Chapter 5

Membrane Cycling between the ER and Golgi Apparatus and Its Role in Biosynthetic Transport

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1. INTRODUCTION

Selective localization and transport of protein and lipid within eukaryotic cells requires the proper functioning of an intercommunicating "endomembrane" system characterized by distinct membrane-bound compartments and membrane transport pathways. Two organelles that play a key role in the generation and maintenance of this endomembrane system, as well as in membrane targeting within it, are the ER and Golgi apparatus. All newly synthesized proteins enter the endomembrane system in the ER and only move to different final destinations in the cell after passing through the Golgi apparatus. This fundamental relationship between the ER and Golgi apparatus in the regulation and sorting events of

Abbreviations used in this chapter: ARF, ADP ribosylation factor; BFA, brefeldin A; BiP heavy-chain binding protein; CGN, *cis*-Golgi network; βCOP, coatomer subunit; ER, endoplasmic reticulum; IC, intermediate compartment; MHC, major histocompatibility complex; MTOC, microtubule organizing center; NEM, *N*-ethyl-maleimide; NSF, NEM-sensitive fusion protein; SNAP, soluble NSF attachment protein; TGN, *trans*-Golgi network.

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biosynthetic transport was first recognized in the 1960s (Palade, 1975). Only recently, however, has insight into the underlying mechanisms of membrane traffic between the ER and Golgi apparatus been achieved, because of the development of new biochemical, pharmacologic, genetic, and morphologic approaches.

Of particular significance to our understanding of membrane transport between the ER and Golgi apparatus has been the recognition of the role of membrane cycling in this process (Pelham, 1991; Lippincott-Schwartz *et al.*, 1990; Hsu *et al.*, 1991). Recent studies suggest that membrane traffic between the ER and Golgi apparatus is not unidirectional but appears to be a finely regulated bidirectional highway connecting two steady-state systems (i.e., ER and Golgi apparatus) whose respective size and structure depend on membrane input and efflux rates (Klausner *et al.*, 1992). Given this apparent phenomenon, several important questions regarding ER/Golgi trafficking must be addressed. These include: What is the nature of the pathways connecting the ER and Golgi apparatus? What is the biochemical machinery that regulates transport within this system? How is selectivity of the membrane components moving within this system achieved?

In this review, membrane transport within the intercommunicating ER/Golgi membrane system will be discussed in light of these questions. It begins with a description of ER and Golgi structure/function and then focuses on the characteristics of anterograde (forward) and retrograde (reverse) membrane transport between the ER and Golgi apparatus. It concludes with a discussion of how regulation of transport within this bidirectional membrane transport system may be achieved.

2. CHARACTERISTICS OF THE ER/GOLGI MEMBRANE SYSTEM

2.1. Structure and Function of the ER

A number of important cellular processes occur within the ER. These include: biosynthesis and assembly of proteins, compartmentalization of the nucleus, lipid biosynthesis and metabolism, regulation of ion gradients, drug detoxification, protein degradation, and certain types of bulk cytoplasmic movements (Palade and Siekevitz, 1956; Helenius *et al.*, 1993; Wilgram and Kennedy, 1963; Terasaki and Sardet, 1991; Bonifacino and Lippincott-Schwartz, 1991; Kachar and Reese, 1988; Terasaki and Jaffe, 1991). Since the ER is the sole site of synthesis of protein and of most lipid comprising the endomembrane system, an additional, constitutive function of this organelle is in membrane export.

The morphology of the ER reflects these diverse functions. As a network of intercommunicating tubules and lamellae extending throughout the cell, the ER

consists of both smooth and rough (studded with ribosomes) portions (see Figure 1). Tubular extensions of the ER reach out to the cell periphery, utilizing microtubules to maintain their peripheral distribution (Terasaki *et al.*, 1986; Dabora and Sheetz, 1988). By contrast, regions of the cell near the microtubule organizing center (MTOC) are largely devoid of ER membrane. Membrane tubules of

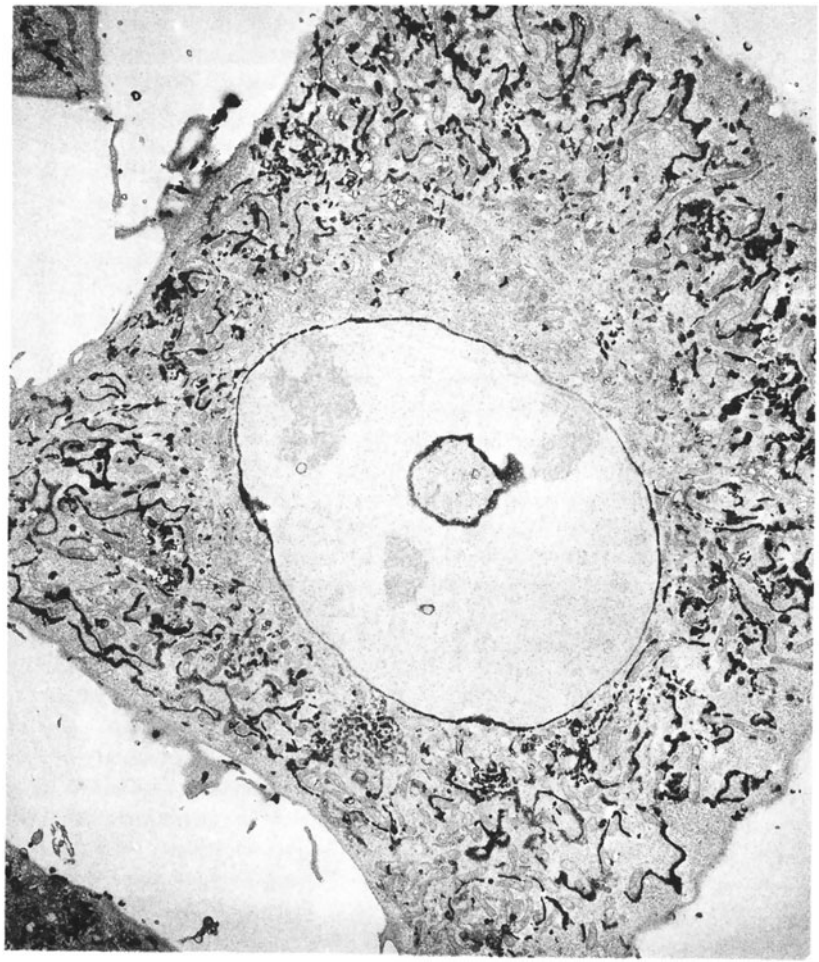

FIGURE 1. Distribution and morphology of the ER. The extensive tubular and peripheral distribution of the ER is revealed in this mouse fibroblast transfected with and immunoperoxidase stained for the ER-retained T cell antigen receptor alpha chain. Note the relative absence of ER membrane (marked by the black reaction product) in central regions of the cell adjacent to the nucleus where the Golgi resides. Photograph courtesy of Lydia Yuan (CBMB, NICHD, NIH).

the ER are extremely dynamic and selectively fuse with each other but not with other membranes (Dabora and Sheetz, 1988).

The rough ER is the site where newly synthesized proteins enter the ER from the cytoplasm. Proteins are targeted here via a complex machinery uniquely associated with the ER that recognizes hydrophobic signal sequences (see Klappa *et al.* and Simon and Blobel, this volume). Once within the lumen of the ER, newly synthesized proteins are enmeshed in a milieu ideal for folding and assembly into higher-order structures (see Rowling and Freedman, this volume). Protein concentrations in the lumen of the ER have been estimated to be as high as 100 mg/ml giving it a gellike consistency (Booth and Koch, 1990). Much of the ER lumenal contents (comprising the ER matrix) is made of resident ER proteins which participate in the folding and assembly of proteins (Helenius *et al.*, 1993).

2.2. Structure and Function of the Golgi Apparatus

All membrane transported out of the ER is conveyed uniquely to the Golgi apparatus before being sorted to different final destinations in the cell. This involves routing of newly synthesized proteins from multiple peripheral sites in the ER to the centrally located Golgi complex (Saraste and Svensson, 1991; Lippincott-Schwartz *et al.*, 1990), which consists of organized stacks of cisternae localized near the MTOC (Rambourg and Clermont, 1990).

Overall, the Golgi complex is organized into three distinct polarized domains: the *cis*-Golgi network (CGN), the Golgi stack, and the *trans*-Golgi network (TGN) (Mellman and Simons, 1992). Membrane and protein enter this complex in the CGN and then move through the Golgi stacks before entering the TGN. The CGN includes the *cis*-most cisternae of the Golgi stacks together with vesicles and an array of tubules extending from this region. Recent data suggest that the CGN is primarily involved in the selective recycling of protein and lipid back to the ER with only a limited role in glycosylation (Hsu *et al.*, 1991; Pelham, 1991). The Golgi stack, comprising the cisternal and tubular structures in the middle of the Golgi complex, by contrast, functions primarily in glycosylation events, including the ordered remodeling of *N*-linked oligosaccharide side chains of glycoproteins. The third domain of the Golgi complex, the TGN, appearing on the *trans* side of the Golgi stack as a sacculotubular network, mediates the sorting and final exit of proteins (Griffiths and Simons, 1986).

A consequence of the polarized (*cis*-to-*trans*), multicisternal organization of the Golgi complex is that newly synthesized proteins must be transported from one Golgi cisternal compartment to the next. This is widely believed to occur via transport vesicles that bud from the rims of one Golgi cisterna and then fuse with the next (Rothman and Orci, 1990). Isolation of the putative Golgi transport vesicles from mammalian cells has revealed their membrane to be coated with an electron-dense material containing several unique cytosolic components ("COPs") that form a large complex called coatomer (Malhotra *et al.*, 1989;

Serafini *et al.*, 1991). One of the COPs, βCOP, was previously identified as a 110-kDa peripheral protein associated with the Golgi apparatus (Allan and Kreis, 1986; Duden *et al.*, 1991). Coatomer (detected by antibodies to βCOP) has been shown to rapidly cycle on and off Golgi membrane in an energy- and GTP-dependent manner, and on membranes interacts with ADP ribosylation factor (ARF), a low-molecular-weight GTP binding protein (Donaldson *et al.*, 1991a,b; Serafini *et al.*, 1991). Significantly, coatomer binding is not restricted to the Golgi apparatus. ER-to-Golgi intermediate structures also contain coatomer (as judged by βCOP binding) (Duden *et al.*, 1991), suggesting a role for coatomer binding in ER-to-Golgi as well as intra-Golgi transport.

What unique functions of the Golgi apparatus dictate its separate compartmentalization within the cell? As mentioned above, the Golgi apparatus enzymatically functions as a carbohydrate factory engaged in the biosynthesis of glycolipids and of the oligosaccharide portions of glycoproteins and proteoglycans. Many of these posttranslational processing events occurring in the Golgi stack are needed for proteins to function properly. The characteristic cisternal morphology of the Golgi stacks may serve to enhance the efficiency of the glycosylating enzymes localized here by increasing the membrane surface-to-lumenal volume ratio (Mellman and Simons, 1992).

A significant feature of the Golgi apparatus is its localization at the center of the cell near the MTOC (Rambourg and Clermont, 1990). Why the Golgi complex is localized here is not known but is probably related to its tight association with microtubules (Thyberg and Moskalewski, 1985). Disruption of microtubules with agents such as nocodazole and colchicine, which bind tubulin and inhibit their polymerization, results in the reversible fragmentation and dispersal of the Golgi complex (Turner and Tartakoff, 1989; Ho, *et al.*, 1989). Dispersed Golgi fragments in nocodazole-treated cells can still function in the processing and secretion of proteins. However, secretory vesicles insert in the plasma membrane randomly under these conditions. This is in contrast to cells with an intact microtubular system where membrane moving through the secretory pathway inserts in the plasma membrane in a polarized fashion at sites closest to the central Golgi complex (Rogalski and Singer, 1984). This type of polarized secretion has been proposed to play a role in cell-cell signaling and in directed cell migration (Kupfer, *et al.*, 1982).

3. TRANSPORT AND TARGETING FROM THE ER TO THE GOLGI APPARATUS

3.1. Control of Export from the ER

Early studies of protein export from the ER showed that different membrane and secretory proteins exit the ER at distinct rates, ranging from minutes to

several hours (Fitting and Kabat, 1982; Lodish *et al.*, 1983; Williams *et al.*, 1985). This indicated that export is a selective process and led to the proposal that transport of molecules out of the ER required a signal or a recognition motif on the exported protein that enabled it to interact with a transport apparatus (Rose and Bergmann, 1983). Efforts to identify discrete signals on proteins required for export out of the ER have thus far been unsuccessful. Moreover, when an "inert" molecule within the ER lumen, an *N*-acyl glycotripeptide (which presumably lacks any "export" signal), was followed in cells to measure the rate of intracellular bulk flow transport, the half-time for its secretion was significantly faster than that for transport of most proteins (Wieland *et al.*, 1987). This suggested that transport out of the ER is a nonselective process, occurring by default.

The nonselective or "bulk flow" model for egress of protein and lipid from the ER is now widely accepted. According to this view, lumenal and membrane proteins are free to flow out of the ER into transport structures unless retarded in some way (Pfeffer and Rothman, 1987). The lumenal space of the ER, according to this model, functions like a two-phase system: a mobile or aqueous phase and a relatively immobile gel or matrix. The permanent resident proteins of the ER, including chaperone proteins and folding enzymes, would make up the gel/matrix. The degree of adsorption of newly synthesized proteins to this matrix by electrostatic and/or hydrophobic interactions would determine the rate at which these proteins enter the fluid phase which leads out of the ER, analogous to the process of adsorption chromatography. Thus, conditions promoting interaction with the gel/matrix, including aggregation, binding to BiP, or exposure of free sulfhydryl groups, result in the protein being excluded from the mobile phase and its retention within the ER lumen (Helenius *et al.*, 1993). Membrane proteins, in addition to lumenal ER proteins, could be retained by virtue of their interaction with the ER matrix, although additional retention mechanisms for membrane proteins can be envisioned (Poruchynsky and Atkinson, 1988; Klausner, 1989; Jackson *et al.*, 1990).

How are lumenal ER matrix components themselves retained in the ER? The most likely explanation is that they are unable to enter the mobile phase leading out of the ER because of extensive low-affinity interactions among themselves (Rothman and Orci, 1992). Interestingly, many ER resident proteins that are abundant components of the ER lumen and envisioned as components of the ER matrix contain the sequence KDEL at their C-terminus. Deletion of this KDEL sequence results in enhanced secretion of these proteins, while addition of this sequence to lysozyme results in their retention in the ER (Munro and Pelham, 1987). This suggested that an additional retention mechanism involving the KDEL sequence might be operating to retain this class of proteins within the ER (Pelham, 1990).

Since the number of proteins with KDEL sequences retained in the ER is far too numerous for there to be a specific receptor for this sequence in the ER,

Pelham (1990) suggested that KDEL receptors might be acting downstream, functioning to retrieve KDEL-containing proteins from a post-ER site. Consistent with this, biochemical evidence of Pelham (1988) showed that a fraction of KDEL-lysozyme cycles from the Golgi back into the ER, and putative KDEL receptors have been identified and localized to pre-Golgi and Golgi compartments (Lewis *et al.*, 1990; Vaux *et al.*, 1990).

Retrieval of KDEL-containing proteins from post-ER sites, however, may only play a backup role in the mechanism by which the cell retains KDEL proteins in the ER (Rothman and Orci, 1992). KDEL-containing proteins have never been morphologically detected in pre-Golgi and Golgi compartments (Bole *et al.*, 1989), even when recycling was slowed by either low temperature or nocodazole treatment, which results in a significant accumulation of other cycling proteins in the Golgi apparatus (Hsu *et al.*, 1991). Furthermore, removing KDEL sequences from ER-retained proteins only results in their slow secretion (Zagouras and Rose, 1989). Thus, it is more likely that multiple low-affinity interactions among KDEL-containing proteins within the ER rather than a post-ER retrieval process is the primary mechanism of retention of these molecules in the ER.

3.2. Genetic and *in Vitro* Reconstitution Studies of ER-to-Golgi Transport

There is a growing consensus among cell biologists that transport from the ER to the Golgi apparatus is a highly regulated process involving interactions of multiple gene products. Diverse approaches to studying the cellular machinery responsible for conveying proteins from the ER to the Golgi apparatus have ruled out the possibility that secretory proteins directly traverse from the ER to the Golgi apparatus, and support the view that this process occurs through topologically distinct transport intermediates. *In vitro* reconstitution studies following protein transport between the ER and Golgi complex in both mammalian and yeast cells have revealed that this process shares many components with intra-Golgi transport (Beckers *et al.*, 1987; Baker *et al.*, 1988; Ruohola *et al.*, 1988; Groesch *et al.*, 1990; Plutner *et al.*, 1991).

A genetic approach for dissecting the underlying mechanisms involved in ER-to-Golgi transport has produced many temperature-sensitive mutants in yeast *Saccharomyces cerevisiae* defective in this process. The secretory mutants sec 7, 12, 13, 16, 17, 18, 19, 20, 21, 22, and 23, bos 1, bet 1, sar 1, and ypt 1 mutants are all implicated in transport processes between the ER and the Golgi apparatus (Schekman, 1992; Kaiser and Schekman, 1990; Nakano and Muramatsu, 1989; Segev *et al.*, 1988; Newman and Ferro-Novick, 1987; Shim *et al.*, 1991). In most of these mutants, upon incubation at the nonpermissive temperature newly synthesized proteins accumulate in the ER and an extensive network of ER

membrane is formed (Hicke and Schekman, 1990). A subclass of these mutants, including sec 17, 18, 20, and 22, also accumulate numerous 50-nm vesicles at the restrictive temperature. These vesicles are believed to be ER-to-Golgi intermediates because their generation requires the action of sec 12, 13, 16, and 23 (Kaiser and Schekman, 1990).

Functionally active transport vesicle intermediates can be produced from the ER in crude yeast lysates that fuse with Golgi *in vitro* (Baker *et al.*, 1988; Rexach and Schekman, 1991; Groesch *et al.*, 1990). Formation of the transport intermediate requires ATP and GTP hydrolysis, cytosol, the 21-kDa GTP binding protein, sar 1p, and involves the action of sec 12p, 13p, 16p, and 23p (d'Enfert *et al.*, 1991; Rexach and Schekman, 1991). Targeted fusion of the intermediate requires ATP, cytosol, Ca^{2+}, the GTP binding protein rab 1/ypt as well as sec 17p, 18p, 22p (Segev, 1988; Rexach and Schekman, 1991). The *in vivo* accumulation of transport vesicles in sec 17, 18, and 22 cells at the nonpermissive temperature is consistent with these biochemical observations.

Recent studies have demonstrated the conservation of the biochemical machinery involved in protein transport between the ER and Golgi apparatus. sec 18 and sec 17 gene products have been shown to be homologous to mammalian factors NSF (NEM-sensitive fusion protein) and SNAP (soluble NSF attachment protein) identified biochemically in a mammalian *in vitro* transport assay (Clary *et al.*, 1990; Wilson *et al.*, 1989; Kaiser and Schekman, 1990). Whereas NSF (homologous to sec 18p) is proposed to be involved in transport vesicle fusion, SNAP (homologous to sec 17p) is thought to be involved in NSF binding to membranes (Malhotra *et al.*, 1989; Wilson *et al.*, 1989; Clary *et al.*, 1990).

Cell fractionation and DNA sequence analysis have begun to define the biochemical characteristics of the yeast gene products necessary for ER-to-Golgi transport. Two sec mutant gene products shown to be integral membrane proteins are sec 12p and sec 20p. sec 12p has been proposed to have a role in promoting vesicle assembly/budding through the interaction of its cytosolic domain with sar 1p (d'Enfert *et al.*, 1991). sec 20p (50 kDa) is the only transmembrane protein known to contain the sequence HDEL at its C-terminus (Sweet and Pelham, 1992). In addition, sec 20p shows genetic interaction with an allele of the sac 1 gene, which in turn shows interactions with actin mutants. This, together with the observation that depletion of sec 20p from cells results in an elaboration of ER and clusters of small vesicles, has led to the suggestion that sec 20p might provide the connection between transport vesicles and some component of the cytoskeleton (Sweet and Pelham, 1992).

At least three sec gene products implicated in ER-to-Golgi traffic (sec 13p, sec 23p, and sec 7p) are present in the cytosol in high-molecular-weight complexes that can loosely associate with the cytoplasmic surfaces of membranes. sec 23p (85 kDa) is bound to a 105-kDa protein (Hicke *et al.*, 1992), and sec 13p (34 kDa) is part of a 400-kDa complex (Pryer *et al.*, 1990). Ultrastructural

localization of the sec 23p mammalian homologue in exocrine and endocrine pancreatic cells showed a specific distribution to the cytoplasmic face of the ER near the Golgi apparatus (Orci *et al.*, 1991a). sec 7p has been observed by immunoelectron microscopy to be associated with the cytoplasmic surface of ER-to-Golgi transport vesicles (Franzusoff *et al.*, 1992) and is a large (230 kDa) cytosolic phosphoprotein recovered in both soluble and sedimentable forms. It is not yet known if any of these sec gene products are similar to components of the above-mentioned coatomer complexes observed in mammalian cells.

3.3. Role of the Pre-Golgi Intermediate Compartment in ER-to-Golgi Transport

The properties and characteristics of the transport intermediates that deliver membrane and protein from the ER to the Golgi apparatus have recently been examined. In mammalian cells, these intermediates take the form of small (90 nm) vesicles in addition to larger tubulovesicular structures (up to 200–500 nm in diameter) (Saraste and Svensson, 1991), and are largely devoid of resident components of the ER and Golgi apparatus (Hauri and Schweizer, 1992). At 16°C, however, newly synthesized viral glycoproteins accumulate in these structures indicating that they represent an intermediate compartment (IC) through which proteins moving from the ER to the Golgi apparatus must pass (Saraste and Kuismanen, 1984).

A number of cellular processes have been postulated to occur in the IC including: fatty acylation of proteins (Rizzolo and Kornfeld, 1988; Bonatti *et al.*, 1989), the first step of *O*-glycosylation (Tooze *et al.*, 1988), and the first enzyme step in the generation of the mannose-6-phosphate signal for lysosomal protein targeting (Kornfeld and Mellman, 1989). In addition, budding of coronavirus and murine leukemia virus is believed to occur at this site (Tooze *et al.*, 1988; Ulmer and Palade, 1991).

Lodish *et al.* (1987) and Paulik *et al.* (1988) were among the first to isolate low-density membrane fractions with characteristics expected for the IC. Further characterization and purification of the IC has been made possible with the availability of antibodies that preferentially recognize the IC in mammalian cells. Antibodies to two integral membrane proteins of 53 kDa (p53) and 58 kDa (p58) (Schweizer *et al.*, 1988; Saraste *et al.*, 1987) label a tubulovesicular membrane system extending from the *cis* side of the Golgi apparatus. At 16°C, structures containing p53 and p58 colocalize with sites of accumulation of newly synthesized membrane viral proteins (Schweizer *et al.*, 1990; Saraste and Svensson, 1991). These structures are scattered throughout the cytoplasm (Schweizer *et al.*, 1990) and have βCOP bound to their cytoplasmic surfaces (Duden *et al.*, 1991).

The biochemical characteristics of the IC have been pursued by Schweizer *et al.* (1991) who have isolated subcellular fractions enriched 40-fold for p53.

The purified IC fractions displayed a unique polypeptide pattern distinct from rough ER and *cis*-Golgi. Thus, they did not contain the ER proteins ribophorin I/II, BiP, and protein disulfide isomerase nor the *cis*-Golgi enzymes involved in generating the lysosomal targeting signal mannose-6-phosphate.

The observation that the structures comprising the IC at 16°C are scattered throughout the cytoplasm (Schweizer *et al.*, 1990; Saraste and Svensson, 1991) strongly suggests that sites of exit from the ER are not restricted to regions positioned near the Golgi apparatus as originally envisioned. The question of how membrane and protein within these peripheral structures are delivered to the centrally located Golgi complex is therefore significant. This could be accomplished by two different mechanisms. One possibility is that vesicles shuttle back and forth between the ER, the IC, and the Golgi complex with the IC acting as a stable intermediate. The alternative possibility is that the tubulovesicular structures and surrounding vesicles comprising the IC translocate as a unit through the cytoplasm and fuse with the Golgi complex. Under this mechanism, the IC would be only a transient structure continuously fusing with the Golgi complex and being re-formed from the ER.

Morphologic evidence favoring the latter model has been provided by Saraste and Svensson (1991) (but see also Bonatti and Torrisi, this volume). These authors demonstrated that p58 accumulates in large peripheral IC structures at 16°C. Rather than remaining peripherally distributed, however, these structures redistribute into the Golgi region when cells are briefly warmed to 37°C. This suggests that peripheral p58-containing structures are only transient structures that fuse with the Golgi complex upon transport into the region of the cell near the MTOC.

What directs the movement of IC structures from peripheral sites in the cytoplasm toward the cell center where the Golgi apparatus resides? Saraste and Svensson (1991) have proposed that microtubules might facilitate this movement. These authors showed that in addition to being inhibited at temperatures below 16°C, movement of p58-containing structures into the central Golgi region at 37°C requires intact microtubules (Saraste and Svensson, 1991). This conclusion is supported by studies of Lippincott-Schwartz *et al.* (1990) who followed the relocation of Golgi membrane proteins out of the ER in cells washed free of the drug brefeldin A (BFA), which redistributes Golgi membrane proteins into the ER (Lippincott-Schwartz *et al.*, 1989; Doms *et al.*, 1989). Within 5 min of washout of this drug, Golgi proteins appeared in punctate structures widely distributed within the cytoplasm. These punctate structures appeared to aggregate into larger structures by 10–15 min after washout but were still peripherally distributed in the cell. At 30 min after washout the structures became larger and remarkably had a distribution very similar to that of Golgi fragments in nocodazole-treated cells. After longer periods the Golgi aggregates moved into a perinuclear location resembling the distribution of the Golgi complex in control

cells. This step could be inhibited by nocodazole (Lippincott-Schwartz, unpublished observations). These studies suggest, therefore, that sites of protein exit from the ER are widely distributed within this membrane network and that the subsequent long intracellular distances traveled by intermediate structures en route to the Golgi apparatus may involve an interaction with microtubules.

4. SORTING AND RECYCLING AT THE CGN

At some point in its movement toward the MTOC or cell center, the membranes comprising the IC fuse with the Golgi complex, delivering their content and membrane to this organelle. This is believed to occur at the CGN, which by electron microscopy appears as an array of vesicles and tubules that connect to the first one or two cisternae at the *cis* face of the Golgi stack (Rambourg and Clermont, 1990).

4.1. Distinction between the IC and CGN

Because the IC migrates toward and eventually fuses with the CGN, the two compartments have overlapping membrane constituents. p53, p58, and rab 2, for instance, are concentrated in both subcompartments (Schweizer *et al.*, 1990; Saraste and Svensson, 1991; Chavrier *et al.*, 1990). This has led to some confusion regarding the distinction between these compartments. At least one protein, the E1 glycoprotein of avian coronavirus, however, appears to be specifically localized to the CGN (Machamer *et al.*, 1990).

There are several reasons for conceptualizing the IC and CGN as distinct compartments. The IC consists of structures into which protein leaving the ER first accumulates. In many cells these structures are peripherally located and translocate toward the cell center where the Golgi complex resides (Saraste and Svensson, 1991; Lippincott-Schwartz *et al.*, 1990). They are therefore only transiently existing structures. The CGN, by contrast, is stably localized at the cell center where it interacts with the rest of the Golgi complex (Mellman and Simons, 1992). The CGN is involved not only in receiving but also in sorting components arriving from the ER. Sorting at the CGN involves the decision either to transfer molecules deeper into the Golgi stacks or to recycle them back into the ER.

4.2. Evidence for Golgi-to-ER Recycling

It has long been hypothesized that some mechanism must exist for returning lipid to the ER in the face of the continuous flow of membrane out of this organelle. Indeed, attempts at estimating the rate of lipid loss from the ER caused

by bulk flow into the secretory pathway suggested that the rate of loss of lipid from the ER vastly exceeds the rate of lipid biosynthesis (Wieland *et al.*, 1987). How might lipid be retrieved to the ER? Two possibilities have been most widely considered: cytosolic transfer of nonvesicular lipid back into the ER, and membrane recycling from the Golgi apparatus into the ER. While there is at present no evidence for lipid retrieval into the ER by monomeric diffusion, compelling evidence for a membrane transport pathway from the Golgi complex to the ER has come from studies of cells treated with the fungal metabolite brefeldin A, BFA.

Within minutes of adding BFA to cells, all membrane and content of the CGN and Golgi stacks are transported into the ER and export out of the ER is inhibited (Lippincott-Schwartz *et al.*, 1989; Fujiwara *et al.*, 1989; Doms *et al.*, 1989; Young *et al.*, 1990; Strous *et al.*, 1991). That the pathway followed by Golgi components in the presence of BFA operates at some level in untreated cells for the selective recycling of molecules is supported by several studies. The normal steady-state distributions of p53 and p58 include the ER, IC, and CGN. To investigate whether these molecules constitutively cycle between these compartments, Lippincott-Schwartz *et al.* (1990) cooled cells to 16°C whereupon p53 became concentrated in the IC. A synchronized population of p53 molecules could then be followed upon warming. Brief warming to 37°C resulted in the movement of p53 first into the CGN (1 to 2 min) and then into the ER (10 min) where it colocalized with ER resident markers. Continued incubation at 37°C (30 min) returned p53 to its steady-state distributions in the ER, IC, and CGN. These temperature manipulations had no effect on the distributions of resident ER or Golgi proteins.

Similar results have been obtained from morphologic experiments following the intracellular dynamics of p58 (Saraste and Svensson, 1991), and MHC class I molecules in a mutant cell line (Hsu *et al.*, 1991), suggesting that these molecules also constitutively cycle between the ER and Golgi apparatus. Ultrastructural studies showed that MHC class I in the mutant cell line moved only as far as the CGN and no farther into the Golgi stacks before recycling to the ER (Hsu *et al.*, 1991). Consistent with this, biochemical experiments in the same study showed no processing to endo H resistance of the *N*-linked oligosaccharides of MHC class I.

Evidence for the recycling of lipids from the Golgi complex to the ER has recently been provided by Hoffman and Pagano (1993). These authors examined the fate of nonexchangeable fluorescent lipid analogues after delivery to the Golgi apparatus in semi-intact cells. The lipids were shown to redistribute from the Golgi to the ER within 30 min, with no obvious changes in the overall morphology of the Golgi or ER. Lipid redistribution into the ER was temperature and energy dependent, NEM sensitive, and required cytosol. Since these results were obtained in the absence of added drugs (i.e., BFA) and without temperature perturbations, they add further support to the idea that retrograde flow of mem-

brane from Golgi to ER is a normal process. Future studies need to address the question of the extent of membrane cycling along this pathway.

4.3. Possible Roles of Membrane Cycling between the ER and Golgi Complex

A variety of functions could be served by a constitutive Golgi-to-ER recycling pathway. In addition to maintaining the membrane surface area of the ER in the face of continuous bulk flow of membrane out, recycling has been proposed to act as a "second line of defense" in preventing the loss of KDEL-containing ER resident proteins (Rothman and Orci, 1992; Pelham, 1991). According to this model, interaction of KDEL-containing proteins that have escaped the ER retention system with KDEL receptors in the Golgi complex would direct the escaped proteins into the retrograde retrieval pathway leading them back into the ER (Pelham, 1991; Rothman and Orci, 1992).

A different function of the Golgi-to-ER retrograde pathway could be to provide the cell with an additional quality control system to that of the ER for preventing molecules that have failed to assemble properly from being transported to the cell surface. An example of this is the MHC class I complex in mutant cells (Hsu *et al.*, 1991), where failure of the heavy and light chains to assemble properly leads to their failure to be transported to the cell surface. Rather than being retained in the ER, MHC class I molecules remain trapped within the ER/Golgi system futilely cycling between the ER and Golgi.

In addition to these functions, the Golgi-to-ER retrograde pathway may be utilized for remodeling of the Golgi apparatus during specific periods of a cell's lifetime, including the dispersal of the Golgi during mitosis (Lucocq *et al.*, 1989), alterations in Golgi location during myogenesis (Tassin *et al.*, 1985), and during microtubule disruption. Finally, use of the retrograde pathway might be one way the cell regulates net membrane inflow into the Golgi stacks.

4.4. Characteristics of the Golgi-to-ER Retrograde Transport Pathway

Insight into the characteristics of the retrograde recycling pathway into the ER has come from detailed studies using the drug BFA. Within minutes of adding BFA, the Golgi apparatus undergoes major structural alterations with the result that the CGN and Golgi stacks fuse into larger structures and give rise to an extensive array of long tubular processes that extend along microtubules to the cell periphery where they eventually fuse and mix with the ER (Lippincott-Schwartz *et al.*, 1990). Concomitant with these morphologic effects, anterograde transport including protein secretion is blocked (Misumi *et al.*, 1986). Remarkably, upon washing out BFA, Golgi proteins along with secretory proteins move out of the ER into IC-like structures, subsequently re-forming the Golgi appara-

tus and refilling the rest of the biosynthetic pathway (Lippincott-Schwartz *et al.*, 1989).

The membrane tubules that mediate "retrograde" transport of Golgi components into the ER in BFA-treated cells have distinct properties (Lippincott-Schwartz *et al.*, 1990; Donaldson *et al.*, 1991a). Fusing uniquely with the ER, these Golgi-derived tubules are about 90 nm in diameter and can extend up to 10–20 μm in length. Unlike membrane structures mediating anterograde transport, which are coated with COPs, retrograde tubules are devoid of coatomer or "uncoated." The tubules appear to utilize microtubules (moving toward the plus poles) and are not observed when cells are treated with microtubule-disrupting agents like nocodazole. In addition, the tubules are not formed at temperatures below 16°C, during ATP depletion, or in the presence of aluminum fluoride or GTPγS when added before BFA.

Do membrane tubules arising from the CGN mediate retrograde transport from Golgi to ER in cells under normal conditions? Transient tubules emanating from the Golgi are observed in living cells at 37°C (Cooper *et al.*, 1990). Evidence that such tubules might mediate retrograde transport comes from studies of Lippincott-Schwartz *et al.* (1990). These authors showed using immunofluorescence that at 16°C and after brief warm-up periods, the recycling molecule p53 appears in tubular processes emanating out of the CGN. Golgi resident proteins (including galactosyl transferase) were normally excluded from the p53-containing tubular processes, but upon BFA treatment at 16°C became colocalized with p53 in the tubular processes. This suggested that the tubular processes containing p53 were normally utilized for retrograde traffic since they were observed in the presence or absence of BFA.

The idea that "noncoated" membrane tubules mediating retrograde transport provide an alternative transport mechanism to "coated" structures mediating anterograde transport has emerged from studies revealing the biochemical basis of BFA's effects. Donaldson *et al.* (1990) observed that within 30 s of adding BFA to cells, the coatomer protein βCOP comes off Golgi and Golgi-associated membranes. This proximal effect results from the inability of coatomer to bind to membrane in the presence of BFA (Donaldson *et al.*, 1991a; Orci *et al.*, 1991b). As mentioned above, association of coatomer with membranes is a controlled process utilizing GTP and is believed to be essential for both ER-to-Golgi and intra-Golgi anterograde traffic (Klausner *et al.*, 1992; Rothman and Orci, 1992). That BFA prevents membrane binding of coatomer provides a simple explanation for the ability of BFA to block anterograde movement into the Golgi. That retrograde tubule trafficking continues in the presence of BFA is consistent with this pathway not requiring coatomer binding.

The relationship between "coat"-mediated anterograde traffic and tubule-mediated retrograde traffic was examined using BFA in a cell-free intra-Golgi transport system (Orci *et al.*, 1991b). When isolated Golgi membranes were

treated with BFA, coatomer (detected by βCOP binding) was no longer associated with these membranes. Nevertheless, transport of protein from donor to acceptor Golgi fractions occurred with normal kinetics. This indicated that transport is not absolutely dependent on coatomer binding. To obtain an explanation for the *in vitro* "transport" under BFA conditions, ultrastructural examination of BFA-treated Golgi membrane preparations was performed. An extensive network of "uncoated" tubules that connected previously separate cisternae and stacks was observed. These tubule networks were proposed to represent the *in vitro* equivalents of membrane tubules mediating retrograde transport *in vivo*, allowing the mixing of contents of Golgi cisternae with each other. The parallels between tubule network formation of purified Golgi stacks and of tubules mediating retrograde transport *in vivo* were consistent with this interpretation: both required ATP to form and could be prevented from forming with pretreatment with GTPγS; and both mediated transport or mixing of molecules (Orci *et al.*, 1991b; Lippincott-Schwartz *et al.*, 1990; Donaldson *et al.*, 1991a).

These results give strong support to the idea that there are two alternative pathways of membrane traffic operating within the ER/Golgi system: one mediated via "coated" structures and the other mediated via "uncoated" tubules (Orci *et al.*, 1991b) (see Figure 2). BFA's effects on the ER Golgi system can then be rationalized in terms of its disruption of a normal balance between these two pathways that is regulated at the level of coatomer binding (Klausner *et al.*,

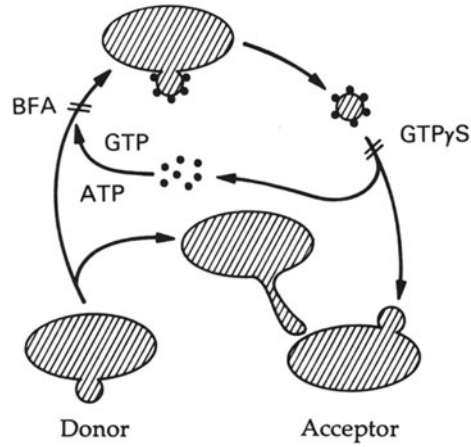

FIGURE 2. Two alternative membrane transport pathways for membrane trafficking. Two pathways, one mediated by discontinuous "coated" structures and the other by continuous tubules, are proposed to operate within the ER/Golgi system. Both pathways require the functioning of a constitutive budding process. Binding of coatomer and other cytosolic factors (i.e., ARF) to the bud would result in transport by the coat-mediated pathway. In the absence of coatomer binding, budding would give rise to tubule structures. Both pathways would require additional components for the recognition and targeting events necessary for the final fusion of donor and acceptor membranes. Regulation of transport along the "coated" vesicle versus noncoated tubular pathway would depend on the status of coatomer binding, with conditions favoring coatomer binding (i.e., GTP) resulting in enhanced "coat-mediated" transport and conditions favoring coatomer dissociation from membranes (i.e., BFA) resulting in enhanced tubular transport. GTPγS, which prevents coatomer dissociation from membranes, would inhibit both pathways, since coatomer-free buds are required for tubular traffic, and coatomer dissociation is required for vesicle fusion along the coat-mediated pathway.

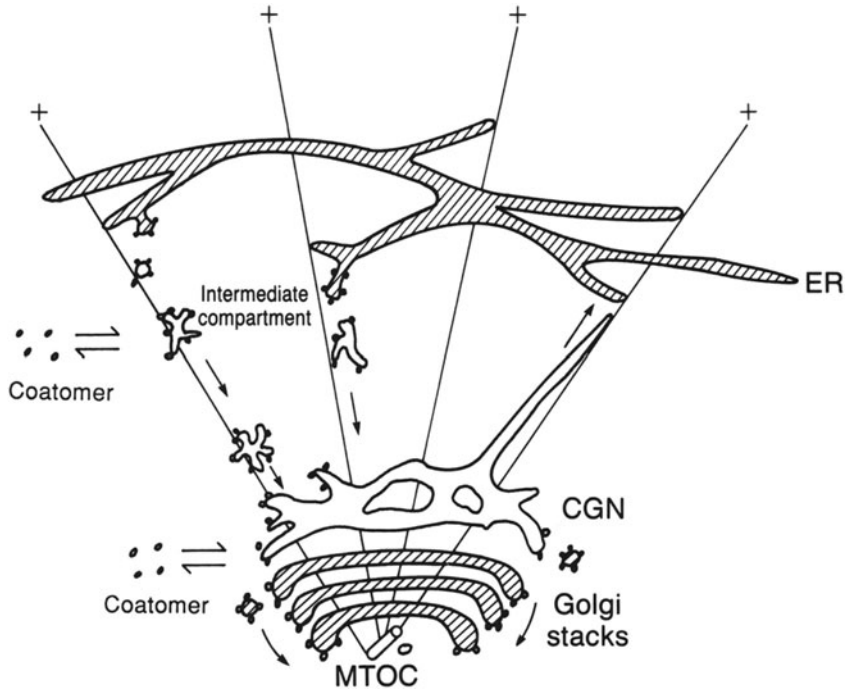

FIGURE 3. Hypothetical model for ER-to-Golgi membrane trafficking. Newly synthesized proteins leave the ER in membrane structures, which can arise from multiple sites throughout the cytoplasm. These peripheral transport intermediates (intermediate compartment) move toward the MTOC in a microtubule-facilitated manner. In addition to being relatively large (200–500 nm wide), the intermediates have coatomer bound to their cytoplasmic surfaces. As the intermediates reach the Golgi region they fuse with the CGN. Dissociation of coatomer would allow membrane buds to form tubular structures which utilize microtubules to move toward the cell periphery where they fuse with the ER. Sorting mechanisms for localizing membrane and protein into tubules versus coated structures would operate throughout this cycling system.

1992) (see Figure 3). Anterograde movement into the Golgi complex, which requires coatomer binding onto membrane, would be inhibited by BFA. Release of coatomer by BFA would be associated with Golgi tubulation and retrograde transport into the ER. The additional profound effect of BFA on Golgi structure suggests that maintenance of this organelle depends on a controlled balance of membrane input and output through these pathways.

What is the advantage to the cell of having two pathways—one leading into the Golgi and the other leading back to the ER—that exhibit such distinct structural characteristics? Such a system may provide the physical basis for sorting at the site(s) of recycling by the segregation of structures that maximize

volume-to-surface area ratio (vacuoles) from structures that minimize volume-to-surface area ratio (tubules). This would be analogous to sorting of fluid-phase molecules from recycling membrane receptors in the endocytic system (Geuze *et al.*, 1983). At the level of the ER/Golgi system, anterograde-moving vesicles from the ER (which maximize volume content) would carry bulk flow lumenal constituents forward through the biosynthetic pathway while tubules (which minimize volume) would recycle membrane components back to the ER. This process coupled with the selective movement of membrane components into different transport structures would allow for efficient sorting of bulk flow from recycling components early in the biosynthetic pathway.

5. MECHANISMS FOR REGULATING MEMBRANE CYCLING BETWEEN THE ER AND GOLGI

5.1. Role of Coatomer Binding in ER-to-Golgi Trafficking

Assuming that ER-to-Golgi traffic involves two distinct transport mechanisms (i.e., coatomer-mediated anterograde transport and tubule-mediated retrograde transport), what normally regulates the balance between the two? An important clue to this question has come from the observation that under conditions that favor membrane accumulation of coatomer (incubation with GTPγS), retrograde tubules do not form, whereas when association of coatomer is prevented (by BFA), tubules predominate (Donaldson *et al.*, 1991a; Orci *et al.*, 1991b). This reciprocal relationship between BFA-sensitive coatomer binding and tubule production suggests that both pathways share a common regulatory system tied to the membrane assembly/disassembly of coatomer.

How might the status of coatomer binding result in different transport processes? Since budding is required for the formation of both tubules and "coated" vesicular structures, coatomer binding may not be required for budding *per se* as previously proposed (Rothman and Orci, 1990), but be superimposed on a budding process to serve other roles (Klausner *et al.*, 1992). For instance, binding of coatomer to membrane could inhibit or slow bud growth into a tubule by masking sites on microtubule motor proteins required for the extension of the tubule along microtubules. Extending this thinking to the level of the ER, if one considers the ER as a dynamic tubulating system utilizing microtubule motor proteins to move to the cell periphery, then blocking motor activity at specific sites (perhaps by coatomer binding) would inhibit tubule growth. Fission of the coated tubules would then result in detached structures free to be transported toward the cell center where the Golgi complex resides. Another role of coatomer binding might be to provide specificity to the membrane content in the regions of binding (Klausner *et al.*, 1992).

5.2. Role of GTP-Binding Proteins in Regulating Coatomer Binding

Insight into the biochemical machinery that regulates coatomer binding is only just beginning. The observation that aluminum fluoride and GTPγS both promote association of βCOP with Golgi membranes implicates one or more GTP-binding proteins in initiating the association of coatomer with membranes (Donaldson *et al.*, 1991b). Both the low-molecular-weight GTP-binding protein, ARF, and a heterotrimeric G protein have been proposed to be involved in this process (Donaldson *et al.*, 1991b).

ARF is an abundant cytosolic protein that reversibly associates with Golgi membranes (Donaldson *et al.*, 1991b). Like βCOP, association of ARF with Golgi membranes can be enhanced in the presence of GTPγS and is inhibited by BFA. Current evidence suggests ARF plays an essential role in regulating βCOP (i.e., coatomer) binding (Donaldson *et al.*, 1992). Not only does Golgi membrane binding of cytosolic βCOP require the presence of ARF, but interaction of recombinant, myristoylated ARF, GTPγS, and Golgi membrane is sufficient to make the Golgi membrane fully competent for βCOP binding at a subsequent step (in the absence of free ARF and GTPγS). This initial ARF–membrane interaction step has been proposed to be the site of BFA action (Donaldson *et al.*, 1992).

Like other GTP-binding proteins, ARF can be envisioned to cycle between GDP- and GTP-bound forms. During activation, cytosolic ARF–GDP would interact with a membrane binding site, whereupon GDP/GTP exchange would convert it into the active GTP-bound form. This would initiate the process of coatomer binding. Hydrolysis of GTP would reverse activation with release of coatomer from the membrane. Hydrolysis-resistant GTP analogues like GTPγS would persistently activate ARF, resulting in persistent coatomer binding and a resistance to BFA action.

A role of heterotrimeric G protein(s) in coatomer binding to Golgi membrane was first suggested by Donaldson *et al.* (1991b) based on the effect of aluminum fluoride in promoting βCOP association with membrane. Aluminum fluoride is a potent activator of G proteins, but not of ARF and other low-molecular-weight GTP-binding proteins (Kahn, 1991). Support for this hypothesis was provided by experiments using βγ subunits of G proteins. Many effects of G proteins are mediated via free Gα subunits and addition of βγ subunits may inhibit these effects. When Donaldson *et al.* (1991b) added βγ subunits of a G protein to Golgi membranes and cytosol, GTPγS-dependent association of both βCOP and ARF to Golgi membranes was inhibited. Using a permeabilized cell system, Ktistakis *et al.* (1992) have provided further support for the role of a G protein in coatomer binding, showing that coatomer binding to membrane can be modulated by the G protein stimulator, mastoparin. The precise role that G proteins might play in the membrane association/dissociation cycle of coatomer

is unknown, but Donaldson *et al.* (1992) suggested that they might enhance the formation or stability of active ARF–GTP complexes. One G protein, Giα3, has been localized to the Golgi apparatus and its overexpression results in the inhibition of proteoglycan secretion (Stow *et al.*, 1991).

Since the paradigm for G protein function on the plasma membrane is that they act as signal transducers, it is possible that the functioning of G proteins in regulating binding of coatomer to the Golgi complex is coupled to receptors that respond to signals within the cell. One molecule that might serve as such a receptor is ERD2 (the putative receptor for KDEL-containing proteins) (Semenza *et al.*, 1990; Hsu *et al.*, 1992). Lowering ERD2 levels in cells results in a block in normal transport through the secretory pathway and an accumulation of Golgi membrane (Semenza *et al.*, 1990). By contrast, overexpression of ERD2 or an ERD2 homologue in mammalian cells results in: the redistribution of Golgi membrane into the ER, loss of coatomer binding to membrane, and a block in secretion, all similar to the effects of BFA (Hsu *et al.*, 1992). Based on these observations, Hsu *et al.* (1992) have proposed that one function of KDEL receptors in the Golgi apparatus might be to regulate membrane traffic between the ER and Golgi apparatus. Interaction of KDEL receptors in the Golgi complex with KDEL proteins that have leaked out of the ER, according to their model, could provide a detection mechanism for the overall regulation of retrograde transport or recycling of proteins from the Golgi back into the ER. Overexpression of these receptors might lead to a constitutively "on" signal, resulting in an imbalance in membrane traffic with retrograde transport now favored, analogous to the effects of BFA.

6. SUMMARY

Membrane traffic between the ER and Golgi is now recognized as a carefully regulated process controlled by distinct anterograde (to the Golgi) and retrograde (to the ER) pathways. These pathways link two organelles with different morphologies, structures, and localizations within the cell. The ER, which is involved in multiple cellular functions including protein biosynthesis and folding, extends to the cell periphery and forms a dynamic tubule reticulum. By contrast, the Golgi apparatus, which functions in membrane sorting and recycling events, is localized at the center of the cell near the MTOC and is comprised of compact cisternal units. The required transport into the Golgi apparatus of newly synthesized proteins exported from the ER offers a twofold advantage to the cell. First, the rate of movement of membrane and protein through the biosynthetic pathway can be controlled by the selective use of a recycling pathway. Second, membrane moving through the biosynthetic pathway enters a structure specialized for sorting of membrane to different final destinations in the cell.

Control of biosynthetic transport within the ER/Golgi system involves the utilization of two alternative transport pathways: anterograde (ER to Golgi) and retrograde (Golgi to ER). These two pathways share a common regulatory system involving membrane assembly/disassembly of cytosolic coatomer proteins. Thus, conditions that favor irreversible coatomer binding (i.e., GTPγS) inhibit retrograde transport while producing anterograde transport intermediates. Conditions that prevent coatomer binding (i.e., BFA) inhibit anterograde transport and enhance retrograde transport.

The underlying biochemical machinery that normally balances anterograde and retrograde membrane traffic between the ER and Golgi is only just beginning to be understood. Any model to explain this system, however, must account for the morphologic characteristics of the membranes involved. Whereas anterograde traffic involves discontinuous "coated" structures moving from peripheral sites in the ER toward the central Golgi, retrograde traffic utilizes continuous "noncoated" tubule structures that move from a central site (i.e., the CGN) to the peripheral ER (see Figure 3). Such a system maximizes volume transport (utilizing vacuolar structures) in the anterograde direction and membrane transport (utilizing tubules) in the retrograde direction. It is therefore ideal for sorting of bulk flow lumenal contents from recycling membrane early in the biosynthetic pathway.

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