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Take the 'A' train: on fast tracks to the cell surface

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Abstract. Cholesterol, certain lipids, membrane-bound and soluble proteins, as well as viruses that are synthesized in the endoplasmic reticulum (ER), reach the plasma membrane (PM) via non-classical pathway(s) that remain poorly understood. Typical for this transport is (i) its insensitivity to brefeldin A (BFA), which dissociates selected coat complexes from membranes, resulting in the disassembly of the Golgi apparatus; (ii) its rapid kinetics as compared to the classical secretory pathway; and (iii) its role in the trafficking of lipid raft components. Based on results showing that the intermediate compartment (IC) at the ER-Golgi boundary constitutes a stable tubular network that maintains its dynamics in the presence of BFA, we propose that two bidirectional Golgi-bypass pathways to the PM exist, a direct route from early IC elements, and another, reminescent of the yeast secretory pathway, from late IC elements via the endosomal system. These pathways have implications for the organization of the secretory processes in different cell types. (Part of a Multi-author Review)

Keywords. Secretory pathway, brefeldin A, pre-Golgi compartments, endosomes, rafts, Rab1, yeast Ypt1.

Classical and non-classical secretion

It can be estimated that about one-third of the proteins encoded by the eukaryotic genome are synthesized on ER-bound ribosomes. The ER is also the major site of cellular sterol and lipid synthesis. The secretory pathway is primarily responsible for distribution of the newly synthesized products among the endomembrane compartments, as well as delivery to the exterior of the cells. The prevailing view is that soluble and membrane-bound cargo is transferred vectorially from the ER to the cell surface [1]. Cargo molecules are first transported from ER exit sites to the entry (*cis*) side of the Golgi apparatus by mobile elements of the intermediate compartment (IC) [2, 3], and then pass through the polarized Golgi stacks, consisting of *cis*-, *medial*- and *trans*-cisternae, via cisternal maturation (progression), vesicular and tubular traffic, or a combination of both [4, 5].

The Golgi apparatus is a key organelle of the classical secretory pathway due to its central positioning and numerous functions. Most proteins and many lipids undergo post-translational modifications in the Golgi system, including glycosylation (attachment or terminal modification of sugars), proteolytic processing, sulfation and phosphorylation. In the case of proteins these modifications are required for their maturation, oligomerization and/or trafficking [5]. Sequential modification of the itinerant cargo molecules can be determined by the polarized organization of the Golgi stacks or the specificities of the Golgi enzymes themselves [6]. The Golgi apparatus is also regarded as the central station for the sorting of cargo molecules; however, the topology of the sorting processes in this system is still not completely understood [7]. A common idea is that two main sorting compartments, referred to as *cis*- and *trans*-Golgi networks (CGN and TGN), operate at the entry and exit faces of the organelle, respectively. An important function of the

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CGN is to recycle molecules back to the ER to maintain the functional integrity of this organelle. The TGN again is considered as the principal distribution center, where the lipids and proteins reaching the *trans*-Golgi are sorted to pleiomorphic carriers for further transport to various cellular destinations, including different domains of the PM, the endosomal system, as well as earlier Golgi compartments [8, 9, 9a]. The TGN has also been suggested as the site of formation of cholesterol and sphingolipid-rich membrane microdomains (rafts), which participate in membrane traffic [10–12]. However, recent studies of yeast and mammalian cells provide evidence that raft assembly also takes place in pre-Golgi compartments and endosomes [13–19].

Membrane-bound coat proteins (COPs) play important roles in protein sorting at the ER-Golgi boundary, in the Golgi apparatus and endosomes, as well as at the PM [20]. In addition to concentrating cargo into vesicular and tubular transport carriers, COPs also organize membrane domains in large-sized transport intermediates and create organelle architecture [21]. COPII coats function at ER exit sites in the initial export of cargo from the ER [22, 23], COPI coats operate in forward transport and recycling in the IC and *cis*-Golgi membranes [24], while clathrin and associated adaptor proteins (AP1, AP3, AP4, GGA) localize variably to trans-Golgi/TGN and endosomes [25]. Notably, whereas the COPII and COPI coat complexes have been well characterized [21, 26], our current knowledge of the multiple coat machineries that operate in the *trans*-Golgi/TGN remains limited [27].

It has been known for some time that the export of proteins from cells can also occur via mechanisms that are distinct from the above-outlined classical secretory pathway. The term 'non-classical secretion' is often linked to proteins (e.g. growth factors, cytokines, and extracellular matrix components) that lack signal sequences and remain in the cytosol, but nevertheless can be released to the extracellular space [28]. It appears that the export, as well as import [29], of some of these proteins also involves membrane traffic [28]. However, here we focus on another type of non-classical secretion, namely, the trafficking of molecules that enter the secretory pathway in the ER, but instead of taking the classical route to the PM, follow pathway(s) that bypass the Golgi apparatus. This type of transport is characterized by its insensitivity to the fungal metabolite brefeldin A (BFA), which blocks the activation of a subset of ADP-ribosylation factors (ARFs). These GTPases regulate the binding of COPs – such as COPI and clathrin-AP1 – to IC, Golgi and endosomal membranes [30]. The release of the COPs by BFA results in rapid disassembly of the Golgi apparatus and the redistribution of Golgi proteins to the ER and the endosomal system [31, 32].

Early studies with BFA showed that this compound blocks the transport of many proteins, such as the vesicular stomatitis virus G glycoprotein (VSV-G) and secretory proteins in hepatocytes [33, 34]. However, subsequent work revealed that the effects of BFA are often variable, depending on the cell type, protein examined and concentration of the drug [31, 32]. Moreover, BFA-resistant cell types were identified, such as the epithelial MDCK cells and PtK1 cells, in which the Golgi apparently remains intact [35–37].

COP-free subways?

The first evidence for BFA-insensitive pathways that bypass the Golgi apparatus was obtained by Urbani and Simoni, who compared the biosynthetic transport of cholesterol and VSV-G [38]. They showed that the transport of both molecules is inhibited at 15°C, a temperature that blocks the transport of newly synthesized proteins from the IC to the Golgi stacks [39]. Moreover, at 15°C cholesterol and VSV-G were found in the same low-density vesicle fraction, suggesting that they both accumulate in the IC. However, in contrast to VSV-G, which is transported via the Golgi [40], the transport of cholesterol to the PM after shift from 15°C to 37°C was not inhibited by BFA, suggesting that it employs a non-conventional pathway [38]. The rate of transport of cholesterol to the PM (~10 min) is considerably faster than that of VSV-G (~30 min), and resistant to monensin, an ionophore that inhibits Golgi function and blocks VSV-G transport [41]. More recently, Heino et al. followed the movement of cholesterol from ER to the PM using a high-resolution transport assay, and obtained similar results regarding its rate of transport, as well as the differential effects of BFA on cholesterol and protein (influenza virus hemagglutinin) transport. Importantly, like biosynthetic protein trafficking, cholesterol transport was shown to be dependent on an intact microtubule (MT) system [14].

We carried out a literature search resulting in an interesting list of molecules that, like cholesterol, are transported to the PM in a BFA-insensitive manner (Table 1). Although certainly not complete, even this short list reveals the diversity of molecules that display this type of non-classical trafficking. In addition to cholesterol, a number of lipids are included, such as sphingolipids (sphingomyelin), glycolipids (ganglioside GD3), and phosphatidylethanolamine. The situation with lipids is more complex since their intracellular movements also involve non-vesicular transport through the cytosol [42], possibly occurring at

specialized membrane contact sites [137, 139, 140]. The proteins display different membrane topologies, including lumenal (calsequestrin), integral (e.g. CD45), peripheral (flotillin-1), and GPI-anchored (F3/contactin) proteins. They fall into different functional categories: cell adhesion proteins (E-cadherin, integrin PS1, metalloproteinase), channel proteins (connexin-26) and signalling proteins (heterotrimeric G-protein, Ras). Interestingly, in addition to Golgi-independent trafficking, a common denominator between the membrane-bound molecules is their association with lipid rafts.

The biochemical and topological diversity of the molecules listed in Table 1, as well as their typical association with lipid rafts, indicate that their unconventional trafficking does not involve pathways that are artificially created by BFA. This conclusion is supported by the results obtained with the two glycoforms of protein-tyrosine phosphatase CD45. These variants of the same polypeptide are transported to the PM with different kinetics displaying differential sensitivities to BFA. Moreover, the drug does not affect the transport rate of the BFA-insensitive, high-mannose form of CD45, which reaches the PM more rapidly than the BFA-sensitive, terminally glycosylated form of the protein [43].

There also exists other types of evidence for non-conventional secretory pathways. The AE1 anion exchanger is a channel protein that first reaches the PM in high-mannose form, apparently bypassing the Golgi, and then is endocytosed and receives terminal modifications on its sugars [44]. Similarly, the subunits of the sodium channel (ENaC) located at the apical surface of epithelial cells contain predominantly high-mannose sugars, which could be explained by Golgi-independent trafficking [45]. Evidence for non-classical transport of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel, was obtained in biochemical and immunolocalization studies. The latter showed the presence of the protein in the ER, IC, and peripheral *cis*- and and *trans*-Golgi tubules, but not in the Golgi stacks [46]. It has also been proposed that the transfer of CFTR to the PM takes place via an 'endosomal intermediate' [47, 48]. Further, immuno-EM localization of fibronectin, an extracellular matrix protein, showed its absence from the Golgi cisternae, but presence in Golgi vacuoles that frequently display an acidic luminal pH [49, 50], correlating with results showing that the secretion of this protein is partially resistant to monensin [51]. Recently, a Golgi-bypass pathway was implicated in the secretion of integrins (αPS1) that link cells via fibronectin to their extracellular matrix [199]. Moreover, rotavirus is released from the apical surface of intestinal cells via a non-conventional pathway that is insensitive to monensin [52]. Virus assembly begins in the ER, whereafter immature particles transit through the IC [53] and reach the *trans*-Golgi region where their assembly is completed. One of the virus coat proteins, VP4, is not detected in the ER or the Golgi stacks [54]. It interacts with flotillin and mediates the association of mature virus particles with lipid rafts, which appear to facilitate their movement from the *trans*-Golgi to the outside of the cell [55].

Finally, results from knockdown of the ARF guanidine nucleotide exchange factors (GEFs), GBF1 and BIG, that control the binding of COPI and clathrin-APs to IC/*cis*-Golgi and *trans*-Golgi membranes, respectively, are compatible with the existence of the Golgi-bypass pathway(s) discussed here. Accordingly, knockdown of GBF1 (like BFA) results in COPI release, but allows the transport of VSV-G from the ER to the IC, where it becomes arrested [56]. By contrast, these cells are still capable of secreting soluble proteins [57]. Unexpectedly, knockdown of BIG does not affect the transport of VSV-G to the PM, indicating that this process is not dependent on a functional TGN [56].

COP-free traffic through the IC network

Different possibilities have been suggested to explain the trafficking of molecules in the presence of BFA. Either the ER – most likely its smooth domain – makes direct contact with the cell surface or, alternatively, the transport involves a Golgi-independent, post-ER vesicular pathway to the PM [38, 43]. So far, the latter possibility has been regarded as unlikely since the IC, an obligatory way station in post-ER trafficking [58, 59], is expected to become non-functional under these conditions. Namely, the binding of COPI coats to the IC elements [60–62, 198] has been considered as a prerequisite for their formation and/or MT-dependent movement to the *cis*-Golgi region [63]. Although earlier studies on the cycling IC protein p58/ERGIC-53 suggested that this compartment, unlike the Golgi, persists in BFA-treated cells [64–66], later work showed that the protein accumulates at the ER exit sites, which remain operational in the absence of COPI binding [67, 68].

However, recent results on the dynamic organization of the IC are changing this picture. It turns out that this compartment consists of structurally and functionally distinct vacuolar and tubular subdomains, which play different roles in trafficking at the ER-Golgi boundary. The vacuolar domain contains anterograde cargo (e.g. VSV-G), the cargo receptor p58 and membrane-bound COPI coats. By contrast, the tub-

Table 1. Molecules employing non-classical secretory pathways.

Molecule	Properties	Pathway characteristics	References
Cholesterol	Membrane constituent Lipid rafts	Temperature-sensitive(15°C) BFA-insensitive	[38]
		Temperature-sensitive (15°C) BFA-insensitive Microtubule(MT)-dependent	[14]
Phosphatidylethanolamine	Membrane constituent	BFA-insensitive	[182]
Sphingomyelin	Membrane constituent Lipid rafts	Temperature-sensitive (< 25°C) BFA and monensin-insensitive	[183]
		BFA-insensitive (ERC to PM) (synthesized in ERC?)	[196]
		BFA-insensitive MT-dependent	[197]
		Fast transport to PM (half-time~10min)	[106]
Glucosylceramide	Membrane constituent Lipid rafts	BFA-independent Temperature-insensitive (15ºC)	[184]
		BFA-insensitive MT-dependent	[197]
Ganglioside GD 3	Membrane constituent Lipid rafts	BFA-insensitive (Golgi to PM) Rab11-independent	[185]
Connexin 26	Gap junction protein Lipid rafts?	BFA-insensitive MT-dependent Temperature-insensitive	[89]
		BFA-sensitive Sar1-dependent Microtubule-dependent	[186]
E-cadherin (uvomorulin)	Cell adhesion (MDCK cells)	BFA-insensitive (to basolateral PM)	[187]
Paranodal complex/F3 contactin	Cell adhesion GPI-anchored protein Lipid rafts	BFA-insensitive EndoH-sensitive sugars	[188]
Syndecan-1 (heparan sulfate proteoglycan)	Cell migration Cell-matrix interactions (MDCK cells)	BFA-insensitive (to basolateral PM)	[189]
Integrin αPS1	Cell adhesion	BFA-insensitive Syntaxin 5-independent	[199]
Membrane type-1 matrixmetallo-proteinase	Tumor cell invasion Lipid rafts	BFA-insensitive Fast transport to PM	[190]
CD45 High mannose glycoform	Receptor protein-tyrosine phosphatase Lipid rafts	BFA-insensitive Transport to PM in ~ 5min	[43]
Heterotrimeric G protein	Signalling molecule Lipid rafts	BFA-insensitive Sar1-independent	[191]
K-Ras	GTPase Signalling molecule Lipid rafts	BFA-insensitive	[86]
H-Ras	GTPase Signalling molecule Lipid rafts	BFA-insensitive Sar1-independent Microtubule-dependent?	[88]
TC10	GTPase (Rho family) Lipid rafts	BFA-insensitive	[192]
Flotillin-1/Reggie-2	Signalling molecule Lipid rafts	BFA-insensitive Sar1-independent	[193]

Table 1 *(Continued)*

Molecule	Properties	Pathway characteristics	References
Cystic fibrosis transmembrane conductance regulator	Chloride channel in apical PM of epithelial cells	Immature ΔF508-CFTR BFA-insensitive	[194]
		Insensitive to dominant negative Arf1, Rab1A and Rab2	[47]
Calsequestrin	Calcium storage in sarcoplasmic reticulum (SER)	Transport from RER to SER Sar1-dependent Arf1-independent	[195]

ular domain lacks the two latter components, but is enriched in the GTPase Rab1 [69, 70]. Visualization of the dynamics of fluorescent Rab1 in living cells showed that the IC tubules are highly dynamic and, in addition to joining peripheral (early) and central (late) IC elements into an interconnected network, function in an MT-dependent Golgi-bypass pathway that extends from the IC to the cell periphery, e.g. the growth cones of neuron-like PC12 cells, or the pseudopodia/lamellipodia of motile fibroblastic cells [70; H. A. D., M. M. and J. S., unpublished results]. The Rab1-containing tubular domain of the IC also contains HMG-CoA reductase, the key enzyme of cholesterol biosynthesis [70], suggesting that it is involved in the BFA-resistant, temperature (15°C)-sensitive and MT-dependent transport of cholesterol to the cell surface [14, 38]. Unexpectedly, live cell imaging revealed that the IC is a long-lived compartment, that maintains its dynamic properties in the presence of BFA. The Rab1-mediated pathway from the IC to the growth cones remains operational in BFA-treated, polarized PC12 cells [70]. Moreover, the topological organization of the early and late IC elements is maintained, and bidirectional communication between them via dynamic tubules continues in the drug-treated cells [Marie et al., submitted]. By removing COPI coats BFA most likely influences the function of the COPI-containing IC vacuoles in ER-Golgi trafficking, whereas the operation of the tubules remains unaffected. Since COPI has been implicated in both forward and retrograde trafficking at the ER-Golgi boundary [24], COPI-independent pathways in both directions should remain operational in the presence of BFA. Evidence for such pathways has been obtained both in mammalian cells [71, 72] and the yeast *Saccharomyces cerevisiae* [73, 74].

The response of the IC to BFA therefore turns out to be similar to that of the endocytic system. Despite causing extensive tubulation of endosomes, BFA does not dramatically affect the function of the endocytic pathway, since both internalization at the PM, membrane recycling, and communication between endosomes and lysosomes continues in the drug-treated cells [31, 75–77]. Regarding post-Golgi trafficking, BFA inhibits both constitutive and regulated transport from *trans*-Golgi compartments to the PM [78]. Notably, the membrane protein TGN38 that cycles between *trans*-Golgi/TGN membranes and endosomes accumulates in a pericentrosomal location in BFA-treated cells [79], either due to the collapse of the TGN membranes around the centrosomes or the arrest of TGN38 in an endocytic compartment.

How to circumvent the central station?

Based on the above results showing that the IC maintains its dynamic properties in the presence of BFA, we propose that different routes that circumvent the Golgi apparatus connect topographically distinct domains (early and late) of this compartment and the PM (see Fig. 1). [*Since it may help to think about the proposed routes by drawing an analogy to traffic in a metropolitan area (see also Acknowledgments), some practical instructions to drivers and eventual passengers are given in square brackets.*]

Route A1-A2. This pathway involves MT-dependent movements of vacuolar and tubular IC elements from ER exit sites to the cell center and direct communication of late IC elements with endocytic compartment(s). [*'Drive uptown on A1, but be careful, the road might be slippery. Make a stop before the central station and take the bypass to A2. It may be a good idea to change your vehicle to one of the E models'.*]

Route A3. A direct connection from early IC to the PM. This pathway, which could also exists in yeast cells [80], may – depending on the cell type – be more rapid and less sensitive to temperature reduction and depolymerization of MTs. From a geometric point of view, the narrow tubules observed [70] would appear as optimal carriers for membrane constituents. [*'Take*

a right at the ER exit, then just follow A3. Watch out! The peripheral A3 exit is still under construction.']

Route A1-A3. This route involves cycling between the early and late IC elements. It would allow cargo to acquire post-translational modifications in the Golgi system, as well as facilitate the assembly of protein complexes from multiple subunits. ['*Take A1 towards the center of the city. Pick up your band in front of the station, change gear in the back of the vehicle, make a U-turn and drive back. Then take a left to A3 at the ER exit. I said left!'*]

Based on the suggested routes, it is possible to re-examine the pathways that some of the molecules listed in Table 1 utilize in their trafficking. In fact, some could follow several routes, depending on the cell type and the prevailing physiological conditions. In light of previous studies [14, 38] cholesterol could move on *route A1* to the late IC and then take the bypass route to the endosomal system (Fig. 1), where Rab11 and Rab8 are known to participate in cholesterol transport [81, 82]. Caveolin, which is transported from the ER to late IC or Golgi, where it oligomerizes [83], has been implicated in cholesterol transport to the cell surface [84]. Direct delivery from the IC to endosomes could explain an observed cholesterol gradient (*cis*-to-*trans*) across the Golgi stacks [85].
Since raft formation is expected to mainly take place in the Golgi region, many of the raft proteins listed in Table 1 could also follow *route A1* to the cell center. As mentioned above, rotavirus VP4 protein, which localizes to the IC and participates in virus maturation in the *trans*-Golgi region [55], interacts with flotillin, suggesting (indirectly) that flotillin and caveolin could utilize similar pathways. Ras proteins, such as H-Ras and K-Ras, associate with ER membranes and seem to employ both classical (ER-Golgi-PM) and non-classical pathways during their trafficking to the PM [86], correlating with the lipid modification(s) (farnesylation, palmitylation) that they receive [87]. However, recent studies indicate that H-Ras associates with the IC, but not with Golgi membranes, and moves to the PM via pathways that are BFA-insensitive, but show variable requirement for intact MTs [88]. It is possible that the trafficking of H-Ras involves alternative vesicular routes (Fig. 1*, A1-A2 and A3*), which may differ in their sensitivity to depolymerization of MTs. The recently described BFA-insensitive and syntaxin 5-independent secretory pathway of integrin αPS1 – from ER/IC directly to the basolateral surface of *Drosophila* follicular epithelial cells [199] – resembles the *route A3* proposed here. Finally, in the presence of BFA connexin-26 is transported to the PM, where it forms functional gap junctions [89]. Since the assembly of connexins takes place in post-ER compartments between the IC and *trans*-Golgi [90, 91], it is possible that connexin-26 uses the Golgi-bypass *route A1-A2* (Fig. 1).

Of yeasts and men

The existence of a putative Golgi-bypass *route A1-A2* (Fig. 1) is also of interest regarding the comparison of the secretory pathways that operate in yeast and mammalian cells. Although these pathways share many similarities, there are also considerable differences. Morphologically, the secretory system of the baker's yeast *S. cerevisiae*, consisting of continuous tubular networks which connect with the ER and form secretory granules, appears simpler than its mammalian counterpart [92, 93]. Whereas *S. cerevisiae* does not contain cisternal Golgi stacks, such structures are present in the fission yeast *Schizosaccharomyces pombe*, correlating with the fact that secretion of model proteins in these unicellular eukaryotes is differentially affected by BFA, continuing in the former but blocked in the latter [94, 95]. Biochemically, the high-mannose structures added to yeast and mammalian glycoproteins are very similar, but subsequent processing steps differ considerably. *S. cerevisiae* builds extensive mannose-based structures but lacks glycosyltransferases of the kind that function in terminal processing of glycoproteins (addition of sialic acid, galactose and fucose) in the mammalian Golgi apparatus [6]. By contrast, N-linked oligosaccrarides are modified by addition of galactose in *S. pombe* [96].
Regarding the transport machineries, *S. cerevisiae* gets along with 11 Ypt/Rab GTPases, of which three – Ypt1 (in humans: IC/*cis*-Golgi Rab1), Ypt 31/32 (*trans*-Golgi/endosomal Rab11) and sec4 (t*rans*-Golgi/endosomal Rab8) – function in the secretory pathway, whereas 60 Rabs are encountered in different mammalian cells [97–99].
In spite of the above differences, a common idea is that *S. cerevisiae*, like human cells, contains a subcompartmentalized Golgi apparatus. The presence of the IC in yeast has remained an enigma, although it has been considered on the basis of the similar phenotypes of COPI and COPII mutants [100]. Notably, the proposed Golgi-bypass *route A1-A2* (Fig. 1) shares striking similarity with the secretory pathway of *S. cerevisiae.* Thus, it could represent a basic secretory system that is shared by the two cell types, whereas additional COP machineries have developed in mammalian cells to enhance protein sorting and to build up a more versatile glycosylation apparatus. First, the rate of transport along this BFA-insensitive route in mammalian cells is comparable to the rapid secretion

in yeast cells, where glycoproteins such as invertase traverse the secretory pathway in ~5 min [101]. Second, the functions of Ypt1 (Rab1) and Ypt 31/32 (Rab11) in yeast are linked at the molecular level by the two switchable forms of the conserved TRAPP complex, which act as GEFs to successively activate these GTPases [102]. Finally, the secretory pathway in yeast seems to consists of two branches, of which one transits through endosomes [103, 104],

Bulk flow revisited

Acyl-tripeptides containing the Asn-Tyr-Ser sequence have previously been used to measure the rate of bulk flow from the ER via the Golgi apparatus to the PM [105]. These tripeptides are taken up by cells by an unknown mechanism – which, however, may be related to the transport routes utilized by certain protein toxins (see below) – and apparently reach the ER, where they become N-glycosylated. Depending on the cell type, the half-time of subsequent secretion of these glycopeptides was found to be 5–10 min, i.e. similar to that of cholesterol and sphingomyelin [14, 41, 106]. Their secretion from cells is blocked at 15°C and by GTP-γ-S, indicating that it involves vesicular trafficking [107]. Depending on the cell type, up to 30% of the glycopeptides are modified by Golgi glycosyltransferases, including addition of terminal galactose and sialic acid by *trans*-Golgi enzymes [108]. Since the secretion of the glycopeptides is not affected by BFA [108], it is possible that they utilize the proposed Golgi-bypass *route A1-A2* (Fig. 1). These small peptides are not expected to contain sorting information, and thus may become initially concentrated in the vacuolar parts of the IC [61, 70]. Their Golgi-specific processing in control cells could be explained in two (or more) ways. Either only ~30% of the molecules pass through the Golgi stacks and become modified, or the peptides are transferred from late IC to endosomal compartments. By receiving newly synthesized enzymes from the ER, and communicating with the Golgi stacks, these peri-Golgi compartments may contain sufficient amounts of the Golgi enzymes to support the observed terminal glycosylation [109–113]. In the presence of BFA the enzymes are redistributed, explaining the inhibition of the terminal processing of the tripeptides [108].

Get back along the same tracks

Like highways and subways, membrane traffic routes are bidirectional. For example, the tubules containing fluorescent Rab1A not only move from centrally located IC elements to the cell periphery, but also in the opposite direction [70]. Studies of bacterial toxins lead to the discovery that the secretory pathway is reversible [114]. The cellular uptake of these protein toxins from the PM involves different endocytic mechanisms. Subsequently, certain toxins (such as Shiga and cholera toxin, as well as ricin) are transported to the Golgi region, and further to the ER from which they (or their subunits) are translocated to the cytosol to exert their toxic effects [115, 116]. The Golgi-to-ER transfer of these toxins involves different mechanisms, but can also occur in a COPI-independent fashion, suggesting the involvement of Golgi-bypass pathways [71, 117–119]. The comprehensive endocytic tracer wheat germ agglutinin (WGA) also reaches the ER in BFA-treated cells; however, only when the drug is added during the later stages of endocytic uptake, when the lectin has accumulated in the *trans*-Golgi region of HepG2 hepatoma cells [120]. In addition, caveolar endocytosis of polyoma virus SV40 [121] and the autocrine motility factor receptor [122] results in their delivery to smooth ER, providing further examples of endocytic processes that may involve the Golgi-bypass routes discussed here [123].

Sorting and transport in the central vacuolar system

The ideas concerning transport routes that bypass the Golgi apparatus go back in time. Based on their studies on the secretory process in pancreatic exocrine cells, Jamieson and Palade concluded that the transfer of secretory proteins from tubulo-vesicular membranes at the *cis*-side of the Golgi apparatus to condensing vacuoles at its *trans*-side occurs without apparent involvement of the cisternal stacks [124]. Similarly, Morré and Ovtracht used the term 'boulevard périphérique' to describe the flow of secretory material in the plant Golgi apparatus [125]. Previously, Alex Novikoff's GERL (Golgi-ER-lysosome) concept had postulated that newly synthesized lysosomal enzymes (and possibly also secretory proteins) are sequestered into specialized ER tubules that make direct contact with the *trans*-Golgi cisternae, suggesting that the biogenesis of lysosomes involves a pathway that bypasses the Golgi stacks [126]. Two decades later, Griffiths and Simons introduced the TGN as a *trans*-Golgi sorting compartment, which also functions in the routing of lysosomal enzymes, contributing to the replacement of the GERL concept [127]. Largely based on biochemical and immuno-EM studies on the intracellular site of accumulation of newly synthesized virus membrane proteins during the 20°C temperature block [39, 128, 129], the TGN

was originally defined as a tubular reticulum extending from the last *trans*-Golgi cisterna [127]. However, since different cargo proteins display variable localization in the 20°C-treated cells, it remained unclear whether they are arrested in a specialized *trans*-Golgi secretory organelle, or within a complex membrane system located at the crossroads of the exocytic and endocytic pathways [127, 130, 9a].

Supporting the latter possibility, the extensive EM studies by Rambourg and colleagues showed that the TGN is variably expressed in different cell types correlating with their secretory and endocytic activities. Cells that are active in endocytosis generally contain well-developed *'trans*-tubular networks', whereas certain professional secretory cells seem to lack these structures altogether but, instead, display procounced *'cis*-tubular networks' [131, 132]. The structure and size of the TGN also varies in individual cell types, and in cells subjected to different experimental manipulations that affect membrane traffic [130, 133–136]. Whereas 'resting' HepG2 cells do not contain a detectable TGN, endocytic uptake of WGA results in the formation of a Golgi-associated membrane network consisting of vacuolar and tubulovesicular domains. This 'endocytic TGN' remains initially a separate structure located at variable distance from the Golgi apparatus, but is subsequently incorporated into the cisternal stacks and decreases in size [134, 135]. In addition, the appearance of the TGN varies depending on the extent of flow of secretory cargo through the Golgi system [127]. For example, recent EM tomographic studies showed that whereas the TGN is poorly developed in cells where IC-to-Golgi transfer of secretory proteins is prevented during incubation at 15°C, it expands considerably as a consequence of the shift of cells to a higher temperature (40°C) that triggers trafficking across the Golgi stacks [136].

EM tomography of the Golgi apparatus has been instrumental in revealing important structural features of the trans-Golgi/TGN and contacts between these membranes and other organelles [133, 135–138]. Studies of NRK cells kept at 20°C showed that the predominant effect of the low-temperature incubation is the bulging of the penultimate *trans*-Golgi cisternae and the disappearence of their tubular extensions, but did not provide evidence for a tubular network connecting with the *trans*-most cisterna [138]. Notably, only the latter contains clathrin coats, suggesting that it specifically communicates with endosomes [137]. 3-D reconstructions have also provided evidence for the close association between *trans*-Golgi membranes and specific ER domains [137], as first proposed by Novikoff. However, the current idea is that these direct ER-*trans*-Golgi contacts correspond to the sites where intercompartmental transport of ceramide (the precursor of sphingolipids) and oxysterol via the cytosolic proteins CERT and OSBP, respectively, takes place [137, 139], bypassing the more proximal Golgi compartments. However, the precise non-vesicular pathways in which these transfer proteins operate remain unknown [140, 141]. If membrane contact sites are indispensable for the trafficking of certain lipids and sterols, they are expected to persist when the Golgi is disrupted by BFA, explaining the ongoing delivery of these membrane constituents to the surface of the drug-treated cells (Table 1).

In conclusion, a number of studies suggest that the TGN is not an autonomous, stable secretory organelle and emphasize its inducible nature at the cell center, where multiple membrane traffic pathways converge [9a]. Also, it has been well established that molecular sorting along the secretory pathway occurs at multiple locations [142–145]. Pre-Golgi sorting takes place both at ER exit sites and in the IC [59, 61, 70, 72, 142], whereas post-Golgi sorting involves endosomal compartments [25, 143, 144, 146]. In particular, studies of both polarized and non-polarized cells have shown that the endocytic recycling compartment (ERC) operates as a way station during the transport of e.g. VSV-G and E-cadherin to the PM [147–149], emerging as a major sorting site for both biosynthetic and endocytic trafficking.

If the TGN is not the principal recipient of ER-derived secretory cargo and the major site of its sorting in the Golgi apparatus, how is this system then organized? The studies discussed below may give additional clues to address this question. First, Orci, Rothman and coworkers generated 'megavesicles', i.e. vacuoles with a diameter of ~400 nm, by depositing aggregates of an exogenous, non-glycosylated protein into the ER and Golgi lumen and then employed the 15° and 20°C temperature blocks to regulate the transport of these membrane-enclosed aggregates across the Golgi stacks [150]. They concluded that transport through the stacks (*cis*-to-*trans*) takes about 10 min. During their journey, the aggregates were mostly (~80%) observed to associate with the dilated ends of the cisternae, but a smaller fraction was present in free vacuoles at the periphery of the stacks. However, since low-temperature treatment can exert a major effect on the morphology of the Golgi apparatus [39, 130], including extensive tubulation [151], it is possible that part of the connections between these peripheral vacuoles and Golgi cisternae could not be detected by the serial section analysis that was carried out.

Second, Saraste and Kuismanen used temperature-shift protocols to synchronize the movement of Semliki Forest virus (SFV) membrane glycoproteins

across the Golgi stacks and obtained a similar estimate (~10 min) for the transit time of the SFV proteins from the 15°C (IC) to the 20°C (*trans*-Golgi/TGN) block site [39, 130]. At both temperatures the proteins were localized by immuno-EM to vacuoles (300–400 nm) at one side of the Golgi stacks. These vacuoles were partly continuous with the Golgi cisternae, but mostly devoid of any apparent attachment. Again, the serial section analysis could have missed possible, narrow tubular connections between the vacuoles and cisternae, or these were broken due to the mild chemical fixation employed. In cells shifted from 15° to 20°C (or 39°C), the apparent transport of the proteins into the Golgi stacks via tubular parts of the IC elements was accompanied by a considerable loss of label from the vacuoles [39]. Based on their studies on the transport of VSV-G in nocodazole-induced Golgi ministacks, employing EM tomography and quantitative immuno-EM, Luini and co-workers arrived at a similar conclusion on the role of tubules in the transfer of cargo from the IC into the *cis*-Golgi cisternae. Moreover, they reported that the synchronized forward movement of cargo across the Golgi stacks (via cisternal progression) is coupled to the formation of intercisternal tubular connections, which mediate retrograde transport of Golgi enzymes [136].

Third, the transport pathways taken by the mainly unglycosylated secretory proteins in resting and stimulated pancreatic acinar cells seem to differ. As mentioned above, in resting cells the proteins move directly from peripheral *cis*-Golgi elements to the *trans*-Golgi condensing vacuoles [124], whereas in stimulated cells they are more readily detected in the dilated rims of the cisternae and also can occupy more central regions of the Golgi stacks. Notably, the kinetics of secretion is not affected by stimulation [152].

Collectively, the above studies suggest that the peripheral, dilated rims and the central cisternal stacks correspond to different subdomains of a single functional unit, having specialized roles in protein transport across the Golgi apparatus. In addition, recycling endocytic traffic from the PM can be directed to the dilated ends of Golgi stacks [153, 154]. Accordingly, the following mechanisms could be discussed in the transfer of different molecules in the Golgi system (Fig. 1). To begin with, centrally located IC elements could generate Golgi organization in a process where their vacuolar domains, containing concentrated lumenal cargo [155], give raise to the dilated cisternal ends. Integral membrane components are sorted from the vacuoles into the central regions of the Golgi stack, whereas soluble cargo is mainly restricted to the vacuolar (saccular) parts. The latter, however, possibly depending on the type of cargo they contain, also move to more central regions of the stacks [156]. Vesicles could shuttle certain components from one cisternae to another, possibly operating together with cisternal progression [4], and tubular connections provide retrograde pathways for the intercisternal transfer of Golgi enzymes [136]. However, the model in Figure 1 proposes that the Golgi stacks do not operate in a vectorial manner regarding the transfer of all cargo molecules. Bidirectional pathways bypassing the Golgi stacks would

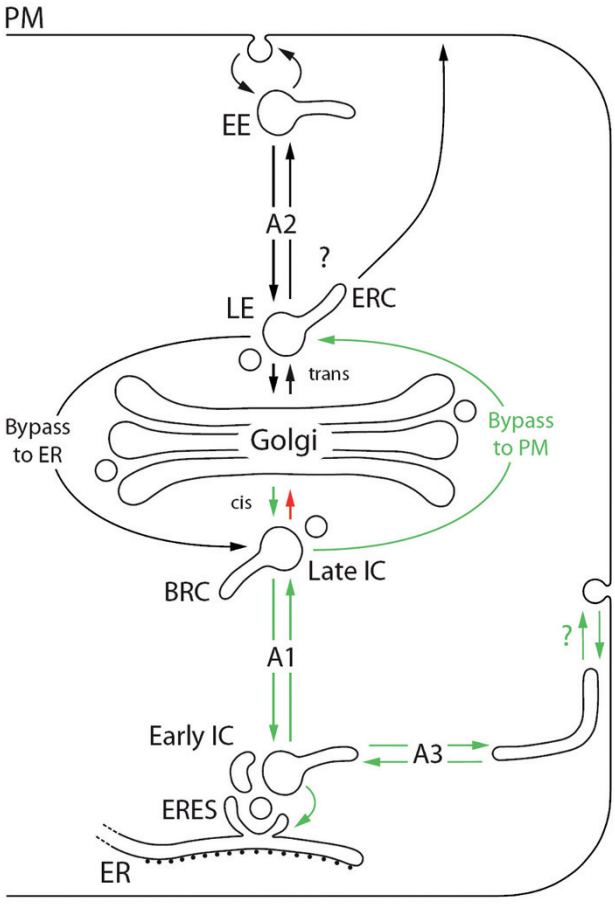

Figure 1. A map of the proposed Golgi-bypass pathways connecting the IC with the PM. Bidirectional pre- and post-Golgi pathways are indicated by arrows. A1 and A2 indicate bidirectional routes between early and late IC elements, and early (EE) and late (LE) endosomes, respectively. A3 designates a direct route from early IC to the plasma membrane (PM) [70]. The function of these pathways depends on various coat proteins (COPs) [20, 21], or possible scaffold proteins [83, 181], which differ in their sensitivity to brefeldin A (BFA). The pre-Golgi pathways that are sensitive or insensitive to BFA, including the proposed route from late IC elements to the endosomal system, are indicated by red and green colour, respectively (see the text for further discussion). For simplicity, the biosynthetic [70, 164] and endocytic recycling compartments (BRC and ERC) extending from IC and endosomal vacuoles, respectively, are shown as short tubules, although they constitute extensive tubular networks [25, 70]. Similarly, the tubular networks at the *cis*- and *trans*-sides of the Golgi stacks (CGN and TGN) are not included in this planar diagram. ERES = ER exit site.

make it possible that entry and exit of molecules can take place at the two poles of the organelle. For example, certain Golgi membrane proteins could bypass the *cis*- and *medial*-Golgi compartments and enter the *trans*-Golgi from endosome-derived tubules, resulting in their polarized distribution within this organelle. Novel live-cell imaging techniques and model systems will hopefully make it possible in the future to study and compare the transport routes of different cargo molecules in the Golgi system at high resolution.

The biosynthetic and endocytic pathways could exert 'opposing forces' on Golgi dynamics (Fig. 1), giving rise to different traffic patterns observed in different cells. This could clarify some previous findings and discrepancies, for example, the variable localization of the cation-independent mannose-6-phosphate receptor (M6PR) that functions in the sorting of lysosomal enzymes. In professional secretory cells, such as pancreatic acinar cells, the M6PR is localized to *cis*-Golgi membranes [157], whereas in other cell types it can be present throughout the stacks, or con-centrate in the *trans*-Golgi/TGN [158]. Different cell types, depending on their relative secretory and endocytic activities, could variably allocate their sorting processes either at the *cis*- or *trans*-side of the Golgi stacks [159]. As another example, in differentiating leucocytes the formation of different secretory granules shifts from one pole of the Golgi to the other [160].

Conclusions and perspectives

In the case of many newly synthesized proteins the transfer time through the Golgi apparatus is propably in the range of 5–15 min [39, 150]; however, some proteins require even longer times for their passage [156]. The Golgi-bypass pathways considered here, by granting direct transport to the cell surface, could have an important role in cell physiology. For example, they could participate in phagocytosis and the delivery of membrane components to the leading edge of migrating cells, such as fibroblasts [161–164]. Although it has been reported that long-term treatment with BFA prevents cell polarization and motility [165], Golgi disassembly by this drug has no immediate effect on these events [H. A. D, M. M. and J. S., unpublished data]. In addition, rapid transport to the cell surface in incompletely processed form, followed by endocytosis and Golgi-specific modification, may be a life style that is shared by many PM proteins, including receptors, channels and pumps. The notion that these pathways constitute a basic transport system that involves the intracellular assembly of membrane microdomains (lipid rafts) implies that they function in signalling events and the coordination of the biosynthetic and endocytic functions of the cell. Finally, these routes may have a role in antigen presentation [166] and intracellular replication of viruses [167–171].

The existence of Golgi-bypass routes in plant cells [172], and in cells equipped with a relatively simple secretory apparatus, such as *Plasmodium* and *Giardia* [173–175], suggests that these pathways belong to the secretory repertoire of a variety of eukaryotic cells. The finding that fibroblasts can express the apical and basolateral routes defined in polarized epithelial cells [176] further suggests that eukaryotic cells share a basic, adjustable secretory system that can adopt various functional forms in specialized cell types. In neurons a pathway that circumvents the Golgi apparatus could give rise to the 'satellite pathway' that functions in local protein synthesis and adaptation of dendritic spines to allow long term information storage in the brain [177–180]. However, a number of questions regarding the operation of the Golgi-bypass routes in different cell types remain unanswered. For example, do they involve the 'new types of coats' (membrane-shaping proteins) that recently have been suggested to operate in organelle formation and membrane traffic [83, 181]? Or, what are the fusion factors (e.g. SNAREs) that function in the required intercompartmental transport events?

The center of a cell is a crowded place, and the Golgi apparatus has many near neighbours. Also, since the residents of many of the packed organelles pay frequent visits to adjacent compartments, it is not always easy to tell how many compartments there are, and who lives where. A cell biologist hoping to map the arrangement and interactions of endomembrane compartments, for example, 'exosomes' [39] and endosomes, could copy a cytogeneticist, who takes a dipteran chromosome and spreads it on a glass slide or EM grid to reveal its puffs and nucleosomes. Indeed, preparations like this can provide important information, but as is the case with chromosomes, the 3-D picture is definitely lost. Fortunately, a living cell, an epithelial cell or a neuron, can help with the mission. The Golgi is in some cells more widespread than in others, but still too compact to reveal its secrets. To understand the traffic plan of this organelle, perhaps one should take another look at the periphery of the cell instead of just looking at the center.

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from eastern Brooklyn, bypassing Grand Central Station, passing through Harlem (Sugar Hill) and ending up in northern Manhattan, moving along the express tracks in Manhattan. (However, since the A train runs on the West Side of Manhattan and goes nowhere near Grand Central Station, which is on the East Side, the comparison of membrane traffic routes with the New York City subway system remains metaphorical.)

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