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Trans-Golgi network sorting

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Abstract

The trans-Golgi network (TGN) is a major secretory pathway sorting station that directs newly synthesized proteins to different subcellular destinations. The TGN also receives extracellular materials and recycled molecules from endocytic compartments. In this review, we summarize recent progress on understanding TGN structure and the dynamics of trafficking to and from this compartment. Protein sorting into different transport vesicles requires specific interactions between sorting motifs on the cargo molecules and vesicle coat components that recognize these motifs. Current understanding of the various targeting signals and vesicle coat components that are involved in TGN sorting are discussed, as well as the molecules that participate in retrieval to this compartment in both yeast and mammalian cells. Besides proteins, lipids and lipid-modifying enzymes also participate actively in the formation of secretory vesicles. The possible mechanisms of action of these lipid hydrolases and lipid kinases are discussed. Finally, we summarize the fundamentally different apical and basolateral cell surface delivery mechanisms and the current facts and hypotheses on protein sorting from the TGN into the regulated secretory pathway in neuroendocrine cells.

Keywords

Trans-Golgi network; coat protein; sorting signal; lipid; lipid kinase; apical targeting; basolateral sorting; regulated secretion

Introduction

In our technology-laden era we are often surprised to realize that our ‘novel’ inventions are actually recapitulations of processes invented by biological systems eons ago. For example, in a recent copy of their in-flight magazine, Delta Airlines claimed to be the first company to implement the ‘hub-and-spoke’ system to sort the more than 500 million airline passengers that fly in the US alone. Passengers from around the world are collected into hub airports like that in Atlanta or Chicago and then sorted to their various destinations. The principal advantage claimed by this hub system is its ability to efficiently collect passengers in a central location and sort them to their final destinations in a timely, efficient, and energy-saving manner. Little did the worlds' airlines realize that they had stumbled on a hub system used for millions of years by the eukaryotic cell secretory pathway, the trans-Golgi network (TGN), to collect, package, and sort numerous molecules to their final cellular destinations. The seminal treatise published just 15 years ago by Griffiths and Simons [1] explained the TGN as a ‘specialized organelle on the *trans* side of Golgi stack that is responsible for the routing of proteins to lysosomes, secretory vesicles and the plasma membrane from the Golgi complex’. During the past several years, this simple concept has been expanded to include identification of the effectors – including proteins, lipids, various small molecules, and components of the cytoskeleton – that control the sorting capacity of the TGN. In this review, we summarize

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recent findings regarding new concepts of TGN structure and molecular dynamics and how they have affected our view of the TGN and the regulation of membrane traffic.

Structure and dynamics of the TGN

Newly synthesized proteins of the secretory pathway, which are not retained in the endoplasmic reticulum (ER), are targeted to the Golgi complex. The Golgi complex of most eukaryotic cells is a series of membrane-bound flattened cisternae arranged in a stack of usually four to six cisternae with numerous vesicles associated with the rims of the stack [2]. The cisternae are generally classified into cis, medial, or trans cisterna. Secretory pathway proteins transported from the ER enter the stack at the cis face, move through the various cisternae and then exit at the trans face. The stacked Golgi cisternae have two tubular-reticular structures associated with them. One is associated with the cis face and is variously known as the cis-Golgi network (CGN), ER-Golgi intermediate compartment (ERGIC), or the intermediate compartment (IC) [3-6]. This compartment is thought to be the entry site for cargo transported from the ER and may arise directly from the fusion of ER-derived transport intermediates known as vesicular-tubular clusters (VTCs) [7,8]. The other tubular-reticular structure is known as the trans-Golgi network (TGN) and is thought to be the organelle responsible for sorting secretory pathway proteins to their final destinations [1].

The presence of a membranous compartment associated with the trans face of the Golgi stack was first suggested through the work of Novikoff and colleagues [9]. These studies demonstrated that membrane elements close to the Golgi apparatus were, like lysosomes, cytidine monophosphate positive, and various data suggested that these structures were specialized regions of the smooth ER [10]. This led to the use of the acronym GERL to describe a structure 'intimately related to the Golgi saccule, that is part of the ER, and that forms lysosomes' [9]. Subsequently, several lines of evidence argued strongly against the continuity of this compartment with the ER [reviewed in ref. 11], which led to a new acronym, the TGN, to describe this compartment [1].

The vast majority of structural and morphological information about the TGN has come from thin- and thick-section electron microscopy (EM) studies on chemically fixed cells. The first three-dimensional view of the TGN came from studies on Sertoli cells by Rambourg and colleagues [12] that revealed flattened saccules that were 'peeling off' from the trans face of the Golgi stack. These saccules were attached to an anastomotic network of membrane tubules, some of which were fragmented into smaller tubules, tubular networks, and vesicles [12].

Further analysis of the TGN structures from 14 different mammalian cell types has shown that TGNs vary widely in both size and configuration [comprehensively reviewed in ref. 13]. In general, cells that do not form large secretory granules but have extensive lysosomal systems also have extensive, multilayered TGN structures. Cells that form small- to medium-size secretory granules have small TGN structures that appear to be residual fragments associated with the trans cisternal elements of the Golgi stack. Cells producing very large secretory granules, on the other hand, do not appear to have TGN structures at all. These observations suggest that TGNs are not stable or permanent structures but are constantly undergoing renewal; moreover, the observed morphology and size of the TGN in a particular cell type appears to depend on the post-TGN intermediates that are formed in those cells.

More recent views of the three-dimensional structure of the entire Golgi complex have come from the elegant studies by Ladinsky and colleagues using dual-axis, high-voltage EM tomography of cryofixed, freeze-substituted normal rat kidney cells [14]. These studies failed to show the presence of any interconnected reticulum of tubules at the trans face of the Golgi stack. The Golgi structure elucidated by this technique is comprised of seven stacked, fenestrated cisternae with polymorphic membranous elements associated with the cis Golgi

cisterna (suggested to correspond to the ERGIC). Interestingly, while the structure lacks a classical TGN-like compartment (in terms of morphology), all three of the trans-most cisternae demonstrate tubules that project into the trans space of the Golgi complex, many of which contain coat-like structures at the tubule tips. Each of these cisternae appears to produce only one type of coated vesicle structure, with clathrin-coated buds present only on the seventh cisterna. These data have raised the possibility that these three cisternae together could constitute what is commonly viewed as the TGN, and that the TGN is not actually an independent compartment; the tubular reticular structures previously observed could have resulted from changes in cisternal architecture due to events during the relatively slower fixation and staining procedures involved in more traditional EM. However, the data presented by Ladinsky and colleagues show the structure of only approximately 5% of the entire Golgi complex, and looked at only one cell type. Therefore, the possibility that TGN structures of the classical tubular-reticular morphology exist elsewhere in these cell types or in different cells cannot be discounted. Whether or not the TGN turns out to be an independent compartment has important consequences for how we understand sorting mechanisms for cargo moving through the secretory pathway. The model from Ladinsky et al. [14] suggests that sorting would occur in sequence with specific cargo budding from distinct cisternae (each of the three trans-most), whereas the traditional view of the TGN would predict parallel sorting with cargo budding into different carrier vesicles from the same 'cisterna' (fig. 1). It will be intriguing to see structural information on the Golgi complexes from the various cell types studied by Rambourg and colleagues, using the technique described by Ladinsky and colleagues. Interestingly Ladinsky and colleagues show an intimate association of ER elements with the trans cisterna of the Golgi, perhaps suggesting that the dismissal of the original GERL hypothesis may have been premature.

Much of the data that support the view of the TGN as a distinct compartment from the main Golgi stack have arisen from studies with the fungal metabolite brefeldin A (BFA). Incubation of cells with BFA was found to inhibit ER-to-Golgi transport, resulting in the rapid redistribution of Golgi markers into the ER [15-17]. This redistribution was correlated with the tubulation of the Golgi stack, with these tubules moving along microtubules and subsequently fusing with the ER [18]. The TGN was shown to undergo similar tubulation in the presence of BFA but these tubules fuse with the endosomal system, and markers of the TGN such as TGN38 are redistributed to a 'collapsed' area around the microtubule organizing center (MTOC) [19-21]. Thus, the different responses of proteins in the Golgi stack and TGN to BFA have been taken to demonstrate the functional and physical differences between these two compartments. However, all the markers of the TGN studied in the presence of BFA [such as TGN38, furin, and mannose-6-phosphate receptor (MPR)] are actively cycling proteins that exit the TGN and can reach the cell surface, after which they enter the endosomal system. All the Golgi stack markers studied in the presence of BFA (such as the numerous glycosylation enzymes), on the other hand, do not commonly exit the trans face of the Golgi and are more stable residents of the Golgi complex. In light of the findings of Ladinsky *et al.* it is conceivable that TGN marker proteins are actually present in the trans cisternae of the Golgi stack, and that BFA allowing their unregulated exit. Such events would allow all these TGN markers to leave the Golgi cisternae, and the subsequent accumulation around the MTOC could be due to an inhibition of endosome-to-TGN recycling pathways. One method to clear up some of the confusion about the true nature of the TGN would be to combine immunolocalization techniques with the structural determination techniques of Ladinsky *et al.* It would be of great interest to see which of the three trans cisternae hold the greatest concentration of TGN markers, such as TGN38 and furin, within the cryo-EM, three-dimensional structure of the Golgi.

Whatever the true nature of the TGN in mammalian cells, various elements of the cytoskeleton are known to play pivotal roles in the structure and function of the whole Golgi complex. The proximity of the Golgi complex to the MTOC demonstrates the close relationship of this

organelle with the microtubule network. Microtubules are known to contribute to maintaining Golgi morphology, transport to and from this organelle, and equal partitioning of the Golgi into daughter cells during mitosis [reviewed in ref. 22]. There is also an emerging body of evidence showing the involvement of actin cytoskeleton components in Golgi morphology and function. One actin cytoskeleton protein, spectrin, is associated with many organelles; spectrin forms extensive multifunctional scaffolds by binding directly or indirectly to numerous membrane proteins, cytosolic proteins, phospholipids, and other cytoskeletal proteins [23]. One particular form of spectrin, $\beta 3$ spectrin, has been shown to associate with the Golgi complex [24]. This Golgi-associated spectrin is thought to be involved in maintaining the structure of the Golgi complex and orchestrating protein traffic in the secretory pathway [comprehensively reviewed in ref. 25]. The association of spectrin with the Golgi complex is regulated by the small GTP-binding protein ARF, probably due in part to ARF-stimulated synthesis of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂], a target of direct interaction for spectrin [26,27]. Currently there are two compatible hypotheses for the involvement of spectrin in Golgi traffic. The first model, known as the mesh hypothesis, states that the Golgi-associated spectrin network forms a template for the Golgi structure and that the ability of the mesh to undergo rapid (ARF-dependent) remodeling allows pre- and post-Golgi transport intermediates to be released through the spectrin mesh [28]. The second hypothesis states that the Golgi spectrin network, including various adapter proteins, forms the basis for the physical capture of integral membrane proteins by direct interaction with their cytosolic domains, and the subsequent inclusion of such cargo into forming transport intermediates [29]. It will be interesting to assess the relative importance of these two models for the involvement of this intriguing Golgi scaffold in the morphology of the TGN and its effect on protein sorting.

Lysosomal sorting, coat components, and sorting signals

Proteins destined for endosomes or lysosomes are generally sorted away from the trafficking pathways followed by secreted proteins at the TGN and are instead targeted to the endocytic pathway compartments. Classical examples of proteins that follow this route are newly synthesized lysosomal hydrolases. These proteins are cotranslationally translocated into the ER and undergo glycosylation similar to normal secretory pathway proteins. However, certain oligosaccharide side chains on these lysosomal hydrolases are further modified in the Golgi apparatus by the addition of 6-phosphomannosyl residues. The post-translational addition of these mannose 6-phosphate (M6P) groups allows the newly synthesized lysosomal hydrolases to be recognized by transmembrane receptors, the MPRs. The MPR-lysosomal hydrolase complexes are then transported from the TGN to endosomal compartments. Lysosomal enzymes dissociate from the MPRs in endosomes due to their acidic environment. From these endosomal compartments, lysosomal hydrolases are subsequently delivered to the lysosomes, while the receptors are returned to the TGN for further rounds of transport [30]. The activity of MPR to cycle between the TGN and endosomal compartments is not unique to MPR, and other transmembrane proteins such as TGN38 and furin also follow similar pathways [31].

To achieve correct sorting of different TGN proteins, vesicle coat proteins can recognize different types of sorting signals contained in the cytoplasmic domain of cargo molecules and sort them into corresponding transport vesicles. Clathrin- and adaptor-coated vesicles have been characterized in the TGN and endosome system [32]. Clathrin/AP-1 coated vesicles are involved in vesicle formation on the TGN and are thought to mediate the sorting of lysosomal hydrolases (fig. 2). Clathrin/AP-2-coated vesicles form at the plasma membrane and mediate clathrin-dependent endocytosis. Two additional adaptor complexes have been identified: AP-3 [33,34] and, most recently, AP-4 [33,35,36]. Whether AP-3 and AP-4 are also associated with clathrin is still unsettled. AP-3 is suggested to mediate the direct transport of some lysosomal proteins to the lysosomes from the TGN, while the function of AP-4 is not yet clear.

Despite the different trafficking pathways in which adaptor complexes are involved, they are all heterotetramers that are composed of two large, one medium, and one small adaptin subunit [37]. AP-1 (composed of γ , β 1, μ 1, and σ 1 adaptins) has been shown to mediate the transport of many proteins, including the MPRs, furin, some viral membrane glycoproteins such as the Varicella zoster virus glycoprotein E (VZV-gE), from the TGN to endosomes [38]. AP-1 is recruited onto the TGN membrane via the small GTP-binding protein ARF1. The interaction of AP-1 with TGN membranes is blocked by BFA and enhanced by GTP γ S, a nonhydrolyzable analogue of GTP [39,40]. The GTP-dependent ARF1 binding on the TGN membrane creates high-affinity binding sites for AP-1 and initiates clathrin/AP-1 coat assembly [41,42]. The cytoplasmic domain of the major cargo molecule MPR is not thought to be involved in the initial ARF1-mediated AP-1 recruitment on the TGN membrane. However, the MPR cytoplasmic domain seems to affect the stability of ARF1-GTP on the membrane, thereby also affecting the formation of the clathrin-coated vesicles [42]. AP-1 has also been observed to be present on endosomes [43]. Although the functional role of endosomal-associated AP-1 is not yet known, the clathrin/AP-1 coat could be involved in endosomal recycling, a suggestion that is bolstered by the observation that clathrin is associated with tubular endosomes [44]. Recent studies on transgenic mice in which the μ 1A gene locus was disrupted showed that the remaining AP-1 adaptins fail to bind to membrane (except in epithelial cells where the epithelial-specific μ 1B is intact). In μ 1 A-deficient cells, localization of MPRs is shifted to an endosomal compartment, while lysosomal enzyme delivery is normal [45]. These data suggest that AP-1 may have important functions in the retrograde transport of proteins from endosomes to the TGN [45].

In the search for answers to the question of the role of AP-1 in TGN sorting pathways, novel proteins have been discovered that are associated with AP-1 or are homologous to subunits of AP-1. For example γ -synergin has been shown to directly interact with γ adaptin by the yeast two-hybrid system [46]. Immunofluorescence analysis shows that γ synergin displays an extended perinuclear localization pattern similar to γ adaptin; γ synergin contains EH (Eps15 homology) domains, a domain involved in protein-protein interaction in intracellular trafficking [47,48]. However, the role of γ synergin in clathrin/AP-1-coated vesicle assembly remains to be discovered. In the search for homologues to γ adaptin and the effectors of ARF1, five groups recently found a new family of proteins termed either Vear or GGA proteins (Golgi-localized, gamma ear-containing, ARF-binding proteins) [49-53]. The GGA protein was functionally identified in a yeast two-hybrid screen through interaction with an activated form of ARF3. GGA proteins contain an N-terminal VHS domain [a domain found in Vps27, Hrs (hepatocyte growth factor receptor-regulated tyrosine kinase) and STAM (signal-transducing adaptor molecules)], a domain found in a number of proteins implicated in membrane traffic, and a C-terminal domain with 70% identity to the C-terminal 'ear' domain of γ adaptin. GGA proteins are localized to the TGN and overexpression of GGA altered the localization of TGN38 and MPR [51]. The membrane-binding domain on GGA (termed GAT) interacts with ARF, and overexpression of the GAT region causes dissociation of a number of coat proteins including AP-1, AP-3, AP-4, and COP-I [52]. In yeast, two homologues of GGA have been found and the deletion of both genes results in the missorting of the vacuolar enzyme carboxypeptidase Y (CPY) [52,53]. Taken together, these studies have demonstrated that in addition to AP-1 adaptor complexes, other proteins appear to be involved in the regulation of sorting from the TGN.

The AP-3 adaptor complex is another coat complex involved in transport to lysosomes (fig. 2). Studies in yeast reveal that yeast cells have two distinguished targeting pathways for transporting proteins to the degradative compartment (the vacuole in yeast). These two pathways are defined by the different trafficking itineraries of the proteins CPY and alkaline phosphatase (ALP) [54]. The delivery of CPY in yeast uses the classical Vps pathway (TGN-endosome-vacuole), similar to the transport of lysosomal enzymes in mammalian cells.

However, the transport of ALP from the TGN to the yeast vacuole bypasses the endosomal compartments; instead, ALP is directly sorted from the TGN to the vacuole. The yeast homologue of the AP-3 complex has been shown to be involved in ALP transport to the vacuole. AP-3 is composed of δ , β 3, μ 3, and σ 3 adaptins. The deletion of yeast AP-3 proteins prevents the appropriate localization of ALP and Vam3p (a vacuolar t-SNARE), two cargo molecules that have been so far found to follow the ALP pathway, but has no effect on the vacuolar delivery of CPY [55,56]. Similar pathways might exist in higher eukaryotic cells. A number of naturally occurring mutations in AP-3 genes have been found in *Drosophila*, mouse, and human cells. They all show common defects in the biogenesis of lyso-some-related organelles such as melanosomes and platelet dense granules [54,57]. In biochemical studies, AP-3 was shown to be involved in the lysosomal transport of several lysosomal integral membrane proteins such as LAMP1 and LIMP-II [57]. At the molecular level, AP-3 was shown to interact with the cytoplasmic tail of the LIMP-II and the melanosome-associated protein tyrosinase, and the binding is dependent on a dileucine sequence on both proteins [58]. Taken together, these data suggest that the mammalian AP-3 complex mediates transport from the TGN to lysosomes (or lysosome-like organelles) and that this pathway is different from the one followed by MPR.

The proper sorting of cargo molecules requires not only the coat proteins but also specific sorting signals on the cytoplasmic domain of these cargo molecules. Adaptor complexes are recruited to the cytoplasmic tails of itinerant membrane proteins through conserved amino acid sequences that constitute specific sorting motifs. Tyrosine-based motifs, a family of sorting signals based on the amino acid sequence YXX \emptyset (X is any amino acid and \emptyset is an amino acid with a bulky hydrophobic side chain), is one of the best-characterized sorting motifs. It has been observed that these motifs direct endosomal and lysosomal targeting, internalization from the plasma membrane, and sorting at the TGN [59]. As mentioned the YXX \emptyset motif has been shown to interact directly with the μ subunits of all known adaptor complexes by yeast two-hybrid analyses and by biochemical interactions [60-63]. However, given that each adaptor resides in distinct subcellular locations and that cargo-adaptor interactions are necessary for proper trafficking between various compartments, additional levels of specificity must exist that govern interactions between specific μ subunits and the various tyrosine-based motifs. Recent crystallographic studies have illustrated a possible mechanism by which the sequence of the tyrosine-based motif could mediate the sorting itineraries by favoring interaction with specific adaptors. Owen et al. [64] cocrystallized the C-terminal two-thirds of μ 2 with peptides containing the tyrosine-based motifs of TGN38 (DYQRLN) and epidermal growth factor (EGF) receptor (FYRALM). Their studies have reiterated that tyrosine at position 1 and a bulky hydrophobic residue at Y+3 are necessary for interaction with adaptors. Both these residues are buried in a hydrophobic binding pocket, while the hydroxyl group of the tyrosine participates in a series of hydrogen bonds within μ 2 residues. Interestingly, the affinity of this interaction is modulated by the amino acids at positions Y+1 and Y+2 within the tyrosine-based motif. The arginine at position Y+2 in TGN38 donates a hydrogen bond to μ 2 and exposes its guanidinium group to the solvent, whereas the arginine at Y+1 in the EGF receptor is highly unordered and makes no significant contacts with μ 2. These preferences could suggest a mechanism by which the large group of tyrosine-based sorting motifs can be divided into specific signals that follow separate sorting itineraries. The μ chains of the different adaptor complexes could have varying affinities for amino acids at the Y+1 and Y+2 position of tyrosine motifs depending upon the nonconserved residues present within the various μ chains. This would allow the recruitment of the different adaptor complexes to different cytoplasmic tails. Indeed, μ 1, μ 2, and μ 3a all have been shown to bind different subsets of YXX \emptyset , favoring nonpolar, arginine-rich, and acidic amino acids at the X positions, respectively [60,63].

Dileucine-based motifs form another class of sorting motifs present in the TGN/endosomal system. These motifs have been shown to be recognized by AP-1, AP-2, and AP-3 probably

through the interaction with β adaptins [65]. A significant degree of overlap exists between the sorting itineraries directed by the presence of tyrosine and dileucine-based motifs: dileucine motifs also mediate internalization from the plasma membrane (PM) and sorting to endosomes [32]. One of the two leucines in the dileucine-based motif can be replaced by isoleucine, valine, or methionine and many dileucine-based motifs require surrounding acidic residues and phosphorylatable residues for their function [38]. For example, the CD3 γ receptor uses SDXXXLI as an internalization signal, and both the aspartic acid and serine phosphorylation are important for efficient internalization [66,67]. Acidic clusters, which are stretches of several acidic amino acids, can act in concert with dileucine- and tyrosine-based motifs to mediate protein trafficking. First identified in the cytoplasmic domain of furin and later found to be common to many other membrane proteins [68], acidic clusters often contain consensus casein kinase 2 (CK2) sites that serve as TGN retrieval signals [69]. Although furin contains both dileucine- and tyrosine-based motifs, efficient TGN localization of furin cannot occur unless the serines of the acidic cluster are phosphorylated [70,71]. Acidic clusters contained in a number of other membrane proteins have also been shown to be important for their TGN localization and sorting, including MPR [72] and MHC-II [73]. Direct interaction between acidic clusters and adaptor complexes has not yet been reported. However a 'connector' protein called PACS-1 has been shown to link phosphorylated acidic cluster sorting motifs to AP-1 adaptor complexes [74,74a] (and see below).

Retrograde transport to the TGN

Lysosomal enzyme-MPR complexes dissociate in the low-pH environment of the late endosomes, allowing the lysosomal hydrolases to be transferred to the lysosomal compartment while the MPRs are recycled to the TGN for further rounds of transport. Other proteins that cycle through the TGN/endosomal system, such as furin and TGN38, are also thought to follow similar transport steps from endosomes to the TGN. Very little is known about the precise transport intermediates and proteins regulating these retrograde transport activities to the TGN. MPRs were shown to return to the TGN from the late endosomal compartment, and this transport is dependent on the small GTP-binding protein rab9 [75,76]. Furin has also been reported to follow the same return pathway, whereas TGN38 seems to return from the early endosomal compartments directly to the TGN [31].

Recently, proteins that appear to be involved in retrograde transport to the TGN have begun to be identified in mammalian cells, including the AP-1 adaptor complex and two novel proteins, TIP47 and PACS-1. Studies on cell lines derived from transgenic mouse embryos with a disrupted μ 1A gene have provided good evidence for the role of AP-1 in retrograde transport to the TGN [45]. However, the molecular mechanisms of AP-1 involvement in such transport have yet to be clearly demonstrated. A new cytosolic protein called TIP47 has been identified and shown to be required for MPR transport from late endosomes to the TGN. Using the cation-dependent MPR (CD-MPR) cytosolic domain as bait in a yeast two-hybrid screen, TIP47 was found to interact with a phenylalanine/tryptophan (FW) motif on CD-MPR [77]. TIP47 was shown to be associated with endosomes and required to mediate CD-MPR recycling from endosomes back to the TGN through a combination of in vitro endosome-TGN transport assays (with TIP47 immunodepletion) and antisense studies. These data suggest that TIP47 is directly required for the efficient sorting of CD-MPR into the transport intermediates destined for the TGN [77]. TIP47 also interacts with the cytosolic domain of the cation-independent MPR (CI-MPR) and is required for the transport of CI-MPR from endosomes back to the TGN [78,79]. However, the CI-MPR cytosolic domain does not contain an FW motif as found in CD-MPR; the binding of TIP47 to CI-MPR seems to depend on the membrane-proximal region, as well as the secondary structure of the CI-MPR tail [78]. Interestingly, the binding of TIP47 to the CI-MPR cytosolic domain is competed by the binding of AP-2 but not AP-1 to CI-MPR, suggesting a mechanism that prevents TIP47's interference with MPR endocytosis but allows

both TIP47 and AP-1 to act in concert [78]. Among all the cytosolic domains of potential protein cargo tested, TIP47 could interact only with CI- and CD-MPR but not with other TGN/endosomal cycling proteins, such as furin, TGN38, or carboxypeptidase D (CPD). These data suggest that the action of TIP47 may be specific to the MPRs [79].

To maintain its steady-state localization in the TGN, furin is actively retrieved from post-TGN compartments back to the TGN in a phosphorylation-dependent manner [69]. Using a yeast two-hybrid screen with a mutant mimicking the phosphorylated form of the furin cytosolic domain, a novel protein termed phosphofurin acidic cluster sorting protein-1 (PACS-1) was discovered. PACS-1 has been shown to interact specifically with the CK2-phosphorylated form of the furin cytosolic domain, and the presence of PACS-1 is also required for the correct TGN localization of furin [74]. While the phosphorylation of the furin acidic cluster motif by CK2 maintains furin's TGN localization, conversely, the dephosphorylation of furin's acidic cluster by PP2A ACB α isoform is required for the transport of furin between endosomal compartments [80]. The interaction of PACS-1 specifically with the CK2-phosphorylated form of furin has revealed a new way by which cellular trafficking machinery can distinguish between phosphorylated and nonphosphorylated motifs within cargo molecules such as furin. PACS-1 behaves as a connector that forms a ternary complex with furin and AP-1, allowing the inclusion of phosphorylated furin into clathrin/AP-1 coated vesicles [74a]. Such a possibility correlates well with a role of AP-1 in retrieval pathways to the TGN. Furthermore, PACS-1 also recognizes acidic cluster-sorting motifs contained in other TGN/endosomal proteins, including CI-MPR, PC6B, CPD, and some viral proteins, such as the human cytomegalovirus glycoprotein B (HCMV-gB), and is required for their TGN retrieval [68,74,81]. Recently, PACS-1 was also found to interact with HIV-1 Nef protein, which contains a similar acidic cluster motif (EEEE₆₅) and this acidic motif on Nef was shown to be required for the Nef-mediated down-regulation of MHC-I. Coimmunoprecipitation and immunofluorescence studies showed that cellular PACS-1 binds to the viral Nef acidic cluster and directs Nef to the TGN, where it is required for the down-regulation of MHC-I [82]. The function of PACS-1 is just beginning to be understood and the current knowledge on PACS-1 suggests an important role of this protein in TGN retrieval of cellular proteins as well as a role in viral pathogenesis (fig. 2).

In yeast, the sorting of CPY by Vps10p (the CPY receptor) is analogous to the sorting of lysosomal hydrolases by the MPR [83,84]. The late Golgi compartment in yeast is defined by the presence of the proteolytic enzymes Kex1p, Kex2p, and dipeptidyl aminopeptidase A (DPAP A), and is considered to be the functional equivalent of the mammalian TGN. Similar to the MPR in mammalian cells, Vps10p cycles between the late Golgi compartment and the prevacuolar compartment (PVC; equivalent to late endosomes in mammalian cells). The retrieval of Vps10p from the PVC to the TGN has been shown to depend on the products of several yeast genes, termed the 'retromer' complex [85,86]. The retromer complex is formed from the assembly of two subcomplexes: the Vps35p-Vps29p-Vps26p and the Vps5p-Vps17p complex. Cofractionation of Vps35p with Vps10p has suggested that the role of the Vps35p/29p/26p complex is cargo selection [85]. Furthermore, it has been recently shown that Vps35p could be coimmunoprecipitated with the cytosolic domain of DPAP A, which supports the model of Vps35p involvement in cargo sorting [87]. Vps5p bears self-assembly capacity and physically interacts with Vps17p [86,88,89]. The Vps5p/17p complex may be involved in further assembly of the retromer complex. In mammalian cells, Vps5p is homologous to sorting nexins, a family of proteins that have been shown to be involved in the retrieval of various cargo receptors [88-90]. The human orthologues of Vps26, Vps29p, and Vps35p have been recently cloned and this study further confirms that human Vps26p-Vps29p-Vps35p forms a complex, with the Vps35 protein serving as a core domain of assembly [91].

Through the study of A-ALP, a chimera of the cytosolic domain of DPAP A fused to the transmembrane and luminal domains of ALP, a new gene *grd19* was found to be required for the proper localization of A-ALP [92]. A *grd19* null mutation causes mislocalization of A-ALP and Kex2p to the vacuole, but the sorting of Vps10p was largely unaffected. Grd19p is localized to the prevacuole compartment and interacts with the DPAP A cytosolic domain. It has therefore been suggested that Grd19p is involved in the retrieval of certain TGN proteins from the prevacuolar compartment. Another new gene, *grd20*, was also found to be involved in protein sorting in the TGN/endosomal system in yeast [93]. Interestingly, perturbation of *grd20p* function results in rapid mislocalization of Kex2p to the vacuole, while the trafficking of AALP and Vps10p remains unaffected. Taken together, it seems that while the retromer complex is involved in the retrieval of all cargo that recycle from endosomes back to the TGN, Grd19p is specifically involved in the retrieval of DPAP A and Kex2p, and Grd20p is specifically involved in the retrieval of Kex2p. These data suggest either that different TGN proteins follow different trafficking routes for their return to the TGN or that they require different mediators for their inclusion into the same retrograde transport vesicles.

In a recent screen of yeast mutants with vacuolar sorting defects, a new multimeric complex composed of Vps52p, Vps53p, and Vps54p has been found to be required for retrograde transport to the TGN [94]. These three proteins are associated in a 1:1:1 stoichiometry, and a *vps52/53/54* triple mutant exhibits indistinguishable phenotype from single mutants of each of these three genes. The mutant strains all fail to transport a number of late Golgi proteins (including Vps10p and Kex2p) back to the TGN, resulting in the mislocalization of these proteins to the vacuole where they are cleaved by vacuolar proteases. These data suggest that Vps52p-Vps53p-Vps54p is yet another complex involved in TGN retrieval. However, it is unlikely that this complex is involved in either cargo sorting or budding from the PVC because it is localized to the late Golgi membrane. Therefore, it has been speculated that this Vps52p/53p/54p complex is involved in the docking and fusion of the retrograde transport vesicles with the late Golgi compartment (fig. 2).

Involvement of lipids and lipid-modifying enzymes in post-Golgi transport

In the complex process of vesicle formation, proteinaceous coats play important roles in initiating cargo sorting and deformation of the donor membranes. However, proteins are not the only constituents of the transport machinery. Lipids, the major component of membranes, have recently been discovered to play an active role in regulating membrane dynamics. Along the secretory pathway, various forms of phosphoinositides (PIs) and the hydrolyzed products from phosphatidylcholine (PC), such as phosphatidic acid (PA), play important roles in post-Golgi transport. The production and the balance of these lipids are controlled by various lipid kinases and lipid transfer proteins, which tightly regulate membrane transport.

The yeast protein Sec14p is a phosphatidylinositol transfer protein (PITP) that is required for post-Golgi transport in *Saccharomyces cerevisiae* [95-97]. A similar role for the mammalian PITP has also been demonstrated [98-100]. PITP is an abundant cytosolic protein that contains a single phospholipid-binding site per protein. This binding site can interact separately with PI and PC with high affinity and PITP functions to transfer PI and PC from one compartment to another. As mentioned, the yeast PITP (Sec14p) is essential for protein transport from the Golgi complex. The *sec14-1ts* mutant shows a Golgi secretory block at the restrictive temperature. Secretory pathway glycoproteins that are blocked in this mutant yeast strain are shown to have acquired their terminal glycosyl modifications, suggesting the block caused in *sec14-1ts* occurs at a late Golgi compartment. EM studies also show an accumulation of cisternate-like tubules at the restrictive temperature in the *sec14-1ts* yeast strain [95,96]. In mammalian systems, PITP has been shown to be involved in many secretory pathway events. These include mediating the Ca²⁺-stimulated exocytosis of secretory granules in

semipermeabilized neuroendocrine cells [101], the budding of the secretory vesicles and immature secretory granules (ISGs) from the TGN in neuroendocrine cells [102], and the formation of post-Golgi, polymeric IgA receptor (pIgR)-containing vesicles [103]. Recently, a PITP mutant that is incapable of PI transfer activity but is still capable of PC binding and transfer was shown to be able to rescue the secretory defect in *sec14-1ts* yeast [104]. This study suggests, then, that the PC binding and transfer activity, rather than that of PI, may be more directly involved in the control of vesicular trafficking. The exact mechanism of how PITP regulates secretory vesicle generation is not yet clear, but most likely it acts as a sensor of the balance between the PC and PI metabolic pathways.

Consistent with the hypothesis that PITP regulates post-Golgi transport by maintaining balanced levels of PC and PI along the secretory pathway, two groups of genes were discovered that are involved in PC or PI metabolism in yeast and are capable of rescuing the *sec14* defects (fig. 3). One such gene is *pld1*, the gene encoding yeast phospholipase D (Pld1p, PLD) [105, 106]. PLD is a lipid hydrolase that converts PC to PA and a hydrophilic head group choline. In eukaryotic cells, PLD has previously been shown to mediate intra-Golgi transport [107, 108] and post-Golgi secretion in the regulated secretory pathway [109]. In permeabilized pituitary GH3 cells, the release of nascent secretory vesicles can be measured following the secretion of growth hormone (GH) and prolactin. Using this system, the addition of mammalian PLD1 or plant PLD could stimulate GH-containing vesicle release. In mammalian cells, PLD1 is a PLD isoform that is activated by ARF1 and localizes to ER, Golgi, and endosomes. The enzymatic activity of PLD to hydrolyze PC to PA is required for GH release, and under conditions of enhanced budding of GH-containing vesicles, a concomitant stimulation of PLD activity is observed [109].

As the increased levels of PA produced by PLD can be metabolized very quickly to diacylglycerol (DAG), the question was raised as to which of the two lipids is actually required for TGN budding. By adding PI-specific phospholipase C (PI-PLC) and DAG kinase to produce DAG and PA independently from PLD activity, Siddhanta and Shields [110] showed that the accumulation of PA, rather than DAG, seems to be the key requirement. To support this idea, when permeabilized GH3 cells are incubated with a primary alcohol, 1-butanol, to compete with PA production, the secretion of growth hormones from the TGN is inhibited [111]. Together, these data suggest a more important role for PA than for DAG in secretory vesicle release and that DAG might instead play a role in signaling events.

The other *sec14* suppressors is a group of enzymes involved in the metabolism of PIs: phosphatidylinositol 4 phosphate (PI4P) and PI (4,5)P₂. In yeast, *Sac1* was recently shown to suppress the *sec14-1ts* defect [112]. The yeast *Sac1* gene encodes a phosphatase that hydrolyzes PI4P and some other PIs. PI4P accumulates in the *sac1* mutant, and the high level of PI4P can rescue the *sec14-1ts* secretory defect. These data suggest that PI4P is a critical lipid involved in secretion. Overexpression of *Pik1p*, one of the two yeast PI 4-kinases, can overcome the secretion defect in the *sec14-ts* strain by producing PI4P, thus bypassing the decreased level of PI4P that occurs at the restrictive temperature. In an independent screen to isolate yeast post-Golgi secretory mutants, *Pik1p* was also found to be involved in post-Golgi release of invertase, a secretory marker [113].

Pik1p is also found to be associated with *Frq1*, a homologue of frequenin in yeast. *Frq1* binds to a conserved motif in *Pik1p* outside its catalytic domain, and stimulates the lipid kinase activity of PIK1 *in vitro* [114]. In mammalian cells, PI 4-kinase activity is associated with small synaptic vesicles and chromaffin granules and has been shown to be required for stimulated secretion [115,116]. Both mammalian and *Drosophila* frequenin also function in neurotransmission. *Drosophila* frequenin is thought to modulate synaptic vesicle releases [117,118], and the mammalian frequenin (also called neuronal calcium sensor-1) is thought to

sense the local Ca^{2+} concentration and promote regulated secretion of dense-core granules in neuroendocrine cells [119]. Hence, the association of PI 4-kinase and Frq1 in yeast suggest that PI 4-kinase could act together with frequenin in sensing and regulating Ca^{2+} -regulated secretion. Consistent with these suggestions, it has been recently reported that overexpressed frequenin coimmunoprecipitates PI4 kinase (beta isoform) in polarized Madin-Darby canine kidney (MDCK) cells and inhibits the delivery of newly synthesized influenza HA from the TGN without affecting other transport steps. These data demonstrate a role of both frequenin and PI4 kinase in apical post-Golgi transport [120].

The phosphorylation and production of particular PIs are also regulated by the PLD and PA pathway, providing additional evidence for the interconnection of PC and PI pathways in the late secretory pathway. In the search for downstream effectors responsive to the raised level of PA, the level of the phospholipid $\text{PI}(4,5)\text{P}_2$ was found to be increased [111]. The addition of recombinant human PLD1 stimulates $\text{PI}(4,5)\text{P}_2$ synthesis due to an increased level of PA. This PA-stimulated $\text{PI}(4,5)\text{P}_2$ synthesis was shown to be required for secretion, as well as for maintaining Golgi integrity and structure. From an independent report, ARF was also shown to stimulate PI4P and $\text{PI}(4,5)\text{P}_2$ synthesis in the Golgi membrane by a new mechanism [27]. It was shown that ARF can directly recruit PI 4-kinase (beta isoform) and PI4P 5-kinase onto the Golgi membranes, where they become activated and cause an increased level of PI4P and $\text{PI}(4,5)\text{P}_2$. It is suggested that the local production of $\text{PI}(4,5)\text{P}_2$ could result in the formation of microdomains that can act as binding sites for spectrin, certain other actin-binding proteins or dynamin. This group of interactions prompts the local disassembly or rearrangement of the actin cytoskeleton, which in turn allows membrane deformation that facilitates the vesicle formation. The authors further show that a mutant form of the PI4 kinase (PI4K β D656A) lacking the kinase activity causes the Golgi to change into tubular and punctate structures, which is attributed to the disrupted interactions with the actin cytoskeleton proteins. Taken together, these data suggested that $\text{PI}(4,5)\text{P}_2$ is an important lipid that plays a pivotal role in coordinating, the recruitment of coat proteins and various actin-binding proteins around the Golgi. Different signalling pathways through PLD and PA, ARF and PI-4 kinase, PI-5 kinase as well as PITP converge to produce and maintain the level of $\text{PI}(4,5)\text{P}_2$ on the Golgi membrane, thereby maintaining the Golgi structure and regulating the formation of the secretory vesicles [28].

Polarized sorting from the TGN

As opposed to nonpolarized cells that have a continuous plasma membrane, a polarized cell surface is divided into separate domains that perform different physiological functions. In polarized epithelial cells such as MDCK cells, apical and basolateral cell surfaces have very different protein and lipid compositions, and the two domains are separated by tight junctions. To achieve and maintain this polarity, the majority of the newly synthesized apical and basolateral proteins are sorted in the TGN and transported to the appropriate surfaces. Indirect routes also exist to transport apical proteins, first to the basolateral surface and subsequently to the apical surface by transcytosis. In MDCK cells, polarity is primarily achieved by TGN sorting, whereas in hepatocytes or enterocytes, the indirect pathways are more frequently used [121-123].

Sorting of proteins to the basolateral surface in polarized cells relies on specific signals found within cytoplasmic domains of cargo molecules. Tyrosine, dileucine, and acidic sequences, as well as other unrelated amino acid motifs, have been shown to mediate the basolateral targeting of various proteins [for reviews see ref. 121-124]. Many of these basolateral-sorting signals are very similar to motifs that can interact with the clathrin-adaptor complexes involved in lysosomal sorting and endocytosis. Given the similarity of basolateral sorting signals to adaptor interaction motifs, the hypothesis that the clathrin-adaptor sorting machinery is involved in

basolateral targeting naturally follows. To support this point of view, it has been recently shown that pIgR could be coimmunoprecipitated with the AP-1 adaptor en route from TGN to the basolateral membrane [125]. However, it is not clear at which stage in the trafficking of pIgR that the clathrin/AP-1 complex is acting. BFA-sensitive clathrin/AP-1 buds containing basolateral cargo have been observed on endosomal tubules, suggesting that AP-1 could be involved in basolateral cargo sorting from endosomal compartments [126]. However an interaction between pIgR and AP-1 could also be observed in the TGN [125]. These potential disparities may be explained by the possibility that basolateral cargo follows different routes from the TGN to the basolateral surface. An indirect route via endosomes has been shown to occur for a number of basolateral receptors such as pIgR [127], vesicular stomatitis virus-G (VSV-G) [128], transferrin receptor [129], asialoglycoprotein receptor H1 [130], and Semliki Forest virus p62 [131]. The possibility of direct transport routes that do not pass through endosomes, however, cannot be ignored. Evidence for such a direct pathway is suggested by the study of Orzech et al. [125], where a mutant form of the pIgR that failed to interact with AP-1 could be transported in a BFA-insensitive manner directly to the basolateral surface with slower kinetics. Transport vesicles that could mediate such a transport step are not known. Recently, an epithelial-specific μ 1 adaptor isoform, μ 1B, was identified [132]. In a few epithelial cell lines, such as LLCPK1, μ 1B does not appear to be expressed, and several proteins such as low-density lipoprotein (LDL) receptor and transferrin receptor that are normally basolaterally located are missorted in these lines. The missorting of these proteins can be rescued with the expression of μ 1B [133]. Similar to the μ chains from other adaptor complexes, μ 1B recognizes tyrosine-based motifs [132]. However, it is unclear precisely where μ 1B is acting and what is the molecular mechanism of μ 1B action. It has been speculated that μ 1B could function at both the TGN and the endosome due to the dramatic redistribution of LDL receptor and transferrin receptor [121].

Apical sorting mechanisms are fundamentally different from those involved in basolateral sorting. Apical membranes are highly enriched in glycosphingolipids, and apical sorting appears to be mainly based upon lipid-lipid and protein-lipid interactions [123]. One working model for apical sorting is that cholesterol-sphingolipid microdomains are formed in the Golgi and the TGN membrane. These microdomains then function as platforms for the inclusion of apical cargo proteins through high-affinity interactions with lipid and sugar modifications found on the apical-directed cargo molecules [123,134-136]. However, it has been reported that the partitioning of proteins into rafts does not guarantee apical transport for such cargo, suggesting that other sorting machinery may well be involved [137,138].

Cholesterol-sphingolipid microdomains are also called membrane rafts or detergent-insoluble glycolipid-rich (DIG) domains. They can be isolated on a sucrose gradient after a cold Triton X-100 extraction of the cell membrane. Using different microscopic techniques and following glycosylphosphatidylinositol-anchored proteins (GPI-APs), components of the rafts have been observed to be rather small (about 70 nm in diameter), but highly dynamic structures in vivo [135]. Various apical targeting signals have been identified over the years, including amino acid sequences located in the luminal, transmembrane, and cytoplasmic domains of cargo molecules. Furthermore, the lipid modification of GPI-anchored proteins and sugar modifications of the N-linked or O-linked carbohydrates have also been identified as apical targeting signals [123,139]. These sorting signals all have high affinity for lipid rafts.

Currently, no potential protein sorting machinery, such as a proteinaceous coat, has been identified for apical targeting. Some proteins, however, have high affinity for membrane rafts and could mediate raft clustering or protein sorting into the rafts. VIP21/caveolin-1 has a high affinity for cholesterol and has been proposed to form homo-oligomers that interact with GPI-anchored protein [140-142]. However, recent evidence showed that in Fisher rat thyroid cells (cells lacking caveolin), GPI-anchored proteins are mistargeted to the basolateral surface, and

transfection of caveolin-1 into these cells does not rescue the apical sorting even though caveolae are formed under these conditions [143]. These data suggest that caveolin-1 probably plays a less direct role in apical sorting. VIP17/MAL, a tetraspanning membrane protein, has recently been found associated with apical transport vesicles, and the expression level of VIP17 affected apical targeting of influenza HA, giving good evidence for VIP17 involvement in apical targeting [144-146]. Annexin13b, a Ca^{2+} -dependent phospholipid-binding protein has also been implicated in apical transport. This protein was recently shown to localize to the TGN, apical membranes, and vesicle carriers. Annexin13b is also associated with lipid rafts [147,148]. Annexin13b interacts directly with an apically targeted protein, Nedd4 (a ubiquitin protein ligase), and the myristoylation of annexin13b was important for this apical transport function [147,148].

The differential sorting of proteins to axons and dendrites in neuronal cells has often been compared to the basolateral and apical sorting pathways in polarized epithelial cells. As with the apical and basolateral membranes of polarized epithelial cells, axonal and dendritic membranes in neuronal cells have distinct protein compositions and cytoskeletal organization [149]. The sorting of neuronal proteins to dendrites is generally accepted to be similar to the basolateral sorting mechanisms discussed above, with targeting motifs such as the tyrosine-based motifs directing dendritic protein traffic [150]. However, to date, there seem to be few, if any, such similarities between apical and axonal sorting [for a review see ref. 151]. Recently a 'smart motor' model has been proposed to play an important role in controlling the sorting into axons and dendrites. This model postulates that the polarized trafficking of proteins and vesicles is mediated by corresponding microtubule-based motors that are segregated to either axons or dendrites [152]. Whether polarized epithelial cells also have different apical- and basolateral-destined motors remains to be investigated.

Sorting into the regulated secretory pathway

In endocrine and neuroendocrine cells, prohormone molecules are efficiently sorted into the regulated secretory pathway where they are processed and packaged into dense-core secretory granules. In response to physiological stimuli and an influx of extracellular calcium, secretory granules are induced to fuse with the plasma membrane and release their complement of stored molecules. Two models have been proposed for regulated pathway sorting – 'sorting by entry' and 'sorting by retention' [reviewed in ref. 153]. In entry-based models, sorting occurs in the TGN either by the intrinsic ability of regulated pathway proteins to aggregate or by the presence of a sorting signal on the prohormones themselves or by one or more TGN sorting receptors. By contrast, in the retention model, sorting is largely a post-TGN event, occurring in the clathrin- and AP-1-coated ISGs that support the proteolytic maturation of prohormone molecules. This model relies on the ability of granule proteins to condense into a core, leaving nongranule proteins in the luminal periphery where they are removed from the maturing granule apparently by clathrin-based coats.

The basis for the entry model was founded on early studies showing that GH peptide sequence could target a fused reporter protein (a truncated VSV G molecule that is normally constitutively secreted) to secretory granules [154]. The ability of human GH (hGH) to target the reporter to the regulated pathway suggests the presence of a TGN-localized, regulated-pathway sorting receptor that operates similarly to the MPR or Vps10p. However, as the concentration of some granule components exceeds 100 mg/ml, and overexpression of prohormones fails to saturate regulated pathway sorting [155,156], it is unlikely this targeting can be explained by a simple stoichiometric sortase.

The sorting of a large number of granule-destined molecules is simplified by a mechanism that allows such molecules to selectively coalesce into an 'aggregate' that can exclude constitutive

pathway molecules. Such a phenomenon was first determined by analysis of amylase secretion in the exocrine pancreas [157] and is further supported by the elegant morphological analysis by Rambourg et al. [158]. Analysis of lactotrophs (endocrine cells that secrete prolactin) from lactating rats showed the presence of electron-opaque nodular 'progranules' that became abundant in the fenestrated trans-most Golgi cisternae. Consistent with this model, exposure of a number of regulated-pathway proteins [including secretogranin II, chromogranins A and B, proPC2, carboxypeptidase E, prolactin, proopiomelanocortin (POMC), and proinsulin] to low pH (below pH 6.5) and millimolar calcium characteristic of the late Golgi results in the aggregation of the targeted molecules and the exclusion of constitutive secretory pathway markers [reviewed in ref. 159]. The ability of calcium ions to coordinate acidic amino acids near the isoelectric points of the studied proteins may explain the selective aggregation.

The regulated pathway targeting of the hGH/VSV-Gt suggests granule-targeted proteins likely contain a positive sorting signal that in some cases may act independent of aggregation. Although a single universal motif has yet to be identified, numerous studies support the presence of such signals. For example, regulated pathway sorting and aggregation of proatrial natriuretic hormone in AtT-20 cells requires the presence of a pair of glutamate residues within its proregion, raising the possibility that for this proprotein, the sorting and aggregation signals may be one and the same [160]. Sorting of chromogranin B in PC12 cells requires a different signal, one composed of an N-terminal disulfide-bonded loop structure [161]. A similar disulfide loop was reported for the N terminus of POMC [162] although this finding has been questioned [163]. One possible explanation for the conflicting results of the prohormone sorting signals may reside in the cell types studied. For example, in GH4C1 cells, the N-terminal loop of chromogranin B does not affect sorting. Rather, in these cells, an alternate sorting motif near the C terminus of the protein is recognized [164].

The entry model also predicts a sorting receptor that may recognize the aggregated cargo molecules. Intriguingly, one of the enzymes that catalyzes prohormone processing, carboxypeptidase E (CPE), was identified as a sorting receptor for entry to the regulated pathway. Binding studies showed that CPE associates with the N-terminal disulfide-rich segment of POMC and also binds proinsulin and proenkephalin [165]. Consistent with these binding studies, Cool et al. [166] reported that POMC is missorted in CPE $-/-$ (fat/fat) mice. The role of CPE as a prohormone sortase is the center of much speculation, since others have reported that although prohormone processing is impaired in the mutant mice, prohormone sorting is unaffected [167]. As the Ser₂₀₂ \rightarrow Pro mutation in CPE in the fat/fat mice greatly inhibits stability of the enzyme [168], it is difficult to uncouple the enzymatic activity of the CPE mutation from its possible activity as a sortase. Additional studies into the role of CPE in prohormone sorting should address these questions.

Two recent reports investigating the sorting of cargo into the regulated pathway indicate that lipid rafts are membrane components that are used for such sorting. Characteristic of raft-associated molecules, both proPC2 and CPE resist extraction from membranes with 1% Triton X-100 at 4 °C [169,170]. By contrast, chromogranin A was readily extracted under these conditions. Interestingly, treatment of AtT-20 cells with fumonisins 1B (a ceramide synthase inhibitor) caused the missorting of PC2 but not chromogranin A. The raft-independent sorting of chromogranin A is consistent with the CPE-independent sorting of this protein in the CPE (fat/fat) mice [166].

The retention model postulates that the initial sorting from the TGN into the regulated pathway is relatively permissive, with the ISG both housing the endoproteolytic processing of prohormone substrates and also selectively retaining some cargo molecules (i.e., peptide hormones) and membrane proteins (e.g., phogrin, [171]) while removing others (e.g., furin, MPR, and syntaxin 6) after the ISG is initially formed [71,172]. In this way, the ISG is viewed

as an extension of the TGN [153]. However, as TGN38 is not sorted into ISGs [71], the sorting of molecules from the TGN to the ISG to the mature secretory granule (MSG) is perhaps more accurately viewed as a selective and highly regulated distillation apparatus. Furthermore, these results support distinct roles for the ISG and TGN in protein sorting.

ISGs undergo a series of homotypic fusions and a net loss of membrane during their maturation to MSGs. The net loss of membrane is consistent with the transient decoration of the ISG with clathrin coats and suggests that the clathrin-based sorting machinery actively sorts membrane proteins from the ISG. A number of membrane proteins, including furin and the CI-MPR, are sorted into ISGs but are removed prior to granule maturation [71,172]. Sorting of the CI-MPR through ISGs enables the removal of lysosomal hydrolases from this compartment and their transfer to endosomal compartments [173]. The functional role for furin in early compartments within the regulated pathway is not known but may include the activation of PC2 by cleavage of its escort protein, 7B2 [174], and also the processing of several granule proteins including parathyroid hormone [175], chromogranin A [176], and pro-ELH [177].

Consistent with the morphological data, ISG-associated AP-1 appears to participate in removal of furin. Binding studies show that the AP-1 recruitment to ISG membranes is ARF1 dependent [178] and is enhanced by phosphorylation of ISGs with CK2 [71]. Consistent with this finding, furin constructs containing a mutation of the CK2 sites within the furin cytoplasmic domain acidic cluster fail to be efficiently removed from the ISG and are missorted into MSGs [71]. The effect suggests a role for PACS-1 in the retrieval of furin from ISGs; consistent with this model, expression of a PACS-1-dominant-negative construct in AtT-20 cells causes a missorting of co-expressed furin to MSGs [74a]. The presence of phosphorylatable acidic-cluster sorting motifs on the CI-MPR cytoplasmic domain raises the possibility of a similar mechanism contributing to the removal of this protein from ISGs along with lysosomal hydrolases bound to the CI-MPR luminal domain. Whether retention of phogrin on ISGs is an active or a passive sorting step remains to be determined.

As well as the removal of selected membrane proteins from the ISG being a selective process, the ability to retain cargo molecules similarly appears to be an active process. For example, processing of proinsulin to insulin in the ISG facilitates the zinc-dependent condensation of the mature insulin hormone and enhances its selective retention in the MSG [179]. Thus aggregation-based events appear to contribute to aspects of both the entry- and retention-based models.

Conclusion

Our understanding of the TGN compartment has grown quickly over the past few years and new aspects of TGN sorting are constantly emerging. First, using new microscopy technology such as the dual-axis, high-voltage EM tomography of cryofixed cells, the three-dimensional structure of the Golgi compartment has been reconstructed. Second, these structural studies have shown that the last several stacks of the Golgi complex all project different types of transport vesicles, with the clathrin coats specifically localized on the last stack. These observations suggest that different TGN proteins might be sorted in physically distinct stacks, with the respective type of transport vesicle associated to each stack. This is an attractive model for TGN sorting and it will be interesting to localize TGN proteins in the three-dimensional structure to see whether different TGN proteins are indeed localized to different stacks.

As more studies are published, several hot spot areas in TGN sorting mechanisms have emerged. Along the classical lysosomal targeting pathway, new molecules related to adaptors, such as the new family of GGA proteins, have been discovered. The precise function of these GGAs needs to be determined, but their discovery suggests that the clathrin/AP-1 sorting machinery might include many other associated components. For lysosomal targeting, research

on AP-3 has revealed a distinct transport route to the lysosomes that utilizes this adaptor complex. Whether AP-3 is associated with clathrin remains unsettled and the molecular composition of the AP-3-coated vesicles remains to be determined.

Very little is known about the transport machinery required for itinerant TGN membrane proteins to be transported from endosomes back to the TGN. Recently, a series of new molecules have been cloned both in mammalian cells and in yeast that appear to be involved in retrograde transport to the TGN. In yeast, the 'retromer' complex has been shown to mediate the retrograde transport of Vps10p, Kex2p, and DPAP A. It is the first potential 'coat' complex discovered for retrograde transport. While the 'retromer' complex seems to be a general vesicle coat-like protein complex for the transport of all cargo, molecules such as Grd19p and Grd20p were also found to be specifically involved in the retrieval of certain but not all cargo. This situation is similar to PACS-1 and TIP47 in mammalian cells. While both molecules were found to be involved in TGN retrieval, PACS-1 recognizes acidic-cluster TGN-sorting motifs contained in a number of molecules, including furin and MPR, but TIP47 appears to recognize signals only on CI- and CD-MPR and mediates their transport back to the TGN. The relative role of each of these molecules remains to be investigated. One speculation is that there are different retrieval pathways 'toward' the TGN and different categories of cargo might utilize different sorting proteins. Mammalian homologues of the yeast 'retromer' complex have been recently cloned [91] and it will be interesting to determine their function in mammalian cells.

Beside coat proteins, another emerging aspect in TGN sorting is the involvement of lipids, lipid transfer proteins, and lipid kinases. Although PITP has long been known to be involved in the late secretory pathway, more lipids and lipid kinases are now found to act synergistically with PITP to regulate secretion. Specific lipids are proposed to interact with different proteins, including coat proteins and PH-domain-containing proteins such as dynamin, spectrin, and certain other actin-binding proteins. PI(4,5)P₂ appears to be a critical lipid on the Golgi membrane that can regulate local rearrangement of the Golgi membrane skeleton through interaction with PH domains [28]. All together, these studies show that sorting motifs, coat proteins, coat-related and associated proteins, lipids and actin cytoskeleton proteins act in concert to regulate the complex event of TGN sorting. Elucidating the interconnection of these components and their orchestration in TGN sorting is the challenge of the present. As new elements are clarified, we hope to have a more global understanding of the TGN and its sorting mechanisms.

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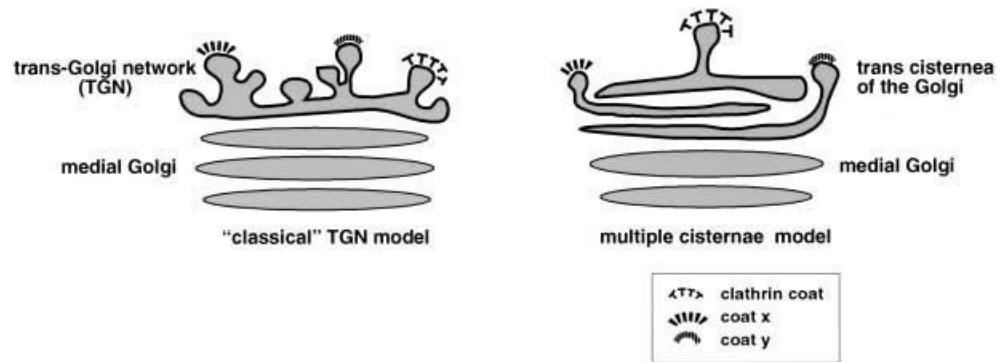
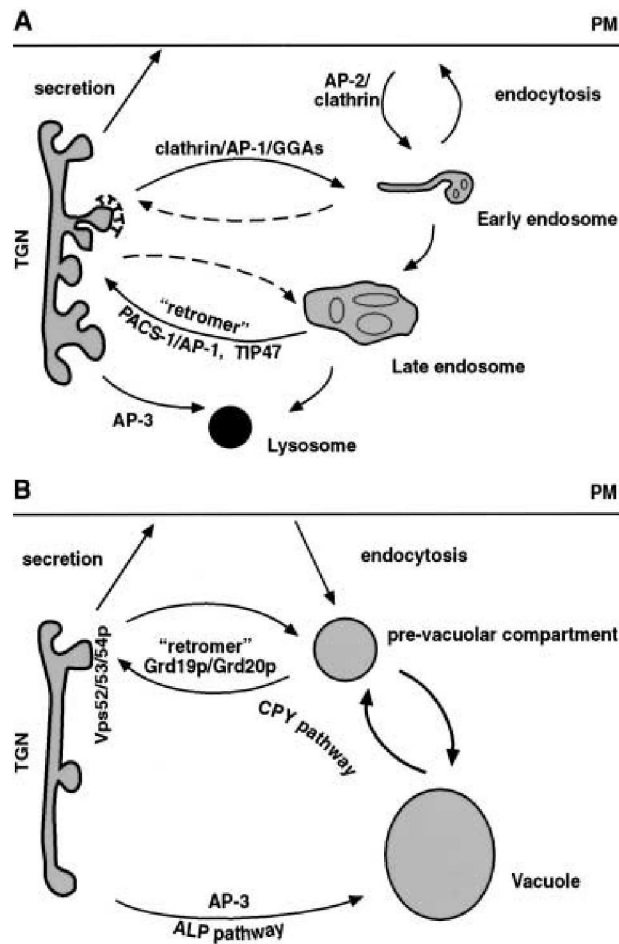


Figure 1. TGN structure. The left panel represents the classical view of the TGN as a separate tubular network at the trans side of the Golgi compartment, with clathrin-coated vesicles or other unknown coats budding from certain areas of the TGN. This model suggests that all proteins are sorted in parallel, in the same TGN compartment. The right panel represents a different view of the TGN: as the trans-most cisternae of the Golgi; each cisterna projects tubules and buds. This model suggests that protein sorting could occur in a sequential manner and in different cisternae of the TGN.

**Figure 2.**

Protein sorting in the TGN/endosomal system. *A* In mammalian cells. From the TGN compartment, secretory proteins are transported to the plasma membrane and extracellular space via the constitutive and regulated secretory pathway. Proteins cycling in the TGN/endosomal system are transported from the TGN to endosomes with clathrin/AP-1-coated vesicles. From the endosomes, the retromer complex, PACS-1/AP1, and TIP47 are involved in retrograde transport. AP-3 vesicles represent a lysosomal delivery pathway that occurs for some proteins that are directly transported to the lysosomes. *B* In yeast cells. From the yeast TGN (late Golgi) compartment, secretory proteins are also transported directly to the cell surface. Similar to the trafficking pathway of lysosomal enzymes and MPR in mammalian cells, yeast CPY and Vps10p (the receptor of CPY) follow the ‘CPY pathway’ to the yeast vacuole. The retrieval of Vps10p to the TGN requires the yeast retromer complex. Grd19p and Grd20p are the two other proteins also involved in the retrieval pathway. In yeast, alkaline phosphatase (ALP) is transported in the ‘ALP pathway’ from the TGN directly to lysosomes using AP-3, bypassing the prevacuolar compartment.

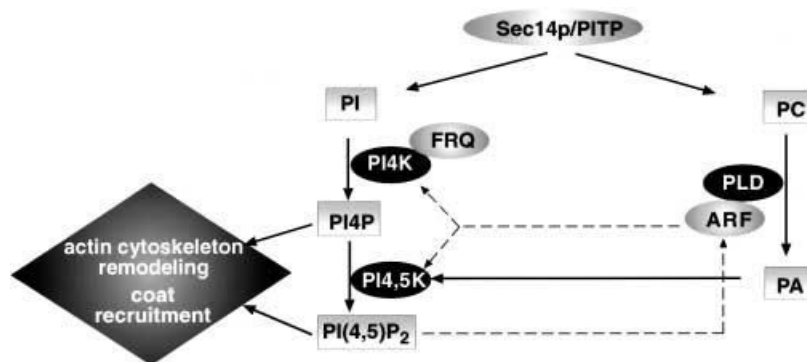


Figure 3.

Lipids involved in TGN trafficking. Lipids from the PI and PC synthesis pathways are involved in post-TGN transport. Sec14/PITP regulates post-TGN transport probably by sensing and balancing between the two pathways. Along the PC pathway, ARF-activated phospholipase D (PLD) hydrolyzes PC to PA. PA may stimulate the recruitment of the protein machinery required for budding. Along the PI pathway, PI4P and PI(4,5)P₂ are important lipids for recruiting coat proteins for budding and for regulating actin cytoskeletal proteins around the TGN. PI4 kinase further interacts with frequenin and is probably involved in sensing Ca²⁺ for regulated secretion. PI(4,5)P₂ is known to be an activator of ARF and conversely, ARF and PA can stimulate the production of PI(4,5)P₂. ARF was recently shown to directly recruit PI4 kinase and PI4,5 kinase to the TGN, an additional way of converging lipid kinases, coat machinery and actin cytoskeleton proteins in post-TGN transport.