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# The Croonian Lecture 1999. Intracellular membrane traffic: getting proteins sorted

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The secretory and endocytic pathways within higher cells consist of multiple membrane-bound compartments, each with a characteristic composition, through which proteins move on their way to or from the cell surface. Sorting of proteins within this system is achieved by their selective incorporation into budding vesicles and the specific fusion of these with an appropriate target membrane. Cytosolic coat proteins help to select vesicle contents, while fusion is mediated by membrane proteins termed SNAREs present in both vesicles and target membranes. SNAREs are not the sole determinants of target specificity, but they lie at the heart of the fusion process. The complete set of SNAREs is known in yeast, and analysis of their locations, interactions and functions *in vivo* gives a comprehensive picture of the traffic routes and the ways in which organelles such as the Golgi apparatus are formed. The principles of protein and lipid sorting revealed by this analysis are likely to apply to a wide variety of eukaryotic cells.

**Keywords:** Golgi; SNARE; yeast; secretion; membrane; syntaxin

## 1. INTRODUCTION

Cell biologists have long been fascinated by the complex internal membrane structures found in eukaryotic cells. Pioneering electron microscopic (EM) studies in the 1950s showed that the internal morphology of cells varies greatly between cell types and species, but that certain common features could always be found, most notably the endoplasmic reticulum (ER) and the Golgi apparatus. This universal morphology is reflected in a universal function: the ER is the site at which newly synthesized membrane and secretory proteins are first assembled and they then pass through the Golgi apparatus, where they undergo a variety of carbohydrate modifications, before being delivered to the cell surface. A related pathway exists for the uptake of proteins and extracellular fluid from the cell surface via endosomes to lysosomes, and traffic can occur in both directions between this endocytic pathway and the exocytic pathway. My talk is concerned with the way in which the organelles of these pathways are formed and their characteristic membrane compositions maintained, and how the directed movement of proteins through them is achieved. Most of our recent studies have used the common budding yeast *Saccharomyces cerevisiae*, but there is every reason to believe that the underlying organizational principles are common to all eukaryotic cells.

Transport of proteins between the organelles of the endomembrane system relies on two processes: the budding of small vesicles or other structures from a parent membrane, and the subsequent fusion of these carriers to another organelle. Each of these processes presents a problem in specificity, because maintenance of the differences between membranes requires first that a particular subset of membrane components is gathered into a forming bud and then that the budded structure fuses only with the appropriate target membrane. Much effort has therefore been devoted to analysing the budding and fusion steps.

The paradigm for vesicle formation is the first step in endocytosis, in which a piece of the plasma membrane is internalized. The membrane that pinches off is covered with a polymeric protein coat formed from clathrin and the AP-2 adaptins, which may help to curve the membrane and certainly helps to select the protein content of the forming vesicle, via direct protein–protein contacts (Hirst & Robinson 1998). Since some of the selected proteins are transmembrane receptors which in turn bind to soluble proteins in the cell medium, the selective internalization of these is also ultimately controlled by the coat proteins. Once a vesicle has formed the coat is disassembled, having served its purpose.

Within the cell several other coat protein complexes are used, including the AP-1–clathrin combination, AP-3, COPI, COPII and a novel putative coat termed retromer (Hirst & Robinson 1998; Schekman & Orci 1996; Seaman *et al.* 1998). These assemble on various organelles, and putative sorting signals—cytoplasmically exposed sequence motifs on membrane proteins that interact, directly or indirectly, with the coat—have been identified for each one. Intracellular sorting receptors for some classes of soluble proteins, notably those in vacuoles or lysosomes and in the ER, are also known (Traub & Kornfeld 1997; Pelham 1995). Thus the principle of coat-mediated cargo selection seems to be a general one.

Our interest has focused on the problem of membrane fusion. This problem became tractable with the identification of specific membrane proteins that are linked to the fusion process, both from yeast genetics (Dascher *et al.* 1991; Hardwick & Pelham 1992) and from biochemical



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analysis of synapses (Bennett & Scheller 1993). These proteins interact with soluble proteins termed NSF and SNAP that are required for membrane fusion *in vitro*, and have been collectively named SNAREs, for SNAP receptors (Söllner *et al.* 1993).

## 2. SNARES AS FUSION PROTEINS AND ORGANELLE MARKERS

Most SNAREs, though not all, are integral membrane proteins with a single C-terminal transmembrane domain (TMD). Adjacent to this is a region with the potential to form a coiled-coil structure. It is the energetically favourable formation of parallel helical bundles containing SNAREs anchored in each fusing membrane that is thought to provide the force that drives the membranes together, though additional components are required for efficient bilayer fusion (Peters & Mayer 1998). The synaptic SNAREs have been studied in most detail; they form an extremely stable four-helix bundle, two of the helices being provided by a single 25 kDa SNARE (called, confusingly, snap-25) which is bound to the membrane only via attached fatty acyl chains, the others by the SNAREs synaptobrevin and syntaxin, each with a TMD (Sutton *et al.* 1998). Despite the high thermal stability of this complex it can readily be dissociated by the action of SNAP and NSF, the energy being provided by NSF-mediated ATP hydrolysis. Thus cycles of association, dissociation and recycling can allow a SNARE to mediate multiple vesicle fusion events.

Yeast vacuoles have the ability to fuse with each other and provide a convenient system for the biochemical analysis of the fusion process. Fusion can readily be followed *in vitro* using vacuoles purified from different strains. Amongst the proteins required are the yeast homologues of NSF (Sec18p) and SNAP (Sec17p), a synaptobrevin homologue (Nyv1p) and a syntaxin homologue (Vam3p). The SNAREs normally exist as a complex, which must be dissociated by Sec18p prior to fusion (see figure 1). Genetic manipulation allows the creation of vacuole-like structures that lack various components, and we were able to show that fusion has an absolute requirement for at least one TMD-containing SNARE in each fusing membrane, consistent with their predicted role (Nichols *et al.* 1997).

The requirement for cognate SNAREs on apposing membranes suggested a simple explanation for the specificity of trafficking events: target organelles would each be defined by a 'target' or t-SNARE, and each vesicle would carry the appropriate partner as a 'vesicle' or v-SNARE. This distinction between v- and t-SNAREs is conceptually convenient but is often blurred, as in the example of vacuole fusion. However, as more SNAREs have been analysed it has become apparent that every fusion event involves a member of the related family of SNAREs called syntaxins and that in many cases these perform the function of a t-SNARE. The syntaxins have a more elaborate structure than the other SNAREs, and a conformational change in syntaxin wrought by NSF is likely to be a key step in the formation and dissociation of the SNARE complex (Ungermann *et al.* 1998; Hanson *et al.* 1995).

A combination of genetics and genome sequencing has given a rather complete view of the SNAREs in yeast:

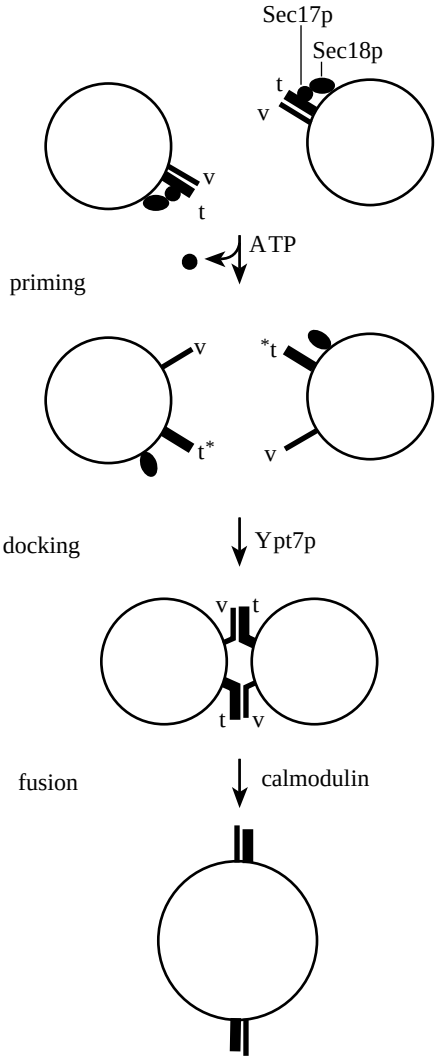


Figure 1. Diagram of the steps in yeast vacuole fusion. During priming SNARE complexes are dissociated and the t-SNARE (Vam3p) activated by the action of Sec17p and Sec18p. Docking involves the GTPase Ypt7p and additional proteins, but results in the binding together of SNAREs in different membranes. After further biochemical steps (which require calmodulin), bilayer fusion is achieved and SNARE complexes are once more present on a single vacuolar membrane. In this simplified diagram only two SNAREs, labelled v and t are shown, but the complexes formed probably contain four different SNAREs.

there are around 20 (depending on the precise definition used) of which eight can be considered syntaxins. By identifying their locations, their associations with each other *in vivo*, and the phenotypic effects of their removal, we have sought to define the steps of the secretory and endocytic pathways and understand more generally how these pathways operate. The results give an overall picture that in some respects is rather different from the one presented in current textbooks.

The approximate locations of the yeast syntaxins have been determined by immunofluorescence and subcellular fractionation. Two of them, Sso1p and Sso2p, are closely related and functionally redundant, and are found on the plasma membrane. Pep12p is on endosomes, Vam3p on

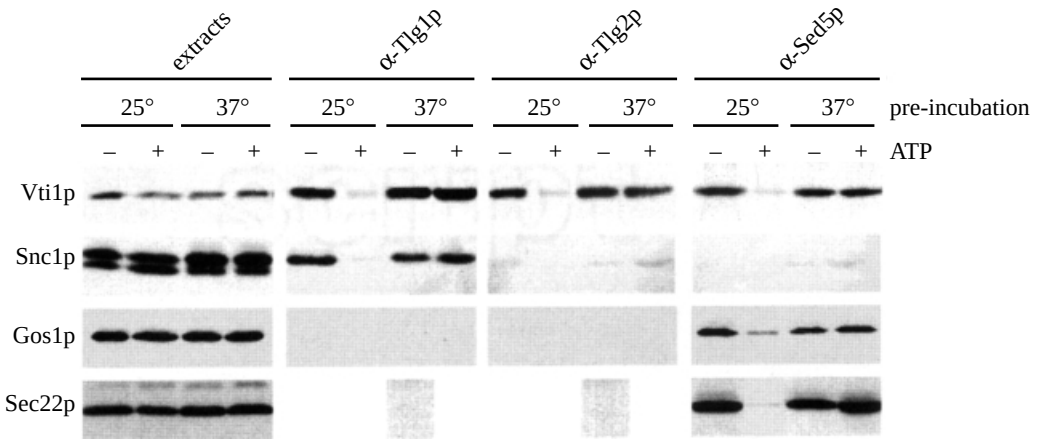


Figure 2. Examples of co-immunoprecipitation of SNAREs. Mutant cells with a temperature-sensitive form of Sec18p were incubated at permissive (25 °C) or non-permissive (37 °C) temperature, lysed either in the presence or absence of ATP, and the syntaxins Tlg1p, Tlg2p and Sed5p precipitated with appropriate antibodies. Samples of the extracts and the precipitates were analysed by gel electrophoresis, and SNAREs detected by immunoblotting. Sec22p, Gos1p and Snc1p are involved in ER–Golgi, intra-Golgi and Golgi–plasma membrane traffic, respectively; Vti1p is involved in multiple steps. The SNARE complexes are disrupted *in vitro* when Sec18p is active and ATP is present.

vacuoles, Ufe1p on the ER, and Sed5p on early Golgi cisternae. Tlg1p and Tlg2p are also associated with the Golgi, but co-localize with late rather than early Golgi markers. Interestingly, the repertoire of syntaxins in higher animals is only slightly more extensive than in yeast (discussed by Steegmaier *et al.* 1998; Pelham 1999), and nematodes seem only to have nine syntaxin genes. Both nematodes and mammals have clear homologues of the Golgi complex syntaxins (Sed5p, Tlg1p and Tlg2p), and around four different plasma membrane syntaxins (some tissue specific, others restricted to one surface of polarized cells). The endocytic syntaxins are somewhat more variable—mammals have at least four, two of which are also present in nematodes, but their precise relationship to the yeast endocytic and vacuolar syntaxins is unclear. In general, it seems that the components of the exocytic pathway are similar throughout the eukaryotes, but curiously no homologue of the ER syntaxin Ufe1p has been found outside the yeasts and fungi. However, a mammalian syntaxin unrelated to Ufe1p has recently been reported to localize to the ER (Steegmaier *et al.* 1998).

The partners of each syntaxin can be identified crudely by the simple approach of co-immunoprecipitation. In the steady state many SNARE complexes exist in cells, and when cells are lysed in the absence of ATP these complexes can be precipitated. Addition of ATP to the lysate leads to disruption of the complexes; this is due to the action of Sec18p (NSF) because it is prevented when a temperature-sensitive *sec18* strain is incubated at the non-permissive temperature (Ungermann *et al.* 1998; Holthuis *et al.* 1998*a*). Examples of typical results obtained in this way are shown in figure 2, in which interactions between the three syntaxins found in the Golgi complex and various other SNAREs were probed. The figure shows that each syntaxin associates, *in vivo*, with a distinct set of putative v-SNAREs. However, it is also apparent that a single v-SNARE can bind multiple syntaxins, the most extreme example being the protein Vti1p, which binds to no fewer than five different syntaxins. Conversely, the syntaxin Sed5p binds to at least seven different SNAREs in at least three separate complexes (Nichols & Pelham 1998). This combinatorial complexity means that unravelling the traffic routes is no easy task, but there are other clues to go on as well.

One of the best-studied steps is that from ER to Golgi, which has been subjected to exhaustive genetic and biochemical analysis. Proteins exit the ER in COPII-coated vesicles which carry several SNAREs including Bet1p, Bos1p and Sec22p (Barlowe *et al.* 1994). These form a complex with the early Golgi syntaxin Sed5p and can thus mediate fusion with the Golgi (Søgaard *et al.* 1994). Export from the ER is selective, but some of the abundant lumenal chaperones escape and are retrieved from the Golgi by a specific receptor (Pelham 1995). Return traffic from the Golgi occurs in COPI-coated vesicles, which selectively incorporate the retrieval receptor and also ferry the SNAREs back to the ER (Letourneur *et al.* 1994; Lewis & Pelham 1996). Fusion is mediated by the ER syntaxin Ufe1p, and by the v-SNAREs Sec22p and (probably) Bet1p (Lewis & Pelham 1996; Lewis *et al.* 1997; Spang & Schekman 1998).

This brief summary illustrates an inherent limitation of the SNAREs as specificity determinants: because v-SNAREs have to be returned to their starting point to be reused, they will be present on vesicles travelling in both directions. In yeast, at least some v-SNAREs function in both forward and reverse transport. Clearly, other proteins must ensure directionality. Such a function is likely to be provided by peripheral membrane proteins—such as GTPases of the rab family and proteins that are recruited to membranes by them—which regulate vesicle docking (Cao *et al.* 1998). I will not discuss these here, but suffice to say the presence of cognate SNAREs, though a prerequisite for successful fusion, is by no means the sole source of specificity.

## 3. THE NATURE OF THE GOLGI COMPLEX

Is the scheme outlined above an accurate representation of ER–Golgi traffic? In answering this it is helpful

first to consider the nature of the Golgi complex and the way in which proteins might be transported through it. In recent years, most textbooks have depicted the Golgi stack as a series of stable compartments with different compositions, proteins transiting through them by means of sequential rounds of vesicular transport (figure 3). One might therefore expect multiple syntaxins, each defining a compartment, and indeed three syntaxins have been localized to the yeast Golgi complex. However, simultaneous deletion of the genes encoding two of these (Tlg2p and Tlglp) has surprisingly little effect on secretion, which argues against multiple obligatory transport steps (Holthuis *et al*. 1998*a*).

An alternative model, based on classical EM studies, assumes that Golgi cisternae are not stable entities but rather are created *de novo* and then mature, eventually breaking down into exocytic vesicles. In this model differences in cisternal composition are achieved by a retrieval process, in which 'resident' proteins are transported in vesicles to earlier compartments, while cargo proteins in transit remain in the cisternae (figure 3; Pelham 1998). This requires no coat-mediated selection of cargo within the Golgi, and can account for the transport of structures such as collagen fibrils that are too large to fit into a conventional vesicle. In contrast to the vesicular transport model it requires all resident proteins, including the syntaxins, to be removed from cisternae as they mature. It also requires a mechanism to generate new cisternae.

In fact, Sed5p does leave the Golgi complex every few minutes—that is, on a time-scale consistent with the calculated lifetime of a maturing cisterna. This can be demonstrated using a temperature-sensitive mutant that blocks COPII vesicle formation from the ER and a version of Sed5p tagged with green fluorescent protein to allow its visualization in living cells. Upon a temperature shift, the Sed5p distribution changes rapidly from the scattered pattern of dots characteristic of Golgi cisternae in yeast to the linear features of the ER (Wooding & Pelham 1998).

Cycling of Sed5p through the ER implies that it is present in the vesicles that bud from the ER and thus it is easy to see how new cisternae could be generated by fusion of these vesicles with each other, just as vacuoles can fuse with each other. Indeed, real-time imaging of green fluorescent protein chimeras in animal cells has shown that proteins leaving the ER at peripheral exit sites accumulate in quite large structures, which then travel en masse to the Golgi region (Presley *et al*. 1997; Scales *et al*. 1997). These structures, which appear to form *de novo*, can be thought of as fragments of new Golgi cisternae.

Maturation requires late Golgi proteins to be delivered to newly formed cisternae in vesicles. One v-SNARE, Sft1p, which is essential for traffic through the Golgi, seems to be specific for this step (Banfield *et al*. 1995). It is found normally mostly in late Golgi cisternae that lack Sed5p, but Sed5p is its only known syntaxin partner. Strikingly, upon shifting a temperature-sensitive *sft1* mutant to high temperature late Golgi markers such as the enzyme Mnn1p rapidly accumulate in small vesicles, whereas early Golgi cisternae are unaffected (Wooding & Pelham 1998). A similar effect is induced by a *sed5* mutation. It is likely that these vesicles are formed by the COPI coat protein, because this coat is found on Golgi cisternae and Golgi enzymes can be found in COPI-coated vesicles in animal cells.

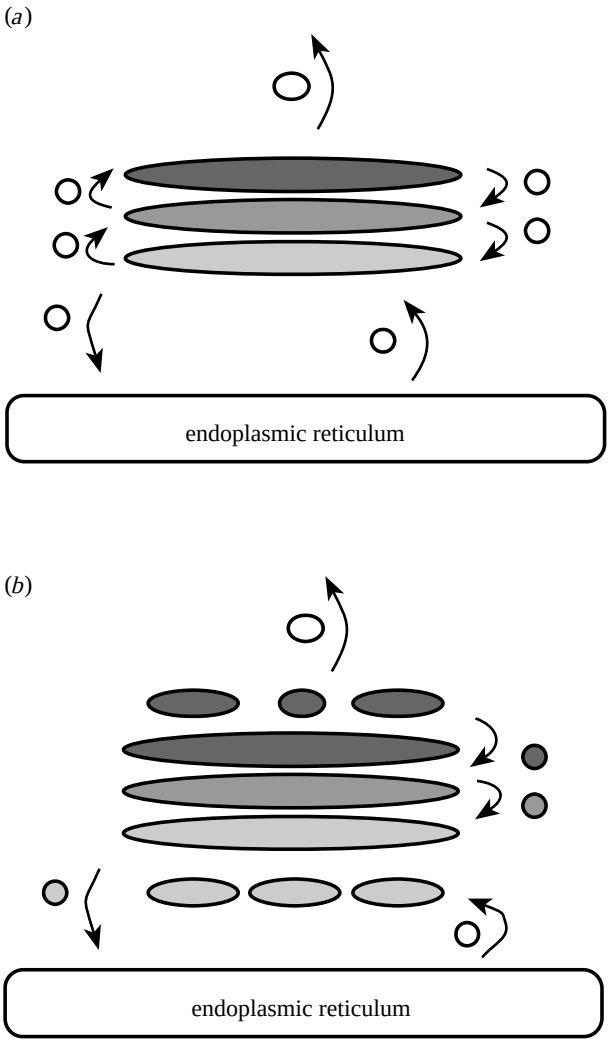


Figure 3. Comparison of the vesicular transport and cisternal maturation models for Golgi transport. In the vesicular transport model, cisternae remain as fixed entities with their associated resident proteins, while secretory and membrane proteins are transported to, between and from them in vesicles. Some proteins, such as v-SNAREs, are selectively retrieved in retrograde vesicles. In the maturation model, cisternae are formed *de novo* from ER-derived vesicles and mature. Secretory proteins remain with a cisterna as it matures and eventually breaks up, while Golgi proteins are continually removed and delivered by backwards-travelling vesicles.

Together, the evidence suggests that new Golgi cisternae can be created from the ER, that resident late Golgi proteins undergo vesicular transport to these new Golgi cisternae, and that early Golgi markers in turn are recycled to the ER (see figure 4). These findings fit closely the predictions of the cisternal maturation model (Pelham 1998). A further prediction is that the entire Golgi complex is a dynamic structure whose existence is dependent on the formation of new cisternae. Mutants that block budding from the ER should cause cisternae to disappear rapidly, Golgi proteins being chased either into vesicles (whose target is no longer present) or back to the ER. This is indeed what is observed, both by

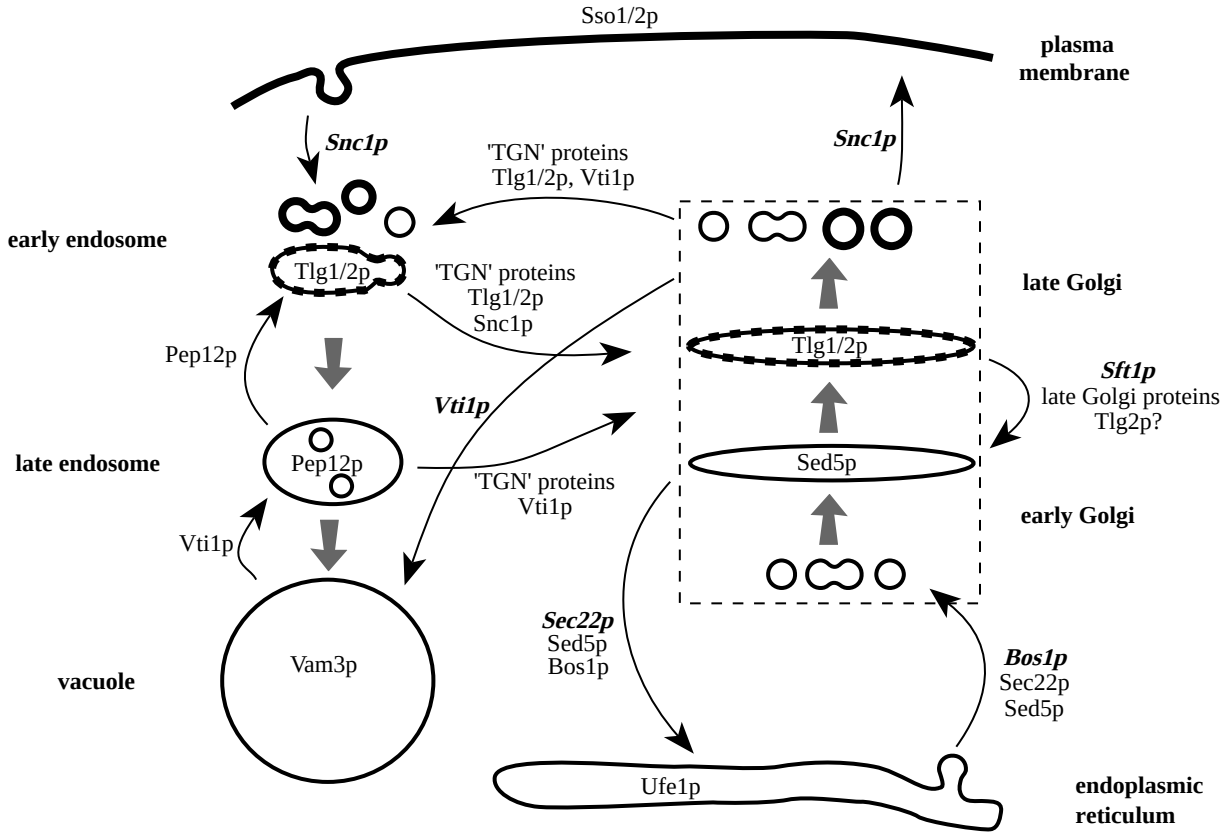


Figure 4. Map of the traffic routes in yeast. Large grey arrows indicate maturation or direct fusion, while thin arrows indicate vesicular steps. The dashed rectangle encloses the Golgi complex. Locations of the syntaxins and some other SNAREs are indicated. Bold italic names are examples of proteins thought to act as functional v-SNAREs for the indicated step, as opposed to merely being transported passively. Not all SNAREs are listed, and the figure is not intended to provide a comprehensive list of SNARE movements and complexes. A rough indication of presumed lipid composition is depicted by line thickness, thick lines representing sterol- and sphingolipid-rich membranes.

immunofluorescence and by EM (Wooding & Pelham 1998; Morin-Ganet *et al.* 1998). Upon restoration of budding from the ER, cisternae reappear within a few minutes. Hence the very existence of the Golgi complex is dependent on traffic through the exocytic pathway.

What happens to a cisterna when its maturation is complete? Such a late Golgi cisterna (or, to use the analogous term from higher cells, the trans-Golgi network or TGN) apparently disintegrates, and at this point there is a major bifurcation in the exocytic pathway. Some of the vesicles which form are destined for the cell surface, while others pass directly to the endocytic pathway. Newly synthesized vacuolar proteins take the endocytic route, together with 'residents' of the TGN such as the syntaxin Tlglp. The separation is clathrin dependent: in temperature-sensitive clathrin mutants almost all proteins go to the plasma membrane (Seeger & Payne 1992). EM of plant and algal Golgi stacks shows the segregation process: late cisternae giving rise to large uncoated cargo-carrying structures and prominent clathrin-coated vesicles. In animal cells that form secretory granules similar events can be seen, with proteins such as the Tlglp homologue syntaxin 6 being removed in clathrin-coated vesicles as the granules form (Klumperman *et al.* 1998).

What determines which route a membrane protein takes? Some proteins have cytoplasmic tails which interact with the clathrin–APl coat, thus allowing them to be selected for transport to endosomes (Traub & Kornfeld 1997). However, this is not the whole story, at least in yeast. Many experiments have shown that transport to the vacuole is not saturable and that no cytoplasmic tail sequences are necessary for a protein to follow this route. Changes within TMDs, in contrast, have a profound effect (Rayner & Pelham 1997). Longer, more hydrophobic TMDs lacking phenylalanine residues allow transport to the plasma membrane, whereas shorter, phenylalanine-rich or relatively hydrophilic ones prevent this. Examination of the TMD sequences of naturally occurring plasma membrane proteins confirms this bias.

TMD-dependent sorting could in principle be mediated by protein receptors, but it more likely reflects the separation of membrane lipids. Vesicles destined for the plasma membrane, like that membrane itself, are rich in sterols and sphingolipids. In contrast, the vacuolar membrane, and presumably also membrane on route to it, largely lacks sterols (Zinser *et al.* 1993). These differences in composition affect the thickness and plasticity of the membrane bilayer, and TMDs would be expected to partition between regions containing different lipids according to their physical characteristics (Bretscher & Munro 1993).

Our view of traffic through the yeast Golgi complex must thus take lipids into account. We imagine that a cisterna forms mostly from ER-derived membrane, which already contains low concentrations of newly made

sterols. As the cisterna matures, Golgi enzymes are delivered in vesicles, and these include the enzymes required for sphingolipid synthesis. Sphingolipids tend to associate with sterols because of their size and shape, and are retained in the cisterna whilst phospholipids are preferentially removed. How this lipid sorting is achieved is not known, but it could simply be that coat proteins impose a tight curvature on the membrane, and that sterol-rich domains, which are relatively inflexible, are preferentially excluded. When the cisterna eventually fragments, the relatively large vesicles that will fuse with the plasma membrane have achieved a lipid composition not very different from their target.

## 4. THE ENDOCYTIC PATHWAY

The Golgi complex is of fundamental importance for cell growth. In contrast, the endocytic pathway is not essential—it is possible to construct viable yeast strains that contain only the ER and plasma membrane syntaxins and Sed5p. These cells lack vacuoles and endosomes; they still internalize their surface, but the primary endocytic vesicles simply accumulate without fusing. Expressing the vacuolar syntaxin Vam3p in such cells creates structures resembling vacuoles, but they lack most of their normal constituents and the normal endocytic pathway is missing (Holthuis *et al.* 1998*b*). This strange state of affairs is explained by the existence of a specialized direct vesicular transport route from the Golgi to the vacuole, mediated by the coat protein AP3, which delivers Vam3p itself and a few other proteins (Stepp *et al.* 1997; Cowles *et al.* 1997). Conversely, expression of Pepl2p is sufficient to create endosomes. In the absence of Vam3p these accumulate vacuolar proteins and form quite large structures that *in vitro* are capable of fusing with vacuoles (Holthuis *et al.* 1998*b*; Nichols *et al.* 1997).

These and other studies suggest that the endocytic pathway shares several features of the exocytic one. Endosomes can evidently form *de novo* by fusion of endocytic vesicles with vesicles carrying Pepl2p, receive traffic from the Golgi, and mature into structures which can fuse with vacuoles (figure 4). As with the Golgi complex, 'resident' endosomal proteins such as Pepl2p are likely to be selectively retrieved to earlier structures by vesicular transport. Endosome maturation also involves the separation of the membranes into distinct domains with different compositions, but rather than budding off in the normal way, membranes containing proteins for destruction bud into the endosome, forming multivesicular bodies whose internal vesicles are in due course delivered to the vacuole (Odorizzi *et al.* 1998). It is not clear what determines the fate of individual proteins, but we know that the TMD is not the major determinant—the TMD of a vacuolar membrane protein is not sufficient to prevent internalization and destruction of a foreign membrane protein.

Sometime before arrival at the vacuole, TGN proteins that were diverted from the secretory pathway to endosomes have to be retrieved. Such retrieval is dependent on cytoplasmic sorting signals, and there is a candidate vesicle coat for this step (Seaman *et al.* 1998). This process, unlike exocytosis, endocytosis and transfer of proteins from the exocytic to the endocytic pathway, is partially dependent on the late Golgi syntaxins Tlglp and Tlg2p (Holthuis *et al.* 1998*a*).

One protein whose trafficking is strongly dependent on the Tlgs is the v-SNARE that mediates fusion of exocytic vesicles with the plasma membrane, Snclp. After arrival at the plasma membrane it dissociates from the t-SNARE Ssolp and is endocytosed. It is then selectively removed from the endocytic pathway and returned to the Golgi. In *tlg* mutants, even those containing very mildly defective alleles of Tlglp, this step fails and Snclp is transported to the vacuole.

The role of Tlglp in this process is quite complex. It is delivered to forming endosomes, remains bound to Snclp after fusion and may serve as a receptor that directs Snclp into vesicles that recycle to the Golgi. Unusually for a syntaxin, Tlglp forms complexes with other syntaxins, namely Tlg2p and Sed5p, and thus may even act as a v-SNARE for the return to the Golgi. The Snclp–Tlglp complex must in any case dissociate before or soon after arrival in the TGN, to allow the two proteins to go their different ways.

Snclp is not the only protein to recycle to the exocytic pathway from the plasma membrane. Another important one is the enzyme responsible for synthesis of the bulk of the chitin in the yeast cell wall, Chs3p. Through much of the cell cycle this enzyme is concentrated at the bud neck, where it lays down a thick layer of chitin. However, Chs3p is not permanently fixed there—it is spread out over the bud membrane in cells with medium-sized buds, and it has to be redirected to the new bud site when one round of budding is complete. Movement of the enzyme is achieved by its endocytosis and redelivery to the growing point of the cell, using the normal secretory process which directs vesicles to the growing point of the cell. Cells lacking Tlglp and Tlg2p, though perfectly capable of endocytosis to the vacuole, fail to localize Chs3p efficiently to the bud neck (Holthuis *et al.* 1998*b*).

Snclp and Chs3p show a particularly strong requirement for Tlg-mediated retrieval, more so than other proteins that cycle through endosomes such as the receptor for vacuolar proteases and the TGN enzyme Stel3p. It seems that these other proteins have a second opportunity for retrieval from late endosomes, in a process that can probably occur without the Tlgs. This second chance is not available to Snclp or Chs3p, probably because they enter the inner vesicular membranes of late endosomes.

## 5. ORGANIZATIONAL PROBLEMS

As I have outlined, the traffic routes within yeast cells are now reasonably well defined, and the analysis of syntaxins and other SNAREs has helped considerably in their elucidation (figure 4). Nevertheless, analysis of SNAREs has not completely solved the problem of specificity in membrane fusion. For example, the v-SNARE Vtilp is thought to be involved in traffic from Golgi to endosomes, from Golgi to vacuole, from endosomes to Golgi and quite possibly several other steps. Furthermore, *in vitro* studies have not shown the degree of specificity in SNARE complex formation that would be needed if they were the prime targeting molecules—the hydrophobic core of the complex is rather well conserved and can be

created from a variety of SNARE combinations, the main sequence differences between SNAREs being amongst residues located on the solvent-exposed surfaces of the helix bundle (Fasshauer *et al.* 1998).

An extreme view would be that SNAREs provide very little specificity and that the main reason for having so many is to provide targeting signals to ensure that one is present in every location. This can be tested by altering the targeting information and testing whether SNAREs can substitute for each other. In some cases this works—mistargeting Vam3p to endosomes allows it to perform some of the functions of Pep12p, for example (Darsow *et al.* 1998)—but in several other cases we have found that mistargeted SNAREs do not function. So what is special about individual SNAREs?

The answer probably lies in the growing number of molecules, many of them not integral membrane proteins, that contribute to the specific docking of vesicles prior to the actual fusion event. These include the rab-type GTP binding proteins, coiled-coil proteins and large protein complexes (e.g. Cao *et al.* 1998; TerBush *et al.* 1996). Directly or indirectly, these molecules interact with the SNAREs, and there is undoubtedly specificity in these interactions. The picture that emerges is one of a series of steps which lead eventually to the formation of appropriate SNARE complexes, specificity being provided by a combination of several different relatively weak interactions. By this means the activities of the SNAREs are regulated, preventing promiscuous fusion of all membrane compartments with each other. SNAREs also interact with other proteins, such as calcium channels, that stimulate bilayer fusion after SNARE complex formation and this too may require organelle-specific features (see Peters & Mayer 1998).

A related specificity problem that arises in a system of maturing membrane compartments and constantly circulating SNAREs is that of defining the 'identity' of a compartment at any given time. What determines when a Golgi cisterna should stop receiving vesicles from the ER and instead export Sed5p? Or an endosome stop receiving traffic from the Golgi and fuse with the vacuole? We do not know the full answers to such questions, but a recurring theme is that global physicochemical properties of individual membrane compartments can determine their fate. A beautiful example of this is the recent discovery that in both yeast and animal cells fusion of endosomes requires the phosphorylation of phosphatidylinositol molecules to form PI3P (Simonsen *et al.* 1998; Burd & Emr 1998). This modification depends on the presence and activity of the appropriate kinase, and the accumulation of the product defines a single topological entity. Docking of the membranes requires the recruitment of a soluble protein (EEA1 in animal cells, Vac1p in yeast), whose binding depends on the presence of both rab5 and PI3P, and thus is restricted to appropriately matured endosomes. EEA1 in turn is thought to interact with endosomal SNAREs, which mediate the actual fusion.

Other global properties are also important for various steps. I have already mentioned the changes in lipid composition that occur in the Golgi, and genetic studies have shown that lipid content is crucial for the generation of post-Golgi vesicles in yeast (Fang *et al.* 1998). Internal acidification is also important for the function of both the Golgi and endosomes. Acid accumulation can be driven by the ATP-dependent 'vacuolar' proton pump, which assembles first in the ER and is probably active throughout the endomembrane system, but it depends on the presence of channels for counter ions, and also the absence of proton channels. Segregation of these components during budding or scission events will thus lead to a different internal pH in the budded structure compared with the parental organelle, which can be used to drive its future behaviour. For example, lumenal ER proteins carry a retrieval signal, KDEL, but do not bind to the KDEL receptor in the ER. However, after budding and fusion of COPII vesicles the lumenal pH drops, triggering both binding of any KDEL proteins that were included in the vesicles to the receptor and also, it seems, the formation of COPI vesicles which carry the receptor back to the ER (Palokangas *et al.* 1998; Wilson *et al.* 1993).

In summary, the feeling I have tried to convey is that the secretory process is driven by a series of physical changes, each of which has an impact on the budding and fusion machinery and triggers the next change. In this view, coat-mediated selection of proteins during exit from the ER leads to acidification, which initiates sorting and retrieval, creating cisternae ready and able to receive vesicles bearing Golgi proteins. Delivery of lipid biosynthetic enzymes results in modification of the lipid bilayer, which in turn causes segregation of proteins and the eventual expulsion of the Golgi components themselves, leaving membranes ready for delivery to the surface. In this way proteins are transferred from the chaperone-filled, phospholipid-rich ER to the impermeant sterol-rich environment of the cell surface. All the components of the intermediates in the pathway are constantly being extracted, recycled and reused, a dynamic form of organization that allows for continuous correction and self-renewal. Many of the same principles apply also to the endocytic pathway. Though the description is far from complete, we have come a long way towards an understanding of how the sometimes bewildering complexity of intracellular membranes is created and maintained.

## REFERENCES

Banfield, D. K., Lewis, M. J. & Pelham, H. R. B. 1995 A SNARE-like protein required for traffic through the Golgi complex. *Nature* **375**, 806–809.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M. & Schekman, R. 1994 COPII: a membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* **77**, 895–907.

Bennett, M. K. & Scheller, R. H. 1993 The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl Acad. Sci. USA* **90**, 2559–2563.

Bretscher, M. S. & Munro, S. 1993 Cholesterol and the Golgi apparatus. *Science* **261**, 1280–1281.

Burd, C. G. & Emr, S. D. 1998 Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol. Cell* **2**, 157–162.

Cao, X., Ballew, N. & Barlowe, C. 1998 Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *EMBO J.* **17**, 2156–2165.

Cowles, C. R., Odorizzi, G., Payne, G. S. & Emr, S. D. 1997 The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell* **91**, 109–118.

Darsow, T., Burd, C. G. & Emr, S. D. 1998 Acidic di-leucine motif essential for AP-3-dependent sorting and restriction of the functional specificity of the Vam3p vacuolar t-SNARE. *J. Cell Biol.* **142**, 913–922.

Dascher, C., Ossig, R., Gallwitz, D. & Schmitt, H. D. 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.

Fang, M., Rivas, M. P. & Bankaitis, V. A. 1998 The contribution of lipids and lipid metabolism to cellular functions of the Golgi complex. *Biochim. Biophys. Acta* **1404**, 85–100.

Fasshauer, D., Sutton, R. B., Brunger, A. T. & Jahn, R. 1998 Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc. Natl Acad. Sci. USA* **95**, 15 781–15 786.

Hanson, P. I., Otto, H., Barton, N. & Jahn, R. 1995 The N-ethylmaleimide-sensitive fusion protein and alpha-SNAP induce a conformational change in syntaxin. *J. Biol. Chem.* **270**, 16 955–16 961.

Hardwick, K. G. & Pelham, H. R. B. 1992 SED5 encodes a 39kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* **119**, 513–521.

Hirst, J. & Robinson, M. S. 1998 Clathrin and adaptors. *Biochim. Biophys. Acta* **1404**, 173–193.

Holthuis, J. C. M., Nichols, B. J., Dhruvakumar, S. & Pelham, H. R. B. 1998*a* Two syntaxin homologues in the TGN/endosomal system of yeast. *EMBO J.* **17**, 113–126.

Holthuis, J. C. M., Nichols, B. J. & Pelham, H. R. B. 1998*b* The syntaxin Tlglp mediates trafficking of chitin synthase 3 to polarized growth sites in yeast. *Mol. Biol. Cell* **9**, 3383–3397.

Klumperman, J., Kuliawat, R., Griffith, J. M., Geuze, H. J. & Arvan, P. 1998 Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein AP-1, clathrin, and syntaxin 6-positive vesicles. *J. Cell Biol.* **141**, 359–371.

Letourneur, F., Gaynor, E. C., Hennecke, S., Demolliere, C., Duden, R., Emr, S. D., Riezman, H. & Cosson, P. 1994 Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* **79**, 1199–1207.

Lewis, M. J. & Pelham, H. R. B. 1996 SNARE-mediated retrograde transport from the Golgi complex to the ER. *Cell* **85**, 205–215.

Lewis, M. J., Rayner, J. C. & Pelham, H. R. B. 1997 A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum. *EMBO J.* **16**, 3017–3024.

Morin-Ganet, M. N., Rambourg, A., Clermont, Y. & Képès, F. 1998 Role of endoplasmic reticulum-derived vesicles in the formation of Golgi elements in sec23 and sec18 *Saccharomyces cerevisiae* mutants. *Anat. Rec.* **251**, 256–264.

Nichols, B. J. & Pelham, H. R. B. 1998 SNAREs and membrane fusion in the Golgi apparatus. *Biochim. Biophys. Acta* **1404**, 9–31.

Nichols, B. J., Ungermann, C., Pelham, H. R. B., Wickner, W. T. & Haas, A. 1997 Homotypic vacuolar fusion mediated by t- and v-SNAREs. *Nature* **387**, 199–202.

Odorizzi, G., Babst, M. & Emr, S. D. 1998 Fablp PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell* **95**, 847–858.

Palokangas, H., Ying, M., Vaananen, K. & Saraste, J. 1998 Retrograde transport from the pre-Golgi intermediate compartment and the Golgi complex is affected by the vacuolar $H^+$-ATPase inhibitor bafilomycin A1. *Mol. Biol. Cell* **9**, 3561–3578.

Pelham, H. R. B. 1995 Sorting and retrieval between the endoplasmic reticulum and Golgi apparatus. *Curr. Opin. Cell Biol.* **7**, 530–535.

Pelham, H. R. B. 1998 Getting through the Golgi complex. *Trends Cell Biol.* **8**, 45–49.

Pelham, H. R. B. 1999 SNAREs and the secretory pathway—lessons from yeast. *Exp. Cell Res.* **247**, 1–8.

Peters, C. & Mayer, A. 1998 $Ca^{2+}$/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. *Nature* **396**, 575–580.

Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J. M. & Lippincott-Schwartz, J. 1997 ER to Golgi transport visualised in living cells. *Nature* **389**, 81–85.

Rayner, J. & Pelham, H. R. B. 1997 Transmembrane domain dependent sorting of proteins to the ER and plasma membrane in yeast. *EMBO J.* **16**, 1832–1841.

Scales, S. J., Pepperkok, R. & Kreis, T. E. 1997 Visualisation of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* **90**, 1137–1148.

Schekman, R. & Orci, L. 1996 Coat proteins and vesicle budding. *Science* **271**, 1526–1533.

Seaman, M. N., McCaffery, J. M. & Emr, S. D. 1998 A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J. Cell Biol.* **142**, 665–681.

Seeger, M. & Payne, G. S. 1992 Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae*. *J. Cell Biol.* **118**, 531–540.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M. & Stenmark, H. 1998 EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394**, 494–498.

Søgaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E. & Söllner, T. 1994 A Rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. *Cell* **78**, 937–948.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. & Rothman, J. E. 1993 SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318–324.

Spang, A. & Schekman, R. 1998 Reconstitution of retrograde transport from the Golgi to the ER *in vitro*. *J. Cell Biol.* **143**, 589–599.

Steegmaier, M., Yang, B., Yoo, J. S., Huang, B., Shen, M., Yu, S., Luo, Y. & Scheller, R. H. 1998 Three novel proteins of the syntaxin/SNAP-25 family. *J. Biol. Chem.* **273**, 34 171–34 179.

Stepp, J. D., Huang, K. & Lemmon, S. K. 1997 The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. *J. Cell Biol.* **139**, 1761–1774.

Sutton, R. B., Fasshauer, D., Jahn, R. & Brunger, A. T. 1998 Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* **395**, 347–353.

TerBush, D. R., Maurice, T., Roth, D. & Novick, P. 1996 The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 6483–6494.

Traub, L. M. & Kornfeld, S. 1997 The trans-Golgi network: a late secretory sorting station. *Curr. Opin. Cell Biol.* **9**, 527–533.

Ungermann, C., Nichols, B. J., Pelham, H. R. & Wickner, W. 1998 A vacuolar v-t-SNARE complex, the predominant form *in vivo* and on isolated vacuoles, is disassembled and activated for docking and fusion. *J. Cell Biol.* **140**, 61–69.

Wilson, D. W., Lewis, M. J. & Pelham, H. R. B. 1993 pH-dependent binding of KDEL to its receptor *in vitro*. *J. Biol. Chem.* **268**, 7465–7468.

Wooding, S. & Pelham, H. R. B. 1998 The dynamics of Golgi protein traffic visualised in living yeast cells. *Mol. Biol. Cell* **9**, 2667–2680.

Zinser, E., Paltauf, F. & Daum, G. 1993 Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J. Bacteriol.* **175**, 2853–2858.