMDS buffer and bound to magnetic beads (m) that had been pre-coated with an anti-Mann II (A) or a control antibody (B). After removing unbound material, the beads were embedded in Epon resin, stained and examined by transmission electron microscopy to visualise the material which had bound (*). Magnification = 4125, Bar = 5µm.

The fact that NAGT I could be precipitated by antibodies specific to Mann II suggested that both enzymes were present in the same structures. These structures were visualised by fixation of the remainder to the beads which were then embedded in Epon resin and processed for electron microscopy. This showed that the beads that had been coated with 53FC3 contained large amounts of material present in the Triton pellet while the control beads contained very little (fig. 4.13). However, the material bound to the beads was contained within large aggregates, possibly due to inadequate resuspension of the Triton pellet. It was therefore possible that Mann II and NAGT I were colocalised to different structures which were both present in these large aggregates. To preclude this possibility, precipitations were carried out using Triton pellets which had been sonicated to disperse these aggregates.

Rebinding was then carried out using varying amounts of Triton pellets and a constant amount of beads to determine whether this binding was saturable. Thus, Golgi membranes were extracted in 50, 100, 200, 300, 400 and 500µg aliquots using TMMDS buffer. Protein assay indicated that 61% of the total Golgi protein had been solubilised by this treatment. The Triton pellets were resuspended in 1ml each of BSA/PBS and the suspensions sonicated three times for 10sec periods with 1min incubations at 4°C between bursts. Sonication was performed using an Ultrasonic Processor (Jencons

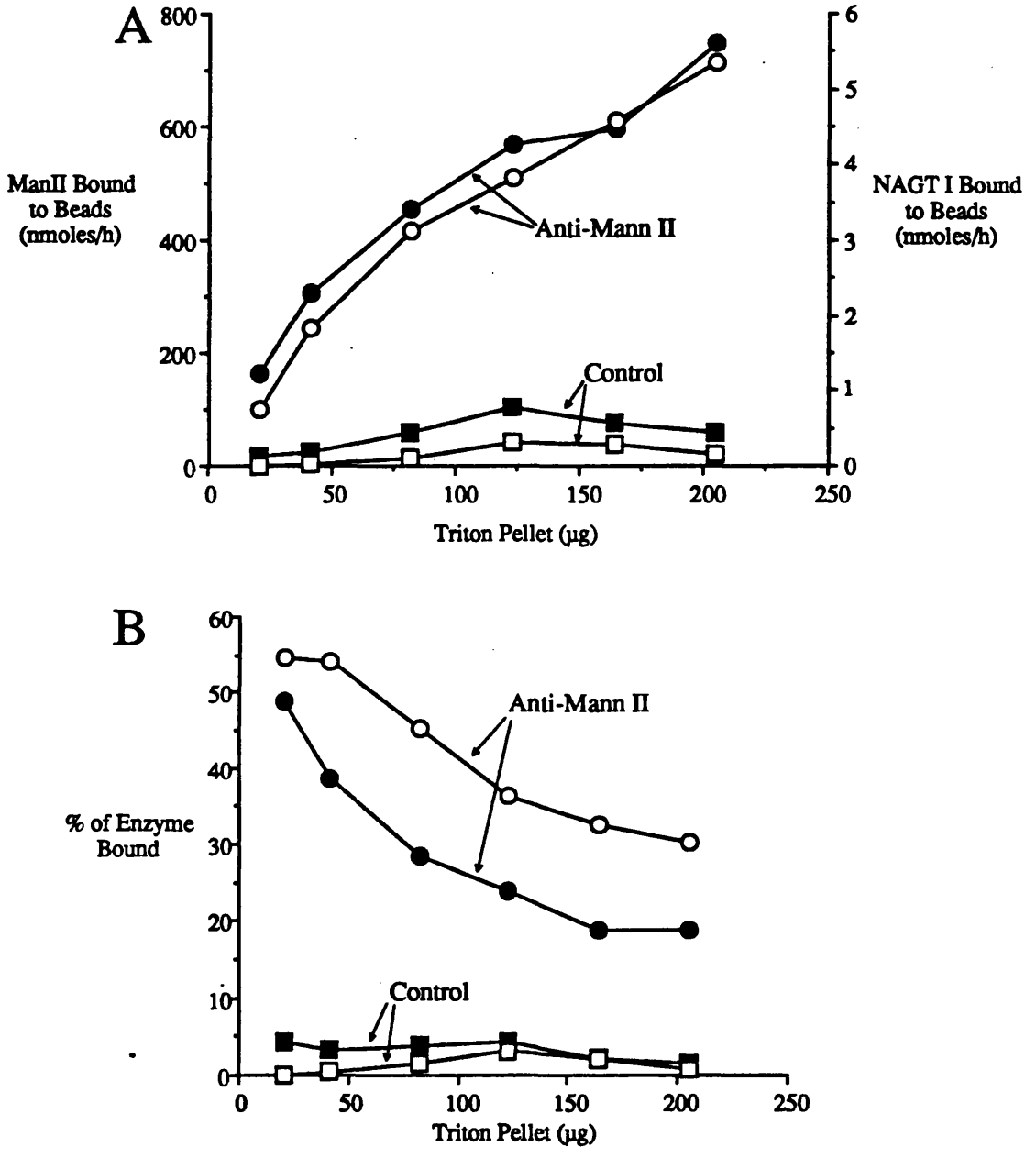

Figure 4.14: Binding of sonicated Triton pellets to magnetic beads. Aliquots of 50, 100, 200, 300, 400 and 500µg of Golgi membranes were extracted with TMMDS buffer and bound to magnetic beads that had been pre-coated with an anti-Mann II (○, ●) or a control antibody (□■). After washing, the beads were assayed for Mann II (○, □) or NAGT I (●, ■). The data are presented either as the enzyme activity bound to the beads (A) or as the percentage of the enzyme loaded that had bound (B).

Scientific Ltd.) at the 60W setting. These sonicated suspensions were then bound to 20μg aliquots of 53FC3 or OKT8 pre-coated beads as before. After binding and washing, each sample and an aliquot of the original Triton pellet was assayed for Mann II and NAGT I activity and the number of nmoles of substrate converted per hour in each sample was calculated.

This showed that both Mann II and NAGT I bound highly specifically to the beads coated with anti-Mann II antibodies, while very little of either enzymes bound to the beads coated with the control antibody (fig. 4.14 A). Additionally, the binding of both enzymes to the beads seemed to be saturable as this curve appeared to be reaching a plateau. This was more evident if the data were plotted to show the percentage of the total enzyme loaded that had bound to the beads instead of the absolute amount bound (fig. 4.14 B). This graph showed more clearly that the beads were being saturated as the percentage of enzyme bound decreased with increased loadings of Triton pellet. The fact that binding was saturable together with the lack of enzyme binding to the control beads showed that the binding was specific.

The fact that NAGT I bound to the 53FC3 coated beads also confirmed that these enzymes were localised to the same structures, though the percentage of NAGT I binding was always slightly less than Mann II (fig. 4.14 B). This was probably due to slight solubilisation of both enzymes in the PBS buffer. This would produce populations of free Mann II and NAGT I which would not interact. The solubilised NAGT I could not, therefore, co-precipitate with Mann II, and the percentage of the total NAGT I that was immunoprecipitated would be less than that of Mann II.

In order to determine which structures contained Mann II and NAGT I, aliquots of beads coated with 53FC3 or OKT8 were incubated with sonicated Triton pellets derived from 500μg of Golgi membranes, washed and processed for electron microscopy. This showed that while the control beads bound very few structures (fig. 4.15 A), the 53FC3 coated beads had (fig. 4.15 B). The fact that sonication had disrupted the large aggregates seen previously (fig. 4.13), but that NAGT I could still be precipitated by anti-Mann II antibodies, showed that Mann II and NAGT I were localised to the same structures and were not co-precipitating due to their presence in large non-specific aggregates.

A more detailed examination of the material bound to the 53FC3 coated beads showed that both the tri-laminar and amorphous structures observed in figure 4.7 were found closely apposed to the beads (fig. 4.16 C-E), suggesting that both contained Mann II and, presumably therefore, NAGT I. The width of the tri-laminar structures remained at 10nm and were comparable to the width of the lipid bilayers in intact Golgi membranes (c.f. fig. 4.16 A with C-E). The fact that the width of the tri-laminar structures was the same as those from an unsonicated sample suggested that sonication had not perturbed

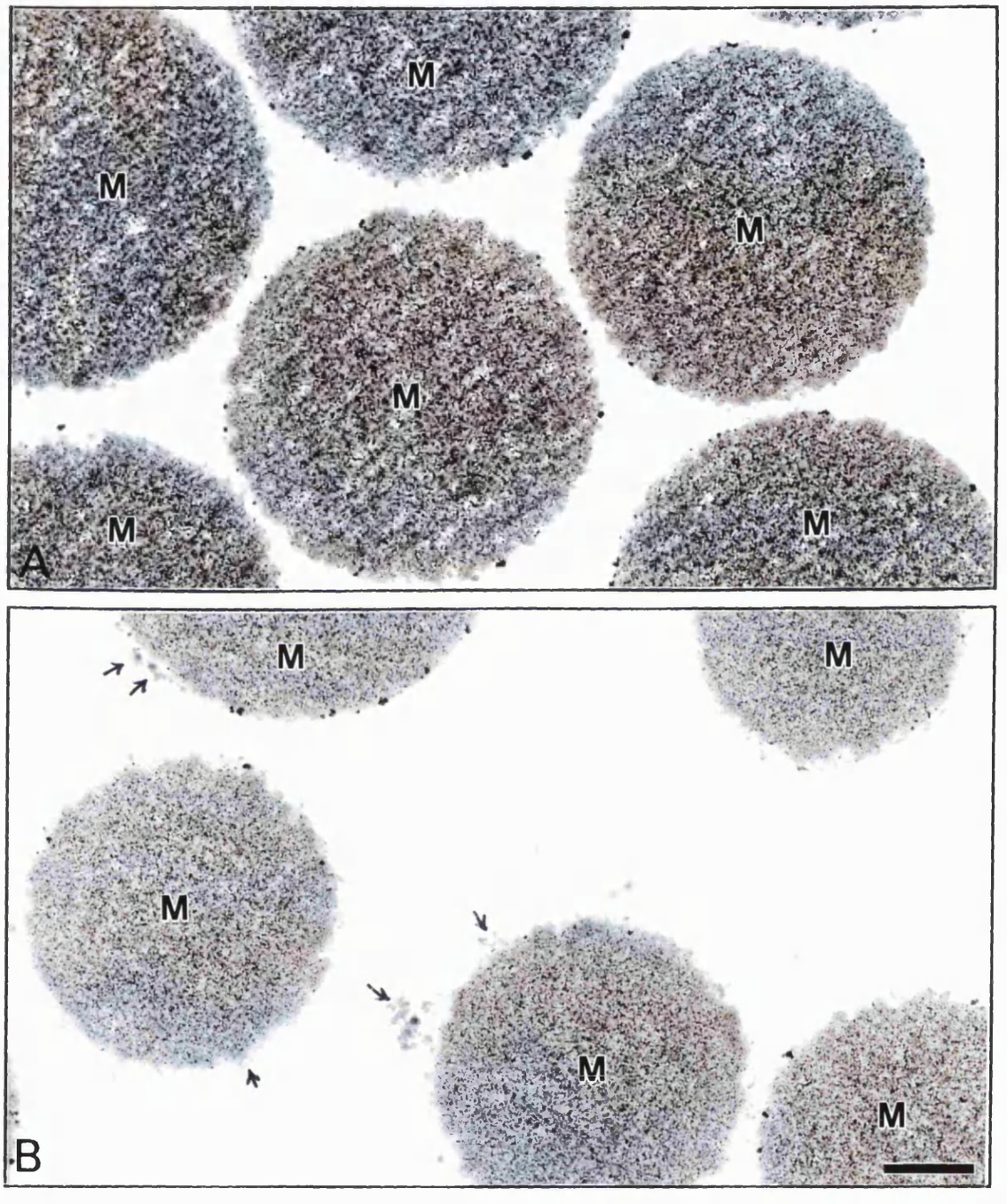

Figure 4.15: Low magnification micrograph of sonicated Triton pellets bound to magnetic beads. Aliquots of 500µg of Golgi membranes were extracted with TMMDS buffer, sonicated and bound to magnetic beads (m) that had been pre-coated with a control antibody (A) or one that recognised Mann II (B). After washing, the beads were fixed and the samples processed for electron microscopy. Magnification = 13,000. Bar = 1µm. Bound material indicated by arrows.

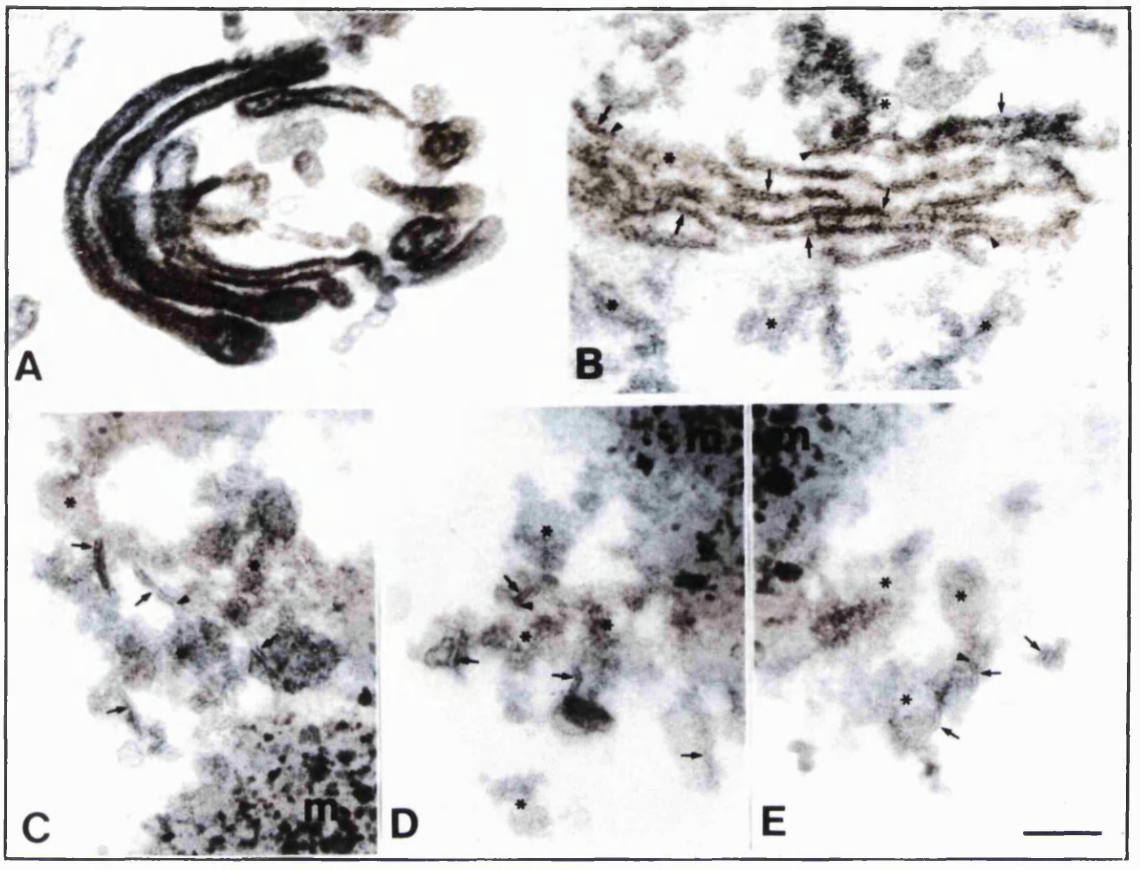

Figure 4.16: High magnification micrographs of sonicated Triton pellets bound to magnetic beads. Aliquots of 500µg of Golgi membranes (A) were extracted with TMMDS buffer and the Triton pellets (B) bound to magnetic beads that had been pre-coated with an antibody that recognised Mann II (C-E). After washing, the beads were fixed and the samples processed for electron microscopy. Note the tri-laminar structures (arrows) and amorphous material (*) which was occasionally seen to emanate from the membrane remnants (arrowheads). Magnification = 106,500. Bar = 0.1µm.

their morphology drastically, though their length did seem to be reduced and they were no longer partially stacked (c.f. fig. 4.7 and fig. 4.16 B with fig. 4.16 C-E). This also suggested that the structures were stable since their morphology was not drastically affected by the sonication.

4.2.5 Effect of Cytoskeletal Disrupting Agents

The insolubility of the *medial*-Golgi enzymes may have been due to their interaction with components of the cytoskeleton which are classically insoluble in non-ionic detergents. To preclude this possibility, Golgi membranes were extracted in the presence of

nocodazole to disassemble microtubules, or cytochalasin B to disassemble actin filaments. This would determine whether the loss of these cytoskeletal elements had any effect on the solubility of Mann II.

Aliquots of 100µg of Golgi membranes were extracted in TMMDS buffer and TMMDS buffer that had been supplemented with 10µM nocodazole or 42µM cytochalasin B. Both of these drugs were dissolved in DMSO to produce concentrated stock solutions of nocodazole and cytochalasin B, and these were diluted into the TMMDS buffer before extraction. As a control for any potential effects of DMSO on the solubility of Mann II, an aliquot of Golgi membranes was also extracted in TMMDS buffer containing DMSO alone at an equal concentration to that in the nocodazole and cytochalasin B buffers.

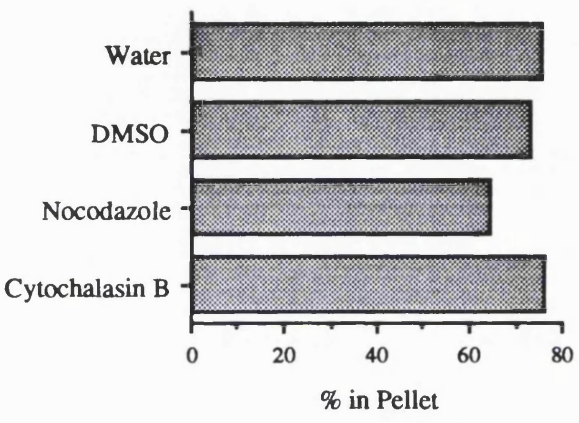

Figure 4.17: Effect of nocodazole and cytochalasin B on Mann II solubility. Aliquots of 100µg of Golgi membranes were extracted with TMMDS buffer or TMMDS buffer supplemented with 213mM DMSO, 10µM nocodazole or 42µM cytochalasin B. The supernatants and pellets assayed for Mann II activity to determine the percentage of Mann II remaining in the pellet.

After extraction, the supernatants and pellets were assayed for Mann II activity, and the percentage insolubility of the enzyme calculated (fig. 4.17). This showed that neither DMSO, nocodazole nor cytochalasin B had any significant effect on the solubility of Mann II. The insolubility of Mann II in TX-100, and presumably the other *medial*-Golgi enzymes, was not caused by interaction with microtubules or actin-based microfilaments.

4.2.6 Solubilisation of Enzymes from the Triton Pellet

In chapter 3 it was discovered that Mann II could be solubilised from Golgi membranes by extraction in TMMDS buffer containing sodium and potassium. It was therefore likely that Mann II could be solubilised from the Triton pellet by a second extraction in TMMDS buffer containing these cations. This was tested by extracting Triton pellets in TMMDS buffer containing increasing concentrations of either NaCl and KCl.

Aliquots of 100µg of Golgi membranes were extracted in TMMDS buffer and the subsequent Triton pellets were extracted in a further 100µl of TMMDS buffer supplemented with 0, 50, 150, 250, 500 and 1000mM concentrations of NaCl or KCl. The supernatants and pellets were separated and assayed for Mann II activity as was an aliquot of untreated Triton pellet. This showed that both NaCl (fig. 4.18 A) and KCl (fig. 4.18 B) were very efficient at solubilising Mann II from the Triton pellet and a comparison of the two solubilisation curves showed that both ions solubilised the enzyme equally well (fig. 4.18 C). This was in slight contrast to their effects on intact Golgi membranes where KCl was slightly less efficient at solubilisation than NaCl (fig. 3.17 A). Complete solubilisation from the Triton pellet occurred at concentrations comparable to those required for solubilisation directly from Golgi membranes (c.f. fig. 3.17 A and 4.18 C).

The solubilisation of Mann II was not due to a second extraction in detergent because virtually no enzyme was released when the second extraction contained no sodium or potassium (fig 3.17 A-C). The enzyme recovery compared to the amount present in the Triton pellet was 92.3±7.9 (±SD) for the NaCl samples and 95.3±9.1 (±SD) for the KCl samples, indicating that the solubilisation of enzymatic activity was a true reflection of protein solubilisation since no significant enzyme activation or inactivation had occurred.

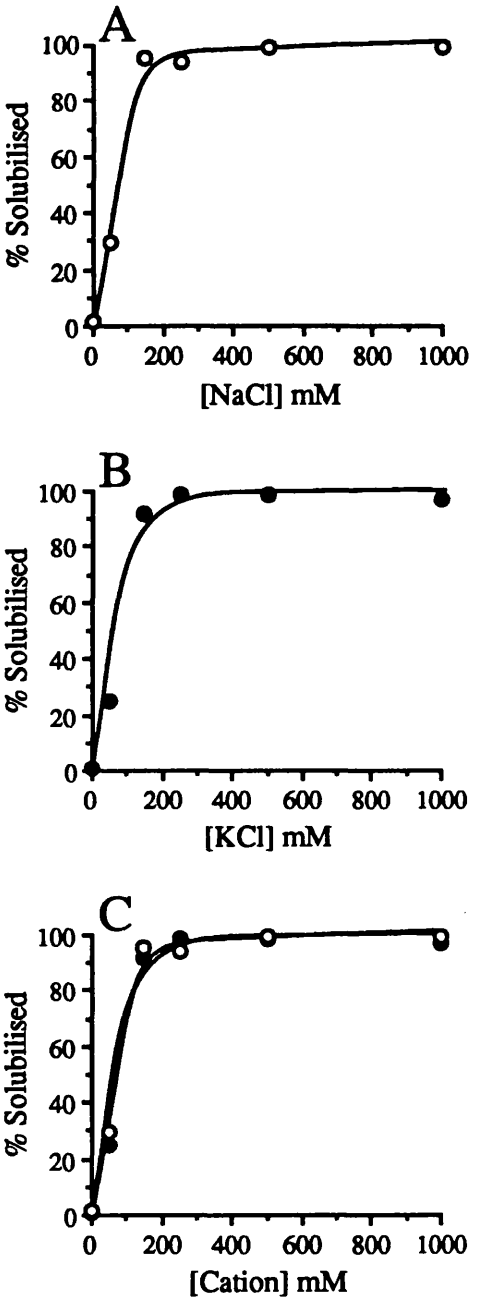

Figure 4.18: Solubilisation of Mann II from the Triton pellet by NaCl and KCl. Aliquots of 100µg of Golgi membranes were extracted with TMMDS buffer supplemented with 0, 50, 150, 250, 500 and 1000mM NaCl (A) or KCl (B). The percentage solubilisation in NaCl (O) and KCl (●) are compared (C).

Since Mann II seemed to be equally soluble in NaCl as KCl, NaCl was chosen arbitrarily to test whether another *medial*-Golgi enzyme, NAGT I, was also solubilised by this cation. NAGT II solubilisation was not assessed due to lack of sufficient substrate with which to perform the assays. Solubilisation was carried out in the 0-150mM range since salt concentrations beyond 150mM caused complete solubilisation of Mann II (fig. 4.18 A). Thus 100µg aliquots of Golgi membranes were extracted in TMMDS buffer and the rinsed Triton pellets were resuspended in 100µl of TMMDS buffer supplemented with 0, 50, 70, 90, 120 and 150mM NaCl. After incubation and centrifugation under the same conditions as during the Triton extraction, the pellets and supernatants were assayed for Mann II and NAGT I activity, as was an aliquot of an untreated Triton pellet. The percentage solubilisation of each enzyme was then determined. This showed that NAGT I was solubilised as efficiently as Mann II by the salt (fig. 4.19 B), with complete solubilisation occurring at 120-150mM NaCl. The recovery of both enzymes in each sample compared to the amount in the Triton pellet was close to 100% (fig. 4.19 A). This indicated that the solubilisation of the enzyme activity was a true reflection of the protein solubilisation and was not an artefact caused by activation or inactivation of the enzymes in the presence of salt.

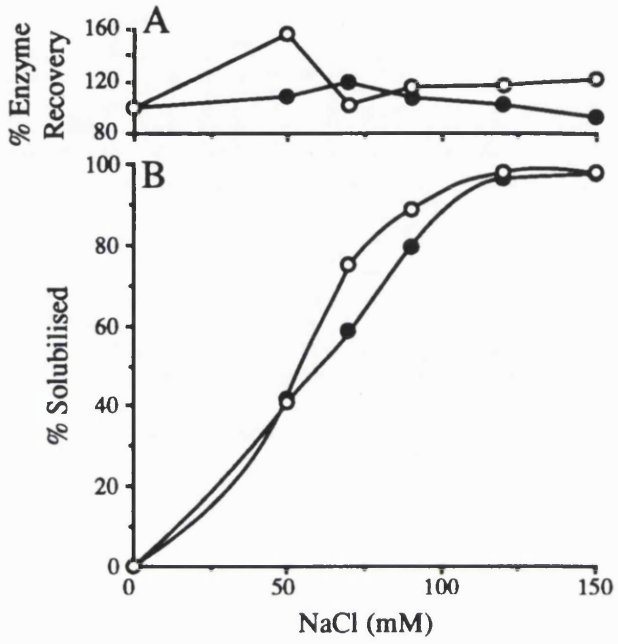

Figure 4.19: Solubilisation of Mann II and NAGT I from the Triton pellet by NaCl. Aliquots of 100µg of Golgi membranes were extracted with TMMDS buffer and the resulting Triton pellet further extracted in TMMDS supplemented with 0, 50, 70, 90, 120 and 150mM NaCl. The percentage enzyme recovery compared to the Triton pellet (A) and the percentage enzyme solubilisation (B) are plotted. Mann II = ○, NAGT I = ●

After centrifugation of the 150mM salt extract, it was noticed that a small pellet was still visible at the bottom of the tube although the enzyme assay indicated that all the Mann II and NAGT I had been solubilised. This opened up the possibility that it was the binding of the *medial*-Golgi enzymes to the material in this pellet that was responsible for

their insolubility in TX-100, and that this binding was abolished in the presence of salt. The protein composition of this pellet was therefore examined by SDS-PAGE.

Aliquots of 100µg and 500µg of Golgi membranes were extracted with TMMDS buffer followed by a second extraction in TMMDS buffer supplemented with 150mM NaCl. The protein in the supernatant and pellet from the 100µg sample and the pellet from the 500µg was precipitated and subjected to SDS-PAGE. This showed that the material in the 150mM salt pellet constituted only a small amount of the original Golgi protein as very few bands were detected in the 100µg sample (fig. 4.20, lane 3), and protein assays showed that the salt pellet constituted only 10% of the original Golgi protein. However, several proteins spanning a molecular weight range of 10-200kD were visible in the higher loading containing the salt pellet from the 500µg sample (fig. 4.20, lane 4). This gel also showed that all the Mann II had been solubilised in the salt extraction, confirming the data obtained by enzyme assay (c.f. lanes 2 and 3 in fig 4.20).

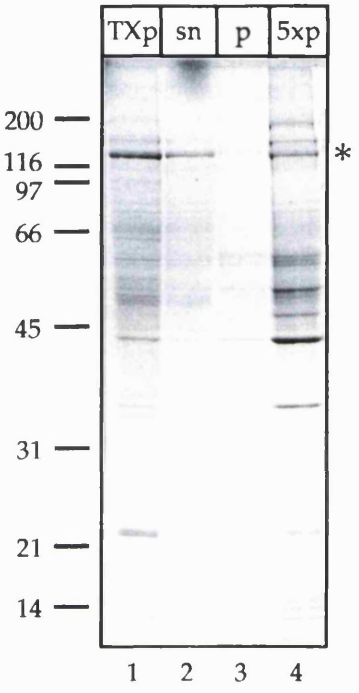

Figure 4.20: SDS-PAGE of the 150mM salt pellet. A Triton pellet derived from 100µg of Golgi membranes (TXp) was extracted with TMMDS buffer supplemented with 150mM NaCl to yield a supernatant (sn) and pellet (p). A five times loading of this pellet is shown in lane 4 (5xp). Positions of molecular weight markers are indicated on the left in kD, and of Mann II on the right (*).

4.3 Summary

After extraction of purified Golgi membranes with TX-100, the *medial*-Golgi enzyme Mann II was found to be the major insoluble protein while the *trans*-Golgi enzyme GalT was almost completely solubilised. In order to determine the extent of solubilisation of other Golgi resident proteins, Golgi membranes were extracted and the amount of solubilisation several Golgi markers was determined.

The classical localisation of these markers within the Golgi spanned the entire stack from the CGN, through the cisternae and to the TGN. Quantitation of the solubilisation of these markers would determine whether TX-100 insolubility was a unique property of Mann II or whether other enzymes from the same or different regions of the stack were also insoluble after this treatment. The markers chosen were p58 for the CGN, Mann I for the *cis*-Golgi,

NAGT I, Mann II and NAGT II for the *medial*-Golgi, GalT for the *trans*-Golgi and TGN38 for the TGN.

After extraction of Golgi membranes in TX-100, it was found that only the *medial*-Golgi markers were significantly insoluble after this treatment. All the non-*medial* markers were present at levels of only 20% or less in the Triton pellet with the exception of p58 which was 35% insoluble. This however contrasts with insolubilities of 60-85% for NAGT I, Mann II and NAGT II. Thus it seemed that extraction in TMMDS buffer selectively solubilised all the subcompartments of the Golgi except the *medial*-cisterna, leaving an insoluble pellet with a high density which equilibrated at 68% (w/v) sucrose after isopycnic sedimentation.

Electron microscopic examination of the immunoprecipitated material showed that both the tri-laminar membrane remnants and the amorphous material had bound to beads coated with anti-Mann II antibodies. This indicated that both structures contained Mann II and NAGT I and supported the supposition that the amorphous material was derived from the more ordered membrane-like remnants.

The *medial*-enzyme insolubility was not caused by binding to microtubules or microfilaments because Mann II remained insoluble after extraction in the presence of nocodazole or cytochalasin B, conditions which disrupt these cytoskeletal structures. Mann II and NAGT I could be solubilised from the Triton pellet by a second extraction in TMMDS buffer supplemented by NaCl, with complete solubilisation occurring at a salt concentration of 120-150mM. This solubilisation was definitely caused by the presence of salt and not because of the second extraction in TX-100 because omission of the NaCl in the TMMDS buffer during the second extraction caused very little solubilisation of either enzyme.

After extraction at 150mM salt, an insoluble pellet was still present after centrifugation. This pellet contained only 10% of the original Golgi protein as determined by protein assay and analysis by SDS-PAGE showed that it reproducibly contained a number of distinct proteins which spanned a molecular weight range of 10-200kD.

The salt-insoluble pellet was therefore a candidate for the Golgi matrix as defined in the original parameters described at the beginning of chapter 3 i.e. that it was insoluble in detergent and salt. It seemed possible that after TX-100 extraction, the *medial*-Golgi enzymes remained bound to this matrix while markers from other regions of the Golgi did not. The reason for this selective insolubility is unclear, but may be due to a difference in the binding properties of these other markers to the matrix, or because they are bound to other structures which are soluble under these conditions.

If the above hypothesis held true, it would be technically possible to reconstitute the matrix-enzyme complex by rebinding the *medial*-enzymes to the salt pellet after the

removal of salt by dialysis. In the next chapter, I describe such experiments and a further characterisation of the material in the salt pellet.

Chapter 5

Characterisation of the Salt Pellet

5.1 Introduction

After extraction of Golgi membranes in TX-100, only the *medial*-Golgi enzymes remained insoluble. These could be released by a further extraction in Triton buffer containing relatively low amounts of NaCl.

The enzymes were completely solubilised at a salt concentration of 150mM. However, after centrifugation a small insoluble pellet still remained. It was hypothesised that this pellet contained a Golgi matrix which bound the *medial*-enzymes and rendered them insoluble in detergent but that this binding was abolished by the addition of salt.

To determine if this was the case, it was necessary to show that the enzymes could rebind to the matrix after dialysis to remove the salt. Furthermore, it was also necessary to show that such effects were specific. Such an interaction would strongly suggest that the material in the salt pellet constituted a biochemically defined matrix which would have a physiological role.

If the salt pellet did contain such a matrix, it would also be desirable to determine its topology. It could be conceivable that the matrix was lumenal and bound the enzymes via their catalytic domains, or alternatively, it could be cytoplasmic and bound to the Golgi enzymes via their cytoplasmic domains or via a membrane-spanning protein which bound the enzymes by their lumenal domains.

In this chapter, I describe experiments that address these critical questions by providing a characterisation of the interaction of the *medial*-enzymes with the salt pellet and by determining the topology of the matrix.

5.2 Results

5.2.1 Velocity Sedimentation of Solubilised Mann II

After extraction of Triton pellets with TMMDS buffer containing 150mM NaCl, both Mann II and NAGT I were released from the pellet. Mann II was shown to be a dimer in solution when it was purified (Tulsiani *et al.*, 1977). To determine whether the enzymes released from the Triton pellet by salt were truly dimers or whether they were present in larger oligomeric structures which were too small to sediment at the relatively low centrifugation velocities used during the extraction, solubilised Mann II was subjected to velocity centrifugation, and its sedimentation characteristics compared to a relevant marker. The marker chosen was catalase, since this was used for molecular weight determinations for the purified enzyme (Tulsiani *et al.*, 1977).

A 500μg aliquot of Golgi membranes was extracted in TMMDS buffer and the resulting Triton pellet extracted in TMMDS buffer containing 150mM NaCl. The supernatant containing the solubilised Mann II was removed and 25μg of beef liver

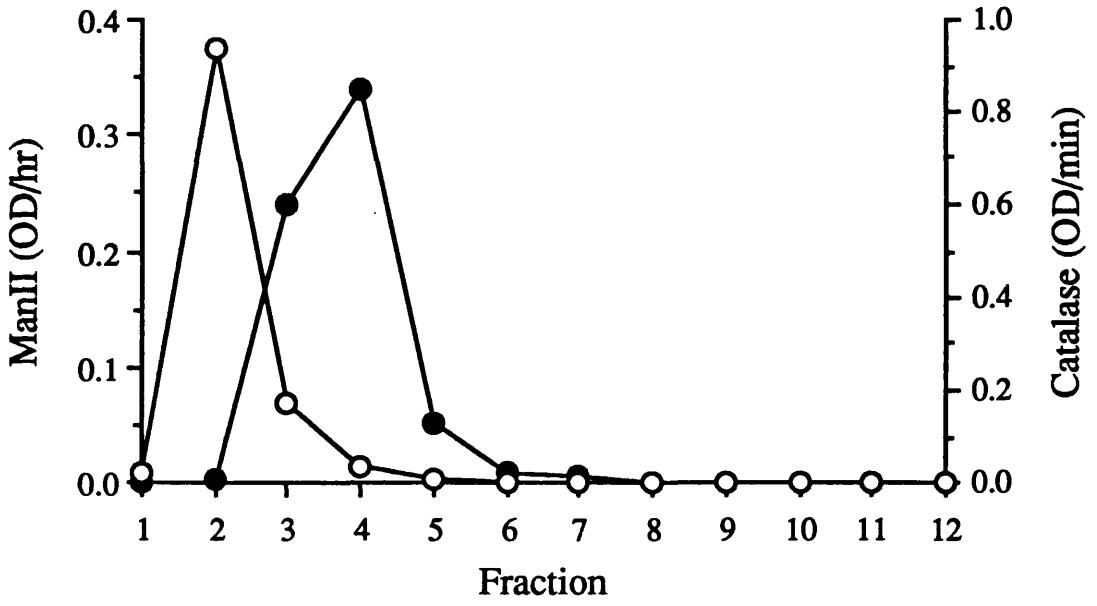

Figure 5.1: Velocity sedimentation of solubilised Mann II. A 150mM salt supernatant derived from 500µg of Golgi membranes and 25µg of beef liver catalase were overlaid onto a continuous 20-40% (w/v) sucrose gradient at centrifuged for 16hrs at 40,000rpm and 4°C. The gradient was divided into 1ml fractions from top to bottom, and these were assayed for Mann II (○) and catalase (●) activity .

catalase (Boehringer) added. This sample was overlaid onto a 20-40% (w/v) continuous sucrose gradient in TMMDS buffer containing 150mM NaCl. The sample was centrifuged for 16hr at 40,000rpm and 4°C using a SW40 rotor and the gradient divided into 1ml fractions from top to bottom. Each fraction was then assayed for both Mann II and catalase activity to determine the degree of migration of both proteins.

This showed that Mann II sedimented less rapidly than catalase (fig 5.1), as reported previously (Tulsiani *et al.*, 1977). Although in its native dimeric form, Mann II has a greater molecular weight than catalase (250kD as a native dimer compared to 240kD as a native tetramer), this was not reflected in their relative sedimentation characteristics. This highlighted the problem of using velocity sedimentation in the presence of detergents as a method for estimating molecular weight (Tanford *et al.*, 1974), and careful analysis of the data is required to obtain a valid result. In fact, this technical problem initially led to an incorrect determination for the oligomeric form of purified Mann II which was thought to be a tetramer (Tulsiani *et al.*, 1977).

It was not possible to determine the actual molecular weight of the solubilised Mann II by the methods of Tanford *et al.* (1974) due to the unavailability of a suitable analytical ultracentrifuge. However, the ratio of the sedimentation mobility of catalase to Mann II in

this experiment was 2.6. This compares favourably with the value of 2.0 reported for the purified enzyme (Tulsiani *et al.*, 1977). This suggested that Mann II was in fact a dimer in the salt supernatant, although this did not exclude the possibility that other molecules were bound to the Mann II which were small enough not to cause a large increase in the apparent molecular mass of the dimer.

5.2.2 Dialysis of the Salt Supernatant

Before performing any experiments regarding the rebinding of the *medial* enzymes to the salt pellet or proposed matrix, it was necessary to determine whether they became insoluble after dialysis in the absence of the salt pellet. If the salt pellet did constitute a matrix which bound these enzymes, it was possible that some of its components would be partially solubilised in 150mM salt, which would cause partial re-sedimentation after dialysis. Alternatively, it was also possible that removal of salt from these supernatants would induce the non-specific aggregation of the enzymes. To determine whether removal of salt from the supernatant caused any resedimentation of the enzymes, supernatants were produced by the extraction of Triton pellets in increasing concentrations of salt. These were dialysed, and the amount of Mann II which became insoluble was determined. Supernatants containing differing amounts of salt were chosen because if resedimentation occurred after dialysis for either of the above reasons, it may have been reduced at lower salt concentrations either due to a decreased amount of solubilisation of matrix components, or due to a lower concentration of enzyme in the supernatant.

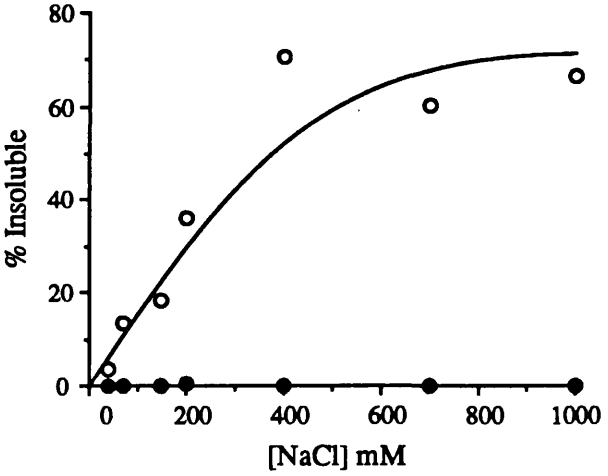

Figure 5.2: Mann II resedimentation after dialysis of salt supernatants. Salt supernatants derived from the extraction of Triton pellets in TMMDS buffer containing varying concentration of NaCl were either dialysed against TMMS buffer (O) or incubated at 4°C (●) for 16hrs. These samples were recentrifuged and the supernatants and pellets assayed for Mann II activity to determine the amount of enzyme which resedimented.

Aliquots of 100µg of Golgi membranes were extracted with TMMDS buffer and the Triton pellets further extracted in TMMDS buffer supplemented with varying

concentrations of NaCl ranging from 0 to 1M. The supernatants were removed and dialysed overnight against TMMS buffer to remove the salt, or incubated at 4°C as a control. The samples were then centrifuged under the same conditions as during the extraction. The supernatants and pellets were separated and assayed for Mann II activity and the percentage of the enzyme that resedimented was calculated.

This showed that Mann II was capable of resedimentation after dialysis, but remained soluble after incubation at 4°C (fig. 5.2). Thus the re-sedimentation was solely due to the removal of salt from the samples and not to the long incubation. Furthermore the amount of re-sedimentation appeared to increase linearly with increasing salt concentrations up to a concentration of 400mM after which it began to reach saturation. If self-aggregation of Mann II were the cause of the resedimentation, it would be dependent on the concentration of the enzyme. This does not seem to be the case since re-sedimentation continued to increase beyond the maximum Mann II concentration which occurred at 150mM NaCl. Mann II solubilisation, and therefore concentration, in the supernatant was maximal at 150mM NaCl while the amount of re-sedimentation continued to increase up to a concentration of 400mM.

It seemed more likely that this was due to the increased solubilisation at higher salt concentrations of molecules involved in maintaining Mann II insolubility. Since resedimentation became maximal at about 400mM, it was possible that the 150mM pellet did still contain some of these components. Since the 150mM pellet did not contain any detectable Mann II activity, it would be possible to attempt to rebind solubilised Mann II to the salt pellet and to detect even very small amounts of enzyme rebinding.

Furthermore, since supernatants derived from lower salt concentration extractions were still capable of resedimenting Mann II after dialysis, presumably due to the partial solubilisation of material from the salt pellet, it was necessary to use low salt supernatants in such experiments to lower the background values of resedimentation in the absence of a salt pellet.

5.2.3 Rebinding of Mann II and NAGT I to the Salt Pellet

The binding of both Mann II and NAGT I to the 150mM salt pellet was assessed by dialysing salt supernatants in the presence or absence of salt pellets and determining the amount of enzyme activity bound. The salt supernatants used were derived from extractions of Triton pellets in TMMDS buffer containing 70mM salt. Under these conditions 60-70% of Mann II and NAGT I were released from the pellet (fig. 4.19), while less than 15% of Mann II was resedimented after dialysis of the supernatant alone (fig. 5.2). This yielded a supernatant containing comparatively large amounts of solubilised Mann II and NAGT I, and in which the Mann II was still relatively soluble

after dialysis. This would give a low background level of resedimentation and allow the determination of any enzyme rebinding to the salt pellet.

An aliquot of 500µg of Golgi membranes was extracted with TMMDS buffer and the Triton pellet extracted in TMMDS buffer containing 70mM NaCl to yield the 70mM supernatant. Additionally two 150mM salt pellets which were derived from 100µg of Golgi membranes each were produced. The pellets were each resuspended in 100µl of the 70mM salt supernatant. One suspension was dialysed overnight at 4°C against 300ml of TMMS buffer while the other was incubated at 4°C without dialysis. Additionally, 100µl of salt supernatant which did not contain any salt pellet was also dialysed and a further aliquot was incubated at 4°C. After dialysis or incubation, the samples were centrifuged as during the extraction procedures and the supernatants and pellets assayed for Mann II and NAGT I activity, as was an aliquot of 70mM salt supernatant which had been kept at 4°C during the incubation but had not been centrifuged. This showed that the enzymes in both the salt supernatant and supernatant/ pellet mixtures remained almost completely soluble without dialysis to remove the salt (fig. 5.3). After dialysis of the salt supernatant alone, the insolubility of both enzymes increased slightly in agreement with previous observations (fig. 5.2), but still the majority of both still remained soluble. In contrast, dialysis of the salt supernatant/ pellet mixture caused a dramatic increase in the insolubility of Mann II and NAGT I with approximately 50% of both enzymes becoming insoluble (fig. 5.3). This effect could not be accounted for because of activation or inactivation of enzymatic activity upon rebinding because enzyme recoveries were 96.0%±11.3 for Mann II and 92%±7.2 for NAGT I (±SEM, n=3)

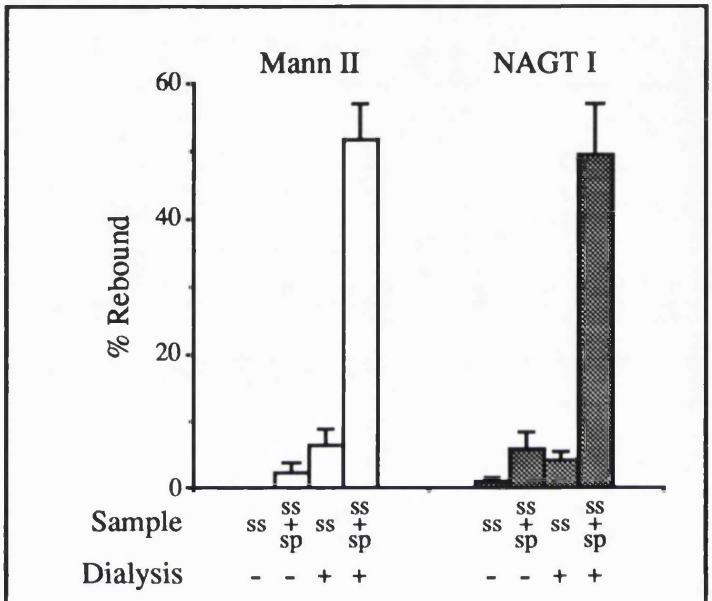

Figure 5.3: Rebinding of Mann II and NAGT I to the salt pellet. 70mM salt supernatants (ss) derived from 100µg of Golgi membranes were dialysed or incubated at 4°C in the absence or presence of 150mM salt pellets (sp) also produced from 100µg of Golgi membranes. After centrifugation, the supernatants and pellets were assayed for Mann II and NAGT I activity and the amount of rebinding calculated. The results are shown as the mean of three separate experiments ±SEM.

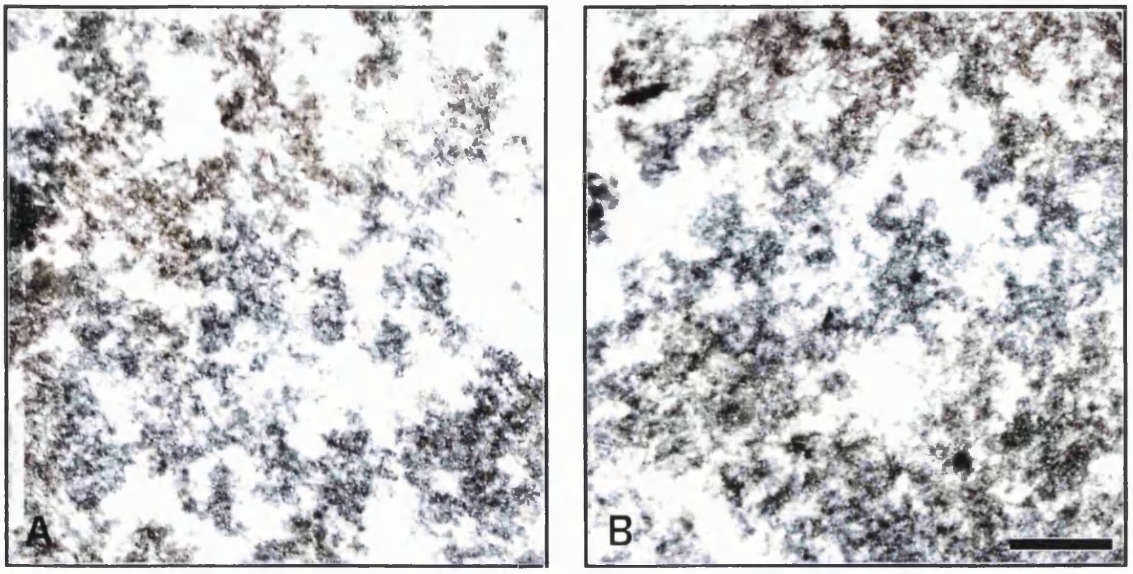

Figure 5.4: Morphology of the salt pellet before and after binding of the enzymes. A 200µg aliquot of Golgi membranes was extracted to produce a 150mM salt pellet (A). to which enzymes from a 70mM salt supernatant derived from 200µg of membranes was bound (B). The samples were fixed and examined by electron microscopy. Magnification = 52,500 Bar = 0.25µm.

when compared to the original 70mM supernatant.

In order to determine whether the reassembled material morphologically resembled the structures in the Triton pellet, 200µg aliquots of the 150mM salt pellet both before and after rebinding of *medial*-Golgi enzymes from a 70mM salt supernatant were fixed and processed for electron microscopy. Examination of these samples indicated that all the material in both the untreated salt pellet and the salt pellet which had rebound the enzymes contained only amorphous material (fig. 5.4), which resembled that found in the Triton pellet (c.f. fig. 4.7 with fig. 5.4), while the tri-laminar structures had disappeared. This suggested that second extraction had disrupted these more organised structures and that they couldn't be reconstituted after enzyme rebinding.

These data showed that both enzymes were capable of rebinding the pellet upon removal of the NaCl and suggested that it was the binding of the enzymes to this 150mM salt pellet, or matrix, that was responsible for their insolubility in detergent. It was possible that this rebinding was caused by a non-specific aggregation of the enzymes upon removal of the salt by dialysis, but this did not seem likely since the resedimentation of both enzymes did not occur efficiently in the absence of the pellet. However, a possibility did exist that the pellet did somehow induce an aggregation of all

soluble proteins on dialysis, and that the rebinding effect was not specific for Mann II and NAGT I.

To confirm that the salt pellet did constitute a matrix which exclusively bound these *medial*-Golgi enzymes, it was necessary to show that the binding was specific. This problem was addressed by three approaches which are described in the following sections.

5.2.4 Dose Dependence of the Matrix on Rebinding

If enzyme binding to the matrix was specific, it would have to be dose-dependent. Thus by incubating a fixed amount of Mann II with increasing amounts of matrix, there should be a concomitant increase in the amount of enzyme binding until a plateau was reached where all the enzyme is rebound.

To test this, 100µl aliquots of 70mM salt supernatants were dialysed in the absence or presence of 150mM salt pellets derived from 10, 25, 50, 100 or 200µg of Golgi membranes. After centrifugation, the supernatants and pellet were assayed for Mann II and NAGT I activity and the amount each of these enzymes which rebound in each sample was calculated. The amount of protein in the salt pellet was also assayed and found to be 10% of that in the original Golgi membranes.

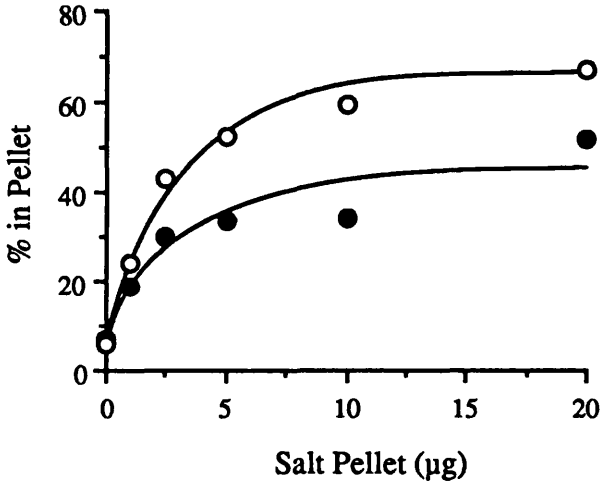

Figure 5.5: Dose dependence of the matrix for reassembly. Aliquots of 100µl of a 70mM salt supernatant were dialysed in the absence or presence of 150mM salt pellets derived from 10, 25, 50, 100 or 200µg of Golgi membranes. After centrifugation, the supernatants and pellets were assayed for Mann II (O) and NAGT I (●) activity, and the amount of enzyme rebinding calculated and plotted against amount of protein in the salt pellet.

This demonstrated that the amount of enzyme binding was dependent on the amount of matrix present (fig. 5.5). This dose dependence was linear for a 70mM salt supernatant and for up to 2.5µg of matrix (or that derived from 25µg of Golgi membranes). Beyond this, the curves began to plateau, as would be expected as the concentration of soluble free enzyme dropped at higher matrix concentrations. Note that the amount of NAGT I

rebinding was lower than that of Mann II (fig. 5.3 and fig. 5.5), which reflected it slightly greater solubility in TX-100 alone (see chapter 4).

This dose-dependence suggested that binding was specific because it indicated that the salt pellet contained a finite number of binding sites. It could not in itself, however, be considered proof of the specificity of binding .

5.2.5 Rebinding of GalT to the Salt Pellet

If the rebinding observed above was due to a non-specific aggregation of the solubilised proteins in the salt supernatant which was induced by removal of NaCl in the presence of the matrix pellet, then it would be expected that other soluble proteins would also become insoluble after dialysis. Since GalT had previously been shown to be soluble in TX-100 (see fig. 4.5 A), this molecule would prove a good marker to examine the specificity of the rebinding. If binding of Mann II and NAGT I was specific, it would be expected that GalT would remain soluble after removal of salt by dialysis, since TX-100 would still be present in the buffer. If GalT did rebind, it would be likely that the rebinding was non-specific since GalT would normally be soluble in TX-100 alone.

It was shown previously that Mann II and GalT (and presumably NAGT I) could be completely solubilised directly from Golgi membranes by extraction directly with TMMDS buffer containing 150mM NaCl (fig. 4.15). It was reasoned, therefore, that since a crude 150mM salt extract of Golgi membranes would contain all the components necessary for rebinding of the enzymes, then it would be possible reconstitute the binding by dialysis of this crude extract. Since the extract also contained solubilised GalT, it would then be possible to determine whether this enzyme would rebind upon dialysis and to compare the amount of GalT rebinding to that of Mann II and NAGT I.

To this effect, two 100µg aliquots of Golgi membranes were extracted in TMMDS buffer supplemented with 150mM NaCl. These crude extracts were then either dialysed against TMMDS buffer or incubated at 4°C and then centrifuged as during normal extraction procedures. The supernatants and pellet were then assayed for Mann II, NAGT I and GalT activity, as was an aliquot of untreated 150mM extract, and the amount of rebinding of each enzyme determined.

This showed that without dialysis, all the enzymes remained completely soluble, as would be expected, and confirmed that NAGT I could also be solubilised directly from Golgi membranes by 150mM NaCl in TMMDS buffer (fig. 5.6). After dialysis, however, the insolubility of both Mann II and NAGT I increased dramatically with approximately 30% of each enzyme being found in the pellet, while in contrast GalT insolubility rose to only 5%, indicating that only the *medial*-Golgi enzymes were rebinding to the matrix. The fact that some GalT rebound was not surprising because a small proportion of this

enzyme is insoluble in TX-100 alone (see fig. 4.5 A). Nevertheless, the fact that much more of the Mann II and NAGT I rebound demonstrated only the *medial*-Golgi enzymes could bind to the matrix. This rebinding of enzymatic activity was a true reflection of the protein rebinding because the recoveries of the enzymes when compared to the untreated 150mM extract were 108%±2.4 for Mann II, 119%±3.6 for NAGT I and 106%±1.6 for GalT (±SEM, n=3).

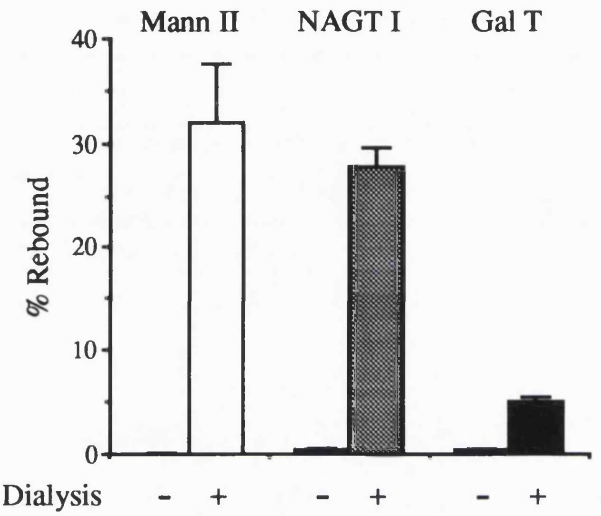

Figure 5.6: Resedimentation of Mann II, NAGT I and GalT after dialysis. Aliquots of 100µg of Golgi membranes were extracted in TMMDS buffer containing 150mM NaCl. These crude extracts were either dialysed against TMMDS buffer or incubated at 4°C. After centrifugation the supernatants and pellets were assayed for Mann II, NAGT I and GalT activity and the amount of rebinding of each enzyme determined. The results are shown as the mean of three separate experiments ±SEM

The amount of reassembly of both Mann II and NAGT I were lower than when rebinding was performed with a 70mM salt supernatant and 150mM salt pellet (c.f. fig 5.3 with fig.5.6). This lower value probably represented a more accurate estimate of the maximal amount of reconstitution possible in this system because the earlier experiment did not contain the same ratio of enzymes to matrix that was present in intact Golgi membranes.

This experiment demonstrated that a *trans*-Golgi enzyme did not bind to the matrix, and although it suggested that the binding of the *medial*-Golgi enzymes was specific, it did not demonstrate it directly. It was perfectly possible that other non-*medial* proteins aggregated non-specifically upon dialysis and that GalT was an exception, or that Mann II and NAGT I were the only enzymes to bind but did so with a low affinity that was not physiologically relevant and would not occur *in vivo*.

To distinguish between these possibilities, it was necessary to show that both enzymes were binding to the matrix with high affinity. A high affinity binding constant would prove that rebinding was specific and reflected a physiologically relevant function.

The affinity of binding of Mann II and NAGT I for the matrix was determined by Scatchard analysis.

5.2.6 Scatchard Analysis

By binding ligands at varying concentrations to constant amounts of receptor and determining the amount of ligand bound at each concentration, it is possible to determine the affinity of the receptor/ligand interaction. This is classically achieved by means of a Scatchard plot in which the concentration of bound ligand in plotted against the ratio of bound ligand and free ligand. If the curve obtained is linear, the ligand binds to the receptor with an affinity which is defined by the negative of the slope of the line, while a bipartite curve indicates that the receptor contains multiple ligand binding sites with different affinities. The point at which the line crosses the y-axis determines the number of ligand binding sites per receptor, though knowledge of the concentration of receptor is required for this calculation. Such analysis were performed to determine the affinity of binding of both Mann II and NAGT I the matrix. This was achieved by dialysing increasing concentrations of solubilised Mann II and NAGT I in the presence of a fixed amount of matrix in the form of a 150mM salt pellet and determining the amount of enzyme bound in each sample. The enzyme concentrations could be determined from their activities by using the specific activities of Mann II and NAGT I which had been reported when the enzymes were purified.

5.2.6.1 Affinity of Mann II for the Matrix

In performing Scatchard analyses, it was necessary to ensure that the salt supernatants containing the Mann II that was to be bound to the matrix did not contain any solubilised matrix components i.e. that it was not capable of reassembly in the absence of matrix. If the supernatant did contain such components, the concentration of matrix in each sample would vary. This would interfere with the analysis which should be performed with constant amounts of matrix. This was achieved by rebinding Mann II from a 25mM salt supernatant to the matrix. This concentration was chosen because at such low concentrations reassembly in the absence of matrix was negligible (fig. 5.2), indicating that few matrix components were present.

Since these supernatants contained such low amounts of Mann II, rebinding was carried out with aliquots of matrix that had been produced from only 25 μg of Golgi membranes. Low amounts of matrix were chosen because it is usual to choose rebinding conditions where the receptors are close to saturation when performing Scatchard analysis. To achieve conditions where the matrix was reaching saturation with

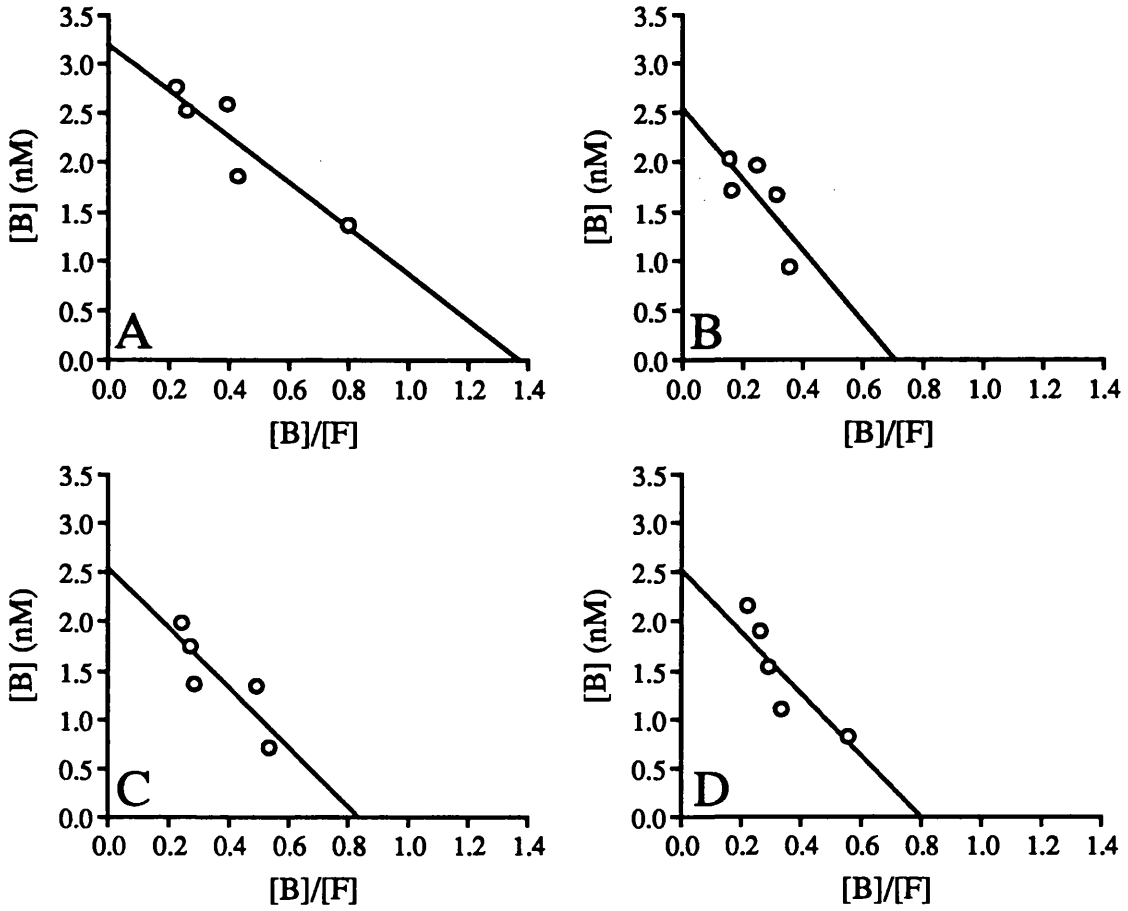

Figure 5.7: Scatchard analysis of Mann II binding to the matrix. Aliquots of 20, 40, 60, 80 or 100µl of a 25mM salt supernatant were made up to 100µl with TMMDS buffer containing 25mM NaCl and dialysed in the presence of 150mM salt pellets from 25µg of Golgi membranes. After centrifugation the supernatants and pellets were assayed for Mann II activity and subjected to Scatchard analysis. The experiment was repeated four times and the slopes of the lines gave K_a values of 2.3nM (A), 3.6nM (B), 3.0nM (C) and 3.2nM (D).

Mann II and NAGT I, it was necessary to use small amounts of matrix because of the low amounts of enzyme in the salt supernatant.

Five 25µg aliquots of Golgi membranes were used to produce 150mM salt pellets and 350µg of membranes were used to produce a 25mM salt supernatant. The supernatant was divided into 20, 40, 60, 80 and 100µl aliquots and the volumes made up to 100µl with TMMDS buffer containing 25mM NaCl. Each matrix pellet was resuspended in one of these supernatant samples and dialysed overnight a 4°C. The remaining salt supernatant was stored at 4°C. The dialysates were centrifuged and the supernatants and pellets were assayed for Mann II activity as was an aliquot of the undialysed salt

supernatant. The amount of Mann II bound was determined from the amount found in the pellet, while the amount remaining free was calculated as being the amount loaded minus the amount bound, as is usual in Scatchard analysis. This experiment was carried out four times to yield four different Scatchard plots (fig. 5.7). The average value obtained for the affinity of a Mann II dimer for the matrix was 3.0nM±0.3 (±SEM, n=4), indicating that the binding was of high affinity and therefore specific. The average recovery of Mann II during these experiments compared to the undialysed salt supernatants was 97.4%±3.7 (±SEM, n=20), indicating that no activation or inactivation had occurred. This also showed that the approach of calculating the amount of free enzyme as being the amount loaded minus the amount bound was a valid one.

Furthermore, the fact that the Scatchard plot was linear indicated that the binding of Mann II was saturable as would be expected of a specific interaction. This was confirmed by replotting the data from one of the Scatchard plots to display the percentage of Mann II bound to the matrix at each enzyme concentration (fig. 5.9).

5.2.6.2 Affinity of NAGT I for the Matrix

Scatchard analysis was carried out in an identical manner to Mann II. One problem that arose however was the relative insensitivity of the NAGT I assay. Because of the low concentrations of NAGT I in the salt supernatants, it was necessary to carry out the NAGT I assays on the entire 100µl sample by the addition of a highly concentrated assay mixture (see chapter 2).

Four experiments were carried out exactly as for the determination of Mann II affinity except that NAGT I was assayed, and four Scatchard plots were produced (fig. 5.8). The recovery of enzyme compared to the undialysed supernatant was 100.4%±4.4 (±SEM, n=4), and the average slope of the lines was 70pM±21.4 (±SEM, n=4). This showed that NAGT I also bound to the matrix with high affinity, and seemed to have a higher affinity for the matrix than Mann II. This was surprising because all previous experiments indicated that NAGT I was more soluble than Mann II in detergent and so suggested that it had a lower affinity for the matrix. The value of 70pM should, however, be taken with some caution for two reasons.

Firstly, the specific activity of rat-liver NAGT I has not been previously reported. This value was required to determine the concentration of enzyme in any given sample. The specific activity of rabbit-liver NAGT I was used instead (Oppenheimer and Hill, 1981), and this is likely to have introduced an error into these calculations. Secondly, the reported specific activity for the rabbit liver enzyme was reported for the purified ovalbumin oligosaccharide and not the intact protein as was used in these experiments. It has been previously shown that the kinetics of N-glycan processing enzymes varies

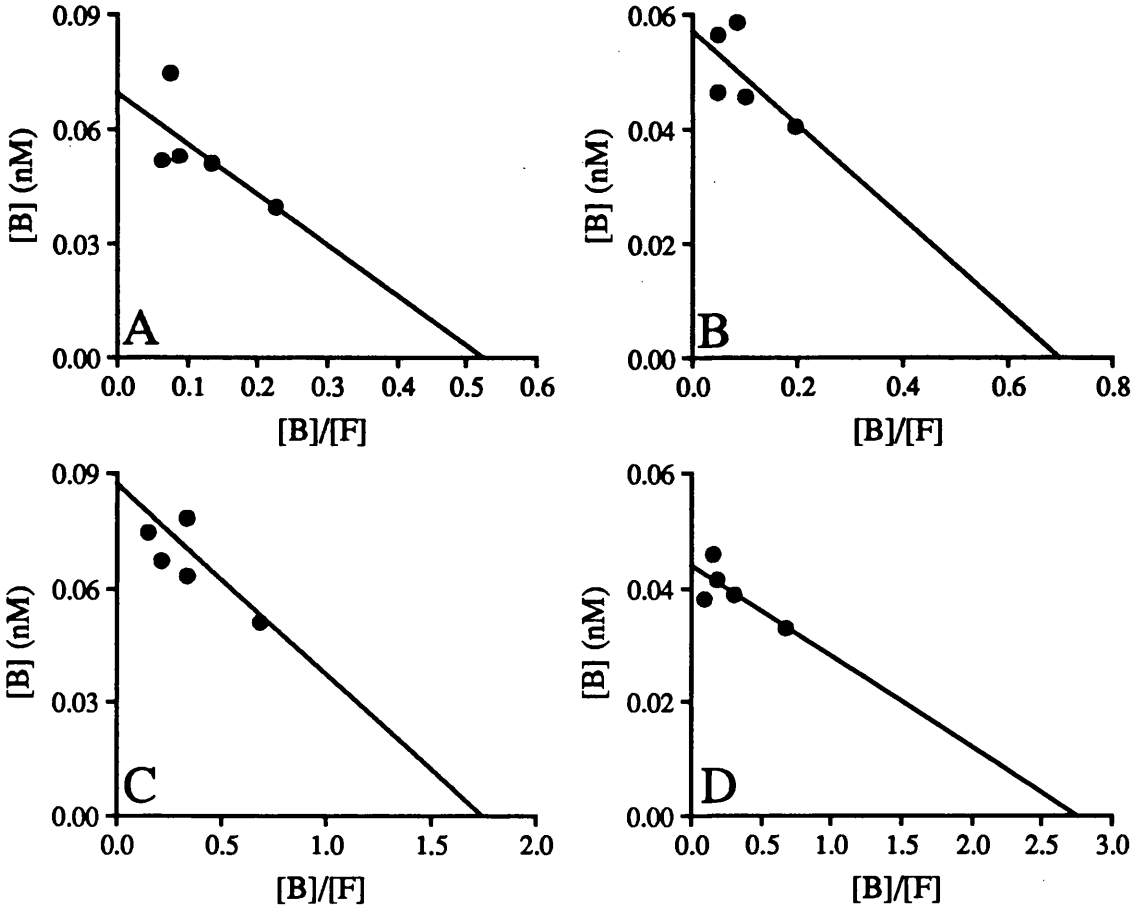

Figure 5.8: Scatchard analysis of NAGT I binding to the matrix. Aliquots of 20, 40, 60, 80 or 100µl of a 25mM salt supernatant were made up to 100µl with TMMDS buffer containing 25mM NaCl and dialysed in the presence of 150mM salt pellets from 25µg of Golgi membranes. After centrifugation the supernatants and pellets were assayed for NAGT I activity and subjected to Scatchard analysis. The experiment was repeated four times and the slopes of the lines gave K_a values of 132pM (A), 82pM (B), 50pnM (C) and 16pM (D).

depending on the substance used as an acceptor (Tabas and Kornfeld, 1979; Harpaz and Schachter, 1980a), and thus because of this a second error would been introduced into the calculations. Thus, although it was not possible to calculate the exact affinity of NAGT I for the matrix, the 70pM value did show that it was in a similar range to that of Mann II, and that binding was specific.

Because of the relative insensitivity of the NAGT I assay, the linearity of the plots was not as convincing as for Mann II. To demonstrate that binding was saturable, the data obtained for figures 5.7 A and 5.8 A were replotted to show the percentage of the total amount of Mann II and NAGT I loaded that had bound to the matrix at each enzyme

concentration (fig. 5.9). This showed that at higher concentrations a lower percentage of the enzymes bound to the matrix compared to the lower concentrations, indicating that binding sites on the matrix were becoming saturated.

Thus the combination of the dose-dependence of the matrix on enzyme binding, the inability of GalT to bind, the high affinity of *medial*-enzyme binding and the saturability of the matrix combined to strongly suggest that the enzyme binding phenomenon was specific.

5.2.7 Digestion with Proteases

Once the fact that the matrix bound the *medial*-Golgi enzymes with a high affinity was established, the next question addressed regarded its intracellular topology. It was not yet known whether the matrix was present within the lumen of the cisternae or, as was initially hoped, in the cytoplasm. This problem was addressed by utilising protease protection experiments. If the matrix were lumenal, it would be resistant to the presence of exogenously added protease and would still be able to rebind enzymes after such treatment. Conversely, if the matrix were cytoplasmic, it would be sensitive to proteolysis of intact Golgi stacks, and enzyme rebinding would be abolished after such treatment.

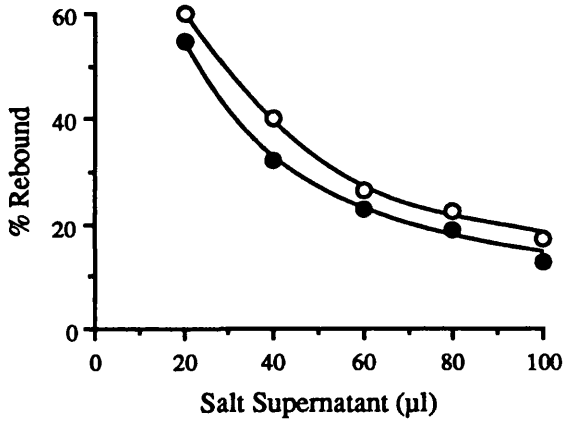

Figure 5.9: Saturability of enzyme binding. Aliquots of 20, 40, 60, 80 or 100µl of a 25mM salt supernatant were made up to 100µl with TMMDS buffer containing 25mM NaCl and dialysed in the presence of 150mM salt pellets from 25µg of Golgi membranes. After centrifugation the supernatants and pellets were assayed for Mann II (○) and NAGT I (●) activity and the percentage of the amount loaded that had bound was calculated.

The effect of digestion of intact Golgi membranes with trypsin and proteinase K was tested. Such membranes were extracted with TMMDS buffer and the solubility of both Mann II and NAGT I tested. If the matrix was cytoplasmic, its removal would render the enzymes soluble in TX-100. Thus solubilisation of the enzymes by detergent after proteolysis of Golgi membranes would indicate that the matrix was cytoplasmic.

5.2.7.1 Trypsin

Trypsin was used to digest Golgi membranes and these membranes were extracted in TMMDS buffer under the usual conditions to determine whether any change in the enzyme solubilities had occurred. To test this, 100µg aliquots of Golgi membranes were digested with trypsin concentrations of 0, 0.5, 1, 2, 5 and 10mg/ml and a Golgi protein concentration of 1mg/ml (obtained by dilution with MMDS buffer), in the presence or absence of 0.1% (w/v) TX-100. After incubation at 25°C for 30min, the protease was quenched with PMSF. The samples that were digested in the absence of detergent were extracted in TMMDS buffer and the supernatants and pellets assayed for Mann II and NAGT I activity as was an aliquot of untreated Golgi membranes. The samples that were digested in the presence of TX-100 were simply assayed for NAGT I activity to determine the effectiveness of proteolysis.

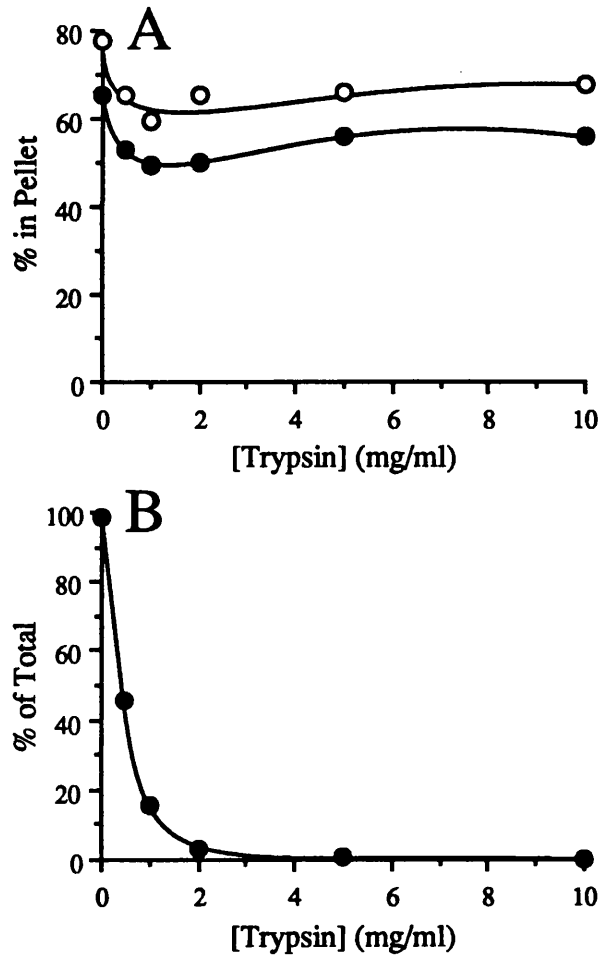

Figure 5.10: Effect of trypsin digestion on Mann II and NAGT I solubility. 100µg aliquots of Golgi membranes were mock-treated or digested with trypsin concentrations of 0.5, 1, 2, 5 and 10mg/ml in the absence or presence of 0.1% (w/v) of TX-100. The samples which were digested in the absence of TX-100 were extracted in TMMDS buffer, the supernatants and pellets assayed for Mann II (○) and NAGT I (●) activity, and the percentage remaining in the pellet determined (A). The other samples were assayed for NAGT I without extraction to determine the extent of proteolysis (B).

This showed that proteolysis by trypsin was occurring, with virtually all NAGT I activity being lost at a protease concentration of 2mg/ml (fig. 5.10 B). This proteolysis had little effect on the solubility of either Mann II or NAGT I (fig. 5.10 A).

The recovery of Mann II activity was determined to be 92.6%±10.3 (±SD, n=6) for Mann II and 103.2%±7.4 (±SD, n=6) for NAGT I, indicating that no change in enzyme activity had occurred during digestion and that the membranes did not allow the protease access to the lumen of the cisternae.

Although the solubility of both enzymes increased by approximately 20% upon the addition of 0.5mg/ml of trypsin, the solubility could not be further increased by the presence of more concentrated protease concentrations and Mann II remained approximately 65% and NAGT I remained 55% insoluble at higher trypsin concentrations. This slight effect, however, suggested that the enzyme insolubility may have been dependent on the presence of cytoplasmic factors, and so the effect of digestion with a different protease was examined to ascertain whether the solubility of the enzymes could be increased further.

5.2.7.2 Proteinase K

Since trypsin is not a broad specificity protease, it was possible that cytoplasmic components of the matrix were resistant to this protease. Proteinase K was chosen for digestion of Golgi membranes because this displays a larger substrate specificity than trypsin and would therefore be more likely digest any such trypsin-resistant cytoplasmic components of the matrix.

Aliquots of 100µg of Golgi membranes were digested at a protein concentration of 1mg/ml in the presence and absence of 0.1% (w/v) TX-100 in much the same way as for the trypsin digestions described above, except that proteinase K was used at concentrations of 0, 0.5, 1.0 and 2.0mg/ml. The samples that were digested in the presence of detergent were assayed for NAGT I activity while the other samples were extracted in TX-100 and the supernatants and pellets assayed for both Mann II and NAGT I. Proteinase K appeared to efficiently digest NAGT I with complete activity being lost even at a protease concentration of 0.5mg/ml in the presence of detergent (fig. 5.11 B). At proteinase K concentrations of 0.5 and 1mg/ml, the solubility of Mann II and NAGT I increased upon TX-100 extraction with 20-35% extra solubilisation occurring (fig. 5.11 A). The recovery of Mann II activity was determined to be 95.1%±10.3 (±SD, n=4) for Mann II and 93.6%±7.4 (±SD, n=4) for NAGT I, indicating that no change in enzyme activity had occurred during digestion. Thus proteinase K seemed to be slightly more efficient at solubilising the enzymes that trypsin (c.f. 5.10 A and 5.11 A), though this increase in solubilisation was not as great as was desired. Furthermore, at a protease concentration of 2mg/ml, the insolubility of the enzymes increased again. This was possibly due to the protease precipitating in the MMDS buffer

at this concentration which may have induced non-specific aggregation of the enzymes to the precipitate, thought this was not shown to be the case.

Since proteinase K appeared to be more effective than trypsin at solubilisation of the *medial* enzymes, this enzyme was chosen for further experiments. However, it was not possible to use protease concentration greater than 1mg/ml due to its precipitation under these conditions. Further solubilisation was attempted by using lower concentrations of Golgi membranes during the digestion with a constant protease concentration. This meant that the protease:Golgi ratio (and therefore the extent of proteolysis) could be increased without using high proteinase K concentrations.

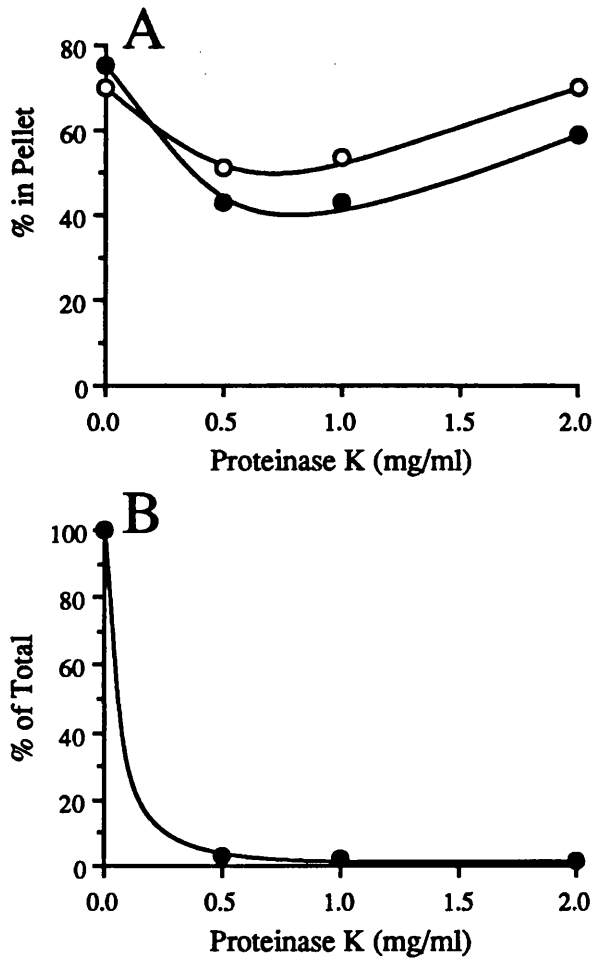

Figure 5.11: Effect of proteinase K digestion on Mann II and NAGT I solubility. 100µg aliquots of Golgi membranes were mock-treated or digested with proteinase K concentrations of 0.5, 1 and 2mg/ml in the absence or presence of 0.1% (w/v) of TX-100. The samples which were digested in the absence of TX-100 were extracted in TMMDS buffer, the supernatants and pellets assayed for Mann II (○) and NAGT I (●) activity, and the percentage remaining in the pellet determined (A). The other samples were assayed for NAGT I without extraction to determine the extent of proteolysis (B).

Thus 25, 33, 50 and 100µg aliquots of Golgi membranes were made up to 100µl with MMDS buffer containing proteinase K at a final concentration of 0.5mg/ml and incubated at 25°C for 30min. After the protease in the samples was quenched by the addition of PMSF, the membranes were extracted with TMMDS buffer at a protein concentration of 1mg/ml of original Golgi membranes. The supernatants and pellets were assayed for both Mann II and NAGT I activity. This

showed that the amount solubilisation of both enzymes was related to the concentration of the Golgi membranes during the digestion (fig. 5.12). As the protease:Golgi ratio increased i.e. at lower Golgi concentrations, larger amounts of both *medial* enzymes were solubilised. At a Golgi concentration of 0.25mg/ml only 10-20% of these enzymes remained insoluble, indicating that digestion of the cytoplasmic faces of the membranes removed components of the matrix that were required to maintain the insolubility of the enzymes.

To determine whether this effect was reproducible, 25µg aliquots of three different preparations of Golgi membranes were made up to 100µl with MMDS buffer, i.e. a protein concentration of 0.25mg/ml, and proteinase K either omitted or added to a final concentration of 0.5mg/ml and incubated as described above. After quenching with PMSF, the membranes were extracted with TMMDS buffer and the supernatants and pellets assayed for Mann II and NAGT I activity as was an aliquot of untreated membranes.

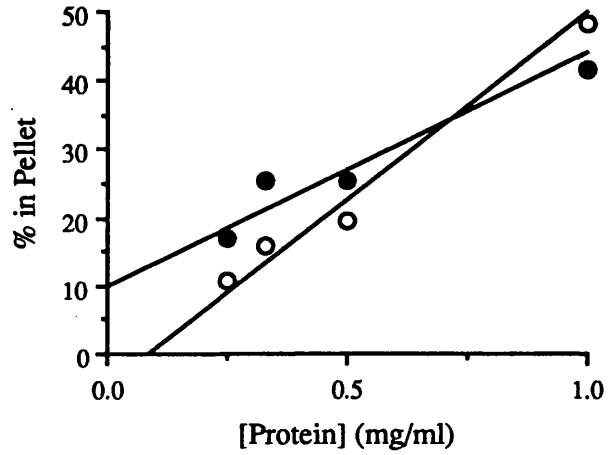

Figure 5.12: Effect of Golgi concentration during proteinase K digestion on Mann II and NAGT I solubility. Golgi membranes were digested at protein concentrations of 0.25, 0.33, 0.50, 1.00mg/ml, and a proteinase K concentration of 0.5mg/ml. The samples were then extracted in TMMDS buffer, the supernatants and pellets assayed for Mann II (O) and NAGT I (●) activity, and the amount of each remaining in the pellet determined.

This showed that both enzymes remained relatively insoluble upon mock treatment (fig. 5.13), indicating that no increase in solubility occurred upon membrane dilution and incubation at 25°C or upon addition of PMSF. However, after proteolysis, both enzymes became highly soluble in TMMDS buffer, showing that the effect was reproducible. The solubility of the *medial*-enzymes in detergent alone was now comparable to that of the non-*medial* Golgi markers (c.f. fig. 5.13 with fig. 4.5 A). The enzyme recoveries when compared to untreated Golgi membranes were 76.0%±2.5 for the mock treated and 79.3%±1.0 for the digested sample (±SEM, n=3) for Mann II and 75.1%±0.9 for the mock treated and 66.4%±3.2 for the digested sample (±SEM, n=3) for NAGT I, showing

that the protease had little access to the lumen of the Golgi cisternae during this experiment.

5.2.8 Topology of the Matrix

The fact that proteinase K treatment of intact Golgi membranes caused both Mann II and NAGT I to become soluble on TX-100 extraction strongly suggested that the matrix was cytoplasmic. However, another possibility existed which meant that the matrix could still be lumenal. These alternative possibilities are outlined in figure 5.14.

The first possibility was that the matrix was cytoplasmic, and that its removal by proteinase K would leave nothing for the enzymes to bind, and thus render them soluble upon detergent extraction (fig. 5.14 A). The second possibility was that the protease may have removed the cytoplasmic domains of the enzymes, thereby inducing a conformational change in their lumenal domains which prevented them from binding to a lumenal matrix (fig. 5.14 B). This would cause them to be solubilised after extraction although an intact matrix was still present in the sample.

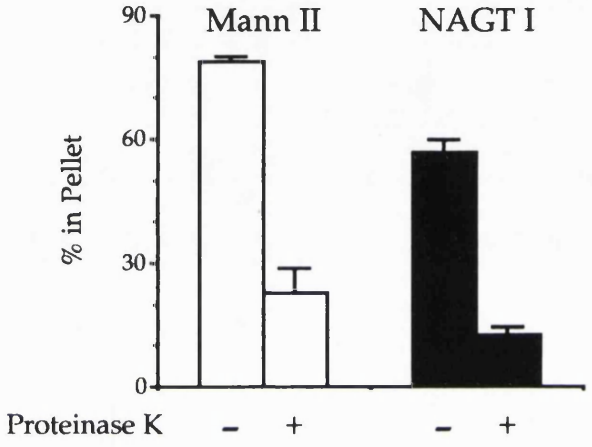

Figure 5.13: Solubilisation of Mann II and NAGT I after proteinase K digestion of Golgi membranes. Three 25µg aliquots of different Golgi membrane preparations were diluted to a protein concentration of 0.25mg/ml, and incubated in the absence or presence of proteinase K at a concentration of 0.5mg/ml. The samples were then extracted in TMMDS buffer, the supernatants and pellets assayed for Mann II and NAGT I activity, and the amount of each remaining in the pellet determined. Results are shown as the average of three experiments ±SEM (n=3).

In order to distinguish between these possibilities, it was necessary to determine whether the solubilised enzymes which were produced by digestion and extraction of Golgi membranes were capable of rebinding a matrix which had been prepared from untreated Golgi stacks. If such enzymes could rebind to the fresh matrix, they could not have undergone a conformational change and this would indicate that the matrix was cytoplasmic. Conversely, if such enzymes did not rebind, it would not be possible to state categorically whether the matrix was cytoplasmic or lumenal (since proteinase K

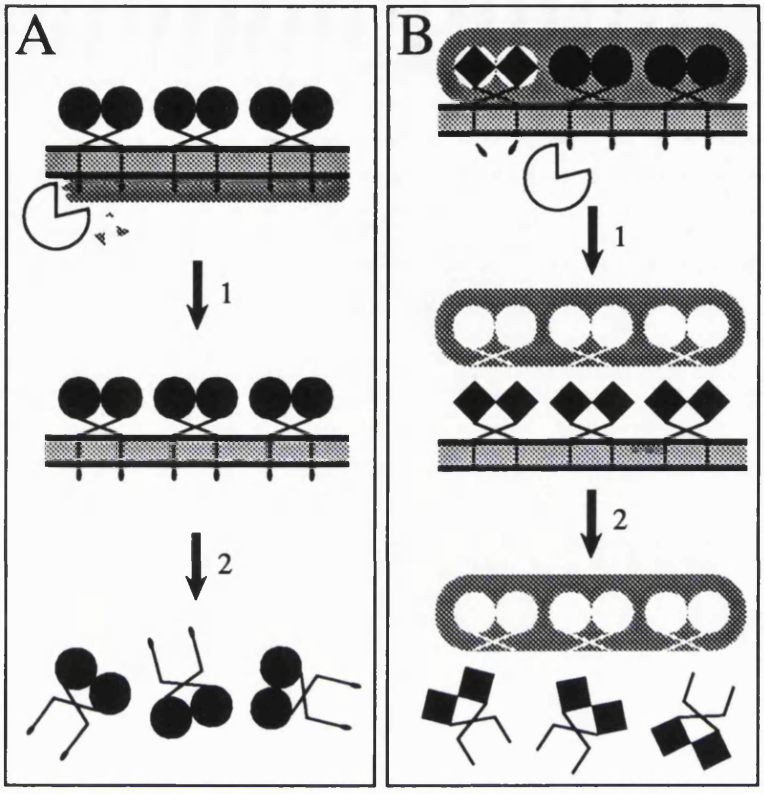

Figure 5.14: Models for enzyme solubilisation after proteinase K digestion. Possible mechanisms of enzyme solubilisation after proteinase K treatment (1) and TX-100 extraction (2) for a cytoplasmic (A) and lumenal (B) matrix. It should be noted that this figure is purely schematic and the cytoplasmic matrix is shown binding to the cytoplasmic tails of the enzymes. It was not known whether this was the case, and the diagram was only drawn this way for convenience.

treatment could also have digested a cytoplasmic matrix and removed the cytoplasmic tails which may have been required for binding). These possibilities were tested in the following experiment.

To this effect, one 100µg aliquot of Golgi membranes at a protein concentration of 0.25mg/ml was mock treated while two 100µg aliquots were digested with 0.5mg/ml proteinase K. These samples were then extracted in TMMDS containing 150mM NaCl and these extracts dialysed overnight. Additionally, one of the extracts prepared from digested membranes was supplemented with a 150mM salt pellet produced from 100µg of untreated Golgi membranes during the dialysis. After the dialysis, the samples were centrifuged under the same conditions as during the extraction protocol and the supernatants and pellets assayed for Mann II and NAGT I activity and the amount of both enzymes remaining in the pellet was determined.

This showed that the enzymes from the mock-treated samples were capable of rebinding the matrix present in the crude extract, while the enzymes from the digested membranes could not (fig. 5.15). However, if the latter sample was supplemented with fresh matrix, the binding activity of Mann II could be restored almost completely (94% of the mock-treated level). NAGT I rebinding increased significantly though not to the same extent (56% of the mock-treated level). The recoveries in the digested samples compared to the mock-treated were 102.8%±3.6 (±SEM, n=3) for Mann II and 106.2%±3.1 (±SEM, n=3) for NAGT I, showing that no protease induced inactivation had occurred. This clearly showed that the enzymes produced from the protease-treated membranes were capable of rebinding to the matrix and thus showed that the protease-induced solubility was not due to a conformational change caused by removal of the enzymes' cytoplasmic domains. Thus the possibility of the matrix being lumenal (as depicted in figure 5.14 B) was eliminated and the solubilisation had to be due to the removal of cytoplasmic components of the matrix which were crucial to the enzyme-binding function of the matrix.

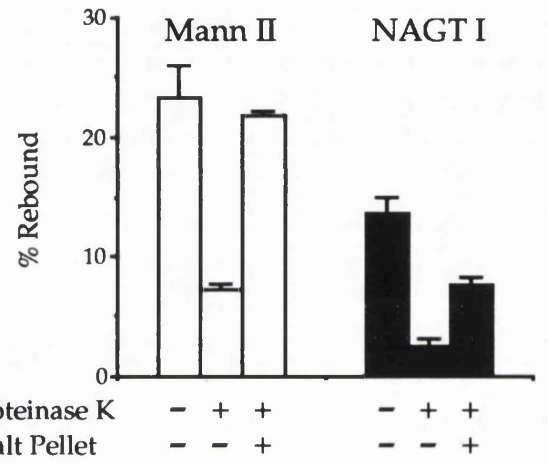

Figure 5.15: Binding of Mann II and NAGT I from proteinase K treated Golgi membranes to the matrix. Aliquots of 100µg of Golgi membranes were mock treated or digested with proteinase K and then extracted with TMMDS containing 150mM NaCl. These samples were then dialysed in the absence or presence of a 150mM salt pellet derived from 100µg of untreated Golgi membranes. Following centrifugation, the supernatants and pellets were assayed for Mann II and NAGT I activity. The experiment was carried out on three different Golgi membrane preparations and the average results are shown ±SEM (n=3).

5.2.9 Rebinding Without Dialysis

Since the *medial*-Golgi enzymes could be solubilised by TX-100 in the absence of salt after digestion with proteinase K, and that these enzymes could rebind the matrix, it seemed likely that they would also rebind if they were solubilised in the absence of NaCl.

This would allow rebinding studies to be carried out without dialysis step for each experiment.

Thus two aliquots of 100µg of Golgi membranes were digested with proteinase K and then extracted with TMMDS buffer. The supernatant was then incubated at 4°C for 1hr in the presence or absence of matrix that had been prepared from 100µg of untreated Golgi membranes. These samples were then centrifuged as during the extraction procedure and the supernatants and pellets assayed for Mann II and NAGT I activity as was an aliquot of supernatant which had not been subjected to the second centrifugation step. This showed that both enzymes could indeed rebind the matrix under these conditions, while very little resedimentation could be observed in the absence of fresh matrix (fig. 5.16). The average enzymatic recoveries compared to the original Triton supernatants were 96.6% for Mann II and 105.2% for NAGT I, indicating that negligible changes in the activity of either enzyme had occurred during rebinding.

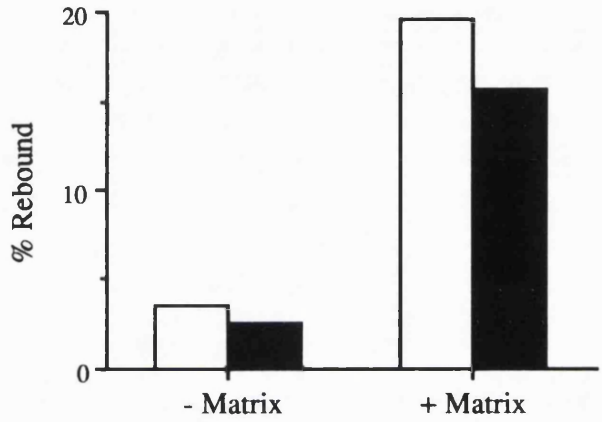

Figure 5.16: Rebinding of Mann II and NAGT I from proteinase K treated Golgi without dialysis. Aliquots of 100µg of Golgi membranes were digested with proteinase K and then extracted with TMMDS and centrifuged. The supernatants were then incubated at 4°C for 1hr in the presence or absence of matrix produced from 100µg of untreated membranes and centrifuged again. The supernatants and pellets were assayed for Mann II (❑) and NAGT I (■) activity, and the amount of rebinding determined.

5.3 Summary

Mann II could be released from the Triton pellet by a second extraction in both TX-100 and 150mM NaCl, and the solubilised enzyme appeared to behave as a dimer when analysed by velocity centrifugation. If the Mann II insolubility in Triton was indeed caused by a salt-sensitive interaction with the matrix, then this suggested that a single binding site existed on the matrix for each Mann II molecule. Alternatively, it was possible that the Mann II dimers could interact with each other to form small oligomers and that this interaction was also sensitive to NaCl. This would mean that less than one

binding site would exist for each Mann II dimer, with each small oligomer binding to one receptor in the matrix.

To test whether the 150mM salt pellet contained a matrix to which the *medial*-Golgi enzymes could bind, the solubilised enzymes were rebound to the salt pellet after dialysis to remove salt. Dialysis of salt supernatants derived from second extractions containing varying amounts of NaCl showed that Mann II became re-sedimentable when the salt was removed and that this insolubility increased in supernatants derived from extractions containing high levels of salt. This increase in re-sedimentation could have been due to partial solubilisation of putative matrix components in the higher salt extractions. At 150mM salt, the resedimentation was still relatively low compared to samples extracted in 400-1000mM, suggesting that the 150mM pellet still contained significant amounts of the proteins responsible for the Mann II insolubility. However, 20% of the Mann II from the 150mM salt supernatant could still re-sediment after dialysis. Thus rebinding studies were carried out using a 70mM salt supernatant which contained significant amounts of Mann II and NAGT I that did not re-sediment as readily. These supernatants were rebound to 150mM salt pellets which contained no detectable Mann II activity but presumably still contained functional matrix components.

Such rebinding studies showed that both Mann II and NAGT I remained almost completely insoluble without dialysis or upon dialysis in the absence of the salt pellet. Upon dialysis in the presence of the pellet, however, large amounts of both enzymes became insoluble, indicating that they had rebound to the material in the salt pellet. Morphologically, the material containing the rebound enzymes seemed indistinguishable from an untreated salt pellet. These structures resembled the amorphous material observed in the Triton pellet (see fig. 4.7), but the tri-laminar structures were no longer present. Thus, although enzyme rebinding could be constituted *in vitro*, it did not seem possible to reconstitute the morphology of the Triton pellet. This was presumably due to the loss of components during the second extraction which were responsible for maintaining the long-range order of the matrix-enzyme complex. Alternatively, these components could still have been present, but may have been unable to function under these conditions. The simplest interpretation is that this component is the lipid bilayer. Low levels of detergent do not remove all the lipids from the membrane (Helenius *et al.*, 1979), explaining how the Triton pellet contained the tri-laminar structures. The second extractions would have removed more lipid, however, which could not reform bilayers during the rebinding and therefore not provide a structural template for the enzyme-matrix. Thus the tri-laminar structures in the Triton pellet may represent an enzyme-matrix complex which is still apposed to the remnants of a lipid bilayer, while the

amorphous material would represent structures from which enough lipid had been removed to disrupt the membranes.

The rebinding Mann II and NAGT I was shown to be specific in three ways. Firstly, rebinding of the enzymes showed a strong dose-dependence when constant amounts of enzymes were rebound to varying amounts of matrix pellet. Secondly, the *trans*-Golgi/TGN enzyme, GalT, could not rebind to the matrix after the removal of salt, indicating that a general non-specific protein aggregation was not occurring during dialysis in the presence of the salt pellet. Thirdly, and most convincingly, the affinity constant of the rebinding of both enzymes was shown to be high by Scatchard analysis. Both enzymes rebound the matrix with an affinity in the nM range for Mann II and in the pM range for NAGT I (though this value could not be determined as accurately), comparable to the affinity of a weak antibody/antigen interaction (Dunphy *et al.*, 1985). The fact that the Scatchard plots were linear indicated that the enzyme binding was saturable, again demonstrating that it was a specific interaction.

Finally the matrix was shown to contain cytoplasmic components because of its accessibility to exogenously added proteinase K. Such treatment of intact Golgi membranes caused both Mann II and NAGT I to become soluble, suggesting that the matrix had been removed by the proteinase. This increase in solubility was not due to a conformational change in the enzyme due to removal of their cytoplasmic domains, since enzymes derived from digested membranes could readily rebind a matrix produced from an untreated Golgi preparation. This demonstrated that at least some of the functional part of the matrix that was required to maintain enzyme insolubility was present on the cytoplasmic face of the Golgi membranes. Furthermore, the fact that both Mann II and NAGT I are present throughout the entire length of the cisternae in which they reside strongly suggested that it was intercisternal (Burke *et al.*, 1982; Nilsson *et al.*, 1993a).

It was, therefore, established that the 150mM salt pellet contained an intercisternal matrix which bound the *medial*-Golgi enzymes. However, the mechanism of binding of these enzymes was unknown, and three possibilities existed. Firstly, the enzymes could bind directly to the matrix via their cytoplasmic tails. Secondly, it was possible that the enzymes bound via their membrane-spanning domains by interaction with a protein that bound the matrix and also entered the lipid bilayer. Finally, the enzymes could be bound by their lumenal domains, via a membrane spanning protein which cross-linked them to the matrix.

In the next chapter, I describe experiments which were designed to distinguish between these possibilities.

Chapter 6

The Mechanism of *medial*-Enzyme Binding

6.1 Introduction

In the previous chapters, evidence was presented to suggest that a Golgi matrix was present in purified rat-liver Golgi stacks and that this matrix specifically bound *medial*-Golgi enzymes with a high affinity. It was this interaction that conferred the property of detergent-insolubility upon the *medial*-enzymes and it was this insolubility that allowed the identification of the putative matrix. Furthermore, by virtue of its accessibility to exogenously added proteinase K, it was shown that at least some of the functional part of this matrix was present on the cytoplasmic face of the Golgi membranes.

However, these experiments did not reveal the mechanism by which the matrix bound the enzymes. Three possible mechanisms could be envisaged for this interaction, none of which could be distinguished in the previous experiments. Firstly, the enzymes could interact directly with the matrix via their cytoplasmic domains. Secondly, the enzymes could interact via their membrane-spanning domains. Thirdly, the enzymes could indirectly interact with the matrix via a cytosolic adapter protein, or by means of a second membrane spanning protein which bound the enzymes by their lumenal domains and the matrix by its cytoplasmic tail.

In this chapter I describe experiments which were designed to differentiate between these possibilities and which addressed the problem by three independent approaches. In the first, the effect of artificial post-translational modification of the cytoplasmic face of the Golgi membranes on the ability of the enzymes to rebind the matrix is examined. In the second, I describe competitive inhibition studies of the effect of artificial peptides corresponding to the cytoplasmic tails of the enzymes on the ability of the enzymes to bind the matrix. Finally I describe the purification of the chymotryptic fragment of Mann II, which lacks the cytoplasmic and membrane-spanning domains, and experiments on its ability to rebind the matrix, and compare this with the rebinding of purified, intact Mann II.

6.2 Results

6.2.1 Effect of SPITC Treatment on Enzyme Rebinding

Digestion of Golgi membranes with proteinase K removed the matrix to which the *medial*-Golgi enzymes were bound. These enzymes could then be rebound to a fresh matrix which had been produced from untreated membranes. The next question that was addressed was whether other cytoplasmic components in the Golgi membranes which had not been removed by the protease were also required for the reconstitution of enzyme rebinding to the matrix. Such components could either be the cytoplasmic tails of the enzymes themselves, cytosolic adapter proteins which were resistant to protease or the

cytoplasmic domains of membrane-spanning proteins which cross-linked the enzymes to the matrix.

The effect of treatment of the cytoplasmic face of proteinase K treated Golgi membranes with the reagent 4-sulphophenyl isothiocyanate (SPITC) on the rebinding of Golgi enzymes was therefore examined. The structure of this molecule is shown in figure 6.1 A. SPITC is a derivative of Edman's reagent (phenyl isothiocyanate) which is classically used in protein sequencing, the difference occurring in the substitution of a negatively charged sulphite group at the *para* position of the benzene ring.

Because of this modification, SPITC in impermeable to lipid bilayers while its reaction chemistry remains unaltered. The molecule specifically reacts with primary amines by virtue of a electrophilic attack on the deshielded carbon on SPITC by the free electron pair on the amine. This yields a product in which the primary amine is modified with the SPITC moiety. The reaction mechanism is detailed in figure 6.1 B.

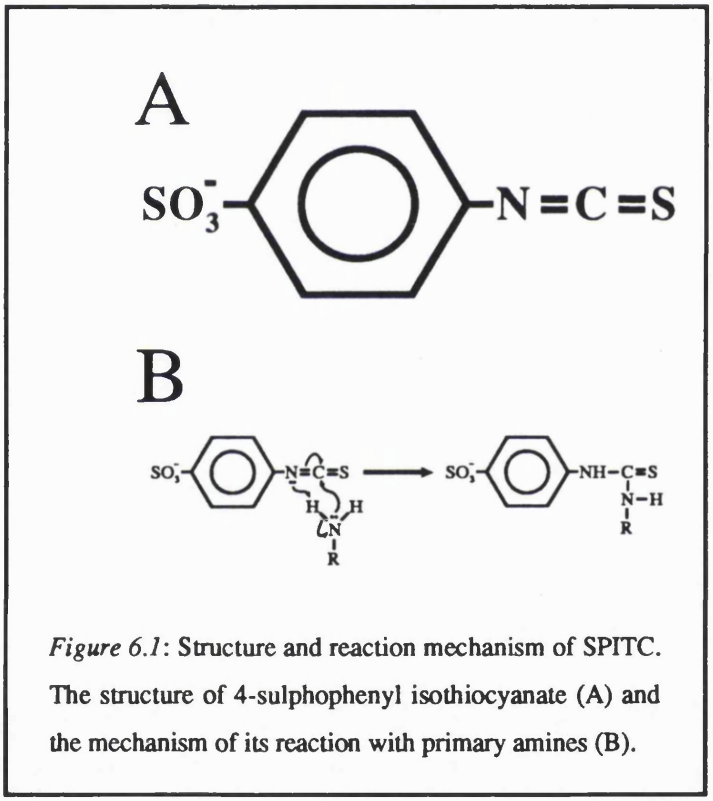

Figure 6.1: Structure and reaction mechanism of SPITC. The structure of 4-sulphophenyl isothiocyanate (A) and the mechanism of its reaction with primary amines (B).

The reaction can only proceed at alkaline pH since the nitrogen on the amine group must be deprotonated in order to allow nucleophilic attack on the carbon of SPITC. Thus reactions were carried out in HMS buffer (50mM HEPES pH8.0, 0.1mM $MgCl_2$, 10% (w/v) sucrose). HEPES was chosen as the buffer because it contains no amines which would react with SPITC.

To test whether the reaction was occurring, SPITC was reacted with a synthetic nonapeptide corresponding to the cytoplasmic and part of the membrane-spanning domain of Mann II (MII-CT), and having the sequence MKLSRQFTV. The peptide was synthesised by the ICRF Peptide Synthesis Facility. Since MII-CT contained one lysine, it could react with two SPITC molecules, one at this residue and one at its N-terminus. Changes in mass of MII-CT due to reaction with SPITC were detected by mass spectrometry.

MII-CT was made up as a 5mM solution in HMS buffer and divided into two aliquots. SPITC was added to one at a final concentration of 15mM and both samples incubated at 25°C for 30min. They were then analysed on a Lasermat MALDITOF (Matrix Assisted Laser Desorbtion Time of Flight) mass spectrometer (Finnigan Mat) by Dr. Darryl Pappin in the ICRF Protein Sequencing Laboratory.

The results of these analyses are shown in figure 6.2. The peak at a molecular mass of 3496.9 (fig. 6.2 A and B, peak d) is that of oxidised bovine insulin B-chain (Sigma) which is used as an internal calibration control. In the absence of SPITC, MII-CT was detected at a molecular mass of 1110.8 Daltons (fig.6.2 A, peak a), only 1.5 Daltons less than its predicted molecular mass based on its amino acid composition. This peak disappears completely upon treatment with SPITC (c.f. fig. 6.2 A and B) and is replaced by two peaks with molecular masses of 1324.5 and 1540.0 respectively (fig. 6.2 B. peaks b and c). The mass differences between these two peaks and that of MII-CT was therefore 213.7 and 215.5 respectively. In both cases, this was very close to the predicted mass increase of 214.2 from the addition of a SPITC moiety, and the differences were within the error range which could be expected from machine being used.

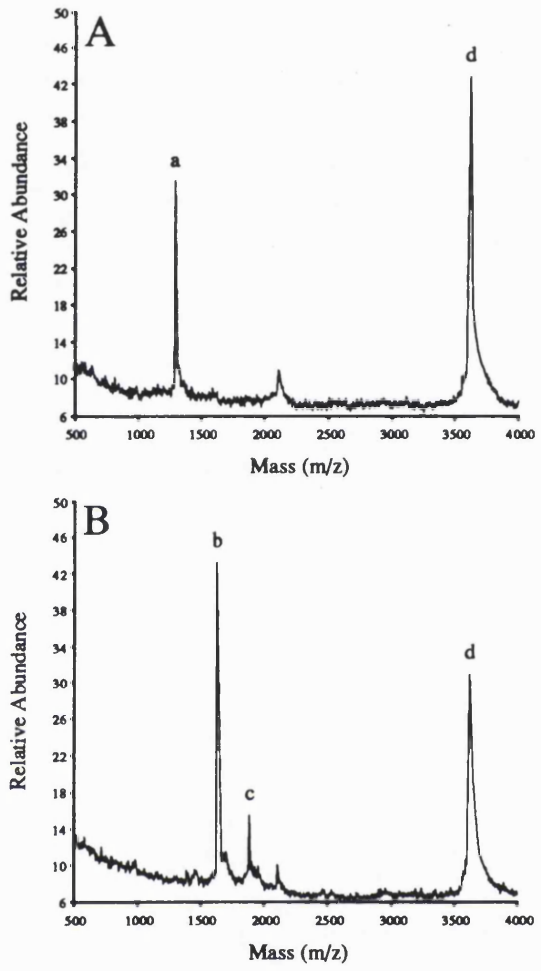

Figure 6.2: Reaction of SPITC with a synthetic peptide. A 5mM solution of a synthetic peptide with the sequence MKLSRQFTV was incubated at 25°C for 30min in the absence (A) or presence (B) of 15mM SPITC. The samples were then analysed by time of flight mass spectrometry. The spectra show the intact (a), mono- (b) and di-substituted (c) peptide and the internal insulin B-chain standard (d).

This demonstrated that all of the peptide had undergone reaction with SPITC. Only a small percentage of peptide had undergone modification at both the lysyl residue and the N-terminus, with the majority of product being in the mono-substituted form (fig. 6.2 B, c.f. peaks b and c). This may have been due to the fact that only a 3-fold excess of SPITC was added to the reaction and that the reagent had become limiting, because of a difference in the reaction chemistry between the lysine and the N-terminus, or because reaction at one amine site prevented reaction at the other. However, these data clearly showed that SPITC was capable of reaction with primary amines in a protein under these conditions.

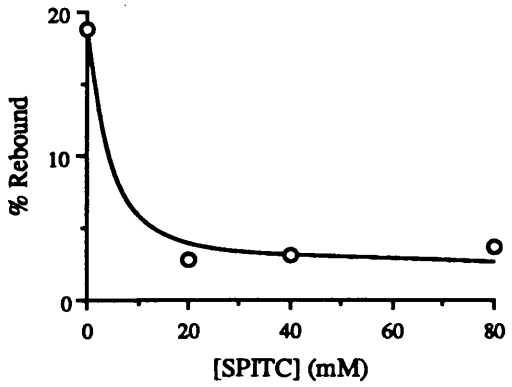

Figure 6.3: Effect of SPITC treatment on Mann II rebinding to the matrix. Four 100µg aliquots of Golgi membranes were digested with proteinase K and then treated with 0, 20, 40 or 80mM SPITC. The samples were extracted with TMMDS buffer and the supernatants incubated with 150mM salt pellets produced from 100µg aliquots of untreated Golgi membranes for 1hr at 4°C. After centrifugation the supernatants and pellets were separated and assayed for Mann II activity and the amount of rebinding determined.

To determine whether treatment of Golgi membranes which had been digested with proteinase K with treated with SPITC would have an effect on the ability of the Mann II from such samples to rebind the matrix, 100µg aliquots of membranes were digested with proteinase K and quenched with PMSF. The membranes were recovered by centrifugation and resuspended in 100µl of HMS buffer containing 0, 20, 40 or 80mM SPITC and incubated at 25°C for 30min. The SPITC was quenched by the addition of a 20µl aliquot of 1M glycine followed by a 5min incubation at 25°C. These membranes were then extracted in TMMDS buffer and the supernatants were incubated for 1hr at 4°C in the presence of 150mM salt pellets which had been produced from 100µg aliquots of untreated membranes. The samples were centrifuged and the supernatants and pellets were then assayed for Mann II activity to determine the amount which had rebound.

This showed that even at a concentration of 20mM, SPITC was effective in preventing the rebinding of Mann II to the matrix (fig. 6.3). The degree of inhibition was 6.5-fold at 20mM and this seemed to remain constant at higher SPITC concentrations. The recovery of Mann II activity in the SPITC samples was 91.5%±5.8 (±SD, n=3)

compared to the mock-treated sample, showing that no enzyme inactivation had occurred upon SPITC treatment.

6.2.2 Peptide Inhibition Studies

The fact that SPITC could inhibit binding of Mann II to the matrix after treatment of the cytoplasmic face for proteinase K treated Golgi suggested that components required for the rebinding of the Golgi enzymes were still present on the cytoplasmic face of the membranes after proteolysis. One possibility was that these components were the cytoplasmic tails of the enzymes themselves and that these were responsible for binding the enzymes to the matrix. Reaction of SPITC with these cytoplasmic tails could account for the loss of binding. The cytoplasmic tail of rat Mann II consists of only five amino acids (Moremen and Robbins, 1991) while that of human NAGT I is only six amino acids long (Kumar *et al.*, 1990). Thus it was possible that proteinase K treatment did not remove these tails due to the steric hindrance of the lipid bilayer.

If the enzymes' cytoplasmic tails were required for the rebinding, it was possible that this could be inhibited by the addition of synthetic peptides corresponding to these regions of the proteins. Such peptides might compete for the binding sites of the native cytoplasmic tails on the matrix and therefore, if used at sufficiently high concentration, could inhibit the binding of the enzymes.

Two peptides were synthesised for this study by the ICRF Peptide Synthesis Facility. They were a pentapeptide, MII-C, corresponding to the Mann II cytoplasmic tail with the sequence MKLSR, and a hexapeptide, NA-C, corresponding to the NAGT I cytoplasmic tail with the sequence MLKKQT (the human sequence was used because no sequence information was available for the rat enzyme).

6.2.2.1 Effects of Peptides During Extraction

If the peptides could indeed compete with the *medial*-Golgi enzymes for binding sites on the matrix, it was possible that their inclusion in the TMMDS extraction buffer could also induce the solubilisation of the enzymes. This was tested by extracting intact Golgi membranes with TMMDS buffer containing increasing concentrations of MII-C or NA-C and determining the degree of solubilisation of Mann II and NAGT I at each peptide concentration.

Thus 100µg aliquots of Golgi membranes were extracted with TMMDS buffer supplemented with 0, 1, 2, 5 and 10mM concentrations of MII-C and NA-C. After centrifugation, the supernatants and pellets were assayed for Mann II and NAGT I activity and the percentage solubilisation of each enzyme was determined.

This showed that the addition of either peptide had little effect of the solubility of either Mann II or NAGT I (fig.6.4). Mann II solubility increased very slightly at a 2mM concentration of either peptide but decreased again at higher concentrations while NAGT I solubility seemed unaffected by MII-C and increased only very slightly in the presence of NA-C, and this only at a concentration of 10mM. All these effects were very small, however, and well within the bounds of experimental error. The recovery of Mann II in the samples containing peptide when compared those which were extracted in TMMDS alone was 108.9%±11.3 (±SD, n=8) and the recovery of NAGT I was 98.2±5.8 (±SD, n=8), showing that the peptides had not affected the activity of the enzymes.

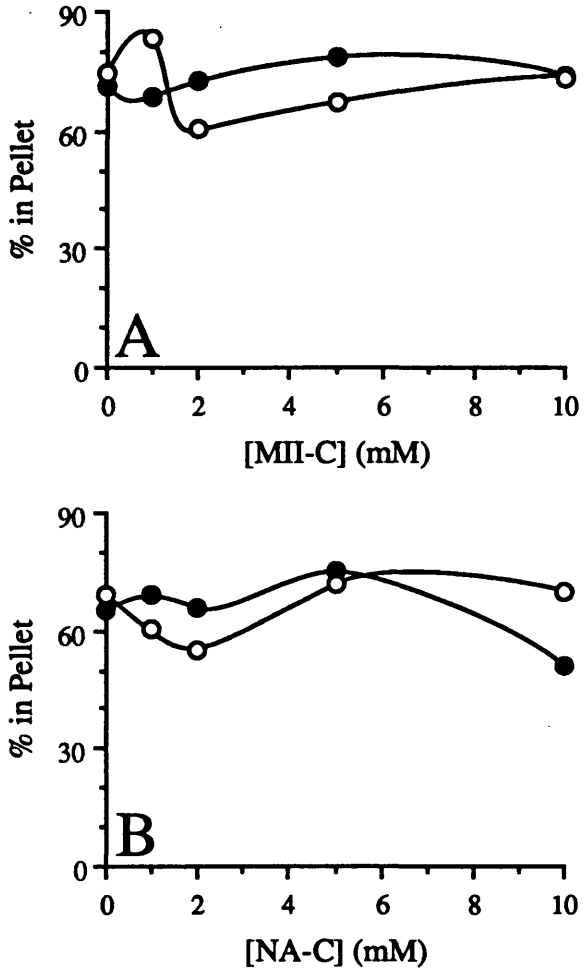

Figure 6.4: Effect cytoplasmic tail peptides on the solubility of Mann II and NAGT I during detergent extraction. Aliquots of 100µg of Golgi membranes were extracted with TMMDS buffer containing 0, 1, 2, 5 and 10mM concentrations of MII-C (A) or NA-C peptide (B). After centrifugation, the supernatants and pellets were assayed for Mann II (○) and NAGT I (●) activity and the amount of solubilisation determined.

In an attempt to increase the solubilisation of both enzymes in the presence of peptide, extractions were performed at an elevated temperature. The rationale being that the elevated temperature would increase the rate of enzyme binding and rebinding to the matrix. At 4°C, it was possible that the enzymes did not dissociate from the matrix very quickly and therefore no opportunity was afforded to the peptides to bind the matrix. If enzymes' on/off rate were increased, the peptides might have more opportunity to inhibit their rebinding.

Thus the effect of extraction of Golgi membranes in the presence of 2mM MII-C or NA-C at 37°C or at 56°C on the solubility of Mann II was examined. The concentration of 2mM was chosen because this seemed to have the maximal effect on the solubilisation of Mann II at 4°C for both peptides (see fig. 6.4).

Thus, two sets of three 100μg aliquots of Golgi membranes were extracted in TMMDS buffer which either lacked or contained MII-C or NA-C at a concentration of 2mM. The samples were incubated at either 37° or 56°C for 30min during the extraction and centrifuged under the normal extraction conditions. The supernatants and pellets were assayed for Mann II activity, as was an aliquot of untreated membranes, and the amount of solubilisation was determined.

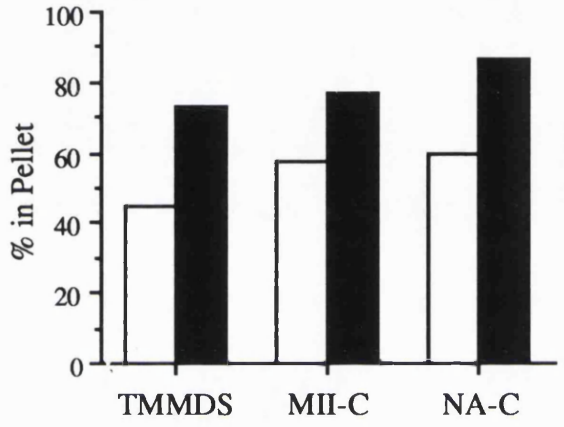

Figure 6.5: Effect of extraction in the presence of Mann II cytoplasmic tail peptide on Mann II solubility at elevated temperatures. Aliquots of 100μg of Golgi membranes were extracted with TMMDS buffer either lacking or containing 2mM MII-C or NA-C. Extraction was carried out at 37°C (❑) or 56°C (■), and after centrifugation, the supernatants and pellets were assayed for Mann II activity and the degree of solubilisation determined.

This showed that at 37°C, the insolubility of Mann II dropped drastically when compared to extraction at 4°C (c.f. fig. 4.5 A with fig. 6.5) in the absence of peptide, with only 45% remaining insoluble at 37°C compared with 85% at 4°C. In the presence of either MII-C or NA-C peptides, there was no apparent increase in Mann II solubility. In fact its solubility seemed to decrease slightly when compared to the sample lacking peptide though this was a small effect and may not have been significant (fig. 6.5). The insolubility of Mann II at 56°C increased when compared to the 37°C samples to levels comparable to extraction at 4°C, though again there was no obvious change in the presence of either peptide. This increase was explained by the fact that the average Mann II recovery was 91.4%±8.9 (±SD, n=3) at 37°C when compared to a control Golgi sample which was kept at 4°C throughout the experiment. However, the recovery at 56°C was only 19.5%±1.1 (±SD, n=3) when compared to the same sample, presumably to temperature-dependent denaturation of the enzyme. This meant that the solubilisation of the protein could not be measured reliably by its enzymatic activity, and also that since

protein denaturation was occurring at this temperature, that it would not be possible to use this temperature in future experiments anyway. This experiment demonstrated that increasing the temperature during extraction had no effect on the peptide's ability to facilitate solubilisation of Mann II in TX-100.

6.2.2.2 Effects of Peptides on Rebinding to the Matrix

Since the peptides seemed to exert only a small effect on the solubilisation of the *medial*-Golgi enzymes in the presence of detergent, their effect on the ability of solubilised enzymes to rebind to the matrix was examined. Since the peptides would be removed during dialysis in a normal rebinding experiment due to their low molecular weight, rebinding was performed without dialysis. This was possible because it had been shown that enzymes which had been solubilised from proteinase K treated Golgi membranes using TX-100 alone could rebind the matrix thereby obviating the need for dialysis (see fig. 5.16).

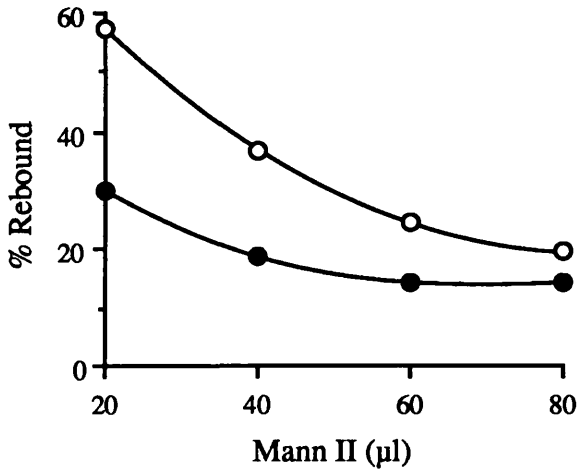

Figure 6.6: Effect of Mann II cytoplasmic tail peptide on the rebinding of varying Mann II concentrations to the matrix. Golgi membranes were digested with proteinase K and extracted with TMMDS buffer either lacking (O) or containing 2mM MII-C (●). Aliquots of 20, 40, 60 or 80µl were then incubated overnight with matrix pellets produced from 25µg of untreated Golgi membranes and then centrifuged. The supernatants and pellets were assayed for Mann II activity and the amount of binding to the matrix determined.

Rebinding conditions were first optimised to maximise the possibility of observing any effects of the peptides on the rebinding of the enzymes. This was achieved by determining the effect of the peptides on the rebinding of varying concentrations of Mann II.

Thus two 300µg aliquots of Golgi membranes was digested with proteinase K and extracted with TMMDS buffer which either lacked or contained 2mM MII-C to yield supernatants containing solubilised Mann II. Aliquots of 20, 40, 60 and 80µl of this supernatant were made up to 100µl with TMMDS buffer which either lacked or contained 2mM MII-C and incubated for 1hr at 4°C in the presence of matrix pellets that had been

produced from 25µg aliquots of untreated Golgi membranes. After centrifugation the supernatants and pellets were assayed for Mann II activity as were aliquots of supernatants that had been incubated in the absence of matrix and not centrifuged.

This showed that rebinding was inhibited by the peptide and that this was most pronounced at lower Mann II concentrations (fig. 6.6). When 20µl of the supernatant was used, binding was inhibited by almost 50%. Thus these rebinding conditions were used in future peptide inhibition experiments i.e. 100µl of a 5-fold diluted TMMDS supernatant from proteinase K treated Golgi was rebound to matrix produced from 25µg of untreated Golgi membranes. Using these conditions, the effect of the MII-C and NA-C peptides on the rebinding of both Mann II and NAGT I was next tested. Thus three 100µg aliquots of Golgi membranes were treated with proteinase K and extracted in TMMDS which either lacked or contained a 2mM concentration of MII-C or NA-C. The supernatants were then diluted 5-fold with the TMMDS which lacked or contained the appropriate peptide and 100µl of this was rebound to matrix prepared from 25µg of untreated Golgi membranes by incubation at 4°C for 1hr. The subsequent supernatants and pellets were assayed for Mann II and NAGT I as was an aliquot of each TX-100 supernatant which had not been incubated with matrix or centrifuged, and the amount of rebinding was determined.

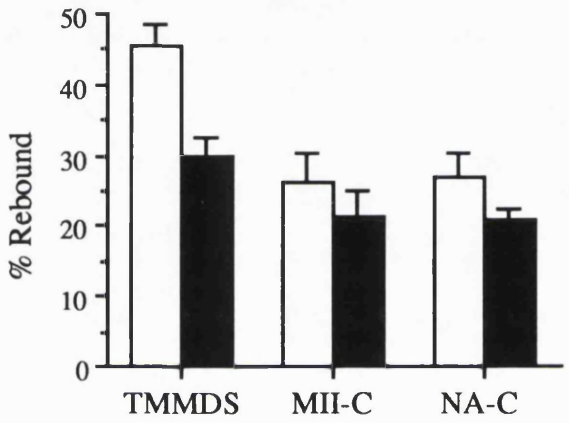

Figure 6.7: Effect Mann II and NAGT I cytoplasmic tail peptides on the rebinding of Mann II and NAGT I to the matrix. Golgi membranes were digested with proteinase K and extracted with TMMDS buffer either lacking or containing 2mM MII-C or NA-C. The supernatants were diluted 5-fold with the appropriate TMMDS buffer and incubated overnight with matrix pellets produced from 25µg of untreated Golgi membranes and then centrifuged. The supernatants and pellets were assayed for Mann II (❑) and NAGT I (■) activity and the amount of binding to the matrix determined. The data are presented as the averages of three experiments ±SEM.

This again showed that MII-C inhibited Mann II rebinding by approximately 45% (fig. 6.7). Surprisingly, however, Mann II rebinding was equally inhibited by NA-C. This effect was also observed for NAGT I in that both peptides seemed to have an equal effect

on inhibiting its binding, though this was not as pronounced as for Mann II, with only a 30% inhibition being observed. The average Mann II recoveries in the TMMDS, MII-C/TMMDS and NA-C/TMMDS samples were 115.1±21.0, 111.1±19.4 and 105.2±11.1 (±SEM, n=3) respectively, and the NAGT I recoveries in the same samples were 118.4±17.2, 110.0±11.3 and 118.3±0.3 (±SEM, n=3) respectively.

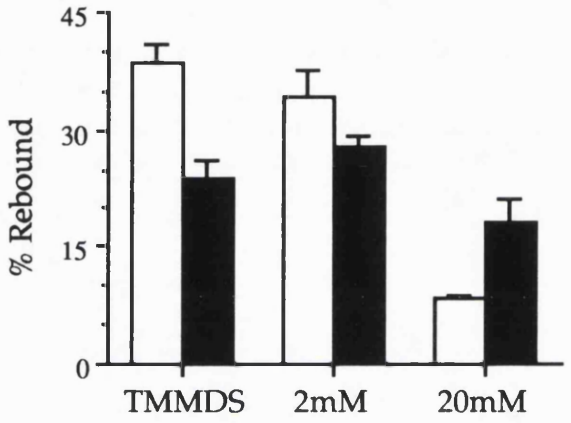

Figure 6.8: Effect lysine on the rebinding of Mann II and NAGT I to the matrix. Golgi membranes were digested with proteinase K and extracted with TMMDS buffer either lacking or containing 2mM or 20mM lysine. The supernatants were diluted 5-fold with the appropriate TMMDS buffer and incubated overnight with matrix pellets produced from 25µg of untreated Golgi membranes and then centrifuged. The supernatants and pellets were assayed for Mann II (❑) and NAGT I (■) activity and the amount of binding to the matrix determined. The data are presented as the averages of 3 experiments ±SEM.

The fact that both peptides could equally well inhibit the binding of both enzymes suggested that this effect may have been non-specific. The concentration of 2mM that was used to observe binding inhibition was six orders of magnitude greater that the association constants of the enzymes and the matrix and therefore far greater that would normally be required to compete for binding if inhibition were specific. Since both peptides contained lysine residues (one lysine in MII-C and two in NA-C), it was possible that the inhibition was a non-specific charge effect induced by these molecules. The effect of the presence of free lysine on rebinding was therefore examined. Rebinding was carried out in the presence of 2mM or 20mM lysine to fully gauge the effects of the presence of this amino acid.

The experiment was carried out exactly as described above for the previous peptide inhibition study (fig. 6.7) except that 2mM or 20mM lysine was substituted for 2mM MII-C or NA-C respectively. This showed that a lysine concentration 2mM, equal to the concentration of lysine in a 2mM solution of MII-C and half the lysine concentration in a 2mM NA-C solution, had little effect on the rebinding of either Mann II or NAGT I to the matrix (fig. 6.8).

However, at the 20mM concentration, rebinding of Mann II was greatly inhibited (approximately 75%). This effect was not so pronounced for NAGT I, with only a 25% inhibition of rebinding being observed. The average Mann II recoveries in TMMDS, 2mM lysine/TMMDS and 20mM lysine/TMMDS samples were 89.2±12.1, 86.5±10.3 and 97.2±14.0 (±SEM, n=3) respectively, and the NAGT I recoveries in the same samples were 104.6±14.8, 105.6±14.8 and 100.6±1.2 (±SEM, n=3) respectively, showing that the presence of lysine had little effect on the enzyme activity of Mann II or NAGT I.

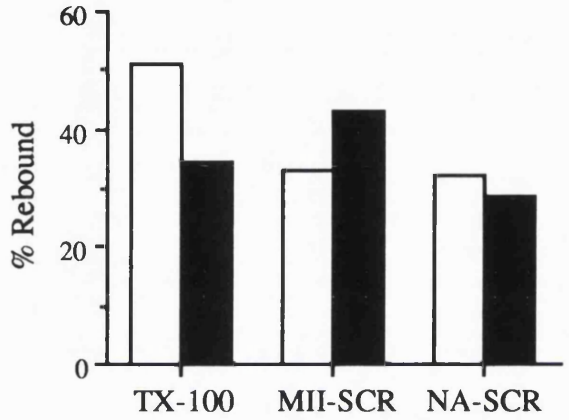

Figure 6.9: Effect scrambled Mann II and NAGT I cytoplasmic tail peptides on the rebinding of Mann II and NAGT I to the matrix. Golgi membranes were digested with proteinase K and extracted with TMMDS buffer either lacking or containing 2mM MII-SCR or NA-SCR. The supernatants were diluted 5-fold with the appropriate TMMDS buffer and incubated overnight with matrix pellets produced from 25µg of untreated Golgi membranes and then centrifuged. The supernatants and pellets were assayed for Mann II (❑) and NAGT I (■) activity and the amount of binding to the matrix determined.

These results were difficult to interpret. It was possible that the inhibition of binding observed in the presence of the peptides was non-specifically caused by the presence of lysyl residues but that higher concentrations of free lysine were required to observe the same effect. Alternatively, it was possible that the presence of lysyl residues in the peptides was not the sole cause of their ability to inhibit enzyme rebinding to the matrix, and that inhibition by free lysine was simply an effect of the increase of the ionic strength of the buffer. Neither of these possibilities could explain why lysine appeared to have little effect on NAGT I rebinding at either concentration.

In order to determine whether the peptide inhibition effect was non-specific or not, two new peptides were synthesised to act as controls. These had the same amino acid composition as MII-C and NA-C by had their amino acid sequence scrambled. If peptide inhibition was specific, such peptides would not be expected to have any effect on the enzyme rebinding. These two peptides were named MII-SCR and NA-SCR and had the same amino acid compositions as MII-C and NA-C respectively. The sequence of MII-

SCR was MLRKS and of NA-SCR was MKTQLK. The only criterion used in scrambling the sequences was that no amino acid should appear in the same position as in MII-C or NA-C except the first methionine.

The experiment was again carried out as for the peptide inhibition experiment (fig. 6.7), except that 2mM MII-SCR and NA-SCR was substituted for MII-C and NA-C respectively. This showed that both scrambled peptides inhibited Mann II rebinding equally well (fig. 6.9) and that the extent of inhibition was almost equal to that of MII-C and NA-C (approximately 40% for the scrambled compared to 45% for the native; c.f. fig. 6.7 with fig. 6.9). The effect of both scrambled peptides on NAGT I rebinding was less pronounced, but comparable still to the effect of the native peptides in the case of NA-SCR. In contrast, MII-SCR seem to enhance NAGT I rebinding slightly. The reason for this effect was unclear but it may have been due to experimental error.

Taken together, these results showed that peptides to the cytoplasmic tails of the *medial*-Golgi enzymes were unable to inhibit specifically their binding to the matrix. This could mean that either the enzyme tails are not involved in binding the enzymes directly to the matrix, or that the synthetic peptides did not possess the same three-dimensional conformation as the cytoplasmic tails of the intact proteins, leaving them unable to compete for the matrix binding sites. These data could not, therefore, determine the mechanism of enzyme binding. One point of interest arising from them, however, was that for the first time a difference in the properties of Mann II and NAGT I binding to the matrix was observed in that NAGT I binding seemed to be less sensitive to the presence of either peptides or lysine. The nature of this difference, however, is unclear.

6.2.3 Purification of the Mann II Chymotryptic Fragment

As described in chapter 3, Mann II is very resistant to proteolysis by chymotrypsin. Such treatment clips the Mann II molecule in the lumenal stem region and releases a soluble, catalytically active fragment which lacks the cytoplasmic and membrane-spanning domains and part of the stalk region. This fragment, which shall hence be referred to as CT-Mann II, can be purified relatively simply by chromatography on a cation exchange column followed by a gel filtration step (Moremen *et al.*, 1991).

If it could be shown that the purified chymotryptic fragment could rebind the matrix, then this would demonstrate that the binding was occurring via either the catalytic domain or the remainder of the stalk region. Conversely, if this molecule could not rebind under conditions where intact Mann II could, it would suggest that rebinding was occurring via the cytoplasmic or membrane-spanning domains, or again via part of the stalk region.

6.2.3.1 Chromatographic Purification

Two Golgi membrane preparations were pooled and 21mg of membranes were extracted with TMMDS buffer and the Triton pellet was further extracted with TMMDS supplemented with 150mM NaCl. This yielded a supernatant which contained solubilised Mann II. Chymotrypsin was added to this supernatant to a final concentration of 25µg/ml and incubated at 15°C for 50min before addition of PMSF to a final concentration of 40µg/ml. After concentration of this sample to a volume of 5ml by ultrafiltration, the sample was diluted to 50ml with FFS buffer (20mM Tris pH7.5, 0.1% (w/v) TX-100) and loaded onto a FFS column which had been equilibrated with the same buffer. After washing the column with 10ml of FFS buffer, the Mann II was eluted with an 80ml linear gradient of 0-150mM NaCl in FFS buffer. Fractions of 1ml were collected and 20µl of these assayed for Mann II activity. Protein assays were also attempted, but the fractions were too dilute for any to be detected reliably.

Mann II eluted as a symmetrical peak centred around a NaCl concentration of 78mM (6.10 A), in close agreement with the value of ~70mM reported by Moremen and colleagues (1991), The slight difference of 8mM was probably due to the different resin being used in this protocol (FFS vs. Mono-S).

Fractions 35-49 were pooled and concentrated to 200µl, again by ultrafiltration. This sample was then loaded onto a Superose 6 column that had been equilibrated with 50mM NH_4HCO_3 and eluted with 50ml of the same buffer. Fractions of 1ml were collected and again assayed for Mann II activity which was detected in 5 fractions (fig. 6.10 B) which were pooled to yield the final CT-Mann II preparation.

In order to determine the extent of purification, aliquots of the original Golgi membranes, the 150mM salt supernatant before and after chymotryptic digestion, the FFS pooled fraction and the final CT-Mann II preparation were assayed for Mann II activity. Additionally, the Golgi membranes, FFS pool and final preparation were also assayed for protein content (the salt supernatant and chymotrypsin digest could not be assayed because of the presence of DTT which interferes with the assay).

These data allowed the compilation of a purification table for the isolation of CT-Mann II (table 6.1). Three points should be noted from this table. Firstly, the amount of protein in the Golgi membranes was judged to be 22.7mg, not 21mg as had been predicted. This difference, however, could easily be attributed to experimental errors between these assays and the ones performed after the purification of the Golgi membranes. Secondly, there was a slight drop in the purification after the Superose 6 step. However, in the original Moremen protocol, this step only gave a small increase in the purification (1.4-fold over the FFS pool) and was designed to remove only trace amounts of contaminating proteins. Since the protein concentrations in the FFS and final

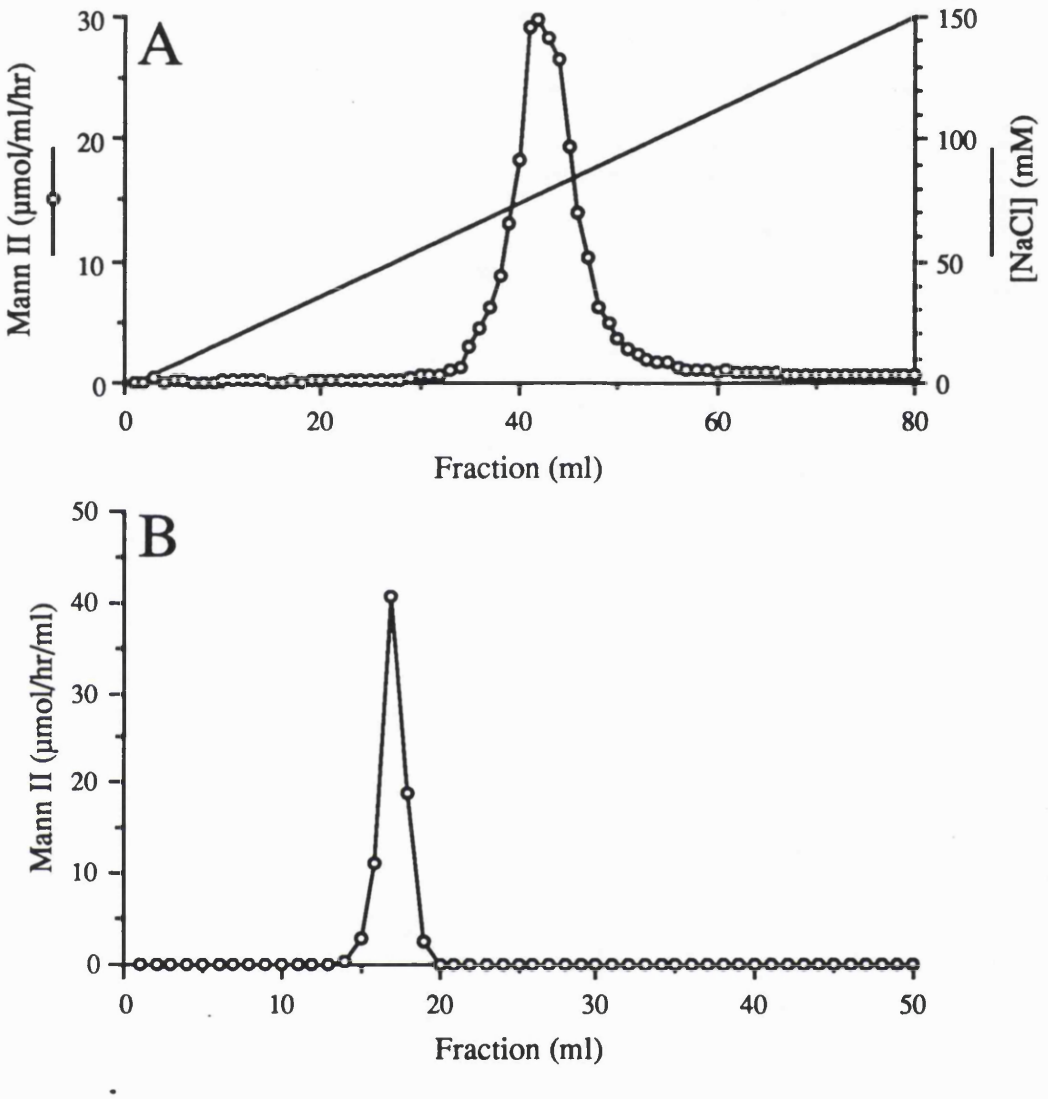

Figure 6.10: Purification of the Mann II chymotryptic fragment. Golgi membranes were extracted with TMMDS followed by TMMDS containing 150mM NaCl to yield salt supernatant containing solubilised Mann II. This was digested with chymotrypsin and the sample bound to a Fast Flow SP-Sepharose column and eluted with a linear 0-150mM NaCl gradient (A). Fractions of 1ml were collected and assayed for Mann II activity. The fractions containing Mann II were then subjected to a gel filtration step using a Superose 6 column and again fractions of 1ml collected and assayed for Mann II activity (B).

Fraction	Volume	[Prot]	Tot Prot	[MII]	Tot MII	S.A.	% Yield	P.F.
	ml	µg/ml	µg	µmol/h/ml	µmol/hr	µmol/h/mg		
Golgi	6.2	3660.0	22700.0	11.9	73.5	3.2	100.0	1
150 s/n	21.0	ND	ND	2.1	44.1	ND	60.0	ND
CT s/n	20.8	ND	ND	2.1	44.1	ND	60.0	ND
FFS	15.0	21.6	324	2.2	32.4	100.0	44.1	30.9
Final	5.0	31.0	155	2.9	14.3	91.9	19.4	28.4

Table 6.1: Purification table Mann II chymotryptic fragment. Golgi membranes, the 150mM NaCl supernatant (150 s/n), chymotryptic digest (CT s/n), FFS pool (FFS) and final CT-Mann II preparation were assayed for Mann II activity and protein content.. The protein content of the 150 s/n and CT s/n sample could not be determined (ND). The specific activity (S.A.), yield and purification fold (P.F.) of CT-Mann II was then determined

samples were on the limits of detection by the assay, it was likely that a small error could have occurred which would account for this, by overestimating the protein concentrations and thus underestimating the specific activities. Finally, the purification-fold of the final preparation was calculated to be approximately 30-fold over the original Golgi membranes. This compares to the reported value of approximately 150-fold reported previously (Moremen *et al.*, 1991), a difference of 5-fold. However, the purity of the Golgi membranes used by Moremen and colleagues was not reported and thus there was no way of knowing whether they were as pure as the ones used in this study. Thus it was possible that if the membranes used here were more pure than those reported previously, the final purification factor would be lower. This however, required that the membranes used by Moremen and colleagues needed to be 5-fold less purified than those used here. This seemed unlikely, and the fact that the specific activity of the purified CT-Mann II in this preparation was 4.6 times lower than previously reported, suggested that the error was more likely due to an inaccuracy in the protein assays of the dilute final preparation.

Thus, in order to determine whether Mann II had indeed been purified to homogeneity, the protein composition of the final CT-Mann II preparation was analysed by SDS-PAGE.

6.2.3.2 Purity of the Fragment

Aliquots of 7.5, 70, 70, 80 and 80µl of the Golgi membranes, 150mM salt supernatant, chymotryptic digest, FFS pool and final CT-Mann II preparation respectively were

subjected to SDS-PAGE after methanol/chloroform protein precipitation and the gel stained with Coomassie Brilliant Blue R. This showed that digestion with chymotrypsin had converted all the intact Mann II into the 110kD fragment (fig. 6.11, c.f. lanes 2 and 3). Furthermore, this treatment had removed many of the contaminating proteins from the 150mM salt supernatant (fig. 6.11, lane 3). Most of these were removed by FFS chromatography with the exception of a single protein with an apparent molecular weight of 200kD (fig. 6.11, lane 4). This was likely to be a Mann II dimer which had not been reduced upon DTT treatment prior to electrophoresis since this protein was not present in the untreated 150mM salt supernatant (fig. 6.11, c.f. lanes 2 and 3), though a slightly higher molecular weight protein was (presumably a dimer of intact Mann II). In addition to this 200kD band, two very high molecular weight bands were faintly visible near the top of the gel (fig. 6.11, lane 4). These could have been larger aggregates of Mann II or contaminating proteins which would be removed by gel filtration.

After Superose 6 gel filtration, however, the 200kD band and the high molecular weight doublet were still present and a smear of protein had appeared which barely entered the resolving gel (fig. 6.11, lane 5). Since these proteins had not been observed in such abundance in the FFS pool, it was likely that they represented aggregates of Mann II. Furthermore, since such large aggregates should not have co-eluted with Mann II in the gel filtration column, it was likely that this aggregation was occurring during processing for SDS-PAGE, presumably because of the high Mann II concentration in the final preparation compared to the samples from earlier in the preparation and/or the absence of other contaminating proteins.

To determine whether these higher molecular weight bands were indeed aggregates of CT-Mann II, the sample was analysed by Western blotting using an anti-Mann II antibody which was kindly supplied by Dr. Kelly Moremen. Furthermore, it was noted that when Moremen and colleagues purified CT-Mann II, they used a SDS-PAGE sample buffer containing 50mM DTT compared with the 7mM concentration which was used here. It was possible that this higher DTT concentration was required to disrupt the higher molecular weight forms, and thus the DTT concentration in the loading buffer used here was increased accordingly.

Thus CT-Mann II was precipitated from 80µl of the final preparation and processed for SDS-PAGE in a loading buffer containing 50mM DTT which was quenched with a 4-fold excess of iodoacetamide. After electrophoresis, one half of the gel was transferred onto Immobilon-C membrane and probed with the anti-Mann II antibody, and the other stained with Coomassie Brilliant Blue R. The Coomassie stained gel showed that most of the high-molecular weight material and the 200kD band had disappeared and there was a concomitant increase in the amount of CT-Mann II on the gel (c.f. fig. 6.11, lane 5 with

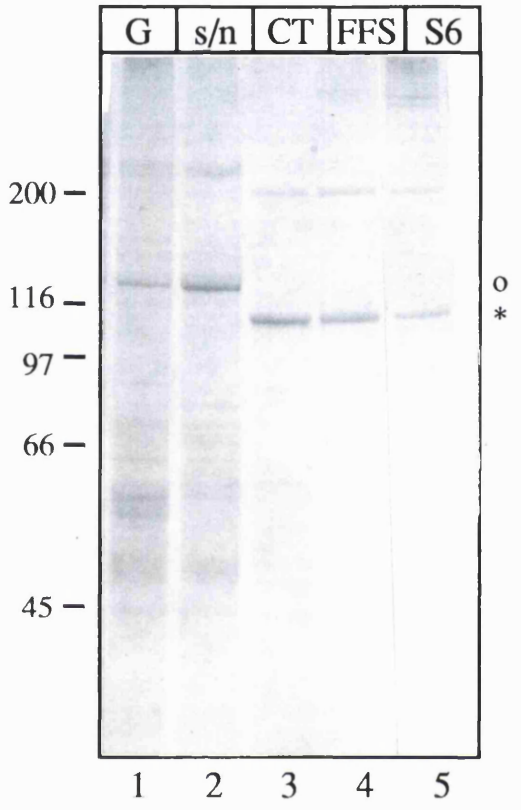

Figure 6.11: SDS-PAGE of samples from the CT-Mann II purification. Aliquots of 7.5, 70, 70, 80 and 80µl of the Golgi membranes (G), 150mM salt supernatant (s/n), chymotryptic digest (CT), FFS pool (FFS) and final CT-Mann II preparation (S6) respectively were precipitated by methanol/ chloroform and subjected to SDS-PAGE. The gel was stained by Coomassie Brilliant Blue R. Positions of molecular weight marker (in kD) are indicated on the left, and of Mann II (o) and CT-Mann II (*) on the right.

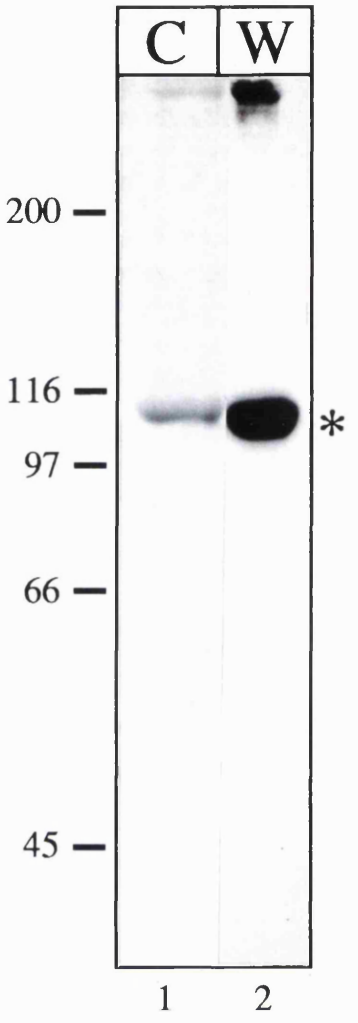

Figure 6.12: Western blotting of CT-Mann II with an anti-Mann II antibody. Two 80µl aliquots of the final CT-Mann II preparation were subjected to SDS-PAGE using a loading buffer containing 50mM DTT. Half of the gel was stained with Coomassie Brilliant Blue R (C) and the other Western blotted and probed with an anti-Mann II antibody (W). Positions of molecular weight markers (in kD) are indicated on the left, and of CT-Mann II (*) on the right.

fig. 6.12, lane 1), indicating that these higher molecular weight bands were in fact dimers and oligomers of CT-Mann II. Furthermore, blotting showed that both the 110kD band and the high molecular weight material contained Mann II (fig. 6.12 c.f. lanes 1 and 2). This demonstrated that the CT-Mann II had been purified to near homogeneity.

6.2.3.3 Oligomeric Nature of the Fragment

In order to be able to perform rebinding experiments with this sample, it was necessary to ensure that the high molecular weight aggregates were not present in the CT-Mann II preparation i.e. that they were formed during processing for SDS-PAGE. It was unlikely that the oligomers were present in the preparations because they should not have co-eluted with non-oligomeric CT-Mann II during gel filtration (see fig. 6.10 B). To be sure that this was the case, the sedimentation velocity of CT-Mann II in the preparation was analysed by velocity centrifugation.

A 200µl aliquot of the CT-Mann II preparation was overlaid onto a 20-40% (w/v) sucrose gradient in 50mM NH_4HCO_3 and centrifuged at 40,000 rpm for 16hr at 4°C using a SW-40 rotor. Fractions of 150µl were collected and 20µl of these were assayed for Mann II activity. This showed that the majority of the Mann II migrated as a single peak near the top of the gradient (fig. 6.13). This was consistent with the sedimentation velocity of a dimer (c.f. fig. 5.1 and fig. 6.13). Two minor peaks of activity exhibiting a higher sedimentation velocity were also observed, but these were very small (6.1% and 1.4% of the total activity on the gradient). These data indicated that the vast majority of CT-Mann II in this preparation was dimeric and that the higher molecular weight forms observed by SDS-PAGE were aggregates induced by sample processing for electrophoresis. Thus this preparation could be used for

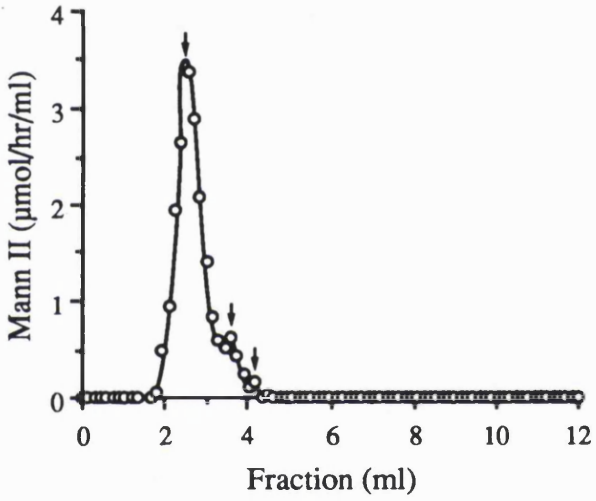

Figure 6.13: Sedimentation of CT-Mann II. A 200µl aliquot of the final CT-Mann II preparation was overlaid onto a 20-40% (w/v) sucrose gradient in a SW-40 rotor tube. After centrifugation, the gradient was divided into 150µl fractions from top (1) to bottom (12), and these were assayed for Mann II activity. The positions of activity peaks are denoted by the arrows.

rebinding studies with no possibility that such aggregates would sediment under the conditions used in such experiments.

6.2.4 Rebinding of CT-Mann II to the Matrix

Once the fact that CT-Mann II had been obtained in a highly purified form was established, its ability to rebind the matrix was tested. In conjunction with this, the rebinding of purified intact Mann II was also tested, since if CT-Mann II could not rebind, it could be argued that this was because of a loss a protein from the 150mM salt supernatant which was required for interaction with the matrix, and not because of the removal of the Mann II cytoplasmic and membrane-spanning domains. Intact Mann II was purified in this laboratory, and generously supplied by Dr. Nobuhiro Nakamura. The enzyme was purified according to the protocol of Moremen *et al.* (1991) except that it was solubilised from membranes by a Triton/salt wash, as for CT-Mann II, and not by using TX-114.

Rebinding was not carried out using dialysis because purified Mann II tends to stick non-specifically to dialysis membrane (Nobuhiro Nakamura, pers. comm.). Since the enzyme preparations were dissolved in buffers which did not contain NaCl (50mM NH_4HCO_3 for CT-Mann II and 100mM potassium phosphate pH7.2, 2% (w/v) TX-100 for Mann II), it was possible to carry out these experiments without dialysis. As a positive control, a 150mM salt supernatant was prepared and dialysed. After centrifugation, the low-salt supernatant, which now contained solubilised Mann II that could not resediment in the absence of matrix, was recovered and used as a Mann II source for rebinding to the matrix.

Finally, purified Mann II also seems to stick to plastics non-specifically (Nobuhiro Nakamura, pers. comm.), and thus a 5mg/ml concentration of BSA was included in all samples to minimise this non-specific interaction. This was made up as a 100mg/ml stock in TMMS buffer and diluted appropriately into the enzyme solutions.

Thus a 150mM salt supernatant was produced from 1mg of Golgi membranes and ten 100µl aliquots were dialysed against TMMS buffer to remove the salt. These samples were pooled and centrifuged under the same conditions used for extraction and the pooled supernatants used for rebinding to matrix. A 10µl aliquot of this supernatant, and 2 and 5µl aliquots of the Mann II and CT-Mann II preparations respectively were assayed for Mann II activity. This showed that the Mann II and CT-Mann II preparations contained 98x and 12x greater concentrations of Mann II activity than the 150mM supernatant. These samples were therefore diluted with TMMS buffer so that they contained Mann II concentrations equal to that of the supernatant, and BSA was then added to all samples to a final concentration of 5mg/ml.

Three matrix pellets were then prepared from 100µg aliquots of Golgi membranes and resuspended in 100µl of 150mM dialysed salt supernatant, Mann II solution or CT-Mann II solution respectively. After incubation at 4°C for 16hr, the samples were centrifuged under the same conditions used for extraction, as were 100µl aliquots of the same solutions that were incubated in the absence of matrix. The supernatants and pellets were then assayed for Mann II activity as were aliquots of supernatant or solutions which had not been centrifuged.

This showed that intact Mann II from both the 150mM salt supernatant and the purified preparation could rebind the matrix, as could CT-Mann II (fig. 6.14). In the absence of matrix, very little resedimentation of Mann II activity from the 150mM supernatant and the CT-Mann II preparation was observed. The resedimentation of intact Mann II in the absence of matrix, however, was 2.5 times greater then in the other samples. This was, however, probably caused by the significant loss of Mann II activity in the sample since the recovery of Mann II activity in this sample was only 32.8% (compared with 93.6% and 68.8% for the 150mM supernatant and CT-Mann II sample respectively). This loss was not observed for the Mann II sample containing matrix where the recovery of activity was 115.6% (and 95.3% and 90.8% for the 150mM supernatant and CT-Mann II preparation). This loss may have been due to the enzyme non-specifically sticking to the centrifuge tube in the Mann II preparation (and to a lesser extent in the CT-Mann II sample), since recoveries were more favourable in the presence of extra proteins in the form of matrix. Nevertheless, Mann II resedimentability was still increased in the presence of matrix, indicating that rebinding was occurring.

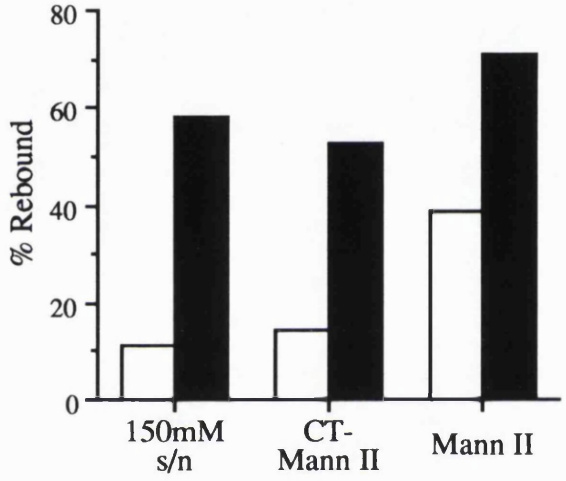

Figure 6.14: Rebinding of purified Mann II and CT-Mann II to the matrix. Aliquots of 100µl of a supernatant from a dialysed 150mM salt supernatant (150mM s/n), a purified CT-Mann II preparation (CT-Mann II) or a purified Mann II preparation (Mann II) were incubated for 16hr at 4°C in the presence (■) or absence (❑) of a 150mM salt pellet derived from 100µg of Golgi membranes. After centrifugation, the supernatants and pellets were assayed for Mann II activity and the amount of rebinding was determined.

This demonstrated that both the intact Mann II and chymotryptic fragment were capable of rebinding the matrix. CT-Mann II rebinding was slightly lower than that of intact Mann II from both the 150mM salt supernatant and the purified preparation, though this difference was small and may not have been significant. The fact that the chymotryptic fragment could rebind to almost the same extent as the intact protein demonstrated that the cytoplasmic and membrane-spanning domains of this enzyme were not required for interaction with the matrix. The fact that the matrix contained functional cytoplasmic components (because it was accessible to proteinase K) suggested that Mann II was connected to it via membrane-spanning protein(s) which bound Mann II via their lumenal and the matrix via their cytoplasmic domains.

6.3 Summary

The purpose of the experiments described in this chapter was to determine the method by which the *medial*-Golgi enzymes were bound to the matrix. Three possible mechanisms of binding existed. In the first, the enzymes could be bound to the matrix directly by their cytoplasmic tails. In the second, the enzymes could interact via their membrane-spanning domains with components of the matrix which protrude into the lipid bilayer. Finally, the enzymes could be indirectly bound to the matrix by cytoplasmic adapter proteins, or by membrane spanning proteins which bound the enzymes by their lumenal domains and connected them to the matrix by their cytoplasmic tails.

Mann II that had been solubilised with TX-100 from Golgi stacks that had been digested with proteinase K and treated with SPITC, which reacts with primary amines on proteins, was found to be unable to rebind a fresh matrix. In contrast, Mann II that had been produced in the same way, but not treated with SPITC, was capable of rebinding. This indicated that upon removal of the matrix by proteinase K, there were still components present on the cytoplasmic face of the membranes that were required to maintain their ability to rebind the matrix. Because SPITC cannot penetrate lipid bilayers, this suggested that the membrane-spanning domain of Mann II was not interacting with the matrix. Thus these data indicated that the SPITC was either modifying the cytoplasmic tails of Mann II, or another component that had not been removed by proteinase K (possibly the cytoplasmic domain of a second membrane-spanning protein that cross-linked Mann II to the matrix, or a cytoplasmic adapter protein).

In order to further investigate the possibility of the *medial*-Golgi enzymes binding directly to the matrix by their cytoplasmic tails, competition experiments were carried out in the presence of synthetic peptides whose sequences corresponded to the cytoplasmic tails of Mann II or NAGT I. Although binding of both Mann II and NAGT I could be partially inhibited in the presence of a 2mM concentration of either peptide, this did not

appear to be a specific effect, as non-specific peptide controls which had the same amino-acid composition but different sequences also exerted the same effect. The fact that this effect was observed at such a high peptide concentration compared to the association constants of the enzymes with the matrix also indicated that the effect was non-specific. These data could not be considered proof that binding did not occur via cytoplasmic tails of the enzymes, because it was equally possible that the three-dimensional conformation of the peptides was not the same as the cytoplasmic tails of the native proteins, and thereby rendering them unable to compete for binding sites on the matrix with the intact proteins.

In the course of these peptide inhibition studies, it was discovered that binding of both enzymes was unaffected in the presence of 2mM lysine. However, at a concentration of 20mM Mann II rebinding was greatly reduced while that for NAGT I decreased only slightly. The reason for this difference, however, was unclear.

The next approach to addressing this problem was to test the ability of purified preparations of intact Mann II, and its chymotryptic fragment, to rebind to the matrix. The chymotryptic fragment of Mann II consists of only the lumenal domain, and possibly part of the stalk region (Moremen *et al.*, 1991). The extent of stalk region remaining on the molecule is not known since the three-dimensional structure of the protein is not known. Thus since the fragment does not possess a cytoplasmic tail or membrane-spanning domain, it would be possible to eliminate these two regions from the binding mechanism if the fragment could rebind to the matrix.

Intact Mann II and its chymotryptic fragment were purified to apparent homogeneity and their ability to rebinding to the matrix was tested. This showed that both proteins were equally capable of rebinding the matrix, and did so to the same extent as the intact protein from a crude solubilised supernatant, though further work will be required to determine the respective affinities of these interactions. The fact that purified, intact Mann II could rebind to the matrix indicated that the salt pellet contained all of the components required for the rebinding, though these would be partially lost from the pellet during salt extraction since it had been already shown that a 150mM salt supernatant also contained all the components required to maintain enzyme insolubility.

The fact that the chymotryptic fragment could also rebind, and apparently to a similar extent as the intact protein, indicated that the membrane-spanning domain and cytoplasmic tail were not required for interaction with the matrix. This suggested that other membrane-spanning proteins were required to cross-link the enzyme to the matrix via its lumenal domain. Alternatively, this could mean that the majority of the matrix was, in fact, lumenal and that it contained membrane-spanning elements which interacted on the cytoplasmic face of the Golgi apparatus to form the larger oligomers. Removal of

these cytoplasmic components would therefore render the matrix soluble in TX-100 by abolishing these interactions.

It was not possible to distinguish between these possibilities using the data presented in this thesis, but they are discussed in more detail in chapter 8.

Chapter 7

Studies on Golgi Retention

7.1 Introduction

The Golgi apparatus contains many membrane-spanning proteins which are required to carry out the post-translational modification of the proteins which pass through it on their way to their final intracellular destinations. These molecules must remain with the Golgi stack (moreover within the correct cisterna(e)) in order to perform their roles, and thus resist being carried away with the bulk flow of membrane which passes through the organelle. The first insight as to the nature of the signal on these molecules which caused their retention in the Golgi apparatus was obtained when it was shown that the first membrane-spanning domain of the M glycoprotein of avian coronavirus was required for its retention in the *cis*-Golgi (Swift and Machamer, 1991). Simultaneously membrane-spanning domains of the endogenous *trans*-Golgi/TGN markers GalT and SialylT were then shown to necessary and sufficient to confer Golgi-residency to a marker protein and thus demonstrated that this region contained the signal for Golgi retention, though some evidence also suggested that some of the flanking amino acids on both sides of the membrane may also play a role (Nilsson *et al.*, 1991; Munro, 1991). Subsequent reports from other workers showed that all the N-glycan processing enzymes which have to date been cloned were also retained in the same manner (Burke *et al.*, 1992; Aoki *et al.*, 1992; Colley *et al.*, 1992; Russo *et al.*, 1992; Tang *et al.*, 1992; Teasdale *et al.*, 1992; Wong *et al.*, 1992). Furthermore, the membrane-spanning domain not only conferred Golgi retention upon the enzymes, but also localised them within the correct cisternae (Nilsson *et al.*, 1991; Burke *et al.*, 1992).

These discoveries posed the problematic question as to how a short stretch of relatively hydrophobic amino acids which were embedded in the lipid bilayer could retain these proteins in their correct positions within the stack. The theory favoured in this laboratory was termed "kin recognition". It was postulated that membrane residents within the same cisterna would associate with each other via their membrane spanning domains to form large "kin oligomers". These oligomers would be linear, since all Golgi enzymes analysed to date have been found to be dimers (Moremen *et al.*, 1991; Navaratnam *et al.*, 1988; Khatra *et al.*, 1974), and would be so large that they would be unable to enter vesicles budding from the dilated rims. Thus the enzymes would be retained by virtue of their inability to enter transport vesicles (Nilsson *et al.*, 1993b). Such membrane-spanning domain mediated oligomers have recently been reported *in vitro* (Weisz *et al.*, 1993). In this study, constructs consisting of VSV G protein fused to the first membrane-spanning domain of avian coronavirus were shown to form large, SDS-resistant aggregates, though their significance *in vivo* has yet to be demonstrated.

According to the kin recognition hypothesis, only the residents of the same cisterna would be able to interact with each other. For example, if *trans*-Golgi enzymes could interact with enzymes present in oligomers in the *medial*-cisterna, they would be retained and would be unable to reach their final destination in the *trans*-Golgi. To test this hypothesis, Tommy Nilsson in this laboratory, constructed a stable cell line termed 4:48 which expressed the p33/NAGT I/myc construct. This consisted of human NAGT I (Kumar *et al.*, 1990) that had been tagged with the *myc*-epitope (Evan *et al.*, 1985) at its C-terminus, to allow its detection with the 9E10 monoclonal antibody, and with the cytoplasmic domain of p33 invariant chain (Lotteau *et al.*, 1990), which contains an ER retrieval signal (Schutze *et al.*, 1994), replacing the NAGT I cytoplasmic domain (fig. 7.1 B). He was able to show that this molecule was localised at steady state to the ER as determined by immuno-fluorescence, immuno-electron microscopy and metabolic labelling studies, and that another *medial*-Golgi enzyme, Mann II, also accumulated in the ER in this cell line. Furthermore, the *trans*-Golgi enzyme GalT did not appear in the ER as judged by immunofluorescence (Nilsson *et al.*, 1994). The redistribution of Mann II was caused by the presence of the NAGT I membrane-spanning domain in the ER because a construct consisting of the p33 cytoplasmic domain, the GalT membrane-spanning domain and the NAGT I lumenal domain could not redistribute Mann II back to the ER (Nilsson *et al.*, 1994).

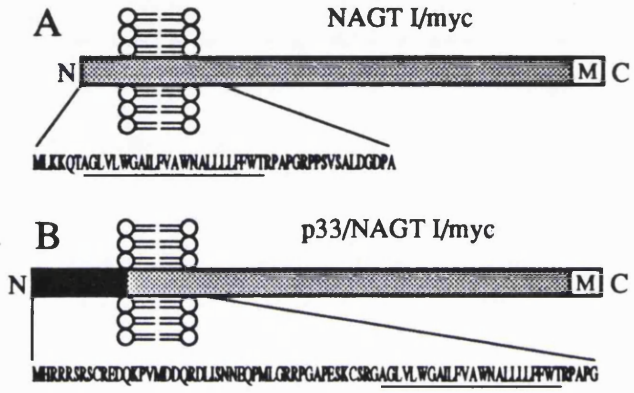

Figure 7.1: Constructs used in the 4:12 and 4:48 cell lines. A construct containing a human NAGT I cDNA (grey) tagged with the *myc*-epitope (M) was stably transfected into Hela cells to produce the 4:12 cell line (A). A construct containing a human NAGT I cDNA tagged with the *myc*-epitope and whose cytoplasmic domain was replaced with that of human p33 invariant chain (black; B) was stably transfected into Hela cells to produce the 4:48 cell line. Amino acid sequences of the cytoplasmic and membrane-spanning domains (underlined) are indicated.

In collaboration with Dr. Nilsson, I undertook a biochemical characterisation of this cell line. This involved the use of isopycnic centrifugation of 4:48 PNS preparations in sucrose gradients to separate the ER from the Golgi membranes, followed by

enzymatic assay to quantitate the amount of redistribution of various Golgi markers in this cell line. As a control, a cell line named 4:12, also produced by Dr. Nilsson, was utilised. This expressed the NAGT I/myc construct which consisted of human NAGT I which had been tagged with the *myc*-epitope (fig. 7.1 A). This cell-line was used in the first study which localised two Golgi markers (GalT and NAGT I) within the same stack by immuno-electron microscopy (Nilsson *et al.*, 1993a). This was achieved by using a polyclonal antibody against GalT in conjunction with the 9E10 monoclonal antibody to visualise the *myc*-tagged NAGT I.

In this chapter I describe experiments which determine the level of expression of NAGT I in these cell lines and also determine the extent of redistribution of a series of Golgi markers in the 4:48 cell lines.

7.2 Results

7.2.1 Expression Levels of NAGT I in 4:12 and 4:48 Cells

In order to draw any valid conclusions from the results obtained from these studies, it was first necessary to determine that the 4:12 and 4:48 cell lines were not greatly overexpressing the NAGT I/myc and p33/NAGT I/myc proteins respectively. Since both of these proteins should possess NAGT I activity, this was tested by measuring the amount of this glycosyltransferase activity in Golgi preparations or post-nuclear supernatants (PNS) which had been produced from these cells and comparing these levels to those of the parental Hela cell line.

Since the NAGT I/myc construct was present in the Golgi apparatus of the 4:12 cell line (Nilsson *et al.*, 1993a), its activity was tested in Golgi membranes preparations derived from the cell line and compared to the activity in a Golgi preparation of the parental Hela cells. These membranes were prepared by Regina Kieckbusch in the laboratory as described previously (Nilsson *et al.*, 1993a). These studies could not be performed on Golgi preparations of 4:48 cells since the p33/NAGT I/myc construct was present in the ER of these cells (Nilsson *et al.*, 1994). Instead, therefore, activity was measured in PNS preparations and compared to the levels of activity in 4:12 PNS preparations.

Since the relative amounts of membranes in the PNS preparations and Golgi preparations would vary from batch to batch, the GalT activity of each sample was also determined. This enzyme's expression level remained constant from cell line to cell line because the specific activity of GalT was very similar in Hela and 4:12 Golgi preparations (1.2 and 1.3 μmoles/hr/mg respectively), and in PNS preparations of 4:12 and 4:48 cells (327 and 213 nmoles/hr/mg respectively). GalT activity was, therefore, used as an internal control for the concentration of membranes in each sample, and the

increase in ratio of NAGT I to GalT activity was used to determine the increase in NAGT I expression.

To this effect, Golgi membrane preparations from parental Hela and 4:12 cells were assayed for GalT activity and undiluted membranes as well as 2, and 5-fold diluted membranes were assayed for NAGT I activity. The concentration of NAGT I activity in each of these samples was then plotted against the GalT concentration for both the Hela and 4:12 Golgi membranes (fig. 7.2 A). This demonstrated that the 4:12 cells did indeed express more NAGT I than the Hela cells. This increase was calculated as being the difference in the slopes of the two lines which was 4.1, indicating that approximately 3 NAGT I/myc molecules were expressed for every endogenous NAGT I.

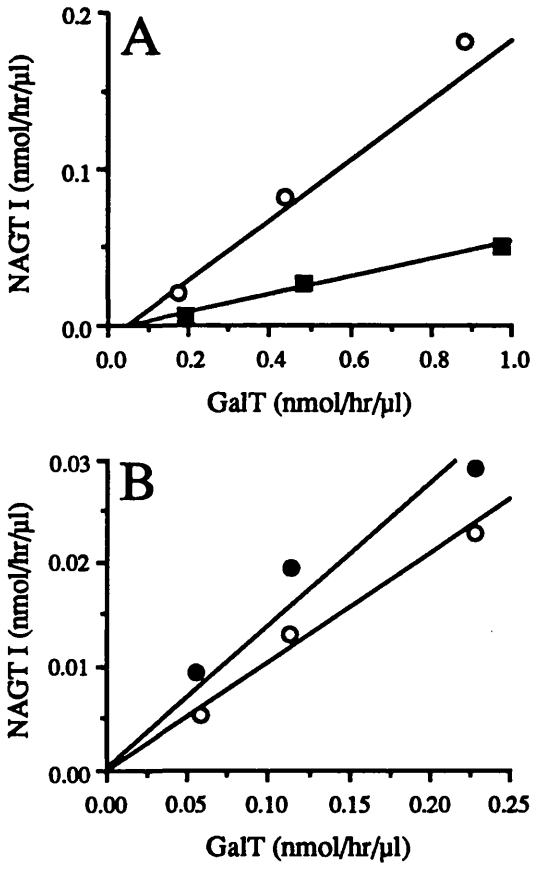

Figure 7.2: Expression levels of NAGT I activity in parental Hela, 4:12 and 4:48 cell lines. Golgi membrane preparations (A) from parental Hela (■) and 4:12 (○) cells as well as post-nuclear supernatants (B) from 4:12 and 4:48 (●) cells were assayed for GalT activity. Increasing dilutions of each sample were then assayed for NAGT I activity and these data plotted against the concentration of GalT present in each sample.

Similarly, PNS preparations produced from 4:12 and 4:48 cells were assayed for GalT activity, and 4, 8 and 16-fold dilutions of these membranes were also assayed for NAGT I activity. This showed that 4:48 cells contained 1.3x the NAGT I activity of the 4:12 cell line (fig. 7.2 B). Thus, 4:48 cells expressed 5.3x more NAGT I than the parental Hela cell line.

These results indicated that the constructs both expressed active enzymes and thus the proteins had folded correctly. The fact that neither cell line grossly over-expressed the constructs with which they had been transfected meant that any observations derived from their study were unlikely to be due to artefacts derived from over-expression. It should be noted,

however, that these results assume that the activity of the NAGT I produced by the constructs was the same as that of the wild-type enzyme. It is difficult to envisage how the replacement of the NAGT I cytoplasmic tail with that of p33 would greatly effect the function of the catalytic domain, particularly since the isolated catalytic domains of other enzymes such as Mann II seem to exhibit identical kinetics as the intact enzyme (Moremen *et al.*, 1991). It was not possible, however, to judge the effect of the presence of the *myc*-epitope at the C-terminus on the activity of the enzyme.

7.2.2 Biochemical Localisation of Golgi Markers

It had been shown by immunofluorescence that p33/NAGT I/myc resided in the ER in the 4:48 cell line (Nilsson *et al.*, 1994) while NAGT I/myc remained in the Golgi apparatus in the 4:12 cell line (Nilsson *et al.*, 1993a). Furthermore, Mann II was also present in the ER in 4:48 and not in 4:12, suggesting that it was interacting with NAGT I.

To confirm that these enzyme were indeed present in the ER biochemically and to determine whether any other Golgi markers exhibited this redistribution, a PNS from 4:48 cells was subjected to isopycnic centrifugation on continuous sucrose gradients and the density of the compartments in which the markers were present was determined by enzymatic assay. As a control for the normal distribution of the markers, a PNS from the 4:12 cell line were used. In this way, it would be possible to assign whether the markers were present in the Golgi or the ER since these organelles have greatly different buoyant densities (Beaufay *et al.*, 1974). Furthermore, such analyses would produce quantitative data and allow the calculation of the degree of redistribution of the various markers in a way that immunofluorescence could not.

The PNS preparations used in these studies were produced from cells that had been treated with cycloheximide and this drug was present throughout the centrifugation steps. This prevented the release of nascent peptide chains from ribosomes and ensured that a maximal number of ribosomes would remain attached to the ER. This would have the effect of slightly increasing the ER density and thus facilitate a maximal separation of Golgi from ER membranes.

These experiments were carried out twice, each time using a different batch of 4:48 and 4:12 PNS preparations. The experiments will be referred to as "A" and "B" and figures containing data from both experiments will be labelled likewise.

Centrifugation was carried out using 20.5-63% (w/v) linear sucrose gradients of 12ml volumes in SW-40 rotor tubes. The gradients in experiment "A" were loaded with 500µl of each PNS while those in experiment "B" were loaded with 700µl. Centrifugation was carried out at a speed of 40,000rpm for 16hr at 4°C and gradients were fractionated into 1ml aliquots from top to bottom.

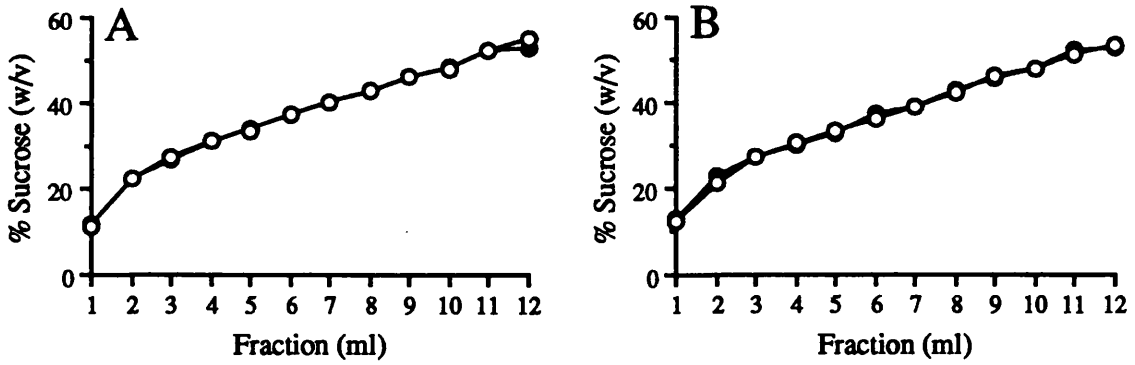

Figure 7.3: Sucrose composition of gradients. Two continuous sucrose gradients were poured consisting of 20.5-63% (w/v) sucrose. After application of the 4:12 (O) or 4:48 (●) PNS preparations and centrifugation to equilibrium, the gradients were fractionated into 1ml aliquots and the sucrose concentration in each determined. This was performed twice using two different 4:12 and 4:48 PNS preparations (A and B).

Immediately after centrifugation, aliquots of each fraction were assayed for each of the chosen markers and for sucrose concentration, and the fractions were then frozen in liquid nitrogen and stored at -80°C.

7.2.2.1 Sucrose Concentrations

In order to be able to validly compare the density shifts of the markers between the 4:12 and 4:48 cell lines, it was necessary to determine the sucrose concentrations in each fraction since the PNS preparations were applied to different gradients. If the density compositions were similar, then fractions from different gradients could be compared directly. Otherwise, comparisons of fractions would have to be by their sucrose densities and not their number. Thus after centrifugation and fractionation of the gradients, 20µl of each fraction was taken and diluted with an equal volume of water. The sucrose concentration of each sample was determined using a densitometer and the units converted to (w/v) using a standard conversion table.

This showed that the density composition of the 4:12 and 4:48 gradients in experiments "A" and "B" were very similar to each other (fig. 7.3) and that direct comparison of the fraction numbers from one gradient to another within the same experiment would be valid, though not necessarily between experiments.

7.2.2.2 NADH-Cytochrome c Reductase Distribution

To determine the position of ER membranes in the gradients, the rotenone-insensitive NADH-cytochrome c reductase activity present in each of the fractions from the gradients was assayed. This activity is also present in mitochondria but is inhibited by rotenone, an

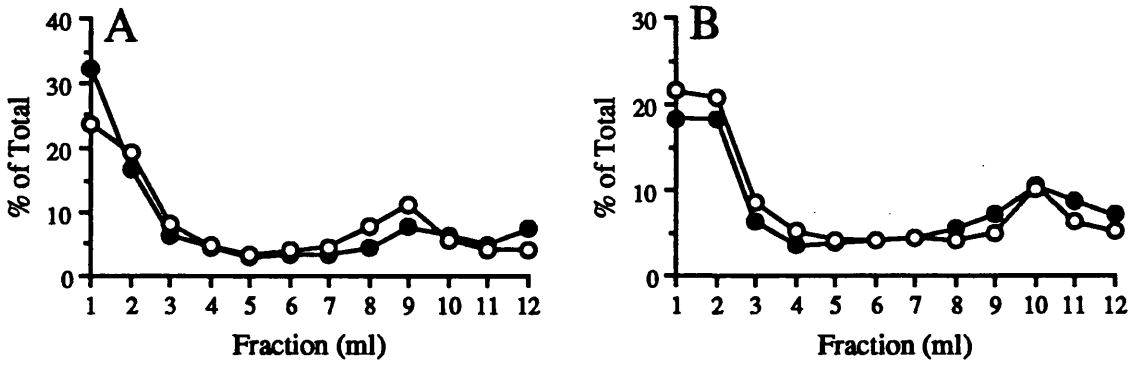

Figure 7.4: Distribution of NADH-cytochrome c reductase activity. NADH-cytochrome c reductase activity was assayed in fractions derived from linear sucrose gradients which had been loaded with PNS preparations derived from 4:12 (○) or 4:48 (●) cells and centrifuged to equilibrium. This was performed twice using two different 4:12 and 4:48 PNS preparations (A and B).

uncoupler of electron transport, leaving just the ER activity present in the assay (see chapter 2). Because of the relatively low sensitivity of this assay, 100µl aliquots of each fraction were assayed for this activity (as opposed to the 50µl samples usually used). The results (and those of subsequent assays) were reported as being the percentage of activity in each fraction relative to the total amount of activity on each gradient.

This showed that the ER peak was found in the fraction 9 of the gradients from experiment "A" and in fraction 10 in the gradients from experiment "B" (fig. 7.4). A large amount of activity was also observed at the top of the gradient. This however was due to the presence of DTT in the PNS preparations applied to the gradient. This reduced the cytochrome c in an enzyme-independent manner and led to a false signal. The fact that this was observed in the first three fractions was probably due to the diffusion of this reducing agent into the gradient during the 16hr centrifugation.

The average sucrose concentrations at which this activity peaked was 46.9% (w/v) ±0.4 (±SEM, n=4) which compared favourably with the value of 51.6% (w/v) previously reported (Beaufay *et al.*, 1974).

7.2.2.3 GalT Distribution

The next marker assayed for on these gradients was GalT. Since this did not appear to redistribute to the ER in 4:48 cells as judged by immunofluorescence (Nilsson *et al.*, 1994), this would provide a good marker for the position of Golgi membranes in the gradients. Thus 20µl aliquots of each fraction were assayed for GalT activity, as were aliquots of the original PNS preparations.

This showed that majority of the GalT was indeed present in membranes with a lower density to those of the ER (fig. 7.5). The peak of maximal activity appeared in between fractions 4 and 5 in all cases. This corresponded to a sucrose density of 32.1% (w/v)±0.2 (±SEM, n=4), which was almost identical to the value of 32.3% reported previously (Beaufay *et al.*, 1974).

To quantitate the amount of GalT in the ER and Golgi in these PNS preparations, fractions 2-7 in experiment "A" were judged as containing Golgi membranes (by virtue of the distribution of the GalT peak) while fractions 8-12 were judged to contain ER membranes (due to the position of the NADH cytochrome c reductase peaks). For experiment "B", fractions 2-8 were judged to be Golgi fractions and fractions 9-12 were designated as being ER. Results were not calculated by extrapolation of the distribution curves since the 1ml fractions collected were judged to be too large for this to be performed reliably. These designations of "Golgi" and "ER" were also used for quantitation of the data obtained for other markers.

There was a small amount of GalT present in the ER fractions in both experiments. For 4:12 cells this amounted to 10.2% of the total (fractions 8-10) in experiment "A", and 9.6 % of the total (fractions 9-11) in experiment "B". The nature of this material was unclear, but it may represent newly synthesised enzyme which has not left the ER or alternatively enzymes that have entered the ER via a recycling pathway. Such a pathway has not yet been conclusively shown to exist for Golgi resident proteins.

The amount of GalT present in the ER fraction in the 4:48 gradients was also small, but significantly greater than that for the 4:12 PNS preparations. Experiment "A" showed 19.9% of GalT present in the ER while in experiment "B" the value was 19.1%. This

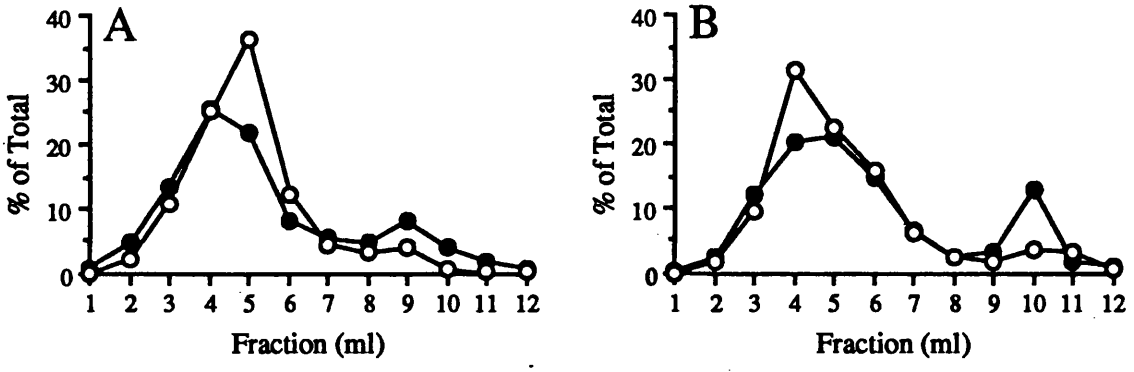

Figure 7.5: Distribution of GalT activity. GalT activity was assayed in fractions derived from linear sucrose gradients which had been loaded with PNS preparations derived from 4:12 (○) or 4:48 (●) cells and centrifuged to equilibrium. This was performed twice using two different 4:12 and 4:48 PNS preparations (A and B).

suggested that the presence of NAGT I in the ER had a small effect on the distribution of GalT and implied that these two enzymes did interact with each other to a small degree.

The recovery for GalT in these gradients when compared to the amount loaded in the PNS preparations averaged 77.8%±3.8 (±SEM, n=4), indicating that enzyme activity was a reliable marker of protein distribution.

7.2.2.4 SialylT Distribution

Aliquots of 6µl of the fractions from the gradients of experiment "A" were assayed for SialylT activity, as were the original PNS preparations, and the distribution of this enzyme determined. This showed that this enzyme was present in the Golgi fraction in 4:12 cells, while in 4:48 cells this activity appeared as a smear across the gradient (fig. 7.6). This may have been due to the activation of this enzyme activity in the gradients since the recovery of activity was 323% in the 4:12 and 436% in the 4:48 gradient when compared to the amount loaded. The reason as to why the 4:12 sample did not migrate as a smear was, however, unknown. This activation meant that SialylT activity could not be used as a reliable marker for the distribution of the protein in the gradients and this assay was not carried out in experiment "B".

7.2.2.5 NAGT II Distribution

The next marker assayed for was NAGT II. Aliquots of 6µl were assayed as was an aliquot of each diluted PNS.

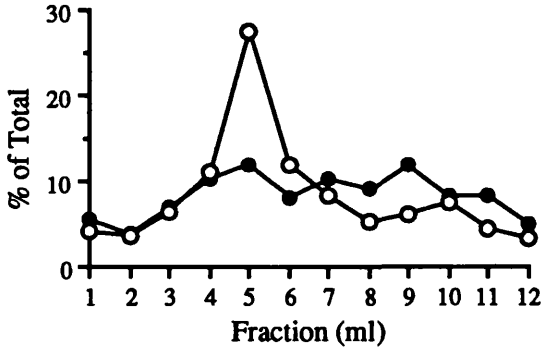

Figure 7.6: Distribution of SialylT activity. SialylT activity was assayed in fractions derived from linear sucrose gradients which had been loaded with PNS preparations derived from 4:12 (○) or 4:48 (●) cells and centrifuged to equilibrium.

This enzyme's activity distribution of the gradients indicated that NAGT II was present in the ER of 4:12 cells (fig. 7.7). In these cells, the ER contained 12.4% of the total activity for experiment "A" and 10.3% in experiment "B", similar to the levels found for GalT. In the 4:48 cells, these levels increased to 31.8% and 25.9% of the total respectively. This again reflected the increase in redistribution observed for GalT, though the degree of redistribution did appear to be significantly greater than that of the *trans*-Golgi enzyme. The recoveries of activity when compared to the amount loaded in the PNS preparations were 91.4%±6.1 (±SEM, n=4),

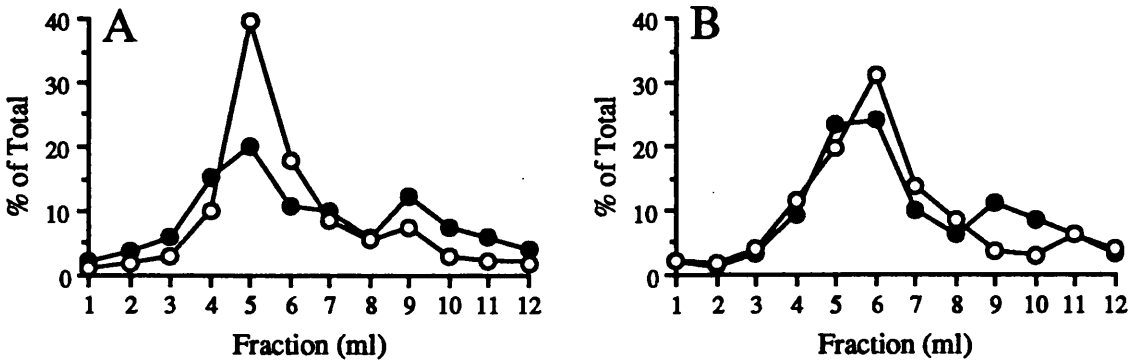

Figure 7.7: Distribution of NAGT II activity. NAGT II activity was assayed in fractions derived from linear sucrose gradients which had been loaded with PNS preparations derived from 4:12 (○) or 4:48 (●) cells and centrifuged to equilibrium. This was performed twice using two different 4:12 and 4:48 PNS preparations (A and B).

indicating that the enzyme activity was a good marker for the actual protein distribution on the gradients.

Thus these results suggested that NAGT II was behaving in a similar way to the *trans*-Golgi marker, GalT, with regards to its interaction with the p33-tagged NAGT I molecules present in the ER, though this interaction appeared to be somewhat stronger.

7.2.2.6 NAGT I Distribution

Aliquots of 20µl of each fraction were assayed for NAGT I activity as were aliquots of the original PNS preparations. This showed that most of the activity was present in the Golgi fractions for 4:12 cells and in the ER for 4:48 cells (fig.7.8) and confirmed the immunofluorescence and immuno-electron microscopic data reported previously (Nilsson *et al.*, 1993a; Nilsson *et al.*, 1994).

The average recovery of activity was 79.7%±5.2 (±SEM, n=4), indicating that little activation or inactivation had occurred after centrifugation. In the 4:12 cells, a small amount of activity was present in the ER, reflecting the amount observed for GalT though these levels were a little higher and amounted to 16.9% of the total in experiment "A" and 18.4% in experiment "B".

In contrast, the majority of this activity in 4:48 cells was present in the ER fractions as would be expected since these cells were expressing greater amounts of the p33-tagged NAGT I than of the wild-type enzyme. Nevertheless, an average of 41.2% of NAGT I activity remained in the Golgi fractions. This presumably corresponded to some of the wild-type enzyme which did not posses an ER retention signal.

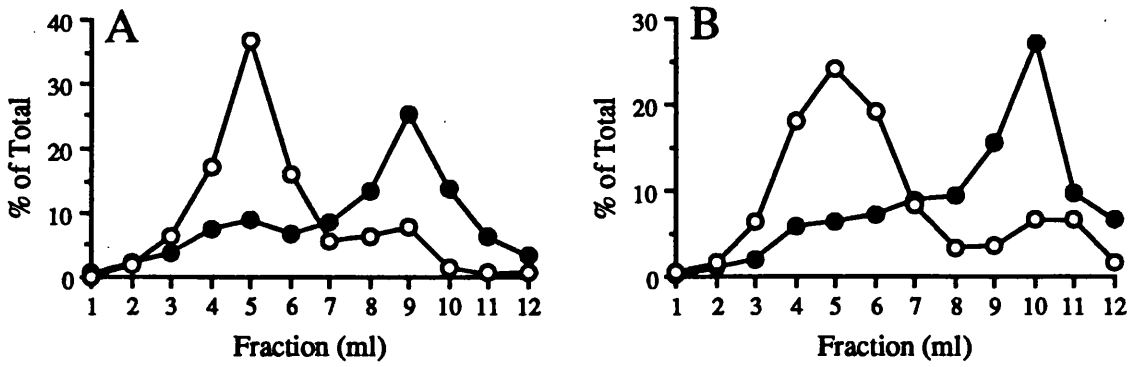

Figure 7.8: Distribution of NAGT I activity. NAGT I activity was assayed in fractions derived from linear sucrose gradients which had been loaded with PNS preparations derived from 4:12 (○) or 4:48 (●) cells and centrifuged to equilibrium. This was performed twice using two different 4:12 and 4:48 PNS preparations (A and B).

However, 80% of the NAGT I activity in these cells should have been due to the p33/NAGT I/myc constructs, while only 58.8% of the activity was present in the ER. This meant that if these values were correct, not all of the hybrid molecules had been retained in the ER. To test this, 100µl aliquots of the fractions from the two gradients in experiment "A" were subjected to methanol/chloroform protein precipitation followed by SDS-PAGE. The gels were then Western blotted and these blots probed with the 9E10 monoclonal antibody which recognises the *myc*-epitope (and therefore both of the constructs being expressed in the 4:12 and 4:48 cells). The amount of NAGT I/myc or p33/NAGT I/myc present in each fraction was then quantitated by laser densitometry.

This showed that the constructs were present only in the Golgi fractions for 4:12 cells and in the ER fractions for 4:48 cells (fig. 7.9). The small amount of enzyme activity in the ER that was observed for GalT, SialylT and NAGT II in the 4:12 cell line was not observed here for the NAGT I/myc construct, and the reason for this was unclear. These data, however, did show that the majority of the p33/NAGT I/myc protein was present in the ER. The discrepancy in the activity distribution mentioned above was possibly due to a combination of errors during enzyme assay, and in the assignment of "Golgi" and "ER" fractions in the gradients due to incomplete separation of the membranes i.e. fraction 7 in experiment "A" and fraction 8 in experiment "B" probably contained a mixture of membranes. Such an error may also have been exaggerated if a significant amount of the hybrid protein was present in the CGN while it was being recycled. This may have been the case in experiment "B" where the Golgi and ER peaks appear to have smeared together.

It was not possible, therefore, to determine with certainty whether any of the wild-type enzyme had redistributed to the ER in the 4:48 cells by interacting with the p33/NAGT I/myc molecules. The enzyme assay data suggested that this was not occurring, but the conflict in the Western blotting and enzyme assay results indicated that the quantitation of the data was not completely accurate, so that it was not possible to state this with confidence.

7.2.2.7 Mann II Distribution

One problem which arose with using the Mann II assay in these studies was caused by the assay's relatively low specificity. As discussed in detail in chapter 2, this assay not only measures Mann II activity, but that of lysosomal and ER mannosidases. This was not a problem in experiments using purified rat liver Golgi membranes because these other organelles were not present in significant amount in those preparations. In this case, however, assays were performed on fractions which may have contained significant amounts of lysosomes or ER and so the Mann II assay would not be reliable.

To circumvent this possibility, Mann II was quantitatively immuno-precipitated from each fraction using a specific antibody. The material bound to the beads would therefore be depleted of the non-Golgi mannosidases and this material was assayed for Mann II. The monoclonal anti-Mann II antibody, 53FC3, could not be used in this study since it only recognises the rodent, not human, enzyme. The antibody used was polyclonal and named TEX-1-3, and was raised against the Triton pellet of extracted rat liver Golgi membranes. This was produced at the ICRF Animal House at Clare Hall. This antibody was used because sufficient amounts of an antibody raised against purified Mann II were not available. Upon Western blotting of rat liver Golgi membranes, TEX-1-3 recognises several different proteins, though

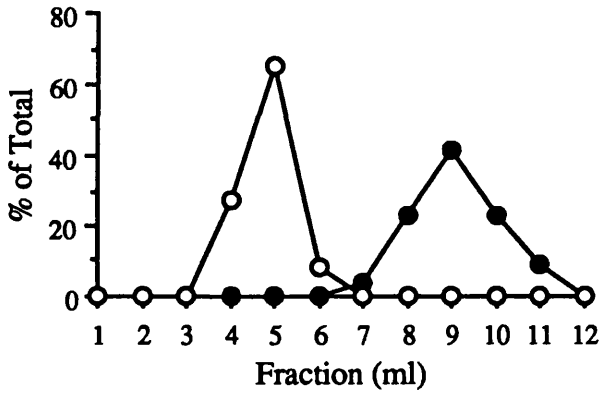

Figure 7.9: Distribution of the *myc*-epitope. Fractions derived from linear sucrose gradients which had been loaded with PNS preparations derived from 4:12 (O) or 4:48 (●) cells and centrifuged to equilibrium were subjected to SDS-PAGE and Western blotting. After probing with the 9E10 antibody, the amount of *myc*-labelled protein in each fraction was determined by laser densitometry.

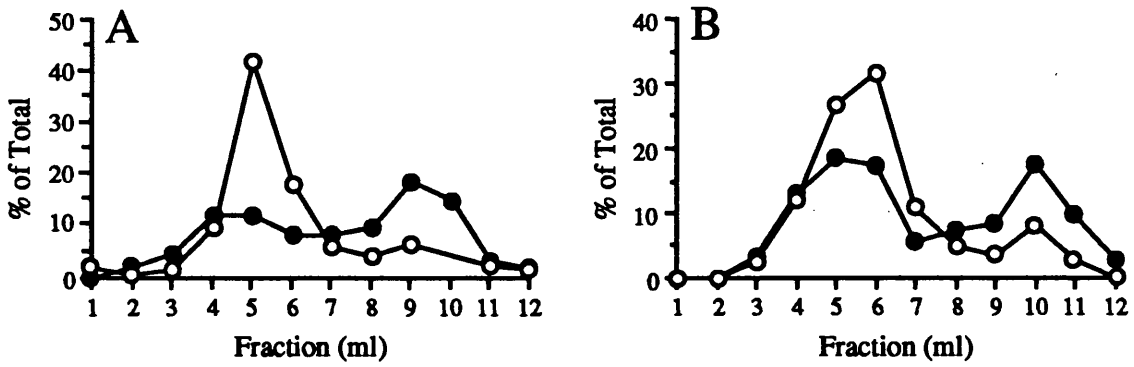

Figure 7.10: Distribution of Mann II activity. Mann II from fractions derived from isopycnic sucrose gradients of 4:12 (○) or 4:48 (●) PNS preparations was immunoprecipitated using protein A-Sepharose beads carrying anti-Mann II antibodies. These precipitates were assayed for Mann II activity and the amount of enzyme in each sample determined. This was performed twice using two different 4:12 and 4:48 PNS preparations (A and B).

immunoprecipitation and SDS-PAGE of radiolabelled PNS from Hela cells demonstrates that it only recognises Mann II in these human cells (Tommy Nilsson, pers. comm.). Thus this antibody, which was readily available, was used for the immunoprecipitation of Mann II from the gradient fractions.

Thus 200µl aliquots of each fraction were immunoprecipitated using TEX-1-3 antibodies which had been bound to protein A-Sepharose. After washing to remove unbound material, the amount of Mann II activity that had bound to the beads was assessed using the Mann II assay.

This showed that Mann II in 4:12 cells was present predominantly in the Golgi fractions while in 4:48 cells there was a significant redistribution to the ER (fig. 7.10), in agreement with the observation that such redistribution occurred as judged by immunofluorescence, immuno-electron microscopy and metabolic labelling (Nilsson *et al.*, 1994). In 4:12 cells, 13.7% of the total Mann II was present in the ER fractions in experiment "A" and 12.7% was in the ER in experiment "B". This again compared favourably with the results for other markers.

In 4:48 cells, however, 49.5% was present in the ER in experiment "A" while 41.5% had redistributed in experiment "B". This represented a much more significant shift than observed for GalT and NAGT II and explains why only a redistribution of this enzyme could be detected by immunofluorescence.

In order for these results to be valid, it was necessary to demonstrate that the immuno-precipitation was truly quantitative i.e. that all the Mann II in each fraction was precipitated by the TEX-1-3 antibody. To demonstrate that this was indeed the case,

Mann II was precipitated from a PNS using differing amounts of the TEX-1-3 antibody. All the preceding results indicated that none of the peak fractions from the gradients contained more than 40% of the total enzyme activity in the PNS. Thus 200µl aliquots of a 2.5-fold diluted 4:12 PNS were immuno-precipitated with 1, 2, 4 or 8µl or TEX-1-3 or with 4µl of the pre-bleed obtained from the rabbit before immunisation (4µl was the volume used for immunoprecipitation from the gradients).

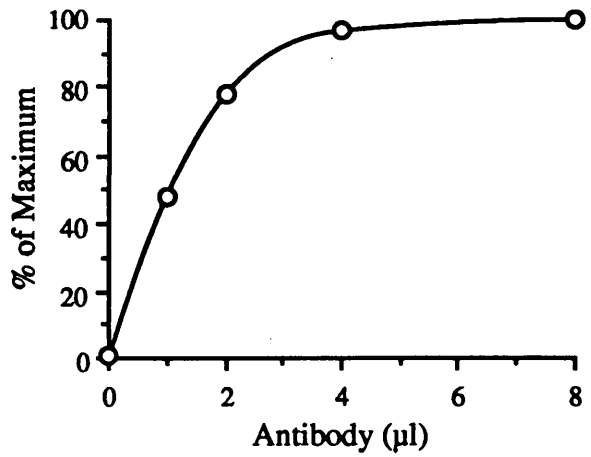

Figure 7.11: Immunoprecipitation of Mann II using TEX-1-3 antibodies. A 4:12 PNS was diluted 2.5-fold and 200µl aliquots were immunoprecipitated using 1, 2, 4 or 8µl of TEX-1-3, or 4µl of the TEX-1-3 pre-bleed (zero) coupled to protein A-sepharose beads. After washing, the Mann II bound to the beads in each sample was assayed and expressed as being the % of the maximal binding.

After washing, the beads were assayed for Mann II activity and the amount that had bound was determined as being the percentage of the maximal amount of binding. This showed that binding was almost complete when using 4µl of TEX-1-3 antibody (fig. 7.11), indicating that under these conditions Mann II was quantitatively immunoprecipitated from the fractions. The binding was specific because none was observed in the presence of the control antibody (fig. 7.11, zero-point).

The effect was not due to a limiting amount of protein A-Sepharose because the amount of beads used would still be in excess of 8µl of antibody, even assuming an antibody concentration of 10mg/ml in the antiserum.

7.3 Summary

The purpose of the experiments described in this chapter was to determine whether retention of Golgi enzymes was caused by oligomerisation of resident membrane-spanning Golgi proteins via their membrane-spanning domains, a hypothesis termed "kin-recognition" in this laboratory.

To test this, biochemical subcellular localisation of a series of Golgi markers was performed on a stable cell line, 4:48, which expressed the *medial*-Golgi enzyme, NAGT I, in the ER. If kin-recognition did occur, it would be expected that other *medial*-Golgi

enzymes would redistribute to the ER by their interaction with NAGT I while enzymes from other regions of the stack would not. As a control, a stable cell line, 4:12, was used which contained NAGT I only in the Golgi apparatus.

The percentage of GalT, NAGT II, NAGT I and Mann II present in the ER in 4:12 and 4:48 cells was quantitated by virtue of the densities of the compartments in which they were contained, as judged by enzyme assay of fractions derived from isopycnic sucrose gradient centrifugation of PNS preparations, and the results are summarised in table 7.1. The *trans*-Golgi marker, SialylT was also assayed but due to a large amount of activation of this enzyme in the gradient, these data were judged to be unreliable. It should be noted that these values may vary slightly from the true distributions of the enzymes because the separation of Golgi from ER membranes in the gradients was not complete.

	% of Total in ER		
Enzyme	4:12	4:48	Difference
GalT	9.9	19.5	9.6
NAGT II	11.4	28.9	17.5
NAGT I	17.7	58.8	41.1
Mann II	13.2	45.5	32.3

Table 7.1: Comparison of enzyme distributions in 4:12 and 4:48 cells. The table depicts a summary of the average percentage of each enzyme in present in the ER of 4:12 and 4:48 cells, and the increase in this percentage in the 4:48 cells.

This showed that each enzyme was present in the ER in small amounts even in 4:12 cells. This level was slightly greater for NAGT I than for the other enzymes, but it was not known whether this was due to an accumulation of newly synthesised enzyme because of the higher expression level or to recycling of enzyme because of less efficient retention in the Golgi.

In 4:48 cells, the proportion of each enzyme present in the ER increased. The greatest increase was that of NAGT I, caused by the efficient retention of the p33/NAGT I/myc construct, which was present entirely in the ER as judged by quantitative Western blotting analysis. It was not possible to determine whether the proportion of endogenous Hela enzyme present within the ER had increased.

Of the other enzymes, GalT showed the lowest increase in redistribution to the ER. This increase was significant, however, and suggested that this *trans*-Golgi enzyme could interact to some extent with NAGT I. Mann II showed the greatest degree of redistribution, and 3.5 times more redistributed when compared to GalT, as would be expected if interactions between *medial*-Golgi enzymes were favoured as predicted by the kin-recognition hypothesis.

NAGT II displayed an intermediate degree of redistribution in 4:48 cells, with just over twice as much being present in the ER compared to GalT. This suggested that NAGT II interacted with NAGT I with a slightly greater affinity than GalT. Although this enzyme has not been localised to the *medial*-Golgi by immunoelectron microscopy, It is thought to be present in a different compartment to GalT as judged by isopycnic centrifugation studies (Dunphy and Rothman, 1983) where it was found to present in slightly higher density fraction than GalT. Since NAGT II acts directly after Mann II, it has been presumed that it is a *medial*-Golgi enzyme. These data were confirmed in this study because GalT in the gradients used here was also present in slightly less dense membranes than NAGT II if the data from experiment "A" were replotted and the actual peak densities of each marker determined by extrapolation (fig.7.12 A). In contrast Mann II and NAGT I appeared to be contained in compartments with the same density as NAGT II (fig. 7.12 B and C) also as reported previously (Dunphy and Rothman, 1983).

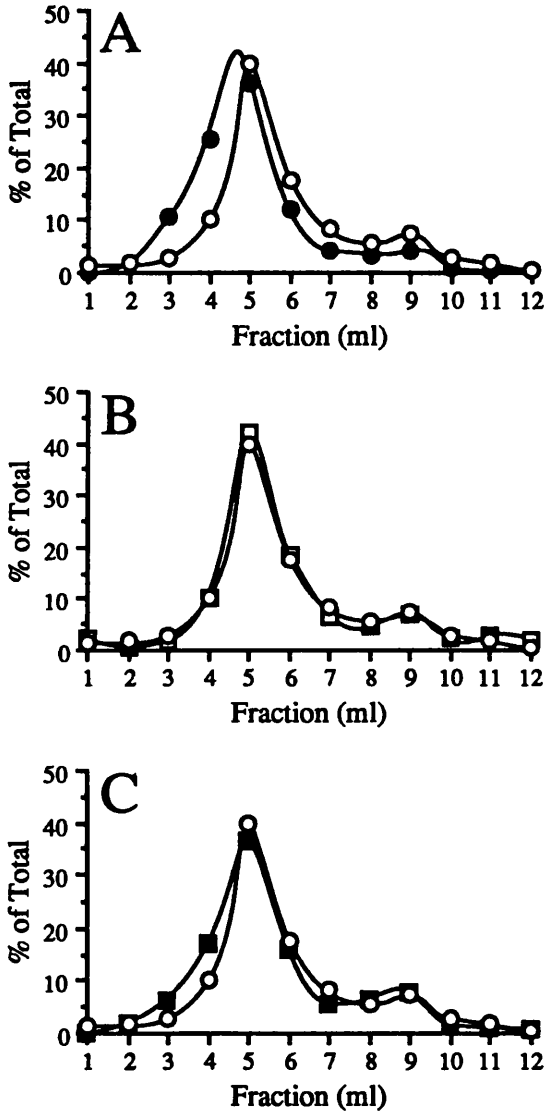

Figure 7.12: Comparison of enzyme distributions in 4:12 cells. The data from experiment "A" were plotted to show the densities of the compartments containing GalT (●; A), Mann II (▽; B) and NAGT I (■; C) relative to NAGT II (○).

These data suggested, but did nor prove, that NAGT II was present in the same compartment as Mann II and NAGT I which was distinct to that which contained the GalT. With this in mind, the simplest interpretation of the redistribution data was that Mann II, NAGT I and NAGT II were present in the *medial/trans* cisterna of 4:12 cells and had a higher affinity for the NAGT I present in the ER of 4:48 cells than the *trans*-Golgi/TGN enzyme GalT.

This interpretation is tentative, however, and needs to be substantiated by an immunoelectron-microscopic study of the distribution of NAGT II in Hela cells once antibodies which recognise this enzyme, or a cloned cDNA which can be *myc*-tagged and stably transfected, become available.

Such compartment-specific affinities are presumably due to interactions of the membrane-spanning domains of the enzymes since a construct consisting of p33/NAGT I/myc in which the membrane-spanning domain of NAGT I had been replaced by that of GalT did not cause redistribution of Mann II as judged by immunofluorescence (Nilsson *et al.*, 1994). The data presented here, therefore, help to support the kin recognition hypothesis. However, the size of the oligomers containing these enzymes has yet to be established as does their existence *in vivo*, and further work will be required to provide direct evidence to support the theory.

Chapter 8

Discussion

8.1 The Golgi Matrix

In this thesis I have presented the results of experiments which have culminated in the isolation of a detergent and salt insoluble proteinaceous matrix which has the ability to bind specifically *medial*-Golgi enzymes. In this final chapter I will summarise these data and speculate about possible functions for the matrix.

8.1.1 Solubility of *medial*-Golgi Enzymes

Extraction of purified Golgi membranes in a TX-100-containing buffer showed that the *medial*-Golgi enzymes NAGT I, Mann II and NAGT II were very insoluble (77, 86, and 60% respectively) when compared to the solubility of markers from other regions of the stack. The non-*medial* markers were generally less than 20% insoluble except for p58, 35% of which remained in the pellet). This insolubility was due to the specific interaction of the *medial*-enzymes with a proteinaceous matrix (see below).

Such differential protein insolubilities have precedents and have been reported previously in other systems. For example, the contents of apical-transport vesicles have been shown to be extremely resistant to extraction in the detergent CHAPS, while other membranes are efficiently solubilised (Kurzchalia *et al.*, 1992). Extraction of synaptic plasma membrane fractions with TX-100 led to the selective insolubility of synaptic proteins (Cotman *et al.*, 1971). Finally influenza virus haemagglutinin was also found to be resistant to extraction in TX-100, but only when it was present in the TGN (Skibbens *et al.*, 1989). The reason for these protein insolubilities was, however, not determined in these studies.

The material containing the insoluble enzymes was found to be very dense and morphologically contained two types of macromolecular structure. The first were generally amorphous while the second resembled lipid bilayers in both their dimensions and appearance. Again such a preservation of bilayer morphology has been previously reported after TX-100 extraction of synaptic plasma membranes, which left an insoluble residue which contained both membrane-like structures and amorphous material very similar to those observed in this study (Cotman *et al.*, 1971). The preservation of the bilayer like-morphology may have been due to the incomplete solubilisation of the membrane phospholipids under these conditions since these should not all be removed by a single mild detergent extraction (Helenius *et al.*, 1979). In fact such extraction of the synaptic membranes cause only 30% solubilisation of the membrane phospholipid (Cotman *et al.*, 1971). Close examination of the insoluble pellet by electron microscopy showed that the amorphous material often seemed to emanate from the ends of the membrane remnants. A simple interpretation would be that this represents a de-stabilised

form of the membrane-remnants, due to differential levels of phospholipid extraction from one region of the pellet to the other, though this would be difficult to prove directly. Removal of phospholipids could destabilise proteinaceous interactions within the membrane-remnants and thus lead to unravelling.

The *medial*-enzymes Mann II and NAGT I were present in the same structures by two criteria. Firstly both enzymes exhibited the same density shift when incubated with anti-Mann II antibodies followed by secondary antibodies coupled to colloidal gold. Secondly, both enzymes could be immunoprecipitated by antibodies specific to only Mann II. Electron microscopy of such immunoprecipitates showed that both the membrane-remnants and amorphous material were adsorbed to the beads. This suggested that both types of structure contained the enzymes and strengthened the idea that the amorphous material constituted a destabilised form of the membrane remnants. Such a conclusion, however, should be treated with a certain amount of caution, because these experiments could not determine the distribution of the enzymes within the structures. Although both should contain Mann II by virtue of their ability to bind the beads, it is possible that only one set of structures contained the NAGT I or that the Mann II/NAGT I ratio varied between the membrane remnants and the amorphous material, which would suggest that the latter was not derived from the former. Such possibilities can only be addressed by a double-immunoelectron microscopic study, which is currently not possible due to the lack of appropriate, functional monoclonal and polyclonal antibodies. Furthermore, these data do not show whether Mann II and NAGT I are even distributed within these structures or present in different sub-domains.

The *medial*-Golgi enzymes could be completely released from the Triton pellet by a second extraction in Triton X-100 and 150mM NaCl or KCl. This produced a second pellet containing material that was insoluble in both Triton and salt. Solubilisation of the enzymes by salt only occurred in the presence of TX-100 and salt, because if the second extraction was carried out in the absence of detergent, little enzyme release was observed. Morphologically, this pellet contained structures that resembled the amorphous material found in the Triton pellet. This opens up the possibility that the amorphous material in the Triton pellet corresponded to the material in the salt pellet, though this seems unlikely since the material in the Triton pellet must have contained some Mann II in order to bind to the beads during immuno-isolation, while the material in the salt pellet contained very little Mann II activity. Nevertheless, the possibility exists that the amorphous material in the Triton pellet did contain small amounts of Mann II which allowed it to bind to the beads and that it is not derived from the membrane remnants. Alternatively, the amorphous material may still be a de-stabilised form of the membrane remnants, but the

de-stabilisation may be induced by the loss of the majority of *medial*-enzymes, not phospholipids, in localised regions of the pellet.

8.1.2 Binding of *medial*-Golgi Enzymes to the Matrix

The fact that release of the *medial*-enzymes, in a dimeric form, from the Triton pellet by a second extraction in Triton and salt resulted in the detection an insoluble material suggested that it might be the binding of the enzymes to this material which is responsible for their detergent in-solubility. If this were the case, the binding would be sensitive to salt in the presence of detergent, but not to TX-100 alone, and the material in the salt pellet would constitute a Golgi matrix, since it contains a large macromolecular structure (based on its rapid sedimentation).

This was confirmed to be the case because dialysis of a salt supernatant to remove the salt caused resedimentation of the enzymes, but only in the presence of the material in the 150mM salt pellet. Rebinding of predominantly *medial*-Golgi enzymes was observed and the *trans*-Golgi marker, GalT, did not rebind in large amounts, suggesting that the material in the pellet did not cause the non-specific aggregation of solubilised proteins after dialysis. Furthermore, the matrix seemed to contain a fixed number of binding sites since addition of a fixed amount of enzyme to a varying amount of matrix eventually led to a saturation of binding. This saturable binding was confirmed in the reciprocal experiment where varying amounts of enzymes were added to a fixed amount of pellet, and these data produced Scatchard plots that were linear (again confirming the saturability of the binding) and showed that the enzymes bound to the matrix with high affinity (3nM for Mann II and approximately 70pM for NAGT I), strongly suggesting that the rebind was specific.

Although the 150mM salt pellet contained enough components to bind, and confer Triton insolubility, to the *medial*-Golgi enzymes, it was clear that some components of the matrix were solubilised at lower salt concentrations. This was observed because enzymes in supernatants of lower salt extracts were capable of resedimentation after dialysis. Because of this effect, it was necessary to carry out rebinding experiments using 150mM salt pellets and low salt supernatants to eliminate signals obtained from supernatants alone. Because of this, it was not possible to determine the stoichiometry of enzyme binding to the matrix since it was not possible to determine how much of the matrix present in the original Golgi membranes was present in the 150mM salt pellet.

The fact that only approximately 30% of Mann II and NAGT I could rebind after dialysis of a crude 150mM salt extract of Golgi membranes could be interpreted in two ways. This may reflect the maximal amount of rebinding possible in this system. Alternatively, this may reflect the possibility that more than one enzyme can bind to a

single binding site on the matrix, but that under the reconstituted system only one enzyme can rebind to this site. This again may reflect an imperfection in the *in vitro* rebinding, or alternatively suggests that the enzymes exist as small oligomers in the Golgi membranes which are disassembled in the salt extraction (since velocity sedimentation analysis revealed that Mann II was a dimer after salt extraction). During reconstitution, the enzymes rebind to the matrix but cannot reform their original oligomers, leading to an inability to become completely insoluble. If this were the case, the fact that 30% rebinding occurs in the crude 150mM salt extract would suggest that these small oligomers would contain 3-4 enzymes each. The stoichiometry of enzyme binding will remain unknown until the molecular composition of the matrix is further characterised.

An alternative explanation of the enzyme insolubility could be that the salt pellet contains nucleating factors which promote the formation of large oligomers of the Golgi enzymes after removal of salt. These oligomers could be large enough to sediment under the conditions used in these studies. Such nucleating factors could be novel proteins or isoforms of the enzymes themselves which are resistant to salt extraction. This interpretation raises several problems which do not arise in a receptor-based hypothesis. Firstly, the nucleating factors would still need to be present in large sedimentable structures, which in themselves would constitute a Golgi matrix. Secondly, if rebinding did occur by nucleation and oligomer formation, binding would not be expected to be saturable unless the oligomers were very small, since in theory, very few nucleation sites would be required to sediment all the enzymes. This is contrary to the Scatchard analyses presented. If such oligomers were very small, they would still have to bind to a matrix to be sedimented, thereby still necessitating the requirement for a large matrix.

Finally, the morphology of the salt pellet to which enzymes had been rebound was the same as before rebinding i.e. both pellets contained the amorphous material which resembled that present in the Triton pellet. No membrane remnant-like structures were reformed. This could reflect the removal of more phospholipids in the second extraction which would have either been lost during dialysis or which could not reconstitute the more ordered structures. Alternatively, this may simply be a thermodynamic effect whereupon the reconstitution of the membrane remnants cannot occur *in vitro* once they have been disassembled. The fact that this ordered morphology cannot be restored may also explain the inability to reconstitute more than 30% of the enzyme binding.

8.1.3 Topology of the Matrix

Although the above experiments established the existence of a Golgi matrix that could bind *medial*-enzymes, they did not address the question of its topology. This was established by means of protease protection experiments utilising proteinase K.

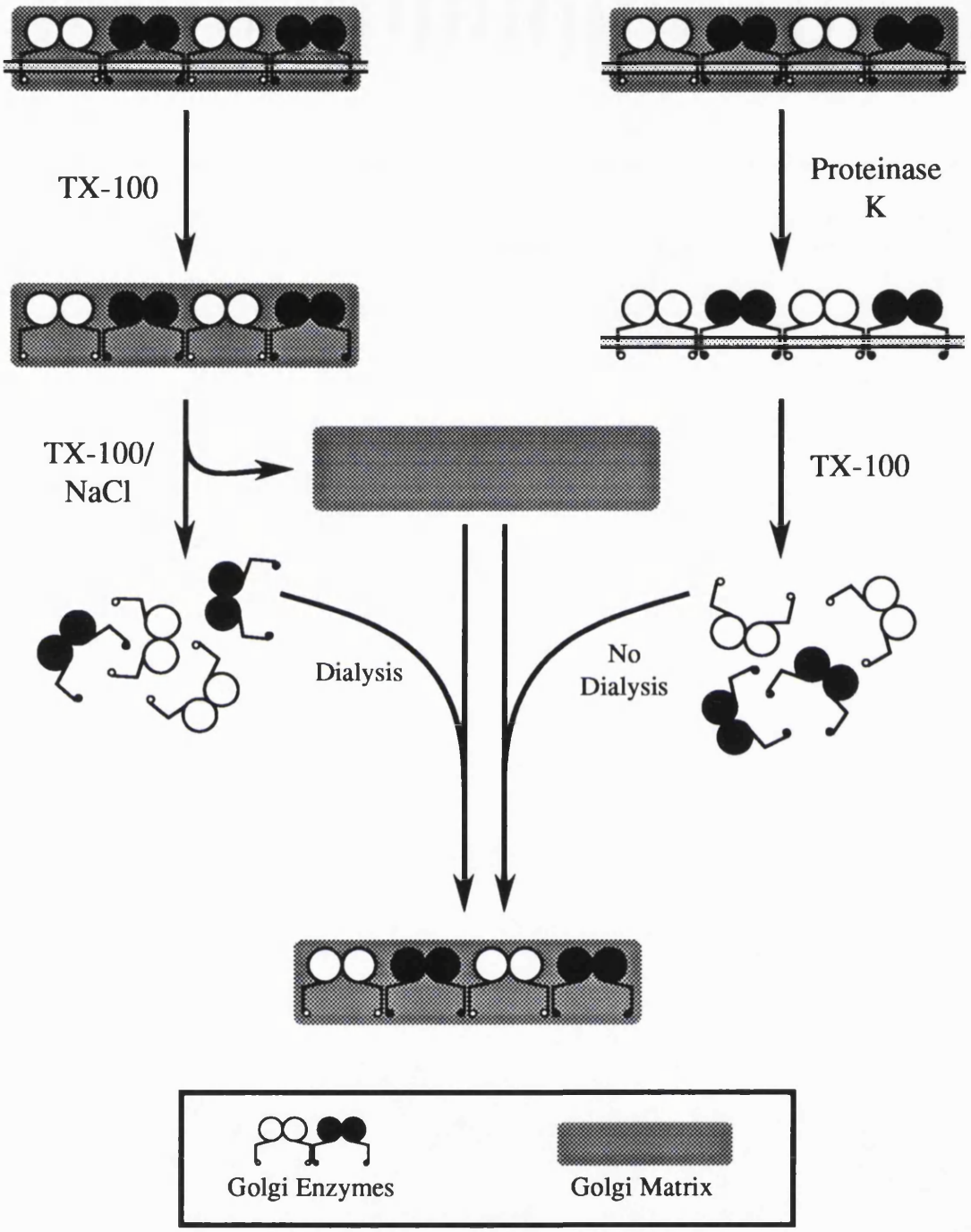

Figure 8.1: Schematic diagram of enzyme solubilisation and rebinding to the Golgi matrix. Extraction of Golgi membranes with TX-100 leaves the enzymes in an insoluble form, bound to the matrix. Pretreatment of membranes with proteinase K removes vital components of the matrix and renders the enzymes soluble in TX-100. The untreated, TX-100 insoluble enzymes can be released from the matrix by extraction in TX-100 and salt. Solubilised enzymes can be rebound to the matrix in the absence of salt.

Digestion of Golgi stacks with this protease resulted in a large increase in the solubility of the enzymes in Triton alone, suggesting that some aspect of the matrix was cytoplasmically exposed. This was not due to a conformational change in the Golgi enzymes induced by the proteolysis of the cytoplasmic domains since enzymes from digested membranes could rebind a fresh matrix produced from untreated membranes.

This experiment could not distinguish the amount of matrix that was present on the cytoplasmic face of the Golgi membranes. It is equally conceivable that the matrix is entirely cytoplasmic or that almost all of its bulk is present in the cisternal lumen. If the latter were the case, the matrix would have to contain membrane-spanning elements which connected it to a cytoplasmic portion. Removal of the cytoplasmic regions would either disassemble the matrix into smaller subunits or induce conformational changes which would prevent enzyme binding. Nevertheless, the protease accessibility of at least some of the matrix, which was required for the maintenance of enzyme insolubility, demonstrated that it was at least partially cytoplasmic. Furthermore, the fact that it can bind at least 30% of the Golgi enzymes *in vitro*, coupled to the fact that both Mann II and NAGT I are distributed throughout the cisternae in which they reside, as determined by immunoelectron microscopy (Burke *et al.*, 1982; Dunphy *et al.*, 1985), suggests that the cytoplasmic portions are intercisternal. Only after further characterisation of the matrix and its components will it be possible to determine its exact distribution across the lipid bilayer.

The method by which the matrix acts upon the solubility of the enzymes is depicted schematically in figure 8.1. In this figure, the matrix is depicted as being present on both sides of the lipid bilayer, though the details of its distribution are left deliberately vague for want on any direct evidence as to its exact topology. Additionally, the enzymes are shown as binding to it as dimers, though as explained above, the stoichiometry of binding is not yet known. Thus after extraction of Golgi membranes in Triton, the enzymes remain insoluble by virtue of their interaction with the matrix. If membranes are pretreated with proteinase K, the matrix is either removed, disassembled or inactivated and the enzymes become soluble in detergent alone. Extraction of the Triton pellet in salt causes release of the enzymes from the matrix and these can rebind after dialysis to remove the salt. Furthermore, solubilised enzymes from Triton-extracted, proteinase K treated membranes can also rebind to a fresh matrix.

8.1.4 Mechanism of *medial*-Enzyme Binding to the Matrix

The enzymes could be envisaged as binding to the matrix in four different ways as depicted schematically in figure 8.2. Firstly, the enzymes could be bound to the matrix directly via their cytoplasmic tails (fig. 8.2A), or by ancillary cross-linking proteins (fig.

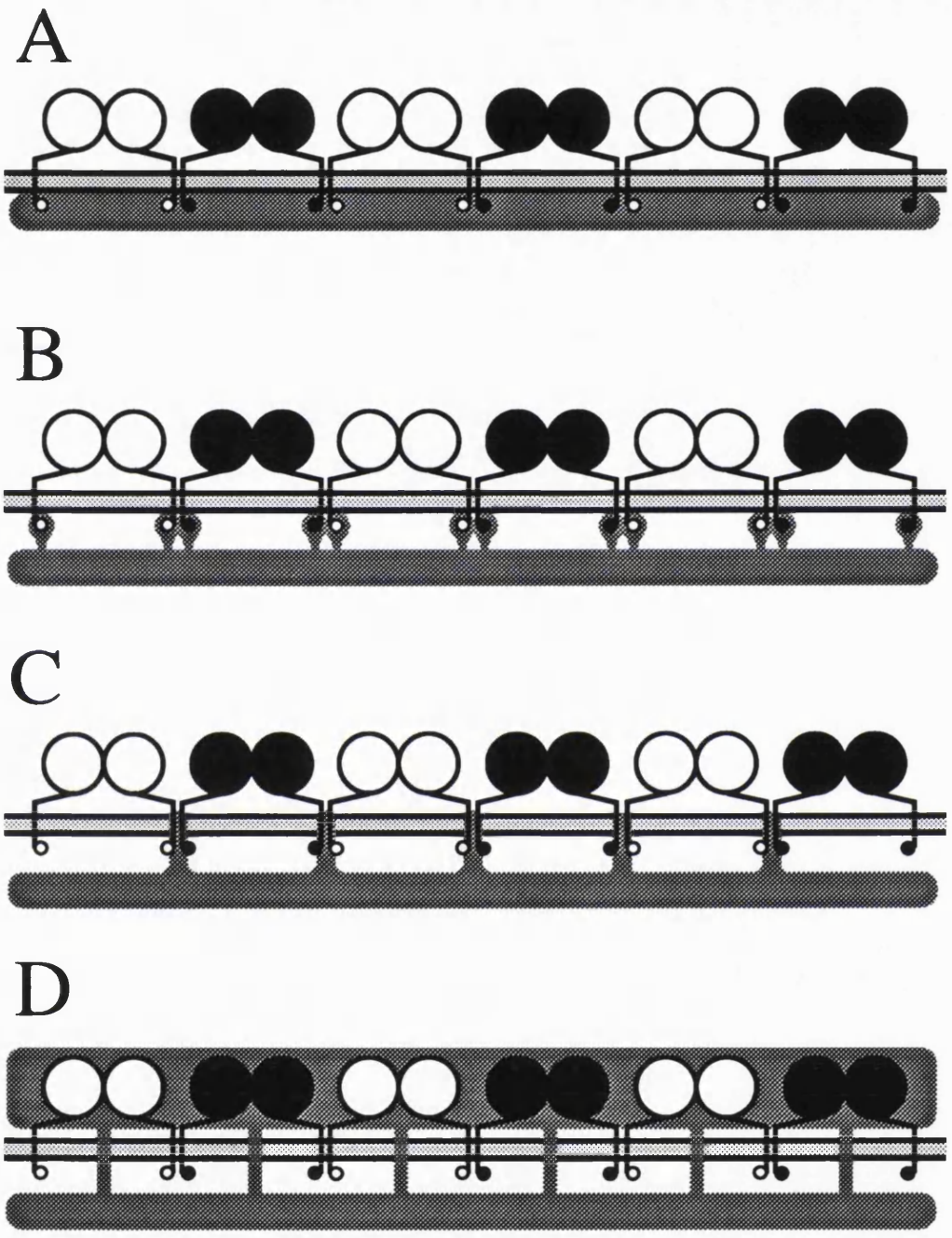

Figure 8.2: Four theoretically possible mechanisms for enzyme binding to the Golgi matrix. Enzyme binding could be direct via their cytoplasmic tails (A) or indirect, utilising ancillary anchoring proteins (B). Alternatively enzymes could bind by their membrane-spanning domains to components of the matrix which penetrate the lipid bilayer (C), or by their lumenal domains to lumenal components of the matrix (D).

8.2B). Both of these possibilities would not have to invoke any lumenal components to the matrix. Alternatively, the enzymes may be bound to components of the matrix which protrude into the lipid bilayer and interact with the enzymes' membrane-spanning domains (fig. 8.2C). This seems unlikely since such an interaction would normally be expected to be sensitive to detergent, but is still a formal possibility, because it has been reported that alpha-helical membrane spanning domains can contain polar faces (Swift and Machamer, 1991), and polar protein interactions could be insensitive to TX-100. Finally, the enzymes could be bound to the matrix by their lumenal domains (fig. 8.2D). This option would require a portion of the matrix to be lumenal. Minimally, this would require membrane-spanning proteins which bind the enzymes by their lumenal domains and cross-link them to the bulk of the matrix in the cytoplasm. In the other extreme, the bulk of the matrix could be lumenal with membrane-spanning components which interact in the cytoplasmic side of the membrane to maintain the matrix as a large macromolecular structure.

Attempts to inhibit enzyme binding to the matrix using synthetic peptides corresponding to the cytoplasmic tails of Mann II and NAGT I were unsuccessful. This negative result did not preclude the possibility that binding did occur via the cytoplasmic tails, however, since the conformations of the peptides may have been different to those of the native cytoplasmic tails.

The fact that the chymotryptic fragment of Mann II, which lacks the cytoplasmic and membrane-spanning domains, is capable of binding to the matrix suggests that binding occurs via the lumenal domain (fig. 8.D). However, care should be taken before dismissing the membrane-spanning and cytoplasmic domains in being involved in some way since the affinity of the fragment's binding has not yet been determined. It is possible that these other regions of the molecule may function in increasing the affinity of enzyme binding.

The fact that enzyme binding appears to occur via the lumenal domain coupled to the fact that enzyme insolubility in TX-100 is abolished by treatment of Golgi membranes with proteinase K implies that the matrix consists of a set of lumenal binding sites and that these sites are connect to cytoplasmic portions of the matrix which are responsible for maintaining the matrix as a macromolecular structure (figure 8.2D). The exact distribution of the matrix between the lumen and cytoplasm will only been known after further characterisation of its components.

8.1.5 Possible Functions for the Matrix

Three obvious functions for the matrix suggest themselves. It may play a role in the retention of Golgi proteins, maintaining cisternal shape or in stacking the Golgi cisternae.

Because retention is now commonly thought to be conferred solely by the membrane-spanning domains of the Golgi proteins suggests that the matrix is not involved in this phenomenon since binding of the enzymes in independent of the presence of this region of the proteins. A very recent study, however, has suggested that the lumenal and cytoplasmic domains of NAGT I also function in the protein's retention (Burke *et al.*, 1994). This study showed that both the lumenal and membrane-spanning domains of NAGT I were capable of retaining the enzyme in the Golgi apparatus in stably transfected cell lines, but that considerable leakage occurred and a significant amount of reporter molecule was present on the cell surface in the presence of the membrane-spanning domain alone. Inclusion of both domains in the reporter molecule led to efficient retention. The cytoplasmic tail alone could not confer retention, but increased the efficiency in the presence of one of the other two domains. These workers, therefore, suggest that all three domains play a role in retention, and that this occurs by oligomerisation which is mediated by all three regions of the protein. An alternative explanation would be that the lumenal domain does not aid retention by promoting oligomerisation, but does so by interaction with the matrix. No direct evidence exists for such an interpretation, but it is intriguing that extraction of transfected cells using the conditions described in this thesis showed that Triton-solubility increased and correlated strongly with loss of Golgi retention. This increase in solubility could be due to an inability of the NAGT I lost from the Golgi apparatus to interact with the Golgi matrix. An alternative explanation is that Triton-insolubility is a property of NAGT I-containing oligomer and that the reduction in this enzyme's ability to form such oligomers confers increased detergent solubility. As discussed above however, this latter interpretation does not account for the ability of NAGT I to bind to the matrix *in vitro*.

The fact that at least some of the components of the matrix are lumenal (by virtue of its ability to bind the lumenal domain of Mann II) suggests that it may also act to maintain the membranes of single cisternae in close proximity and thus maintain the classical flattened cisternal morphology. It can be envisaged that this could be relatively simply achieved by such a matrix if it could simultaneously bind to integral-membrane proteins in both sides of the cisternal membrane. Such a matrix could function to prevent vesicle budding from regions of cisternae other than the dilated rims, or to prevent spontaneous periplasmic membrane fusion as has been suggested by Rothman and Warren (1994).

The third possible function for the matrix is in the maintenance of the Golgi stack. The fact that at least some of the matrix is present on the cytoplasmic face of the Golgi membranes and that it is capable of binding proteins which are embedded in the cisternal membrane means that the matrix could be capable of connecting apposing cisternal

membranes to form the stack. Such an interpretation would have to invoke the existence of other matrices which bind non-*medial* proteins to facilitate stacking of cisternae at the *cis*- and *trans*-faces of the stack. Such matrices may be present in the salt pellet described here but may not be able to bind Golgi proteins under the conditions used, or alternatively may be solubilised under these conditions. It is intriguing to note that the solubility of *trans*-Golgi marker GalT increases at pH values both above and below neutrality (fig. 3.12), suggesting that such matrices may indeed exist, and that the exclusive insolubility of the *medial*-Golgi enzymes observed in this study was a fortuitous consequence of the conditions chosen for extraction.

Such a matrix-based mechanism for cisternal stacking could be simplified by invoking the observed overlapping distribution of Golgi enzymes observed in Hela cells as described in chapter 1 (see figures 1.8 and 1.9), since a single intercisternal matrix would only need to possess binding sites for one subset of Golgi enzymes. Such an interpretation is purely speculative, however, since it has not been shown that overlapping enzymes are restricted to apposing cisternal membranes. If this were not the case, it would be difficult to envisage how the matrix could confer polarity upon the stacking mechanism since both faces of apposing cisternae would contain the same enzymes. Polarity could, however, be conferred by a different mechanism such as the interaction of SNAREs as described in chapter 1, while the matrix could confer structural stability to these interactions and to the cisternae themselves. Such a model could still invoke the existence of several different matrices. but then it would then be necessary to postulate the colocalisation of different matrices in the same cisterna. A simpler model would require only one matrix which is present in all cisternae and can bind to all the Golgi enzymes. If this were the case, only the interaction of the *medial*-Golgi enzymes with the matrix would be preserved in the extraction conditions used in this study. The fact that GalT insolubility increase at pH levels above and below 7.0 (see above) could alternatively be interpreted as demonstrating that the binding of different enzymes to the same matrix is sensitive to the extraction conditions used.

Whether the matrix represents any of the inter- or intracisternal material that has been reported remains, as yet, unknown. The fact that the matrix appears to bind all the *medial*-enzymes suggests that it is present continuously throughout the cisternal length to match the observed distributions of the enzymes, which is not in agreement with the observed discrete nature of intercisternal cross-bridges. If the matrix does represent these structures, it would be necessary to postulate that the enzymes are bound to the cross-bridges in an oligomeric form though this does not seem very likely as outlined above. Alternatively, the cross-bridges may represent subdomains of a continuous matrix which are preferentially stained when observed by electron microscopy, in which case no such

oligomerisation would be required to reconcile the difference in the enzyme/cross-bridge distribution. This would also be the case if the matrix constituted part of the intercisternal elements, though again the relationship between these structures and the cross-bridges is far from clear. The fact that the matrix is also present in the lumen means that it too could constitute the intracisternal cross-bridges and elements which have been reported.

The extent to which the matrix acts in Golgi retention, the existence of other matrices which bind non-*medial* proteins, its possible function in cisternal stacking and maintaining cisternal shape and its relationship to the structures which have been reported in electron microscopic studies are all unresolved question which await the further characterisation of the matrix and the identification of its individual components, as well as a more thorough understanding of the subcompartmentalisation of the Golgi stack. Identification of these components will hopefully lead to the elucidation of the true function(s) of the matrix, and if it is involved in maintaining the stack, may eventually lead to identification of the function of the cisternal stack.

8.2 Golgi Retention

In addition to the above studies, I have also described a series of experiments which were designed to determine the extent to which *medial*-Golgi enzymes interact with each other *in vivo*. It has been proposed that Golgi enzymes are retained in the appropriate cisternae by the formation of large protein oligomers which cannot enter transport vesicles (Machamer, 1991; Machamer, 1993; Nilsson *et al.*, 1993b). Formation of such oligomers has been proposed to be mediated by the membrane-spanning domains of the Golgi proteins, though more recently, other domains of these proteins have also been implicated in this phenomenon (Burke *et al.*, 1994).

By expressing the *medial*-enzyme NAGT I in the ER of Hela cells, it was shown that there was a 2-fold increase in the amount of *trans*-Golgi enzyme GalT also retained in the ER. In contrast the amount of *medial*-Golgi enzymes, Mann II and NAGT II, present in the ER increased 3.5- and 2.5-fold respectively. This indicated that Mann II and NAGT II preferentially interacted with Mann II and so remained in the ER. It was not possible, however, to determine whether these enzyme interacted directly with each other or via as yet unidentified proteins. Furthermore, these studies could not determine the size of the oligomers which contained the *medial*-Golgi enzymes. These are crucial questions which need to be addressed in future studies, since as yet there is no direct evidence that these higher-order oligomers exist or are large enough to prevent enzyme entry into transport vesicles, nor that oligomerisation of Golgi residents alone is enough to confer retention.

The fact that GalT seems to interact with Mann II, albeit to a lesser extent than Mann II and NAGT II, suggests that oligomerisation does not occur exclusively between

medial-enzymes or between *cis*- or *trans*- enzymes. Instead, there appears to be a gradation of affinities of proteins for each other, governed by their position in the stack. One possible explanation is that such overlapping affinities may provide a mechanism for allowing diversity of enzyme distributions within the stack between different cell types. If the cell could modulate the relative affinities of these proteins for each other, by for example modulating the composition of the lipid bilayer within the stack itself, it may be able to control the localisation of each protein within the stack. Such control would facilitate the generation of the pleomorphism observed in both the number of cisternae and the distribution of enzymes within the Golgi apparatus of different species and different cell types within the same organism.

References

Acalde, J., P. Bonay, A. Roa, S. Vilaro and I.V. Sandoval. 1992. Assembly and disassembly of the Golgi complex: Two processes arranged in a *cis-trans* direction. *J. Cell Biol.* **116:** 69-83.

Aebi, U., J. Cohn, L. Buhle and L. Gerace. 1986. The nuclear lamina is a meshwork of intermediate-type filaments. *Nature* **323:** 560-564.

Amos, W.B. and A.V. Grimstone. 1968. Intercisternal material in the Golgi body of *Trichomonas*. *J. Cell Biol.* **38:** 466-477.

Aoki, D., N. Lee, N. Yamaguchi, C. Dubois and M.N. Fukuda. 1992. Golgi retention of a trans-Golgi membrane protein, galactosyltransferase, requires cysteine and histidine residues within the membrane-anchoring domain. *Proc. Natl. Acad. Sci. USA* **89:** 4319-4323.

Ayscough, K., N.M.A. Hajibagheri, R. Watson and G. Warren. 1993. Stacking of Golgi cisternae in *Schizosaccharomyces pombe* requires intact microtubules. *J. Cell Sci.* **106:** 1227-1237.

Ayscough, K. and G. Warren. 1994. Inhibition of protein synthesis disrupts the Golgi apparatus in the fission yeast, *Schizosaccaromyces pombe*. *Yeast* **10:** 1-11.

Baeuerle, P.A. and W.B. Huttner. 1987. Tyrosine sulfation is a *trans*-Golgi-specific protein modification. *J. Cell Biol.* **105:** 2655-2664.

Balch, W.E., W.G. Dunphy, W.A. Braell and J.E. Rothman. 1984a. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* **39:** 405-416.

Balch, W.E., B.S. Glick and J.E. Rothman. 1984b. Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell* **39:** 525-536.

Balch, W.E., K.R. Wagner and D.S. Keller. 1987. Reconstitution of transport of Vesicular Stomatitis Virus G protein from the endoplasmic reticulum to the Golgi complex using a cell-free system. *J. Cell Biol.* **104:** 749-760.

Balch, W.E. 1990. Small GTP-binding proteins in vesicular transport. *Trends Biochem. Sci.* **15:** 473-477.

Balch, W.E., J.M. McCaffery, H. Plutner and M.G. Farquhar. 1994. Vesicular Stomatitis Virus Glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell* **76:** 841-852.

Baron, M.D. and H. Garoff. 1990. Mannosidase II and the 135-kDa Golgi-specific antigen recognised by monoclonal antibody 53FC3 are the same dimeric protein. *J. Biol. Chem.* **265:** 19928-19931.

Baumert, M., P.R. Maycox, F. Navone, P. De Camilli and R. Jahn. 1989. Synaptobrevin, an integral membrane protein of 18000 daltons present in small synaptic vesicles of rat brain. *EMBO J.* **8:** 379-384.

Beardmore, J., K.E. Howell, K. Miller and C.R. Hopkins. 1987. Isolation of an endocytic compartment from A431 cells using a density modification procedure employing a receptor-specific monoclonal antibody complexed with colloidal gold. *J. Cell Sci.* **87:** 495-506.

Beaufay, H., A. Amar-Costesec, D. Thines-Sempoux, M. Wibo, M. Robbi and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* **61:** 213-231.

Beck, K.A. and W.J. Nelson. 1993. Association of a spectrin isoform with the Golgi apparatus. *Mol. Biol. Cell* **4:** A1001.

Bendiak, B. and H. Schachter. 1987a. Kinetic mechanism, substrate specificity, and inhibition characteristics of UDP-N-acetylglucosamine:α-D-mannoside β1-2 N-acetylglucosaminyltransferase II from rat liver. *J. Biol. Chem.* **262:** 5784-5790.

Bendiak, B. and H. Schachter. 1987b. Purification of UDP-N-acetylglucosamine:α-D-mannoside β1-2 N-acetylglucosaminyltransferase II from rat liver. *J. Biol. Chem.* **262:** 5775-5783.

Bendiak, B., L.D. Ward and R.J. Simpson. 1993. Proteins of the Golgi apparatus. Purification to homogeneity, N-terminal sequence, and unusually large Stokes radius of the membrane-bound form of UDP-galactose:N-acetylglucosamine β1-4galactosyltransferase from rat liver. *Eur. J. Biochem.* **216:** 405-417.

Bennett, M.K., N. Calakos and R.H. Scheller. 1992. Syntaxin, a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257:** 255-259.

Bennett, V. 1990. Spectrin-Based Membrane Skeleton: A Multipotential Adaptor Between Plasma Membrane and Cytoskeleton. *Physiol. Rev.* **70:** 1029-1065.

Birky, C.W.J. 1983. Relaxed cellular controls and organelle heredity. *Science* **222:** 468-475.

Bischoff, J. and R. Kornfeld. 1984. The effect of 1-deoxymannojirimycin on rat liver mannosidases. *Biochem. Biophys. Res. Comm.* **125:** 324-331.

Blobel, G. and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine melanoma. *J. Cell Biol.* **67:** 835-851.

Block, M.R., B.S. Glick, C.A. Wilcox, F.T. Wieland and J.E. Rothman. 1988. Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. Natl. Acad. Sci. USA* **85:** 7852-7856.

Bloom, G.S. and T.A. Brashear. 1989. A novel 58-kDa protein associates with the Golgi apparatus and microtubules. *J. Biol. Chem.* **264:** 16083-16092.

Bonatti, S., G. Migliaccio and K. Simons. 1989. Palmitoylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. *J. Biol. Chem.* **264:** 12590-12595.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256:** 1604-1607.

Bos, K., C. Wraight and K.K. Stanley. 1993. TGN38 is maintained in the *trans*-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. *EMBO J.* **12:** 2219-2228.

Bouchilloux, S. 1979. Purification by affinity chromatography and some properties of microsomal galactosyltransferase from pig thyroid. *Biochim. Biophys. Acta* **569:** 135-144.

Brennwald, P. and P. Novick. 1993. Interactions of the three domains distinguishing the Ras-related GTP-binding proteins Ypt1 and Sec4. *Nature* **362:** 560-563.

Bretscher, M.S. and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science* **261:** 1280-1281.

Bretz, R. and W. Staubli. 1977. Detergent influence on rat-liver galactosyltransferase activities towards different acceptors. *Eur. J. Biochem.* **77:** 181-192.

Brown, R.M. and D.K. Romanovicz. 1976. Biogenesis and structure of Golgi-derived cellulosic scales in *Pleurochrysis*. I. Role of the endomembrane system in scale assembly and exocytosis. *Appl. Polymer Symp.* **28:** 537-585.

Burke, B., G. Griffiths, H. Reggio, D. Louvard and G. Warren. 1982. A monoclonal antibody against a 135-K Golgi membrane protein. *EMBO J.* **1:** 1621-1628.

Burke, B., K. Matlin, E. Bause, G. Legler, N. Peyrieras and H. Ploegh. 1984. Inhibition of N-linked oligosaccharide trimming does not interfere with surface expression of certain integral membrane proteins. *EMBO J.* **3:** 551-556.

Burke, J., J.M. Pettitt, H. Schachter, M. Sarkar and P.A. Gleeson. 1992. The transmembrane domain and flanking sequences of β1,2-N-acetylglucosaminyltransferase I specify *medial*-Golgi localisation. *J. Biol. Chem.* **267:** 24433-24440.

Burke, J., J.M. Pettitt, D. Humphris and P.A. Gleeson. 1994. *Medial*-Golgi retention of N-acetylglucosaminyltransferase I. Contribution from all domains of the enzyme. *J. Biol. Chem.* In Press.

Chapman, R.E. and S. Munro. 1994. Retrieval of TGN proteins from the cell surface requires endosomal acidification. *EMBO J.* **13:** 2305-2312.

Cheetham, R.D., D.J. Morré, C. Pannek and D.S. Friend. 1971. Isolation of a Golgi apparatus-rich fraction from rat liver. IV. Thiamine pyrophosphatase. *J. Cell Biol.* **49:** 899-905.

Clary, D.O. and J.E. Rothman. 1990. Purification of three related peripheral membrane proteins needed for vesicular transport. *J. Biol. Chem.* **265:** 10109-10117.

Clary, D.O., I.C. Griff and J.E. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* **61:** 709-721.

Cluett, E.B. and W.J. Brown. 1992. Adhesion of Golgi cisternae by proteinaceous interactions: Intercisternal bridges as putative adhesive structures. *J. Cell Sci.* **103:** 773-784.

Colley, K.J., E.U. Lee and J.C. Paulson. 1992. The signal anchor and stem regions of the β-galactoside α-2,6-sialyltransferase may each act to localise the enzyme to the Golgi apparatus. *J. Biol. Chem.* **267:** 7784-7793.

Collins, R.N. and G. Warren. 1992. Shingolipid transport in mitotic Hela cells. *J. Biol. Chem.* **267:** 24906-24911.

Corthésy-Theulaz, I., A. Pauloin and S.R. Pfeffer. 1992. Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* **118:** 1333-1346.

Cotman, C.W., W. Levy, G. Banker and D. Taylor. 1971. An ultrastructural and chemical analysis of the effect of Triton X-100 on synaptic plasma membranes. *Biochim. Biophys. Acta* **249:** 406-418.

Coull, J.M. and D.J.C. Pappin. 1990. A rapid flourescent staining procedure for proteins electroblotted onto PVDF membranes. *J. Prot. Chem.* **9:** 259-260.

Croul, S., S.G.E. Mezitis, A. Stieber, Y. Chen, J.O. Gonatas, B. Goud and N.K. Gonatas. 1990. Immunochemical visualisation of the Golgi apparatus in several species, including human, and tissues with an antiserum against MG-160, a sialoglycoprotein of rat liver Golgi apparatus. *J. Histochem. Cytochem.* **38:** 957-963.

Dahms, N.M., P. Lobel and S. Kornfeld. 1989. Mannose-6-phosphate receptors and lysosomal enzyme targeting. *J. Biol. Chem.* **264:** 12115-12118.

Dascher, C., R. Ossig, D. Gallwitz and H.D. Schmitt. 1991. Identification and structure of four yeast genes (*SLY*) that are able to suppress the functional loss of *YPT1*, a member of the *RAS* superfamily. *Mol. Cell. Biol.* **11:** 872-885.

De Lemos-Chiarandini, C., N.E. Ivessa, V.H. Black, Y.S. Tsao, I. Gumper and G. Kreibich. 1992. A Golgi-related structure remains after Brefeldin A-induced formation of an ER-Golgi hybrid compartment. *Eur. J. Cell Biol.* **58:** 187-201.

DeBello, W.M., H. Betz and G.J. Augustine. 1993. Synaptotagmin and neurotransmitter release. *Cell* **74:** 947-950.

Doms, R.W., G. Russ and J.W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109:** 61-72.

Donaldson, J.G., J. Lippincott-Schwartz, G.S. Bloom, T.E. Kreis and R.D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *J. Cell Biol.* **111:** 2295-2306.

Donaldson, J.G., D. Finazzi and R.D. Klausner. 1992. Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. *Nature* **360:** 350-352.

Dorling, P.R., C.R. Huxtable and S.M. Colegate. 1980. Inhibition of lysosomal α-mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsonia canescens*. *Biochem. J.* **191:** 649-651.

Duden, R., G. Griffiths, R. Frank, P. Argos and T.E. Kreis. 1991. β-COP, a 110kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell* **64:** 649-665.

Dunn, B., T. Stearns and D. Botstein. 1993. Specificity domains distinguish the Ras-related GTPases Ypt1 and Sec4. *Nature* **362:** 563-565.

Dunphy, W.G., E. Fries, L.J. Urbani and J.E. Rothman. 1981. Early and late functions associated with the Golgi apparatus reside in distinct compartments. *Proc. Natl. Acad. Sci. USA* **78:** 7453-7457.

Dunphy, W.G. and J.E. Rothman. 1983. Compartmentalisation of asparagine-linked oligosaccharide processing in the Golgi apparatus. *J. Cell Biol.* **97:** 270-275.

Dunphy, W.G., R. Brands and J.E. Rothman. 1985. Attachment of terminal N-acetylglucosamine to asparagine-linked oligosaccharides occurs in the central cisternae of the Golgi stack. *Cell* **40:** 463-472.

Dwyer, N. and G. Blobel. 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* **70:** 581-591.

Elazar, Z., L. Orci, J. Ostermann, M. Amherdt, G. Tanigawa and J.E. Rothman. 1994. ADP-ribosylation factor and coatomer couple fusion to vesicle budding. *J. Cell Biol.* **124:** 415-424.

Elferink, L.A., M.R. Peterson and R.H. Scheller. 1993. A role for synaptotagmin (p65) in regulated exocytosis. *Cell* **72:** 153-159.

Evan, G.I., G.K. Lewis, G. Ramsay and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell Biol.* **5:** 3610-3616.

Featherstone, C., G. Griffiths and G. Warren. 1985. Newly synthesised G protein of Vesicular Stomatatis Virus is not transported to the Golgi complex in mitoic cells. *J. Cell Biol.* **101:** 2036-2046.

Fisher, D.Z., N. Chaudhary and G. Blobel. 1986. cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc. Natl. Acad. Sci.* **83:** 6450-6454.

Fleischer, B. and S. Fleischer. 1970. Preparation and characterisation of Golgi membranes from rat liver. *Biochim. Biophys. Acta* **219:** 301-319.

Fleischer, B. 1981. Orientation of glycoprotein galactosyltransferase and sialyltransferase enzymes in vesicles derived from rat liver Golgi apparatus. *J. Cell Biol.* **89:** 246-255.

Franke, W.W., J. Kartenbeck, H. Zentgraf, U. Scheer and H. Falk. 1971. Membrane-to-membrane crossbridges. A means to orientation and interaction of membrane faces. *J. Cell Biol.* **51:** 881-888.

Franke, W.W., J. Kartenbeck, S. Krien, W.J. VanderWoude, U. Scheer and D.J. Morré. 1972. Inter- and intracisternal elements of the Golgi apparatus. A system of membrane-to-membrane cross-links. *Z. Zellforsch. Mikrosk. Anat.* **132:** 365-380.

Franzusoff, A. and R. Schekman. 1989. Functional components of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO J.* **8:** 2695-2702.

Franzusoff, A., E. Lauze and K.E. Howell. 1992. Immuno-isolation of Sec7p-coated transport vesicles from the yeast secretory pathway. *Nature* **355:** 173-175.

Fuhrmann, U., E. Bause, G. Legler and H. Ploegh. 1984. Novel mannosidase inhibitor blocking conversion of high mannose to complex oligosaccharides. *Nature* **307:** 755-758.

Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki and Y. Ikehara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* **263:** 18545-18552.

Gaut, J.R. and L.M. Hendershot. 1993. The modification and assembly of proteins in the endoplasmic reticulum. *Curr. Op. Cell Biol.* **5:** 589-595.

Geisler, N. and K. Weber. 1980. Purification of smooth-muscle desmin and a protein-chemical comparison of desmins from chicken gizzard and hog stomach. *Eur. J. Biochem.* **111:** 425-433.

Gerace, L. and B. Burke. 1988. Functional organisation of the nuclear envelope. *Ann. Rev. Cell Biol.* **4:** 335-734.

Gething, M.-J., K. McCammon and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell* **46:** 939-950.

Glickman, J.N., E. Conibear and B.M.F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate receptor/insulin-like growth factor II receptor. *EMBO J.* **8:** 1041-1047.

Goldberg, D.E. and S. Kornfeld. 1983. Evidence for extensive subcellular organisation of aspargine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* **258:** 3159-3165.

Gonatas, J.O., S.G.E. Mezitis, A. Stieber, B. Fleischer and N.K. Gonatas. 1989. MG-160. A novel sialoglycoprotein of the *medial* cisternae of the Golgi apparatus. *J. Biol. Chem.* **264:** 646-653.

Griffiths, G., G. Warren, P. Quinn, O. Mathieu-Costello and H. Hoppeler. 1984. Density of newly synthesised plasma membrane proteins in intracellular membranes I. Stereological studies. *J. Cell Biol.* **98:** 2133-2141.

Griffiths, G. and K. Simons. 1986. The *trans* Golgi network: Sorting at the exit site of the Golgi complex. *Science* **234:** 438-443.

Hara-Kuge, S., O. Kuge, L. Orci, M. Amherdt, M. Ravazzola, F.T. Wieland and J.E. Rothman. 1994. *En bloc* incorporation of coatomer subunits during assembly of COP-coated vesicles. *J. Cell Biol.* **124:** 883-892.

Harpaz, N. and H. Schachter. 1980a. Bovine colostrium UDP-N-acetylglucosamine:α-D-mannoside β2-N-acetylglucosaminyltransferase I. Separation from UDP-N-acetylglucosamine:α-D-mannoside β1-2 N-acetylglucosaminyltransferase II, partial purification and substrate specificity. *J. Biol. Chem.* **255:** 4885-4893.

Harpaz, N. and H. Schachter. 1980b. Control of Glycoprotein Synthesis. Bovine colostrum UDP-N-acetylglucosamine:α-D-mannoside β2-N-acetylglucosaminyltransferase I. Separation from UDP-N-aceylglucosaminyl:α-D-mannoside β2-N-acetylglucosaminyltransferse II, partial purification, and substrate specificity. *J. Biol. Chem.* **255:** 4885-4893.

Harpaz, N. and H. Schachter. 1980c. Control of glycoprotein synthesis. Processing of asparagine-linked oligosaccharides by one or more rat liver Golgi α-D-mannosidases

dependent on the prior action of UDP-N-acetylglucosamine:α-D-mannoside β2-N-acetylglucosaminyltransferase I. *J. Biol. Chem.* **255:** 4894-4902.

Hasilik, A. 1980. Biosynthesis of lysosomal enzymes. *Trends Biochem. Sci.* **5:** 237-240.

Hatton, M.W.C., L. Marz, L.R. Berry, M.T. Debanne and E. Regoeczi. 1979. Bi- and tri-antennary human transferrin glycopeptides and their affinities for the hepatic lectin specific for asialo-glycoproteins. *Biochem. J.* **181:** 633-638.

Hauri, H.-P. and A. Schweizer. 1992. The endoplasmic reticulum-Golgi intermediate compartment. *Curr. Op. Cell Biol.* **4:** 600-608.

Helenius, A. and K. Simons. 1975. Solubilisation of membranes by detergents. *Biochim. Biophys. Acta* **415:** 29-79.

Helenius, A., D.R. McCaslin, E. Fries and C. Tanford. 1979. Properties of detergents. *Meth. Enzymol.* **56:** 734-749.

Helms, J.B. and J.E. Rothman. 1992. Inhibition by Brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. *Nature* **360:** 352-354.

Helms, J.B., D.J. Palmer and J.E. Rothman. 1993. Two distinct populations of ARF bound to Golgi membranes. *J. Cell Biol.* **121:** 751-760.

Hendricks, L.C., S.L. McClanahan, M. M., G.E. Palade and M.G. Farquhar. 1992. Golgi proteins persist in the tubulovesicular remnants found in Brefeldin A-treated pancreatic acinar cells. *Eur. J. Cell Biol.* **58:** 202-213.

Hicke, L. and R. Scheckman. 1990. Molecular machinery required for protein transport from the endoplasmic reticulum to the Golgi complex. *BioEssays* **12:** 253-258.

Hidalgo, J., R. Garcia-Navarro, F. Garcia-Navarro, J. Perez-Vilar and A. Velasco. 1992. Presence of Golgi remnant membranes in the cytoplasm of Brefeldin A-treated cells. *Eur. J. Cell Biol.* **58:** 214-227.

Hino, Y., A. Asano, R. Sato and S. Shimizu. 1978. Biochemical studies of rat liver Golgi apparatus. I. Isolation and preliminary characterization. *J. Biochem. Tokyo* **83:** 909-923.

Ho, W.C., V.J. Allan, G. van Meer, E.G. Berger and T.E. Kreis. 1989. Reclustering of scattered Golgi elements occurs along microtubules. *Eur. J. Cell Biol.* **48:** 250-263.

Hortsch, M., C. Crimaudo and D.I. Meyer. 1987. Structure and function of the rough endoplasmic reticulum. *Proc. FAOB Congress* **4**

Howell, K.E., R. Schmid, J. Ugelstad and J. Gruenberg. 1989. Immunoisolation using magnetic solid supports: Subcellular fractionation for cell-free functional studies. *Meth. Cell Biol.* 265-292.

Hurtley, S.M. and A. Helenius. 1989. Protein oligomerisation in the endoplasmic reticulum. *Ann. Rev. Cell Biol.* **5:** 277-307.

Jackson, M.R., T. Nilsson and P.A. Peterson. 1990. Identification of a consensus sequence motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* **9:** 3153-3162.

Jackson, M.R., T. Nilsson and P.A. Peterson. 1993. Retrieval of transmembrane proteins to the Endoplasmic reticulum. *J Cell Biol* **121:** 317-333.

Johnston, P.A., A. Stieber and N.K. Gonatas. 1994. A hypothesis on the traffic of MG160, a *medial* Golgi sialoglycoprotein, from the *trans*-Golgi network to the Golgi cisterae. *J. Cell Sci.* **107:** 529-537.

Kahn, R.A. and A.G. Gilman. 1984. Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J. Biol. Chem.* **259:** 6228-6234.

Karrenbauer, A., D. Jeckel, W. Just, R. Birk, R.R. Schmidt, J.E. Rothman and F.T. Wieland. 1990. The rate of bulk flow from the Golgi to the plasma membrane. *Cell* **63:** 259-267.

Khatra, B.S., D.G. Herries and K. Brew. 1974. Some kinetic properties of human milk galactosyl transferase. *Eur. J. Biochem.* **44:** 537-560.

Kornfeld, R. and S. Kornfeld. 1976. Comparative aspects of glycoprotein structure. *Ann. Rev. Biochem.* 217-237.

Kornfeld, R. and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* **54:** 631-664.

Kornfeld, S., E. Li and I. Tabas. 1978. The synthesis of complex-type oligosaccharides. II. Characterisation of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein. *J. Biol. Chem.* **253:** 7771-7778.

Kreis, T.E. and H.F. Lodish. 1986. Oligomerisation is essential for transport of Vesicular Stomatitis Viral Glycoprotein to the cell surface. *Cell* **46:** 929-937.

Kuge, O., S. Hara-Kuge, L. Orci, M. Ravazzola, M. Amherdt, G. Tanigawa, F.T. Wieland and J.E. Rothman. 1993. ζ-COP, a subunit of coatomer, is required for COP-coated vesicle assembly. *J. Cell Biol.* **123:** 1727-1734.

Kumar, R. and P. Stanley. 1989. Transfection of a human gene that corrects the Lec1 glycosylation defect: Evidence for transfer of the strucural gene for N-acetylglucosaminyltransferase I. *Mol. Cell Biol.* **9:** 5713-5717.

Kumar, R., J. Yang, R.D. Larsen and P. Stanley. 1990. Cloning and expression of N-acetylglucosaminyltransferase I, the *medial* Golgi transferase that initiates complex N-linked carbohydrate formation. *Proc. Natl. Acad. Sci. USA* **87:** 9948-9952.

Kurzchalia, T.V., P. Dupree, R.G. Parton, R. Kellenr, H. Virta, M. Lehnert and K. Simons. 1992. VIP21, a 21-kD membrane protein is an integral component of *trans*-Golgi-network-derived transport vesicles. *J. Cell Biol.* **118:** 1003-1014.

Landegren, U. 1984. Measurement of cell numbers by means of the endogenous enzyme hexoseaminidase. Applications to the detection of lymphokines and cell surface antigens. *J. Immun. Methods* **67:** 379-388.

Lazarovits, J. and M. Roth. 1988. A single amino acid change in the cytoplasmic domain allows the Infulenza Virus hemagglutinin to be endocytosed through coated pits. *Cell* **53:** 743-752.

Leelavathi, D.E., L.W. Estes, D.S. Feingold and B. Lombardi. 1970. Isolation of a Golgi-rich fraction from rat liver. *Biochim. Biophys. Acta* **211:** 124-138.

Lewis, M.J., D.J. Sweet and H.R.P. Pelham. 1990. The *ERD2* gene determines the specificity of the lumenal ER protein retention system. *Cell* **61:** 1359-1363.

Lewis, M.J. and H.R.B. Pelham. 1992. Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell* **68:** 353-364.

Li, E., I. Tabas and S. Kornfeld. 1978. The synthesis of complex-type oligosaccharides. I. Structure of the lipid-linked precursor of the complex-type oligosaccharides of the visicular stomatatis virus G protein. *J. Biol. Chem.* **253:** 7762-7770.

Lippencott-Schwartz, J., L.C. Yuan, J.S. Bonifacino and R.D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: Evidence for membrane cycling from Golgi to ER. *Cell* **56:** 801-813.

Lippencott-Schwartz, J., J.G. Donaldson, A. Schweizer, E.G. Berger, H.-P. Hauri, L.C. Yuan and R.D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of Brefeldin A suggests an ER recycling pathway. *Cell* **60:** 821-836.

Lippencott-Schwartz, J. 1992. Bidirectional membrane traffic between the endoplasmic reticulum and the Golgi apparatus. *Trends Cell Biol.* **3:** 81-87.

Lodish, H.F., N. Kong, S. M. and G.J.A.M. Strous. 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. *Nature* **304:** 80-83.

Longmore, G.D. and H. Schachter. 1982. Product-identification and substrate-specificity studies of the GDP-L-fucose:2-acetamido-2-deoxy-β-D-glucoside (Fuc→Asn-linked GlcNAc) 6-α-L-fucosyltransferase in a Golgi rich fraction from porcine liver. *Carbohydr. Res.* **100:** 365-392.

Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S. Schmid, V. Quaranta and P.A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invarient chain. *Nature* 600-605.

Lucocq, J.M., J.G. Pryde, E.G. Berger and G. Warren. 1987. A mitotic form of the Golgi apparatus in Hela cells. *J. Cell Biol.* **104:** 865-874.

Lucocq, J.M. and G. Warren. 1987. Fragmentation and partitioning of the Golgi apparatus during mitosis in Hela cells. *EMBO J.* **6:** 3239-3246.

Lucocq, J.M., E.G. Berger and G. Warren. 1989. Mitotic Golgi fragments in HeLa cells and their role in the reassembly pathway. *J. Cell Biol.* **109:** 463-474.

Luzio, J.P., B. Brake, G. Banting, K.E. Howell, P. Braghetta and K.K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the *trans*-Golgi network (TGN38). *Biochem. J.* **270:** 97-102.

Machamer, C.E. 1991. Golgi retention signals: Do membranes hold the key? *Trends Cell Biol.* **1:** 141-144.

Machamer, C.E. 1993. Targeting and retention of Golgi membrane proteins. *Curr. Op. Cell Biol.* **5:** 606-612.

Mackay, D., R. Kieckbusch, J. Adamczewski and G. Warren. 1993. Cyclin A-mediated inhibition of intra-Golgi transport requirs $p34^{cdc2}$. *FEBS Lett.* **336:** 549-554.

Malhotra, V., L. Orci, B.S. Glick, M.R. Block and J.E. Rothman. 1988. Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell* **54:** 221-227.

Malhotra, V., T. Serafini, L. Orci, J.C. Shepherd and J.E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell* **58:** 329-336.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262:** 10035-10038.

McKeon, F.D., M.W. Kirschner and D. Caput. 1986. Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature* **319:** 463-468.

Melançon, P., B.S. Glick, V. Malhotra, P.J. Weidman, T. Serafini, M.L. Gleason, L. Orci and J.E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell* **51:** 1053-1062.

Mellman, I. and K. Simons. 1992. The Golgi complex: In vitro veritas? *Cell* **68:** 829-840.

Mignot, J.P. 1965. Étude ultrastructurale des Euglèniens: II. A, Dictysomes et dictyocinèse chez *Distigma proteus* Ehrbg. B, Mastigonèmes chez *Anisonema costatum* Christen. *Protistologica* **1 (2):** 17-22.

Misteli, T. and G. Warren. 1994. COP-coated vesicles are involved in the mitotic fragmentation of Golgi stacks in a cell-free system. *J. Cell Biol.* **125:** 269-282.

Misumi, Y., Y. Misumi, K. Miki, A. Takasuki, G. Tamura and Y. Ikehara. 1986. Novel blockade by Brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* **261:** 11398-11403.

Mollenhauer, H.H. 1965. An intercisternal structure in the Golgi apparatus. *J. Cell Biol.* **24:** 504-511.

Mollenhauer, H.H., D.J. Morré and C. Totten. 1973. Intercisternal substances of the Golgi apparatus. Unstacking of plant dichtosomes using chaotropic agents. *Protoplasma* **78:** 443-459.

Mollenhauer, H.H. and D.J. Morré. 1978. Structural compartmentation of the cytosol: Zones of exclusion, zones of adhesion, cytoskeletal and intercisternal elements. *Subcell. Biochem.* **5:** 327-359.

Moremen, K.W., O. Touster and P.W. Robbins. 1991. Novel purification of the catalytic domain of Golgi α-mannosidase II. *J Biol Chem* **266:** 16876-16885.

Moremen, K.W. and P.W. Robbins. 1991. Isolation, characterisation and expression of cDNAs encoding murine α-mannosidase II, a Golgi enzyme that controls conversion of high mannose to complex N-glycans. *J. Cell Biol.* **115:** 1521-1534.

Morré, D.J. and H.H. Mollenhauer. 1964. Isolation of the Golgi apparatus from plant cells. *J. Cell Biol.* **23:** 195-305.

Morré, D.J., R.L. Hamilton, H.H. Mollenhauer, R.W. Mahley, W.P. Cunningham, R.D. Cheetham and V.S. Lesquire. 1970. Isolation of a Golgi-rich fraction from rat-liver. *J. Cell Biol.* **44:** 484-491.

Morré, D.J. and L. Ovtracht. 1977. Dynamics of the Golgi apparatus: Membrane differentiation and membrane flow. *Int. Rev. Cytol.* **5:** 61-188.

Munro, S. and H.R.B. Pelham. 1987. A C-terminal signal prevents secretion of lumenal ER proteins. *Cell* **48:** 899-907.

Munro, S. 1991. Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify Golgi retention. *EMBO J* **10:** 3577-88.

Navaratnam, N., S. Ward, C. Fisher, N.J. Kuhn, J.N. Keen and J.B.C. Findlay. 1988. Purification, properties and cation activation of galactosyltransferase from lactating-rat mammary Golgi membranes. *Eur. J. Biochem.* **171:** 623-629.

Niehrs, C. and W.B. Huttner. 1990. Purification and characterisation of tyrosylprotein sulfotransferase. *EMBO J.* **9:** 35-42.

Nilsson, T., M. Jackson and P.A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reiculum. *Cell* **58:** 707-718.

Nilsson, T., J. Lucocq, D. MacKay and G. Warren. 1991. The membrane spanning domain of β-1,4-galactosyltransferase specifies *trans* Golgi localisation. *EMBO J* **10:** 3567-3575.

Nilsson, T., M. Pypaert, M.H. Hoe, P. Slusarewicz, E. Berger and G. Warren. 1993a. Overlapping distribution of two glycosyltransferases in the Golgi apparatus of Hela cells. *J. Cell Biol.* **120:** 5-13.

Nilsson, T., P. Slusarewicz, M.H. Hoe and G. Warren. 1993b. Kin Recognition. A model for the retention of Golgi enzymes. *FEBS Lett.* **330:** 1-4.

Nilsson, T., M.H. Hoe, P. Slusarewicz, C. Rabouille, R. Watson, F. Hunte, G. Watzele, E.G. Berger and G. Warren. 1994. Kin recognition between *medial* Golgi enzymes in HeLa cells. *EMBO J.* **13:** 562-574.

Nilsson, T. and G. Warren. 1994. Retention and retrieval in the endoplasmic reticulum and Golgi apparatus. *Curr. Op. Cell Biol.* **6:** In Press.

Novick, P., C. Field and R. Scheckman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21:** 205-215.

Novikoff, A.B. and S. Goldfischer. 1961. Nucleosidediphosphatase activity in the Golgi apparatus and its usefulness for cytological studies. *Biochemistry* **47:** 802-810.

Novikoff, P.M., D.R.P. Tulsiani, O. Touster, A. Yam and A.B. Novikoff. 1983. Immunocytochemical localisation of α-D-mannosidase II in the Golgi apparatus of rat liver. *Pro.c Natl. Acad. Sci. USA* **80:** 4364-4368.

Oppenheimer, C.L. and R.L. Hill. 1981. Purification and characterisation of a rabbit liver α1-3 mannoside β1-2 N-acetylglucosaminyltransferase. *J. Biol. Chem.* **256:** 799-804.

Orci, L., R. Montesano, P. Meda, F. Malaisse-Lagae, D. Brown, A. Perrelet and P. Vassalli. 1981. Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. *Proc. Natl. Acad. Sci. USA* **78:** 293-297.

Orci, L., B.S. Glick and J.E. Rothman. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: Its possible role in protein transport within the Golgi stack. *Cell* **46:** 171-184.

Orci, L., V. Malhotra, M. Amherdt, T. Serafini and J.E. Rothman. 1989. Dissection of a single round of vesicular transport: Sequential intermediates for intercisternal movement in the Golgi stack. *Cell* **56:** 357-368.

Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J.G. Donaldson, S.J. Lippencott, R.D. Klausner and J.E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents assembly the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* **64:** 1183-1195.

Orci, L., D.J. Palmer, M. Amherdt and J.E. Rothman. 1993a. Coated vesicle assembly in the Golgi requires only coatomer and ARF proteins from the cytosol. *Nature* **364:** 732-734.

Orci, L., D.J. Palmer, M. Ravazzola, A. Perrelet, M. Amherdt and J.E. Rothman. 1993b. Budding from Golgi membranes requires the coatomer complex of non-clathrin coat proteins. *Nature* **362:** 648-652.

Osafune, T., J.A. Schiff and E. Hase. 1991. Stage dependent localisation of LHCP II apoprotein in the Golgi of synchronised cells of *Euglena gracilis* by immunogold electron microscopy. *Exp. Cell Res.* **193:** 320-330.

Ossig, R., C. Dascher, H.-H. Trepte, H.D. Schmitt and D. Gallwitz. 1991. The yeast *SLY* gene products, suppressors of defects in the essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. Cell. Biol.* **11:** 2980-2993.

Ostermann, J., L. Orci, K. Tani, M. Amherdt, M. Ravazzola, Z. Elezar and J.E. Rothman. 1994. Stepwise assembly of functionally active transport vesicles. *Cell* **75:** 1015-1025.

Ovtracht, D., D.J. Morré, R.D. Cheetham and H.H. Mollenhauer. 1973. Subfractionation of Golgi appratus from rat liver: Method and morphology. *J. Microscopie* **18:** 87-102.

Oyler, G.A., G.A. Higgins, R.A. Hart, E. Battenberg, M. Billingsley, F.E. Bloom and M.C. Wilson. 1989. The identification of a novel synaptosomal associated protein, SNAP-25, differentially expressed in neuronal subpopulations. *J. Cell Biol.* **109:** 3039-3052.

Pääbo, S., B.M. Bhat, W.S.M. Wold and P.A. Peterson. 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell* **50:** 311-317.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science* **189:** 347-358.

Palcic, M.M., L.D. Heerze, M. Pierce and O. Hindsgaul. 1988. The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays. *Glycoconjugate J.* **5:** 49-63.

Palmer, D.J., J.B. Helms, C.J.M. Beckers, L. Orci and J.E. Rothman. 1993. Binding of coatomer to Golgi membranes requires ADP-ribosylation factor. *J. Biol. Chem.* **268:** 12083-12089.

Pâquet, M.R., S. Narasimhan, H. Schachter and M.A. Moscarello. 1984. Branch specificity of purified rat liver Golgi UDP-galactose:N-acetylglucosaminyl β-1,4-galactosyltransferase. Preferential transfer of galactose on the GlcNAcβ1,2-Manα1,3-branch of a complex biantennary Asn-linked oligosaccharide. *J. Biol. Chem.* **259:** 4716-4721.

Pearse, B.M.F. and M.S. Robinson. 1990. Clathrin, adaptors, and sorting. *Ann. Rev. Cell Biol.* **6:** 151-171.

Peat, D. PhD Thesis. Univ. London. 1993

Pelham, H.R.B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* **7:** 913-918.

Pelham, H.R.B. 1989. Control of protein exit from the Endoplasmic reticulum. *Ann. Rev. Cell Biol.* **5:** 1-23.

Pelham, H.R.B. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* **15:** 483-486.

Pfanner, N., L. Orci, B.S. Glick, M. Amherdt, S.R. Arden, V. Malhotra and J.E. Rothman. 1989. Fatty-acyl coenzyme A is required for budding of transport vesicles from Golgi cisternae. *Cell* **59:** 95-102.

Pfanner, N., B.S. Glick, S.R. Arden and J.E. Rothman. 1990. Fatty acylation promotes fusion of transport vesicles with Golgi cisternae. *J. Cell Biol.* **110:** 955-961.

Pfeffer, S.R. and J.E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Ann. Rev. Biochem.* **56:** 829-852.

Ponnambalam, S., C. Rabouille, J.P. Luzio, T. Nilsson and G. Warren. 1994. The TGN38 glycoprotein contains two non-overlapping signals that mediate localisation to the *trans*-Golgi network. *J. Cell Biol.* **125:** 253-268.

Pryde, J. 1986. Triton X-114: A detergent that has come in from the cold. *Trends Biochem. Sci.* **11:** 160-163.

Pryer, N.K., L.J. Wuestehube and R. Scheckman. 1992. Vesicle-mediated protein sorting. *Ann. Rev. Biochem.* **61:** 471-516.

Pypaert, M., D. Mundy, E. Souter, J.-C. Labbe and G. Warren. 1991. Mitotic cytosol inhibits invagination of coated pits in broken mitotic cells. *J. Cell Biol.* **114:** 1159-1166.

Quinn, P., G. Griffiths and G. Warren. 1984. Density of newly synthesised plasma membrane proteins in intracellular membranes II. Biochemical studies. *J. Cell Biol.* **98:** 2142-2147.

Rabouille, C., N. Hui, R. Kieckbusch, E. Berger, G. Warren and T. Nilsson. 1994. Mapping the Golgi apparatus of Hela cells: Immuno localisation of TGN38 and four enzymes involved in the construction of complex, N-linked oligosaccharides. *J. Cell Biol.* Submitted.

Rambourg, A., Y. Clermont and L. Hermo. 1981. Three-dimensional structure of the Golgi apparatus. *Methods Cell Biol.* **23:** 155-166.

Rambourg, A. and Y. Clermont. 1990. Three-dimensional electron microscopy: Structure of the Golgi apparatus. *Eur. J. Cell Biol.* **51:** 189-200.

Rambourg, A., Y. Clermont and F. Képès. 1993. Modulation of the Golgi apparatus in *Saccharomyces cerevisiae sec7* mutants as seen by three-dimensional electron microscopy. *Anat. Rec.* **237:** 441-452.

Rothman, J.E. and L. Orci. 1991. Movement of proteins through the Golgi stack: A molecular dissection of vesicular transport. *FASEB J.* **4:** 1460-1468.

Rothman, J.E. and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature* **355:** 409-415.

Rothman, J.E. and G. Warren. 1994. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* **4:** 220-233.

Russo, R.N., N.L. Shaper, D.J. Taatjes and J.H. Shaper. 1992. β1,4-Galactosyltransferase: A short NH2-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for Golgi retention. *J. Biol. Chem.* **267:** 9241-9247.

Sadler, J.E., J.I. Rearick, J.C. Paulson and R.L. Hill. 1979. Purification of homogeneity of a β-galactoside α2-3 sialyltransferase and partial purification of an α-N-acetylgalactosamine α2-6 sialyltransferase from porcine submaxillary glands. *J. Biol. Chem.* **254:** 4434-4443.

Sandoval, I.V., J.S. Bonifacino, R.D. Klausner, M. Henkart and J. Wehland. 1984. Role of microtubules in the organization and localization of the Golgi apparatus. *J. Cell Biol.* **99:** 113-118s.

Saraste, J. and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest Virus membrane glycoproteins to the cell surface. *Cell* **38:** 535-549.

Saraste, J., G.E. Palade and M.G. Farquhar. 1987. Antibodies to rat pancreas Golgi subfractions: Identification of a 58-kD *cis*-Golgi protein. *J. Cell Biol.* **105:** 2021-2029.

Saraste, J. and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* **100:** 415-430.

Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta and C. Montecucco. 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* **359:** 832-835.

Reaves, B., A. Wilde and G. Banting. 1992. Identification, molecular characterization and immunolocalisation of an isoform of the *trans*-Golgi-network (TGN)-specific integral membrane protein TGN38. *Biochem. J.* **283:** 313-316.

Rogalski, A.A. and S.J. Singer. 1984. Associations of elements of the Golgi apparatus with microtubules. *J. Cell Biol.* **99:** 1092-1100.

Rogalski, A.A., J.E. Bergmann and S.J. Singer. 1984. Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. *J. Cell Biol.* **99:** 1101-1109.

Rose, J.K. and R.W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Ann. Rev. Cell Biol.* **4:** 257-288.

Roth, J. and E.G. Berger. 1982. Immunocytochemical localisation of galactosytransferase in Hela cells: Codistribution with thiamine pyrophosphatase in *trans*-Golgi cisternae. *J. Cell Biol.* **92:** 223-229.

Roth, J., D.J. Taatjes, J.M. Lucocq, J. Weinstein and J.C. Paulson. 1985. Demonstration of an extensive *trans*-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell* **43:** 287-295.

Roth, J., D.J. Taatjes, J. Weinstein, J.C. Paulson, P. Greenwell and W.M. Watkins. 1986. Differential subcompartmentalisation of terminal glycosylation in the Golgi apparatus of intestinal absorptive cells and goblet cells. *J. Biol. Chem.* **261:** 14307-14312.

Roth, J. 1987. Subcellular organisation of glycosylation in mammalian cells. *Biochim. Biophys. Acta* **906:** 405-436.

Rothman, J.E., R.L. Miller and L.J. Urbani. 1984[a] Intercompartmental transport in the Golgi complex is a dissociative process: Facile transfer of membrane protein between two Golgi populations. *J. Cell Biol.* **99:** 260-271.

Rothman, J.E., L.J. Urbani and R. Brands. 1984[b] Transport of protein between cytoplasmic membranes of fused cells: Correspondence to processes reconstituted in a cell-free system. *J. Cell Biol.* **99:** 248-259.

Schutze, M.P., P.A. Peterson and M.R. Jackson. 1994. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO J.* **13:** 1696-1705.

Semenza, J.C., K.G. Harwick, N. Dean and H.R.B. Pelham. 1990. *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* **61:** 1349-1357.

Serafini, T., L. Orci, M. Amherdt, M. Brunner, R.A. Kahn and J.E. Rothman. 1991a. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: A novel role for a GTP-binding protein. *Cell* **67:** 239-253.

Serafini, T., G. Stenbeck, A. Brecht, F. Lottspeich, L. Orci, J.E. Rothman and F.T. Wieland. 1991b. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein β-adaptin. *Nature* **349:** 215-220.

Simon, S. 1993. Translocation of proteins across the endoplasmic reticulum. *Curr. Op. Cell Biol.* **5:** 581-588.

Skibbens, J.E., M.G. Roth and K.S. Matlin. 1989. Differential extractability of influenza virus haemaglutinin during intracellular transport in polarised epithelial cells and nonpolar fibroblasts. *J. Cell Biol.* **108:** 821-832.

Slot, J.W. and H.J. Geuze. 1983. Immunoelectron microscopic exploration of the Golgi complex. *J. Histochem. Cytochem.* **31:** 1049-1056.

Slusarewicz, P., T. Nilsson, N. Hui, R. Watson and G. Warren. 1994b. Isolation of a matrix that binds *medial* Golgi enzymes. *J. Cell Biol.* **124:** 405-413.

Slusarewicz, P., N. Hui and G. Warren. 1994a. Purification of rat liver Golgi stacks. *In* Cell Biology: A Laboratory Handbook., Celis, J.E., ed. Academic Press Inc., Orlando, Florida. pp. In Press.

Smith, C.E. 1980. Ultrastructural localisation of nicotinamide adenine dinucleotide phosphatase (NADPase) activity to the intermediate saccules of the Golgi apparatus in rat incisor ameloblasts. *J. Histochem. Cytochem.* **28:** 16-26.

Söllner, T., M.K. Bennett, S. Whiteheart, R.H. Scheller and J.E. Rothman. 1993a. A protein aasembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion. *Cell* **75:** 409-418.

Söllner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst and J.E. Rothman. 1993b. SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362:** 318-324.

Sottocasa, G.L., B. Kuylenstierna, L. Ernster and A. Bergstrand. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* **32:** 415-438.

Souter, E., M. Pypaert and G. Warren. 1993. The Golgi stack reassembles during telophase before arrival of proteins transported from the endoplasmic reticulum. *J. Cell Biol.* **122:** 533-540.

Spiro, R.G. 1960. Studies on fetuin, a glycoprotein of fetal serum. *J. Biol. Chem.* **235:** 2860-2869.

Spiro, R.G. 1973. Glycoproteins. *Adv. Protein Chem.* **27:** 349-467.

Staehelin, L.A., T.H. Giddings, J.Z. Kiss and F.D. Sack. 1990. Macromolecular differentiation of Golgi stacks in root tips of *Arabidopsis* and *Nicotiana* seedlings as visualised in high pressure frozen and freeze-substituted samples. *Protoplasma* **157:** 75-91.

Starger, J.M. and R.D. Goldman. 1977. Isolation and preliminary characterisation of 10-nm filaments from baby hamster kidney (BHK-21) cells. *Proc. Natl. Acad. Sci. USA* **74:** 2422-2426.

Stenbeck, G., R. Schreiner, D. Herrmann, S. Auerbach, F. Lottspeich, J.E. Rothman and F.T. Wieland. 1992. γ-COP, a coat subunit of non-clathrin-coated vesicles with homology to Sec21p. *FEBS Lett.* **314:** 195-198.

Stuart, R.A., D. Mackay, J. Adamszewski and G. Warren. 1993. Inhibition of intra-Golgi transport *in vitro* by mitotic kinase. *J. Biol. Chem.* **268:** 4050-4054.

Swift, A.M. and C.E. Machamer. 1991. A Golgi retention signal in a membrane-spanning domain of Coronavirus E1 protein. *J. Cell Biol.* **115:** 19-30.

Szumilo, T. and A.D. Elbein. 1985. A simple and reliable assay for glycoprotein-processing glycosidases. *Anal. Biochem.* **151:** 32-40.

Tabas, I. and S. Kornfeld. 1978. The synthesis of complex-type oligosaccharides. III. Identification of an α-D-mannosidase activity involved in a late stage of processing of complex-type oligosaccharides. *J. Biol. Chem.* **253:** 7779-7786.

Tabas, I., S. Schlesinger and S. Kornfeld. 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesised polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. *J. Biol. Chem.* **253:** 716-722.

Tabas, I. and S. Kornfeld. 1979. Purification and charaterisation of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem.* **254:** 11655-11663.

Tanford, C., Y. Nozaki, J.A. Reynolds and S. Makino. 1974. Molecular characterisation of proteins in detergent solution. *Biochemistry* **13:** 2369-2376.

Tang, B.L., S.H. Wong, S.H. Low and W. Hong. 1992. The transmembrane domain of N-glucosaminyltransferase I contains a Golgi retention signal. *J. Biol. Chem.* **267:** 10122-10126.

Tanigawa, G., L. Orci, M. Amherdt, M. Ravazzola, J.B. Helms and J.E. Rothman. 1993. Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi derived COP-coated vesicles. *J. Cell Biol.* **123:** 1365-1371.

Teasdale, R.D., G. D'Agostaro and P.A. Gleeson. 1992. The signal for Golgi retention of bovine β1-4-galactosyltransferase is the transmembrane domain. *J Biol. Chem.* **267:** 4084-4096.

Tezuka, T. and I.M. Freedberg. 1972. Epidermal structural proteins. II. Isolation and purification of tonofilaments of the newborn rat. *Biochim. Biophys. Acta* **263:** 382-396.

Thompson, R.C. 1988. EFTu provides an internal kinetic standard for translational accuracy. *Trends Biochem. Sci.* **13:** 91-93.

Thyberg, J. and S. Moskalewski. 1985. Microtubules and the organization of the Golgi complex. *Exp. Cell Res.* **159:** 1-16.

Tulsiani, D.R.P., D.J. Opheim and O. Touster. 1977. Purification and characterisation of α-D-mannosidase from rat liver Golgi membranes. *J. Biol. Chem.* **252:** 3227-3233.

Tulsiani, D.R.P., T.M. Harris and O. Touster. 1982a. Swainsonine inhibits the biosynthesis of complex glycoproteins by inhibition of Golgi mannosidase II. *J. Biol. Chem.* **257:** 7936-7939.

Tulsiani, D.R.P., S.C. Hubbard, P.W. Robbins and O. Touster. 1982b. α-D-mannosidases of rat liver Golgi membranes. *J. Biol. Chem.* **257:** 3660-3668.

Tulsiani, D.R.P. and O. Touster. 1986. Purification of α1,2-specific mannosidase from rat liver Golgi membranes. *Fe.d Proc.* **45:** 1680.

Tulsiani, D.R. and O. Touster. 1988. The purification and characterisation of Mannosidase 1A from rat liver Golgi membranes. *J. Biol. Chem.* **263:** 5408-5417.

Turner, J.R. and A.M. Tartakoff. 1989. The response of the Golgi complex to microtubule alterations: The roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* **109:** 2081-2088.

Ulmer, J.B. and G.E. Palade. 1991. Effects of Brefeldin A on the Golgi complex, endoplasmic reticulum and viral envelope glycoproteins in murine erythroleukemia cells. *Eur. J. Cell Biol.* **54:** 38-54.

van den Eijnden, D.H., D.H. Joziasse, L. Dorland, H. van Halbeek, J.F.G. Vliegenthart and S. K. 1980. Specificity in the enzymatic transfer of sialic acid to the oligosaccharide branches of bi- and triantennary glycopeptides of α_1-acid glycoprotein. *Biochem. Biophys. Res. Comm.* **92:** 839-845.

Velasco, A., L. Hendricks, K.W. Moremen, D.R.P. Tulsiani, O. Touster and M.G. Farquhar. 1993. Cell type-dependent variation in the subcellular distribution of α-mannosidase I and II. *J. Cell Biol.* **122:** 39-51.

Vischer, P. and C.R. Hughs. 1981. Glycosyl transferases of baby-hampster-kidney (BHK) cells and ricin-resistant mutants. *Eur. J. Biochem.* **117:** 275-284.

von Heijne, G. 1985. Signal sequences. The limits of variation. *J. Mol. Biol.* **184:** 99-105.

Walter, P. and V.R. Lingappa. 1986. Mechanisms of protein translocation across the endoplasmic reticulum membrane. *Ann. Rev. Cell Biol.* **2:** 499-516.

Warren, G. 1985. Membrane traffic and organelle division. *Trends Biochem. Sci.* **10:** 439-443.

Warren, G. 1987. Signals and salvage sequences. *Nature* **327:** 17-18.

Warren, G. 1989. Mitosis and membranes. *Nature* **342:** 857-858.

Warren, G. 1993a. Bridging the Gap. *Nature* **362:** 297-298.

Warren, G. 1993b. Membrane partitioning during cell division. *Ann. Rev. Biochem. Sci.* 323-348.

Waters, M.G., T. Serafini and J.E. Rothman. 1991. 'Coatamer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature* **349:** 248-251.

Weidman, P., R. Roth and J. Heuser. 1993. Golgi membrane dynamics imaged by freeze-etch electron microscopy: Views of different membrane coatings involved in tubulation versus vesiculation. *Cell* **75:** 123-133.

Weidman, P.J., P. Melançon, M.R. Block and J.E. Rothman. 1989. Binding of N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J. Cell Biol.* **108:** 1589-1596.

Weiner, O.H., J. Murphy, G. Griffiths, M. Schleicher and A.A. Noegel. 1993. The actin-binding protein comitin (p24) is a component of the Golgi apparatus. *J. Cell Biol.* **123:** 23-34.

Weinstein, J., U. De Souza-e-Silva and J.C. Paulson. 1982a. Purification of a Gal β1-4 GlcNac α2-6 sialyltransferase and a Gal β3(4) GlcNac α2-3 sialytransferase to homogeneity from rat liver. *J. Biol. Chem.* **257:** 13835-13844.

Weinstein, J., U. De Souza-e-Silva and J.C. Paulson. 1982b. Sialylation of glycoprotein oligosaccharides N-linked to asparagine. Enzymatic characterisation of Gal β1-3(4) GlcNac α2-3 sialyltransferase and a Gal β1-4 GlcNac α2-6 sialyltransferase from rat liver. *J. Biol. Chem.* **257:** 13845-13853.

Weinstein, J., E.U. Lee, K. McEntee, P.-H. Lai and J.C. Paulson. 1987. Primary structure of β-galactoside α2,6-sialyltransferase. *J. Biol. Chem.* **262:** 17735-17743.

Weisz, O.A., A.M. Swift and C.E. Machamer. 1993. Oligomerisation of a membrane protein correlates with its retention in the Golgi complex. *J. Cell Biol.* **122:** 1185-1196.

Wessel, D. and U.I. Flugge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138:** 141-143.

Whaley, M. G. 1975. The Golgi Apparatus. Springer-Verlag/Wien. 190pp.

Whiteheart, S.W., I.C. Griff, M. Brunner, D.O. Clary Nayer, T., S.A. Buhrow and J.E. Rothman. 1993. SNAP family of NSF attachment proteins includes a brain-specific isoform. *Nature* **362:** 353-355.

Whiteheart, S.W., K. Rossnagel, S.A. Buhrow, M. Brunner, R. Jaenicke and J.E. Rothman. 1994. NSF: A trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. *J. Cell Biol.* In Press.

Wiedmann, M., A. Huth and T.A. Rapoport. 1984. *Xenopus* oocytes can secrete bacterial β-lactamase. *Nature* **309:** 637-639.

Wieland, F.T., M.L. Gleason, T.A. Serafini and J.E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell* **50:** 289-300.

Wilson, D.W., C.A. Wilcox, G.C. Flynn, E. Chen, W.-J. Kuang, W.J. Henzel, M.R. Block, E. Ullrich and J.E. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* **339:** 355-359.

Wilson, D.W., S.W. Whiteheart, M. Wiedmann, M. Brunner and J.E. Rothman. 1992. A multisubunit particle implicated in membrane fusion. *J. Cell Biol.* **117:** 531-538.

Wong, S.H., S.H. Low and W. Hong. 1992. The 17-residue transmembrane domain of β-galactoside α2,6-sialyltransferase is sufficient for Golgi retention. *J. Cell Biol.* **117:** 245-258.

Zerial, M. and H. Stenmark. 1993. Rab GTPases in vesicular transport. *Curr. Op. Cell Biol.* **5:** 613-620.

Zucker, R.S. and R.A. Steinhardt. 1978. Prevention of the cortical reactions in fertilised sea urchin eggs by injection of calcium-chelating ligands. *Biochim. Biophys. Acta* **541:** 459-466.