The *trans*-Golgi network: a late secretory sorting station

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Proteins synthesized on membrane-bound ribosomes are transported through the Golgi apparatus and, on reaching the *trans*-Golgi network, are sorted for delivery to various cellular destinations. Sorting involves the assembly of cytosol-oriented coat structures which preferentially package cargo into vesicular transport intermediates. Recent studies have shed new light on both the molecular machinery involved and the complexity of the sorting processes.

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Current Opinion in Cell Biology 1997, 9:527–533

http://biomednet.com/elecref/0955067400900527

 ISSN 0955-0674

Abbreviations

ACTH	adrenocorticotropic hormone
AP	adaptor protein complex
ARF	ADP-ribosylation factor
BFA	brefeldin A
GAG	glycosaminoglycan
HA	influenza virus hemagglutinin
HSPG	heparan sulfate proteoglycan
IG	immature secretory granule
LAMP	lysosome-associated membrane glycoprotein
MPR	mannose 6-phosphate receptor
PKC	protein kinase C
PLD	phospholipase D
TGN	*trans*-Golgi network
VSV G	vesicular stomatitis virus G protein

Introduction

As the last station of the Golgi complex, the *trans*-Golgi network (TGN) plays a pivotal role in directing proteins in the secretory pathway to the appropriate cellular destination. The conventional view has been that protein traffic emerging from the *trans*-Golgi bifurcates at the TGN (Figure 1a). One route, the constitutive or default pathway, delivers proteins to the cell surface while a second, selective pathway sorts protein traffic into the intracellular endosomal membrane system. A variation in the surface-destined constitutive route is seen in polarized cells, where proteins fated for basolateral delivery are differentially sorted and trafficked in carrier intermediates separate from those that shuttle cargo to the apical membrane surface (Figure 1b). This separate routing of proteins ensures the asymmetric distribution of certain proteins at the apical and basolateral surfaces. In cells that undergo regulated exocytosis, the selective endosomal sorting route from the TGN is also modified. This allows the formation and accumulation of mature dense-core secretory granules containing concentrated cargo, poised for exocytotic release (Figure 1c). In many secretory tissues, this is probably the major pathway to the surface. The recent studies discussed below, however, question a clear-cut restriction of specific trafficking routes to specialized cell types, and suggest instead that the protein sorting mechanisms at the TGN in animal cells are both more complex and more numerous than previously anticipated. In this review, we will summarize some of the recent advances made in our understanding of the mechanisms that govern protein sorting at the TGN.

Studying sorting at the TGN

There are several ways to experimentally probe differential sorting at the TGN. The most fruitful method has been to follow the movement of cargo molecules as they exit the TGN in transport vesicles or as they arrive at the cell surface. These assays typically reconstitute multiple complex and sequential sorting steps. Central to the development of many these assays was the discovery that export from the TGN is reversibly blocked at 20°C [1]. The temperature block allows cargo to be accumulated in the TGN at 20°C and then released synchronously upon warming. In fact, using green fluorescent protein tagged cargo, protein export from the TGN can be visualized in real time [2]. A list of common cargo molecules that have been used to follow sorting from the TGN is presented in Table 1.

Table 1

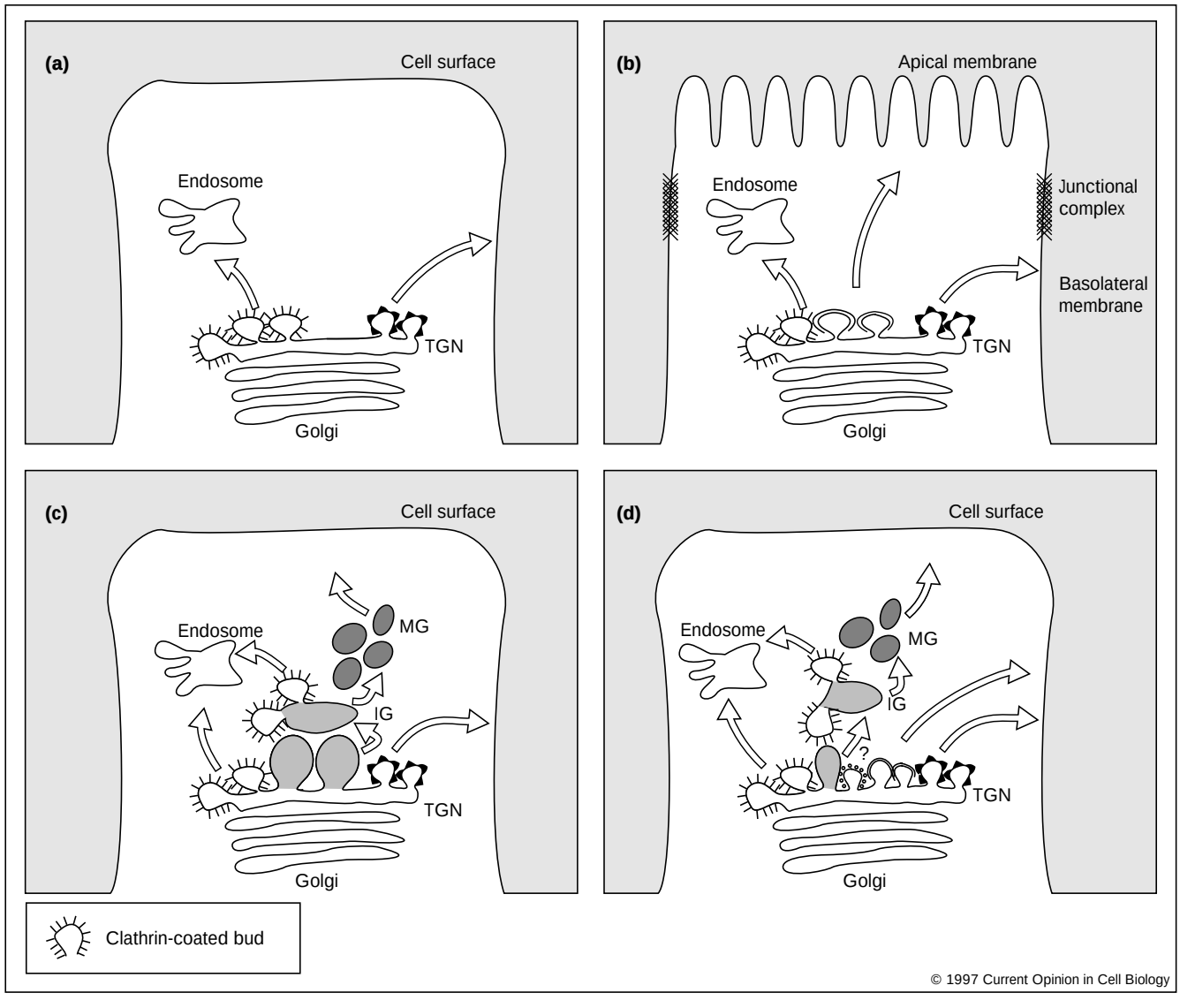
Representative markers used to follow export from the TGN.

Sorting route	Marker	References
Constitutive pathway		
Nonpolarized cells	HSPG	[9,45•,48]
	GAGs*	[43••]
Polarized cells		
Apical surface	HA	[14••,21•,23•]
Basolateral surface	VSV G	[14••,21•,23•]
Selective endosomal pathway	Lysosomal proenzymes	[44•]
	LAMPs	[41•]
Regulated secretory pathway	Proinsulin	[4]
	Secretogranin II	[9,45•]
	Growth hormone†	[8,50•]
	Prolactin†	[8,50•]
	Prosomatostatin†	[8,50•]
	ACTH	[46]

*GAGs are a fluid-phase marker and have been used to follow both the constitutive and the regulated secretory pathways [43••]. †In GH_3 cells, a substantial amount of these polypeptide hormones is secreted via the constitutive pathway.

A second, complementary way of studying protein export from the TGN is to characterize the protein machinery that ensures the differential sorting of cargo, and thereby begin to understand the molecular basis of the sorting

Figure 1



Schematic illustrations of the different trafficking routes from the TGN. **(a)** The simplest model for TGN-mediated sorting differentiates constitutive, surface-destined cargo from that to be delivered to the internal endosomal system via clathrin-coated vesicles. **(b)** In polarized cells, which are characterized by a circumferential band of junctional complexes which physically separate the apical membrane from the basolateral surface, apical- and basolateral-destined proteins are sorted from each other into distinct carriers at the TGN. **(c)** In cells that undergo signal-dependent regulated secretion, proteins incorporated into the secretory granules are diverted from the flow toward the surface and accumulate in immature secretory granules (IG). The IGs, after retrieval to the endosome of excess membrane and mis-sorted proteins in clathrin-coated vesicles, condense to form mature secretory granules (MG). **(d)** Hypothetical diagram of a single cell exhibiting all of the sorting events that can occur at the TGN. The question mark signifies the assembly of coat structures which contain p200, for example, for which a trafficking route still remains to be determined. Biochemically distinct coats are likely to specify protein sorting at the TGN and, to attempt to distinguish the different sorting routes from each other, these coats are indicated by different symbols. Cell surface or basolateral membrane destined buds are surrounded by solid triangular coat subunits; the coat on apical surface bound buds is indicated by a concentric ring; and the spiked coats surround endosome-bound clathrin-coated buds.

process. Studies of this type have been aided by brefeldin A (BFA), a fungal antibiotic which also reversibly arrests protein export from the TGN in most nonpolarized cells examined. Addition of BFA at 20°C overrides the ability of pulse-labeled cargo proteins, which had accumulated in the TGN at the lowered temperature, to exit the TGN on warming to 37°C [3–5]. Because the TGN is not incorporated into the merged endoplasmic reticulum/Golgi compartment that arises in the presence of BFA, this reveals that BFA affects TGN sorting events rather than simply trapping cargo within the modified endoplasmic reticulum [6]. This result also suggests that low temperature arrests vesicular traffic at a point prior to BFA action. As BFA inhibits a nucleotide-exchange factor(s) for a small GTPase termed ADP-ribosylation factor (ARF) [7•], it appears that ARF·GTP might regulate

multiple TGN export sites, and there is now evidence for the involvement of ARF in the sorting of growth hormone [8], heparan sulfate proteoglycan (HSPG) and secretogranin II [9] into secretory vesicles at the TGN.

As all exit routes from the TGN are thought to utilize vesicular intermediates, ARF could affect all these pathways generally by modulating the membrane through the activation of a phosphatidylcholine-specific phospholipase D (PLD) [10–12]. Consistent with this idea, the gene encoding an ARF-activated PLD1 has been cloned and the enzyme has been localized to perinuclear structures which, in part, overlap the Golgi/TGN region [13].

The constitutive pathway

Surprisingly, distinct apical- and basolateral-destined pathways might not be a unique property of polarized cells at all, as fibroblasts also appear to have two analogous trafficking routes to the plasma membrane [14••]. In baby hamster kidney (BHK) and Chinese hamster ovary (CHO) fibroblasts, aluminium fluoride inhibits the surface delivery of only those viral proteins that are sorted basolaterally in Madin–Darby canine kidney (MDCK) cells. Both *N*-ethylmaleimide-sensitive factor (NSF) and rab proteins are important regulators of protein delivery to the basolateral surface. Conversely, in the fibroblasts only an apical-bound molecule (influenza virus hemagglutinin, HA) is found in detergent-insoluble aggregates, and membrane fusion in the apical pathway appears to be independent of NSF activity and is not inhibited by rab guanine nucleotide dissociation inhibitor (rabGDI), as is seen in polarized epithelia [15]. Notably, basolateral sorting signals are not restricted to proteins expressed in polarized cells, so there are candidate endogenous proteins that are likely to be differentially routed to the surface in fibroblasts. Related apical and basolateral trafficking routes might, therefore, exist in all cells, with the default itinerary varying according to cell type. In hepatocytes, for example, the constitutive pathway taken by most migrating transmembrane proteins is to the basolateral (sinusoidal) surface, while in MDCK cells it is to the apical membrane [16].

The precise identities of the proteins that mediate constitutive sorting at the TGN remain to be established. Several non-clathrin coatlike proteins, including p62 [17], p200 [18,19] and p230 [20], have been identified; these proteins cycle onto and off the TGN and are found on coated buds and vesicles at the TGN region. Cell-free systems which can generate Golgi-derived coated transport vesicles that are selectively enriched in certain cargo molecules have also been developed [21•,22]. Unfortunately, the identity of the coat proteins found on isolated coated post-TGN vesicles is still unclear [21•] and so it remains to be seen whether any of the proteins mentioned above are involved in the formation of apical- or basolateral-destined vesicles. It seems that p200 may not be a major protein constituent of either vesicle, however. Depletion of p200 does not perturb either apical or basolateral transport in *in vitro* assays and, in MDCK cells, neither HA nor vesicular stomatitis virus G protein (VSV G) colocalizes with p200 on coated structures on the TGN, although VSV G and HA can be seen on distinct vesicular profiles [23•]. p230 is unrelated to p200 [24•] but, being BFA-sensitive, is another attractive regulatory candidate. p200 and p230 localize to separate clusters of coats within the TGN area [24•] but, because no cargo has been identified yet within the p230-containing structures, it is not yet known whether p230 is involved in basolateral or apical sorting.

As we are faced with more putative coat proteins and sorting routes than our current knowledge of cargo molecules necessitates, is it possible that there might be some redundancy in protein export from the TGN? This idea runs against our notions of sorting specificity, and so it seems more likely that other cargo molecules will utilize the additional itineraries. We already know, for example, that there are at least two parallel routes, which are utilized by different sets of cargo, from the Golgi to the surface in yeast [25], and that, in hepatocytes, membrane and secretory proteins appear to move to the basolateral surface in separate vesicle populations [26].

The selective endosomal route

The differential incorporation of cargo into distinct coated buds at the TGN is an active process. Cytosol-oriented sorting signals direct cargo to the appropriate export site, and much is known about these sorting signals [27]. Curiously, some of the sorting signals both for basolateral targeting and for lysosomal delivery consist of tyrosine- or dileucine-based signals. Given this sequence similarity, how is the sorting specificity preserved at TGN export sites? Although the basolateral and lysosomal sorting signals are similar, they are not identical and may therefore interact with coat components with different affinities for the signals. These differences might be sufficient to prevent the 275 kDa mannose 6-phosphate receptor (MPR), which has a YSKV sequence (single-letter code for amino acids) in the cytosolic domain, from being sorted efficiently into basolateral-destined vesicles. A similar argument would explain why the YTRF internalization signal of the transferrin receptor fails to be sorted into clathrin-coated lysosome-destined vesicles on the TGN. In fact, the μ subunits of the clathrin-associated adaptors AP-1 and AP-2 (adaptor protein complexes 1 and 2) do show different binding affinities for certain sorting signals [28,29]. Another possibility is that the TGN might be organized into discrete subdomains, each dedicated to the assembly of a specific coat population. Some evidence for this idea comes from three-dimensional reconstructions of the TGN based on high voltage electron microscope images [30]. Just how such specialization of the TGN might occur, and how pre-sorting into distinct subdomains could be achieved, is unknown.

Clathrin-mediated sorting of lysosome-destined cargo has not yet been fully reconstituted *in vitro*, but the principal protein components of the vesicle budding machinery are known and we have a better understanding of the early biochemical events that initiate clathrin-coat assembly on the TGN [31,32,33•]. Clathrin-mediated sorting at the TGN is also abolished by BFA, and the identification of ARF1 as the essential, BFA-sensitive regulator of coat assembly [33•,34,35,36•,37] may explain why clathrin-coated vesicles do not assemble on all intracellular membranes containing suitable cargo molecules, such as late endosomes. By restricting ARF recruitment to select membrane sites, determined by the location of a BFA-sensitive nucleotide-exchange factor for ARF, AP-1 recruitment and hence coat assembly are limited. GTP-bound ARF could initiate clathrin-coat assembly on the TGN in one of at least two ways. As there is now little doubt that AP-1 interacts with cargo directly, the GTPase could activate cargo molecules on the TGN, increasing the affinity of these proteins for the μ1 subunit of AP-1. Alternatively, an ARF-activated docking protein could begin the coat assembly process by recruiting AP-1, which is then followed by the preferential incorporation of cargo into the growing lattice.

In trying to distinguish between these two possibilities, it has been found that recruitment of AP-1 onto the TGN of permeabilized fibroblasts derived from MPR-deficient animals is reduced by ~70% and can be restored by transfection of MPR cDNAs [33•]. Together, ARF1 and the MPRs appear to create a high affinity ($K_d \approx 25$ nM) binding site for AP-1 [33•]. Using a vaccinia virus expression system in HeLa cells to produce large quantities of either class II major histocompatibility complexes [38] or the varicella-zoster virus envelope glycoprotein I [39], a modest twofold increase in AP-1 binding can also be seen. When compared with normal MPR-positive cells, the steady-state levels of membrane-associated AP-1 and of AP-1-containing TGN-derived clathrin-coated vesicles in MPR-negative fibroblasts are about three times lower [40•]. Together, these experiments demonstrate the importance of cargo in clathrin-coat assembly and suggest that MPRs are rate-limiting components for clathrin-coat assembly at the TGN, thus supporting the first possibility mentioned above.

Like the MPRs, lysosome-associated membrane glycoproteins (LAMPs) are also predominantly sorted into the endosomal system at the TGN directly. The LAMP1 sorting signal, GYQTI, was shown recently to interact directly with AP-1 and LAMP1 has been identified within clathrin coats assembling on the TGN [41•]. This confirms what has long been suspected, that the LAMPs exit the TGN in AP-1-containing clathrin-coated vesicles, mingling with the MPRs. It is difficult to imagine how the 11 residues of the LAMP1 tail could interact with both ARF and AP-1 simultaneously to initiate coat assembly. Further, if cargo (primarily the MPRs and LAMPs) is the principal AP-1-binding site on the membrane, then one might predict that cross-linking and affinity isolation procedures should reveal these proteins to be prevalent, perhaps stoichiometric, adaptor-binding partners. However, this does not appear to be the case, and, although AP-1 can be shown to bind to several discrete proteins [36•,42•], none correspond in size to either the MPRs or the major LAMPs. The AP-1-binding proteins thus represent candidates for adaptor docking sites, but definitive resolution of this issue requires the isolation and further characterization of the putative membrane docking components.

The regulated secretory pathway

To add even more complexity to the protein sorting story, what appears to be a minor regulated secretory pathway has now been identified in several cells that are not considered to display stimulus-evoked exocytosis [43••]. Identification of this sorting route required prolonged labeling of sulfated glycosaminoglycans (GAGs), as most of this fluid-phase marker was secreted constitutively. The secretory vesicles containing the residual GAGs cofractionated with the small GTPase rab3D, which is considered to be a specific marker of the regulated secretory pathway, and were stimulated to discharge their contents either by elevating cytosolic Ca^{2+} concentrations or by activating protein kinase C (PKC). Conceptually, this pathway could be used for the regulated delivery of select membrane proteins to the plasma membrane but, at present, no known cargo traverses this cryptic pathway and it will be important to establish rigorously whether the fluid-phase tracer used reaches the surface by transiting only through the biosynthetic secretory apparatus or whether it crosses through elements of the endosomal system before being released.

If one views the immature secretory granule (IG) as a functional extension of the TGN [44•], then the identification of a stimulus-dependent secretory pathway in cells that do not display regulated secretion is perhaps not too surprising. Maturation and condensation of IGs seem to occur partly through a sorting process analogous to that seen at the TGN proper. Non-granule proteins certainly enter some IGs passively as the IGs form at the TGN, but these proteins are subsequently removed by selective sorting events [44•]. In fact, AP-1-containing clathrin-coated vesicles play a major role in selectively removing excess membrane and lysosomal proenzymes from the condensing granule [45•]. The biochemical features of clathrin-coat assembly on immature secretory granules are indistinguishable from those features of clathrin-coat formation on the TGN [34,36•], fortifying the idea that the IG really is a specialization of the TGN.

An alternative view, nevertheless, is that sorting of regulated secretory proteins into IGs is selective and receptor-mediated, superimposed upon specific aggregation of regulated secretory proteins in the TGN.

Incorporation into IGs is not dependent on sorting of aggregates, however. In pancreatic β-cells, proinsulin exits the TGN and insulin only condenses (i.e. forms insoluble aggregates) after proteolytic processing of the proinsulin, which occurs after exit from the TGN [4]. Sorting of some peptide hormones in the TGN might be enhanced by secretogranin I, as modest overexpression of this granin in AtT-20 cells improved the intracellular storage of pro-opiomelanocortin-derived adrenocorticotropic hormone (ACTH) but not of β-lipotropin [46], but this activity is more consistent with the role of a helper protein than with the role of a sorting receptor. Although no consensus primary sorting sequence is known, membrane-associated carboxypeptidase E has recently been suggested to be the putative receptor that is common to all regulated secretory proteins [47•]. The phenotype of the *Cpe^{fat}* carboxypeptidase E knockout, however, seems surprisingly benign for deletion of a common prohormone sorting receptor.

Regulation of protein traffic from the TGN

Even though we cannot assign specific coats to several of the sorting routes out of the TGN yet, it is already apparent that traffic is subject to additional levels of regulation. Secretion of HSPG from the TGN via the constitutive pathway is stimulated by the PKC activator phorbol 12-myristate 13-acetate, and is inhibited by calphostin C, a specific inhibitor of the regulatory domain of PKC, and by several other kinase inhibitors [48]. Several isoforms of the PKC family have been localized to the Golgi region. The generation of VSV-G-containing vesicles from the TGN of MDCK cells is also inhibited by PKC inhibitors [21•], but vesicle assembly does not require the kinase activity of the enzyme [49•]. In GH_3 cells, tyrosine phosphorylation has now been shown to influence TGN sorting of polypeptide hormones [50•]. Inhibitors of both tyrosine kinase and tyrosine phosphatase activity suppress packaging into transport vesicles, and a number of tyrosine-phosphorylated proteins have been identified as candidate regulatory elements. Next, it will be important to understand what triggers these regulatory signal cascades and what physiological cues might control sorting at the TGN.

Conclusions

Even as our knowledge of the molecular events that govern sorting at the TGN improves, it is clear that much remains to be uncovered. Instead of the dedicated sorting pathways (Figure 1a–c) being confined to only certain cell types, it now seems possible that all the routes might be operative in some cells (Figure 1d). However, more information on the nature and function of the coat proteins that operate at the TGN is required before we can begin to understand sorting at the molecular level.

Acknowledgements

We would like to thank Ernst Ungewickell and Rosalind Kornfeld for critical comments on the manuscript. This work was supported by an Edward Mallinckrodt Jr research grant.

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Comparing the sorting of procathepsin B and procathepsin L, the authors show that, on exiting the TGN, both the lysosomal proenzymes can enter immature secretory granules in β-cells, as evidenced by stimulus-evoked secretion. Later, only procathepsin B, which has a high affinity for MPRs, can be retrieved, moved to lysosomes and converted to the mature form while procathepsin L remains within the mature secretory granule population.

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Addition of inhibitors of either tyrosine kinase or tyrosine phosphatase activity inhibits the budding of polypeptide-hormone-containing nascent secretory vesicles from the TGN. Using anti-phosphotyrosine antibodies, the phosphorylation of several membrane-associated proteins is indirectly correlated with the inhibition of vesicular transport.