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Unsolved Mysteries in Membrane Traffic

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Golgi complex, membrane traffic, small GTPase, transport carriers

Abstract

Remarkable strides have been made over the past 20 years in elucidating the molecular basis of membrane trafficking (1). Indeed, a combination of biochemical and genetic approaches have determined the identity and function of many of the core constituents needed for protein secretion and endocytosis. But much remains to be learned. This review highlights underlying themes in membrane traffic to help us refocus and solve many remaining and newly emerging issues that are fundamental to mammalian cell biology and human physiology.

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INTRODUCTION

Proteins destined for secretion enter the secretory pathway during their translation at the endoplasmic reticulum (ER). There, they are core glycosylated, folded, and assembled into oligomeric structures before being transported in vesicles to the Golgi complex. Within the Golgi, proteins are further modified by glycosyltransferases prior to their export to either the cell surface, the endocytic compartment, or back to the ER for retrieval.

Transport intermediates utilize coat proteins and include cargo recognition mechanisms to link the collection of the appropriate cargo into a nascent transport vesicle or tubule. Indeed, we have learned a great deal about how certain classes of vesicles form, particularly those en route from the ER to the Golgi complex (see 2) and clathrin-coated vesicles that participate in receptor endocytosis (3–6).

How do transport intermediates identify their targets? Pairs of vesicles and cognate targets contain specific SNARE proteins that form tight complexes to drive membrane fusion. Tethering factors help bring vesicles closer to their targets, and act in concert with SNARE proteins to facilitate docking and fusion. However, very little is known about how tethers work and how SNARE assembly is regulated.

Finally, cells contain a number of distinct, membrane-bound compartments. Why do cells need so many compartments and how are they assembled and maintained? Much remains to be learned about these questions, including how proteins are delivered to distinct compartments and retained within them. Our lack of true molecular understanding is only compounded with genome-wide analysis of requisite protein components: Long lists can be generated with an associated paucity of functional information for most of the implicated proteins (7–9).

UNSOLVED MYSTERIES

A recent study using a genome-wide library of siRNAs (small interfering RNAs) to screen for genes needed by cultured *Drosophila* cells to secrete horseradish peroxidase led to the identification of 104 candidate genes, of which only 26 had been previously identified (10). Thus, *Drosophila* cells encode at least 78 gene products that are potentially important for secretion, but whose roles are completely unknown. Cells depleted of these proteins could be grouped into four morphological classes: those in which Golgi markers were present in the ER, those containing fragmented Golgi membranes, those containing swollen Golgi, and those with normal Golgi morphology.

This study highlights a number of outstanding questions including the following: Which proteins and lipids are responsible for the fact that the Golgi takes the shape of a stack of flattened cisternae in mammalian cells? And why does the Golgi occur as several

ER: endoplasmic reticulum

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins: catalyze membrane fusion of vesicles with target membranes

Tethering factors: participate in the initial process of vesicle attachment to target membranes. Tethers can facilitate SNARE pairing prior to fusion

siRNAs: small interfering RNAs

unconnected stacks in *Drosophila* cells, or as unstacked cisternae in *Saccharomyces cerevisiae*? Swollen Golgi seem to lose their capacity for efficient secretion (see 10). Is this because more energy is required to physically generate transport vesicles from a large, spherical organelle? Are Golgi membranes normally pumped dry of their fluid content to form the flattened stack of pancakes they assume? Channels and pumps are sure to participate in Golgi structure, but it is equally possible that flattened molecular assemblies of proteins yet to be identified stabilize a flattened morphology. These questions deserve continued investigation. A mutant screen for altered Golgi morphology could help identify the requisite players.

Why do cells even need a Golgi complex? The Golgi complex provides well-ordered compartmentalization of oligosaccharide modification reactions that might otherwise compete. The Golgi also provides a sorting station for cargoes destined for different compartments: apical and basolateral plasma membranes, endosomes and lysosomes, or return to the ER. Most importantly, the Golgi serves as a filter to segregate escaped ER residents from those destined for post-Golgi compartments (11). The Golgi also seems to regulate entry of mammalian cells into mitosis, in that its ability to frag-

ment via a series of specific phosphorylation events is essential for this process (12, 13).

A HIERARCHY OF INTERACTIONS REVEALED: SNARES DOWNSTREAM OF RABS AND TETHERS

SNARE proteins mediate membrane fusion (1, 14–17). They lie at the heart of all membrane trafficking reactions and provide significant specificity for membrane traffic (18). Three or four SNARE molecules come together to form a stable SNARE complex comprised of four coiled-coil helices (**Figure 1**). Multiple copies of SNARE complexes are needed to bridge two membranes in close apposition to catalyze bilayer fusion. SNAREs have been categorized into so-called R- and Q-SNAREs: Fusion reactions usually require one R-SNARE (usually on a vesicle, also called a v-SNARE) and three Q-SNAREs, usually contributed by the target membrane (also called t-SNAREs; see 14–17 for review). Mammalian cells express ~36 distinct SNARE proteins (16). Impressively, in the ~10 years since they were discovered, functions have been assigned to most of these proteins. The v-SNARE for Golgi to cell surface transport in mammalian cells is yet to be identified.

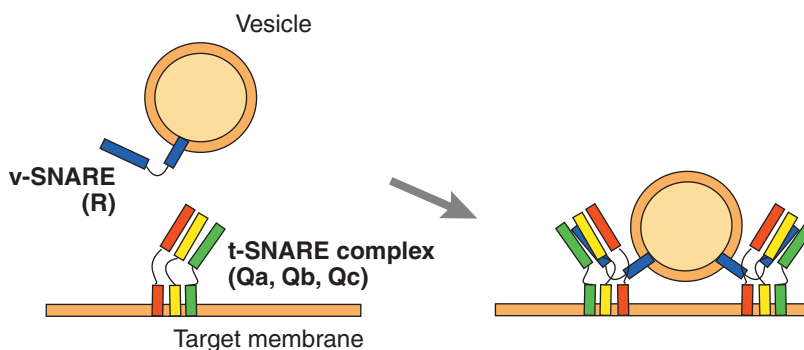


Figure 1

Vesicles carry v-SNAREs that pair with t-SNARE complexes at the target membrane to drive fusion (14–17). Each t-SNARE complex contains one representative of each subtype of t-SNARE (Qa, Qb, or Qc). v-SNAREs are also categorized as R-SNAREs.

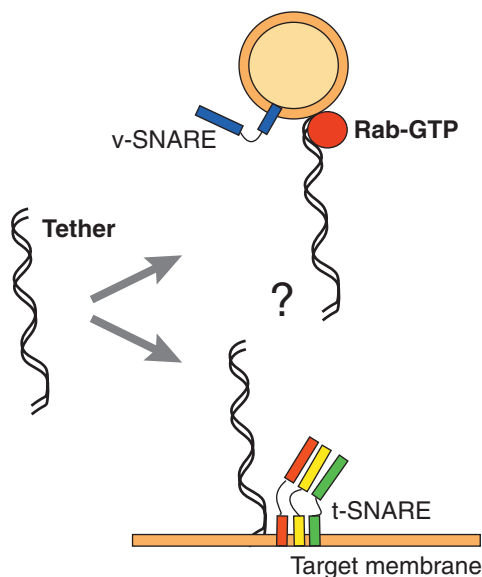


Figure 2

Rab GTPases are present on vesicles and can recruit certain cytosolic tethers to their membranes. Tethers are either long, coiled coils or multimeric assemblies. Certain tethers are also present on target membranes. One tether (p115) has been shown to catalyze the formation of a v-SNARE/t-SNARE complex. Tethers act upstream of SNAREs, but little is known about the precise events that enable tethers to drive fusion. Do tethers help localize SNAREs at target membranes, assembling a “fusion zone”? Do two ends of a tether engage two ends of a fusion reaction? Are tethers needed on the vesicle and at the target?

Although SNARE pairing is relatively specific *in vivo*, SNARE proteins can contribute to distinct SNARE complexes that drive entirely distinct transport events. Moreover, vesicles must recycle v-SNAREs after membrane fusion, from target membranes back to their membranes of origin. SNAREs must not be able to engage each other outside of the context of a properly orchestrated fusion event. Thus, SNARE proteins contribute a layer of specificity that is enhanced by additional, specific, protein:protein interactions. Importantly, these additional layers provide regulation of SNARE pairing so that fusion occurs at the right place and at the right time. Most of the details of SNARE regulation remain unknown.

SNAREs act in concert with tethering factors and Rab GTPases to accomplish vesicle targeting and fusion with high fidelity (19)

(**Figure 2**). Apparently, fidelity is enhanced by multiple layers of specificity. In the simplest model, Rabs on vesicles recruit tethers that bring vesicles to the SNAREs. SNAREs then pair and fusion ensues. Most tethers described to date bind to SNARE proteins (20). In some cases, this may localize a tether to a specific membrane. But tethers are not passive molecules: At least some have the capacity to facilitate SNARE pairing more actively (21) (see below). Thus, membrane traffic involves sophisticated machineries that must be tightly regulated in space and time.

We are accumulating long lists of players: at least 66 Rab GTPases (22), a long list of Rab effectors (19), numerous tethers (20, 23), and other regulatory proteins (7–10). Much work is now needed to refine our understanding of membrane traffic machines. The minimum, core SNARE fusion machine has been reconstituted in liposomes and essential information is being revealed regarding fusion and its regulation (see 24, 25). More work is needed to understand how vesicles engage the target membrane, to elucidate how this machinery functions in a normal, cellular context. What exactly do Rabs and tethers do to facilitate the ultimate SNARE pairing reactions?

THE KDEL RECEPTOR

To understand membrane traffic we need to determine how cellular compartments are provided with distinct sets of constituents, and how those compartments are maintained in the face of a large flux of transiting cargoes. Compartmentalization will involve membrane microdomains that represent function-determining subassemblies of proteins and lipids (26). We need to determine the identity of these subassemblies and learn how they interact with cargoes that interact with them transiently. The story of the KDEL receptor and how it acts to rescue proteins that have escaped from the ER is a satisfying example of our progress in understanding the rules of protein localization in the ER and the

Rab GTPases:

small GTPases that drive vesicle formation, motility, and fusion by recruiting specific effector proteins to form function-specifying membrane microdomains

role of retrograde trafficking in establishing compartment identity.

The experiments that led to the elucidation of the KDEL receptor and its role in ER protein localization represent true classics in biology. Munro & Pelham noticed from gazing at the sequences of soluble ER chaperones that their C termini encoded a conserved KDEL (27). They proved that this sequence participated in ER retention by transplanting it to other secretory proteins (27). Yeast use HDEL rather than KDEL, and a genetic selection for proteins that failed to retain an HDEL-terminating invertase protein revealed two genes, one of which (ERD2) enhanced the ability of yeast cells to retain ER residents (28). In an elegant experiment, Lewis et al. (29) proved the KDEL-receptor function of ERD2 by changing its substrate selectivity: these workers made use of the fact that *Kluyveromyces lactis* uses DDEL instead of HDEL; the ERD2 gene of *K. lactis* was sufficient to change the entire retention specificity of *S. cerevisiae*.

Human cells express ERD2 homologs that also function as KDEL receptors (30). These receptors are localized primarily to the early Golgi complex at steady state (31), but shift in their localization upon coexpression of KDEL-bearing ligands (32). What keeps the KDEL receptor in the Golgi under normal conditions, and why does it fail to be transported to the cell surface? What triggers KDEL receptor retrieval to the Golgi?

We have learned much about COPI-vesicle-mediated retrieval of KDEL receptors (33) but essentially nothing about the basis for KDEL receptor's steady-state Golgi localization. The receptor's 12–13 amino acid cytoplasmic domain seems dispensable for Golgi localization, but is essential for Golgi-to-ER transport (34). The KDEL receptor's cytoplasmic tail mediates interaction with the COPI vesicle machinery. Protein kinase A phosphorylation of a serine may also facilitate COPI binding (34).

Mutagenesis studies implicated a specific charged residue (D193) that lies within the

seventh transmembrane helix, in retrograde transport of the KDEL receptor (35). It is not known how this residue contributes to KDEL receptor transport. Söling et al. (36) suggested that it may mediate interaction between KDEL receptors and the p24 family members that are thought to serve as cargo adaptors for COPI retrograde cargoes (37).

Lewis & Pelham (32) speculated that ligand occupancy might activate the retrograde transport machinery and drive Golgi-ER transport. Indeed, intermolecular association of KDEL receptors was detected by Hsu and colleagues (38), and ligand binding triggered recruitment of cytosolic ArfGAP1 protein to KDEL receptor-containing membranes (38–40). Subsequent FRET (fluorescence resonance energy transfer, or Förster resonance energy transfer) analysis by the late Hans-Dieter Söling and coworkers showed that KDEL receptors aggregate upon ligand binding in the Golgi (36). Moreover, FRET confirmed ligand-stimulated recruitment of Arf-GAP1 to KDEL receptors, and also revealed intimate association of Arf1 and other COPI constituents, with the KDEL receptor in the presence of ligand (36). Aggregation thus appears to be a signal for recognition by the retrograde transport machinery, and would provide enhanced avidity for coat protein:receptor interactions. Aggregation may also recruit protein kinase A (34) to stimulate transport vesicle formation.

How does the KDEL receptor release its retrieved cargo upon return to the ER? KDEL receptor:ligand interactions are pH sensitive (41), with maximal binding between pH 5 and 6. But the early Golgi that houses most KDEL receptors (31) may not be much more acidic than the ER. The ER is pH 7.45, whereas the *trans* Golgi enzyme, galactosyltransferase, resides in a compartment of pH 6.58 (42). The pH is probably higher in *cis* Golgi compartments than in *trans* compartments; nevertheless, the slight difference between the pH of the ER and early (*cis*) Golgi is possibly sufficient to drive ligand binding in the Golgi and release in the ER. This needs to be

ArfGAP1: a GTPase-activating protein that may form part of the structure of COPI coats

TGN: *trans* Golgi network

COPI vesicles: carry KDEL receptors from the Golgi to the ER and also participate in transport within the Golgi complex

GlcNAcT1: N-acetylglucosamine transferase I

tested experimentally. KDEL receptors can retrieve cargo from compartments as distal as the TGN (*trans* Golgi network) (43), in which the tenfold increase in relative proton concentration would ensure receptor:ligand interaction. How frequently KDEL receptors engage cargo in the TGN is not yet known.

If overexpression of KDEL receptor ligands moves the receptor to the ER, what keeps the presumably unoccupied receptor from returning to its normal distribution in the Golgi? Perhaps the extra ligands drive receptor aggregation in the wrong location, and recruit normally Golgi-localized COPI and ArfGAP proteins to the ER. Indeed, such a redistribution could explain the results of Hsu et al. (44) who found a brefeldin-A type phenotype in cells that expressed very high levels of the KDEL receptor. Brefeldin A interferes with Arf recruitment onto Golgi by blocking its activation by a guanine nucleotide exchange factor. This causes much of the Golgi to return to the ER. By driving Arf to excess KDEL receptors in the ER, a brefeldin A-like phenotype could be readily explained.

In summary, signals have been identified that retain proteins in the ER. Most of the soluble ER proteins are involved in protein folding. Why they seem to leave the ER only very slowly is not yet entirely clear, but may simply be due to their association with newly folding protein substrates. ER resident membrane proteins contain dibasic residue-terminating, cytoplasmic domains (45). The dibasic motif is recognized by the COPI coat proteins (46). Thus, there is a means for their retrieval via retrograde, COPI vesicles, but how often does an escape event take place? Do they engage COPI proteins in the ER? Is KDEL receptor-mediated cargo retrieval essential for a normally functioning secretory pathway? At least in yeast, expression of several genes can suppress the essential requirement for the KDEL receptor homolog Erd2p (47, 48).

Although we have learned a great deal about how ER residents are captured if they escape, we know very little about how KDEL

receptors are retained in the Golgi, even in the absence of their own dibasic, COPI-binding motif. As described below, we also know little about the mechanism by which oligosaccharide modifying enzymes are localized to distinct compartments of the Golgi.

ORGANIZATION OF THE GOLGI COMPLEX

The Golgi complex has a striking morphology (49, 50). No other cellular compartment is composed of stacks of flattened cisternae under normal physiological conditions. Glycosyltransferases are compartmentalized across the Golgi stack: Thus, mannosidase II and N-acetylglucosamine transferase I (GlcNAcT1), galactosyltransferase, and sialyltransferase occur in successive but slightly overlapping compartments, defining the *cis*, medial, *trans*, and *trans* Golgi network compartments of the Golgi (**Figure 3**). We still do not fully understand the molecular mechanisms responsible for glycosyltransferase localization within the Golgi complex (51–54).

Golgi enzyme localization may involve retention, retrieval, or both (54, 55). Machamer (56) first proposed that proteins might form oligomers in the specific microenvironments of the Golgi cisternae; residence in a macromolecular structure would preclude access to nascent transport vesicles. Bretscher & Munro (57) noted that glycosyltransferases contain shorter transmembrane domains than plasma membrane proteins. A cholesterol gradient that exists between the ER and the cell surface would result in differences in membrane thickness, and they reasoned that shorter transmembrane domains may “sense” the gradient of cholesterol by partitioning into a membrane in which they are most energetically stable. These authors suggested that such partitioning could exclude Golgi residents from nascent transport vesicles. Rolls et al. (58) showed that a cholesterol gradient was not needed for proper Golgi enzyme localization in insect cells. Recently, Mitra et al. (59) used solution X-ray scattering to measure

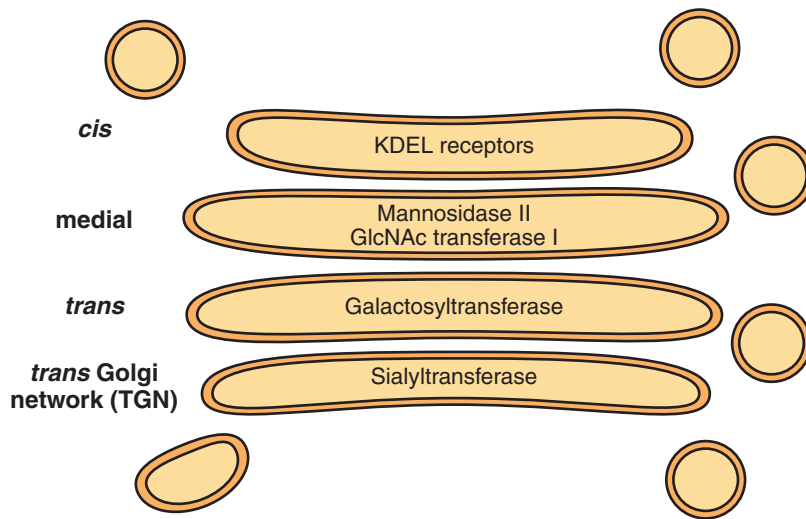


Figure 3

The mammalian Golgi complex occurs as a stack of flattened cisternae. Each layer of the stack contains different enzymes. Although the distribution of enzymes is broader than that depicted here, enzymes occur in different compartments. We know little about how this compartmentation is established or maintained. In addition, the Golgi is surrounded by vesicles, many of which are COPI coated. Static images do not tell us which direction the vesicle is headed. In addition, proteins concentrated at the rims may be about to depart or may have just arrived.

directly the average thickness of ER, Golgi, and apical and basolateral plasma membranes. These were 37.5, 39.5, 35.6, and 42.5 Å, respectively. Cyclodextrin extraction of cholesterol from these membranes had little effect on their thickness; rather, protein content was more important in this regard. Thus, differences in membrane composition may be important, but localization appears not to be strictly linked to cholesterol.

Many labs have carried out domain swap and mutagenesis experiments in an attempt to define the mechanisms of Golgi enzyme localization, and a single definitive mechanism has not been found (51, 60). In support of an enzyme aggregation type of model, Warren and coworkers demonstrated that hetero-oligomers were formed by mannosidase II and GlcNAcT1 of the medial Golgi and that their formation correlated with the localization of these two enzymes in this compartment (61, 62). They proposed that enzymes located in a given compartment rec-

ognize each other and self-assemble in the right compartment; they coined the term kin recognition to describe such a mechanism. Colley and coworkers found that sialyltransferase forms insoluble oligomers at pH 6.3, corresponding to the pH of the late Golgi complex; the ability of this protein to form oligomers correlated with its proper Golgi localization (63). It is not yet known whether perturbation of TGN pH alters the localization of this enzyme *in vivo*. Additional signals may also aid in the initial delivery of this enzyme to the TGN prior to protein oligomerization (60). Gleeson and coworkers could confirm macromolecular complexes for GlcNAcT1 but not for the late-acting Golgi enzymes, 1,4-galactosyltransferase and 1,2-fucosyltransferase, which were readily solubilized in low salt and migrated as monomers or dimers by sucrose density gradient centrifugation (64). Thus, there is increasing evidence for the existence of at least some multienzyme complexes within the Golgi (54).

If enzymes are present in large assemblies, they would be predicted to show a slower rate of lateral diffusion within the Golgi complex. Yet analysis of the mobility of Golgi enzymes using green fluorescent protein-tagged versions of several of the proteins suggested that the enzymes are highly mobile (65). To best understand these findings, it will be important to verify partitioning of tagged proteins into characterized, oligomeric assemblies occupied by the endogenous protein under study. Indeed, a significant challenge associated with the analysis of Golgi protein localization is the recent observation that exogenously expressed, tagged Golgi SNARE proteins distribute across the stack in a manner unlike that of their endogenous counterparts under conditions of ~three to fivefold overexpression (66). This suggests that Golgi localization mechanisms can be saturated, and raises an important red flag regarding how much we can conclude from the behavior of exogenously expressed and tagged glycosyltransferases and other Golgi proteins.

Independent clues to the mobility of Golgi enzymes come from the observations that GlcNAcT1, a medial Golgi enzyme, can acquire sialic acid in the *trans* Golgi network, prior to retrograde transport to the medial Golgi, if it is first engineered to contain an N-linked oligosaccharide addition motif (67). Similarly, GlcNAcT1 can access *cis* Golgi-localized, O-linked glycosylation enzymes after reaching the medial Golgi, as detected using a cleverly designed complementation scheme (68). These experiments indicate that Golgi residents can access other compartments of the Golgi stack, and imply that the proteins are in dynamic equilibria with their macromolecular assemblies.

In summary, no single mechanism can explain glycosyltransferase localization. Membrane composition (if not thickness), molecular aggregation, and kin recognition all remain viable models to explain Golgi enzyme localization. In certain cases, glycosyltransferases can move between cisternae and apparently return to their compartment of origin. This

suggests that glycosyltransferases are retained in compartments by molecular microdomains and can be recognized for retrieval when outside those domains, analogous to escaped ER residents. Clues to the molecular basis of such Golgi enzyme dynamics will come from direct detection of specific enzymes in isolated transport vesicles. Indeed, as discussed below, new tricks to isolate and accumulate transport vesicles are now in hand and are likely to contribute important information regarding these questions.

TRANSPORT THROUGH THE STACK

The past decade has represented a controversial period in terms of conflicting models for the mode by which proteins traverse through the Golgi. Rabouille & Klumperman (69) summarize the controversies that have stemmed from similar electron microscopic experiments carried out in different laboratories and yielding different conclusions. The issue is whether the Golgi stack is a relatively static structure, with secreted cargo moving through it via anterograde-directed transport vesicles, or, in contrast, whether the cisternae themselves move forward in the stack while Golgi enzymes move backward in transport vesicles, allowing the cisternae to “mature” (11, 70).

Two recent papers (71, 72) support the concept of cisternal maturation in yeast, as determined by sophisticated light microscopic, live cell visualization of fluorescently tagged proteins as they progress through the structure. But yeast Golgi compartments are not stacked as in mammalian cells. To complicate (or simplify?) matters, the maturation occurred even in the absence of COPI constituents, albeit with slower kinetics. In another recent report, it was suggested that tubular connections between mammalian Golgi cisternae may permit transfer of cargo between Golgi cisternae (73). Other studies have not detected such intercisternal connections (50), but they may be induced by cargo production (73).

Perhaps a mixture of transport carriers and tubules explains the movement of secretory cargo through the mammalian Golgi complex in a cell type-specific manner (11, 70).

Several labs have focused on the content of COPI coated transport vesicles to distinguish between models of mammalian cell Golgi transport. The Golgi complex is surrounded by numerous tubules and vesicles that are decorated with COPI coat constituents (74). Are COPI vesicles enriched in anterograde cargo or in recycling Golgi enzymes? Elucidation of the precise content of Golgi-derived transport vesicles can tell us much about which proteins are moving and where they are going (based on their Rab, tether, and SNARE content), and will provide essential information to distinguish between a variety of models for how proteins transit the Golgi complex.

A challenge in interpreting protein localizations in and around the Golgi complex is that the presence of cargo and residents does not reveal the transport direction of a vesicle (anterograde versus retrograde) and whether they are about to depart, or instead have just arrived. Analysis of SNARE protein distributions is useful, as we can reasonably assume that v-SNAREs are moving in and out of the Golgi complex and t-SNAREs are marking targets (66). The ER-derived v-SNARE, Bet1, was found to be concentrated at the rims of the *cis* Golgi by immunoelectron microscopy (66), displaying a relative rim-to-center ratio of 11. Similarly, the corresponding t-SNAREs that pair with Bet1 (GS27 and Sec22B) were also concentrated at rims, with a rim-to-center ratio of ~5. Perhaps the t-SNAREs detected at the rims reflect the aftermath of a vesicle fusion event; the enrichment of the v-SNARE could reflect previous arrival as well as pending departure from a given compartment. *Trans* Golgi SNAREs (syntaxin5, GS28, and GS15) were less concentrated at the *trans* Golgi rims than were *cis*-Golgi SNAREs, which may reflect less active trafficking or simple differences in their localization mechanisms or functional requirements. In contrast, the resident glyco-

syltransferases, mannosidase II, and galactosyltransferase were well segregated from the rims and de-enriched from those zones (66).

VESICLES PROVIDE IMPORTANT CLUES

Two classes of COPI coated vesicles were initially identified morphologically in the peri-Golgi region: one class that carried KDEL receptors, probably retrograde carriers en route from the Golgi to the ER as part of KDEL receptor function, and another that carried the Golgi restricted GS28 SNARE protein together with anterograde cargo (33, 74).

A recent paper by Warren and colleagues (75) used Golgi tethers to isolate distinct classes of Golgi-derived transport vesicles. This potentially powerful approach is likely to yield important insight in the future regarding the nature of vesicular transfers within the Golgi and elsewhere. COPI vesicles were generated *in vitro*, and were found to be enriched in Golgin-84. CASP, a Golgin that can bind Golgin-84, was excluded from budding vesicles, and instead localized to the donor, cisternal membranes. Purified CASP, immobilized on glass coverslips, bound a subset of the isolated COPI vesicles, if they were first uncoated *in vitro*. The CASP-isolated vesicles lacked members of the p24 family, but instead were enriched in the Golgi enzymes, mannosidase I and II. Malsam et al. (75) postulate that these vesicles may be retrograde carriers for transport within the Golgi. It will be of interest to deplete CASP from cells and test if similar transport vesicles accumulate.

In contrast, immobilized p115 protein captured vesicles were enriched in p24 family members and the cargo protein, polymeric Ig receptor, but not mannosidase I and II (75). The authors suggest that these latter vesicles are anterograde carriers from within the early Golgi. Others have used antibodies to the p24 tail and isolated vesicles depleted of both enzymes and cargo (76). Vesicle markers also provide valuable antigens as tags for vesicle isolation and characterization.

COG (conserved oligomeric Golgi) complex: a tethering complex that is important for retrograde transport through the Golgi complex

As the biochemical requirements for coat assembly are better defined, it will become more feasible to generate vesicles *in vitro* that have the same composition as vesicles generated in living cells. Early protocols for *in vitro* generation of transport vesicles required salt washes, poorly hydrolyzable GTP analogs, and pipet manipulations now known to bypass the roles of certain key players such as ArfGAP1 (see 77 for discussion). Full fidelity of cargo selection requires ArfGAP1 that may also function as a structural coat component, rather than simply as a coat disassembly factor. Purified tethers should be powerful reagents for the purification of distinct classes of physiologically relevant transport vesicles.

Another important tether that is providing interesting clues regarding Golgi transport is the so-called COG complex (conserved oligomeric Golgi complex) (see 78 for review). The genes encoding the eight COG subunits were identified independently and later found to represent a related complex in yeast and mammalian cells. Null mutants in several subunits led to global alterations in protein glycosylation (79). Subsequent work has revealed that loss of COG subunits leads to hypoglycosylation of multiple classes of proteins due to mislocalization of certain glycosyltransferases (80, 81) and decreased activity of the requisite sugar nucleotide transporters (79). Remarkably, the capacity of the resulting Golgi complex to process proteins for anterograde transport was relatively unimpaired (79, 82, 83).

In CHO cells deficient for COG1 or COG2 proteins, seven type II integral membrane proteins were found to be present at reduced levels (84): CASP, Giantin, and Golgin-84 (three Golgi-associated, coiled coils proposed to be vesicle tethers), the SNARE proteins, GS28 and GS15, a heavily glycosylated protein named GPP130, and the mannosidase II glycosidase. The authors termed these "GEAR" proteins. Loss of proteins is usually due to absence of a stabilizer or mistargeting to the wrong compartment. In this study, the proteins lost their typical Golgi localization and showed enhanced rates of

degradation. Partners of Giantin and Golgin-84, such as p115 and GRASP-65, were not altered in their expression levels or localization in cells lacking COG subunits. Subsequent work (82) has shown that COG3 depletion leads to the codepletion of COG1, 2, and 4 proteins and to the accumulation of small vesicles carrying the GS15 and GS28 SNAREs, together with GPP130. COG3 depletion in yeast also leads to vesicle accumulation (81). Perhaps these small vesicles and their GEAR protein content were targeted for premature degradation. In any case, the ability of COG to bind directly to COPI coat constituents and the Golgi SNAREs implicated in retrograde trafficking, along with the demonstrated block in retrograde transport of Shiga toxin to the ER in COG3-depleted cells (82), represent strong indications that the COG complex serves as a retrograde trafficking tether. It will be of interest to learn of the relationship of the COG-depleted vesicles with those that can be tethered by CASP (75). Purified COG tether could be used to define the constituents of retrograde-directed, Golgi-derived transport vesicles.

After 3 days of COG3 protein depletion, as much as 50% of GlcNAcT1 was detected in vesicles rather than within the Golgi stack (80). Mannosidase II and, to a lesser extent, GlcNAcT2 were also seen in vesicles that are presumed to be the same as the COG complex-dependent vesicles that contain the GEAR proteins (82). Depletion of COG7 also led to mislocalization of GlcNAcT1 and GS15 (80). These results suggest that the codepleted "GEAR" proteins, CASP, Giantin, Golgin-84, GS-28, GS-15, and GPP130, are needed for proper Golgi localization of these glycosyltransferases. Because COG protein depletion alters the level of multiple Golgi protein constituents, this experiment does not yet resolve the mechanism of Golgi enzyme localization. Nevertheless, either GEAR proteins provide a scaffold for Golgi enzyme retention, or retrograde transport is essential for localization. Specific mutant proteins that can block a single aspect of a given protein's

function (see 12, 13, 85) will help clarify this complex pathway.

Malhotra & Mayor (70) note that a strong prediction of the cisternal maturation model is that a block in retrograde traffic should block cisternal maturation and send Golgi-resident proteins to the cell surface or the endosome. An important experiment would be to follow the fate of *trans* Golgi network enzymes such as sialyltransferase in cells depleted of COG proteins.

THE GOLGI IS A HIGHLY DYNAMIC COMPARTMENT

The Golgi is a fragile structure in one sense: Simple depolymerization of microtubules yields dispersed mini-stacks that are nevertheless functional for protein secretion. Upon washout of the depolymerizing agent, mini-stacks reassemble. Thus, the Golgi is poised for homotypic fusion to drive reformation of the stack by lateral fusion; it can also undergo a fission reaction when microtubules depolymerize, separating into mini-stacks. This lateral fission process appears to be countered, under normal conditions, by the mere presence of an intact microtubule cytoskeleton. Thus, the Golgi seems poised for fusion, fission, and probably, vesicle formation.

Putative tethering proteins can also influence the balance between Golgi membrane fusion and fission. Cellular depletion of multiple, putative tethers yields a similar phenotype: generation of mini-stacks. Thus, depletions, for example, of Golgin-84 (86), TMF (87), COG3p (82), and GCC185 (88), share this feature. Similarly, depletion of GM130 (85) yields secretion-competent Golgi mini-stacks. Elegantly, interaction between GM130 and GRASP65 proteins but not with another binding partner, p115, was required for lateral cisternal fusion rescue of the mini-stack phenotype (85). These findings suggest that we should think of Golgi tethers more broadly: Rather than molecules that function solely to capture nascent trans-

port vesicles at their targets (89), Golgi-associated coiled-coil proteins also appear to facilitate cisternal association/homotypic fusion and probably also cisternal stacking (90). Some Golgin proteins may well serve predominantly as a scaffold for tethers, to enable different subsets to facilitate vesicle capture.

An important future challenge will be to dissect the unique and distinct roles of individual Golgins. Their multiple interactions will not make the analysis simple, but we need to define their individual contributions. To date, putative tethers have been categorized into two classes: long, extended coiled-coils and multisubunit assemblies (20). Whyte & Munro (91) noted significant homology between members of the multisubunit class that includes the Exocyst, COG complex, and the GARP complex. With the Exocyst as a prototypical tether that has been shown to engage transport vesicles with their targets, perhaps the multisubunit class will be more conventional and serve entirely a tethering function; coiled-coil Golgins may play more diverse roles in Golgi architecture and trafficking. In analyses of tether function, it will also be essential to monitor depletion of multiple constituents upon siRNA depletion of any given tethering protein. Depletion of individual tethers may possibly disrupt a much larger, Golgi stack-integrity-maintenance complex.

SNARE REGULATION

Several classes of proteins regulate SNARE assembly and function. So-called SM proteins related to yeast Sec1p and mammalian Munc18 also bind to SNAREs (14, 17, 92, 93). Although a number of groups are studying these proteins, more work is needed to clarify their precise and varied roles in regulating SNARE pairing and fusion. In addition, the synapse represents a finely tuned exocytosis machine that must be capable of rapid exocytosis upon calcium entry (94). A number of neuronal proteins regulate SNARE function in presynaptic terminals, including Unc-13, RIM, and synaptotagmin.

Golgins: large, coiled-coil proteins that are Golgi associated. Some may be tethers; others may participate in Golgi cisternal stacking

Synaptotagmin functions as the calcium sensor for synaptic vesicle release, and recent work suggests that it functions with a protein named complexin to “clamp” SNAREs in a fusion-ready manner. Rothman and coworkers have devised a powerful system that permits facile experimental evaluation of SNARE regulator function: SNARE proteins are ectopically expressed on cell surfaces, and their ability to drive cell fusion can be monitored using intracellular, fluorescent marker proteins localized to either the cytoplasm or nucleus (95). Using this system, Giraudo et al. (25) were able to study the mechanism by which complexin proteins regulate SNARE proteins. Complexins bind to a groove that is formed by assembled, and not monomeric, exocytic SNARE proteins (96, 97). When complexin was expressed as a cell surface, GPI-anchored protein, it was a potent inhibitor of cell:cell fusion (25). Remarkably, a relatively rapid, calcium-sensitive–cell fusion reaction could be recapitulated if the calcium sensor, synaptotagmin, was included on the v-SNARE side of a complexin-stalled, cell fusion reaction. The process required the capacity for complexin release from cell surfaces (to remove the complexin “clamp”) by cleavage of the membrane-anchored complexin with phosphatidylinositol-specific phospholipase C. Rothman and colleagues propose that complexins act as a reversible clamp that allows very rapid fusion in a tightly regulated manner as would be required at the synapse (25). Previous reconstituted fusion reactions did not occur on the time scale that a “clamped” SNARE complex allows.

SNARE complex formation is undoubtedly carefully staged *in vivo*. A recent elegant reconstitution study showed that a preassembled, Syntaxin-SNAP25, 1:1 t-SNARE heterodimer is the optimal substrate for synaptobrevin binding, which leads to subsequent rapid fusion (24). In contrast, *in vitro* reactions containing 2:1 complexes of Syntaxin1-SNAP-25 are much slower in fusion. The C terminus of the synaptobrevin v-SNARE could stabilize this 1:1 t-SNARE complex for

subsequent fusion with membranes containing full-length synaptobrevin. Additional cellular regulators may act in a similar manner to stabilize prefusion complexes *in vivo*. Indeed, the proteins tomosyn and amisyn may play a similar role (98; see also 16, 17). Functional assays will continue to provide important clues as to the precise functions of regulatory factors. In addition, structural studies will continue to be essential to understand fully the mechanisms by which SNARE regulators act.

TETHERS CAN CATALYZE SNARE ASSEMBLY

Many tethering factors interact with SNAREs. For example, the COG complex interacts genetically or physically with five intra-Golgi SNAREs: Bet1, Sec22, Ykt6, GS28, and Sed5 (see 14, 16 for review). Tethering factors also facilitate SNARE assembly. In a careful and thorough study, Shorter et al. (21) showed that a specific coiled-coil domain in p115 can catalyze the assembly of at least three distinct SNARE complexes: GS15-Ykt6-GS28-Syntaxin 5, Bet1-Ykt6-GS28-Syntaxin5, and GS27-Bet1-Sec22-Syntaxin5. p115 bound directly to GS28 and Syntaxin5, and stimulated significantly both the rate and extent of complex formation between these proteins. The ability of p115 to act could be inhibited by the addition of a specific coiled-coil segment from within the p115 sequence, and was restricted to SNARE complexes containing Syntaxin5. How p115 and other tethers are themselves regulated in terms of their capacity to drive SNARE complex formation represents another important question.

RABS AND ARLS LOCALIZE TETHERS

At the top of the hierarchy of SNAREs and tethers are the Rab GTPases. This author has written much about these master regulators (see 99, 100) and will be very brief

here. Rabs drive cargo collection into nascent transport vesicles, link vesicles to motor proteins, and recruit tethers to vesicles to facilitate membrane trafficking (19). To accomplish these diverse tasks, Rabs recruit so-called effector proteins to the membrane in which they are localized. Rabs are also stabilized in a given membrane by effector and lipid interactions (see 101). Activation of Rabs at the right time and place can generate a membrane microdomain that possesses unique functionality. Indeed, Rabs are likely to be important contributors to the organization of the Golgi stack and to the control of membrane traffic into and out of the Golgi complex (102, 103).

Identification of the molecules recruited by each Rab to form functional microdomains will be essential for our understanding of the mechanism and regulation of membrane traffic and, also, the establishment of compartment identity. Yet Rabs are not the only small GTPases involved in vesicle tethering and docking. Arl family GTPases are important for the localization of certain Golgi tethers that contain “GRIP” domains at the TGN (103). Rho GTPases also interact with tethering complexes (19): Specifically, the Exocyst that facilitates secretory vesicle docking at the plasma membrane binds to Rho family members in yeast and mammalian cells. Three-dimensional biochemistry will be needed to establish how each of these GTPases are coordinately regulated.

VESICLE FISSION

How do vesicles first bud off of a donor membrane? Assembly of coat proteins on membrane surfaces can contribute significantly to the generation and stabilization of membrane curvature (2, 104, 105). For example, the so-called BAR domain present in proteins such as amphiphysin and endophilin assumes an overall banana shape, the concave face of which is basic and therefore adapted to interact with acidic and curved lipid membranes (105–107). Similarly, ArfGAP1 recognizes defects in lipid

packing that appear when the curvature of a membrane exceeds a baseline level (108). When a membrane is curved by the mechanical force imposed by the COPI coat, ArfGAP1 can become concentrated at the edge of a nascent bud and influence the amount of Arf-GTP that is present there (108).

What triggers pinching off of vesicles? The dynamin GTPase plays a key role in certain cellular membrane fission events and has been reviewed elsewhere (3, 109).

Recent studies of protein export from the TGN provide a satisfying framework that can explain vesicle and tubular budding from that compartment, and turn our focus to the contribution of membrane lipids (110). Malhotra and colleagues propose a “pull and cut” model by which vesicles form. In 1993, these workers identified a compound, ilimaquinone, that causes the Golgi to vesiculate. They went on to show that ilimaquinone vesiculation of the Golgi involved a trimeric G protein and the serine/threonine protein kinase D (PKD), and that these constituents also participate in the normal process of vesicle formation from the TGN. PKD associates with the Golgi complex via diacylglycerol (DAG), and is activated by a Golgi-associated, protein kinase C η . Malhotra’s model for vesicle fission involves cargo-triggered stimulation of PKD to increase the local concentration of DAG. DAG-induced curvature of the membrane would proceed until fission ensued.

Obviously, many key aspects of this model remain to be tested. How is the cargo recognized and which G protein-coupled receptor participates? Which enzymes and substrates are used to generate DAG in a spatially restricted manner? How is this reaction terminated to prevent the vesiculation of the entire compartment? Are BAR domain proteins involved, and how is this machine coupled to incorporation of SNAREs and Rabs into the nascent vesicle? Is pulling mediated by the cytoskeleton, and how are the motor proteins localized and controlled? It is hoped that these questions will be tackled with great gusto.

DAG: diacylglycerol

FUTURE PERSPECTIVES

Is membrane trafficking solved? Certainly not! Consider the analogy of nucleic acid polymerases that were discovered in the late 1950s. The subsequent half century has focused on understanding regulation of DNA

replication, repair, and transcription. Now we must dive headfirst into determining the regulation of membrane traffic, undeterred by a long list of basic, unanswered questions that require and deserve our very full attention.

SUMMARY POINTS

1. Although a great deal has been learned in the past 20 years about membrane traffic and long lists of players have been assembled, many fundamental questions remain and most mechanistic information is lacking.
2. Much has been learned about SNARE proteins; much more needs to be learned about their assembly and regulation.
3. KDEL receptor trafficking provides an elegant example of retrieval-based maintenance of a membrane compartment.
4. We still do not understand how glycosyltransferases are compartmentalized within the Golgi complex.
5. Golgin proteins are important for Golgi architecture and vesicle docking.
6. The use of tethers and anticargo antibodies will be important tools to permit isolation of distinct transport vesicle types; characterization of such vesicles will be key to our elucidating the mechanisms of protein transport through the Golgi complex and between membrane-bound compartments.
7. Proteins and lipids contribute to the process of vesicle fission.

FUTURE DIRECTIONS/UNRESOLVED ISSUES

1. How is the structure of the Golgi formed, and how are the enzymes within it organized?
2. How do vesicles engage target membranes, and how is the docking and fusion machine assembled, disassembled, and recycled in a temporally and spatially regulated manner?
3. How do cells maintain membrane compartment size, shape, and content in the face of large volumes of transiting cargoes?

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