

Illuminating the secretory pathway: when do we need vesicles?

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Journal of Cell Science 114, 1053-1059 © The Company of Biologists Ltd

Summary

Recent studies using GFP-tagged markers and time-lapse microscopy have allowed direct visualisation of membrane traffic in the secretory pathway in living mammalian cells. This work shows that larger membrane structures, 300-500 nm in size, are the vehicles responsible for long distance, microtubule-dependent ER-to-Golgi and trans-Golgi to plasma membrane transport of secretory markers. At least two retrograde transport pathways from the Golgi to the ER exist, both of which are proposed to involve a further class of long, tubular membrane carrier that forms from the Golgi and fuses with the ER. Together, this has

challenged established transport models, raising the question of whether larger pleiomorphic structures, rather than small 60-80 nm transport vesicles, mediate long-range transport between the ER and Golgi and between the Golgi and plasma membrane.

Movie available on-line:

<http://www.biologists.com/JCS/movies/jcs2220.html>

Key words: Life cell imaging, Green fluorescent protein, Transport, Membrane traffic, Golgi complex

Introduction

Proteins destined for secretion, the plasma membrane or membranous cellular organelles enter the secretory pathway in the endoplasmic reticulum (ER) and move via the Golgi complex to the trans-Golgi network, where they are sorted and transported to their final destination. Malfunction in this pathway may lead to the breakdown of cellular organelles, cell polarity, overall cellular architecture and ultimately cell death. A fundamental question in the understanding of the secretory pathway is how the identity of the membrane-bounded structures involved is maintained. Each transport step between adjacent membranes must be tightly controlled at at least four basic levels: (i) sorting of secretory cargo from residents, which have to remain behind at the donor membrane; (ii) formation and transport of the cargo carriers; (iii) delivery of the cargo at the donor membrane; and (iv) recycling of the transport machinery to the donor membrane, which is essential for subsequent rounds of transport.

Most of our knowledge of these processes is based either on ultrastructural studies that aimed to localise molecules precisely to individual membrane structures of the secretory pathway, or on in vitro studies using simplified systems to identify the key players and characterise their biochemistry. Together these approaches have given rise to widely accepted transport models in which small (60-80 nm), coated vesicles act as carriers that mediate uni- or bi-directional transport between two adjacent membranes in the secretory pathway (Schekman and Orci, 1996; Rothman and Wieland, 1996). Transmembrane proteins, together with cytosolic coat protein complexes and additional regulating factors, are thought to mediate sorting of cargo from residents and the formation of transport vesicles. Vesicle targeting and docking with acceptor membranes is mediated by a number of proteins, including fibrous 'tethering' molecules such as p115 and giantin, which, through specific interactions, provide a first level of specificity

for these transport steps (Pfeffer, 1999). v-SNARES on the vesicles specifically bind t-SNARES at the target membrane and have been proposed to facilitate delivery of cargo by fusion (Jahn and Sudhof, 1999; Scales et al., 2000).

Here, we discuss recent in vivo work that has challenged the vesicular transport model. Visualisation of different molecules and transport steps in the secretory pathway in living cells shows that larger (300-500 nm) membrane aggregates or tubular membrane structures rather than small vesicles function as the long-range transport intermediates between the ER and Golgi complex or between the TGN and plasma membrane. This raises the question of the precise role of vesicles in these transport steps.

Traffic between the ER and Golgi complex COPI and COPII and their vesicles

At least two vesicular coat protein complexes, COPI and COPII, are involved in transport between the ER and Golgi complex (Rothman and Wieland, 1996; Schekman and Orci, 1996). Both coat protein complexes interact with the cytoplasmic tails of membrane proteins ending in specific amino acid sequences – for example, KKXX-type motifs, which interact with COPI, and DxE-type motifs, which interact with COPII (Cosson and Letourneur, 1994; Letourneur et al., 1994; Rothman and Wieland, 1996; Schekman and Orci, 1996; Nishimura and Balch, 1997; Nishimura et al., 1999). These interactions have been implicated in the sorting and concentration of cargo and in the subsequent formation of functional transport vesicles. Recruitment of the coat components to donor membranes, sorting of cargo into vesicles, and the regulation of coat release at the target membrane are regulated by the small GTPases ARF1 (for COPI vesicles) and Sar1 (for COPII vesicles) (Rothman and Wieland, 1996; Schekman and Orci, 1996; Springer et al.,

1999; Wieland and Harter, 1999). Strong data from yeast genetics and biochemical studies has led to a simple transport model (Fig. 1A), in which COPII vesicles mediate ER-to-Golgi transport and COPI vesicles mediate Golgi-to-ER transport (Gaynor et al., 1998; Barlowe, 1998).

The role of the ERGIC in mammalian cells

Although the above model is attractive and consistent with most of the existing experimental data, the situation in mammalian cells appears to be complicated by the existence of a complex membrane structure at the interface of the ER-Golgi boundary that is absent in *Saccharomyces cerevisiae*. The organisation of this ER-Golgi intermediate compartment (ERGIC) has remained controversial since its identification (Hauri and Schweizer, 1992). The ERGIC has been suggested to be continuous with the ER (Sitia and Meldolesi, 1992;

Krijnse-Locker et al., 1995) or the cis-Golgi (Mellman and Simons, 1992). It has also been proposed to be a distinct organelle separated from the ER and Golgi by two vesicular transport steps (Hauri and Schweizer, 1992; Pelham, 1989). The latter view would directly imply that COPII and COPI mediate distinct anterograde and retrograde transport steps, respectively; alternatively COPII would mediate ER-to-ERGIC transport, and COPI would mediate ERGIC-to-Golgi transport, a modified COPI complex returning the transport machinery back to the donor membranes (Orci et al., 1997). Also, alternative vesicular coat complexes, which would remain to be identified, could be involved in traffic between the ER and Golgi complex (Fig. 1B).

Direct visualisation of a GFP-tagged secretory marker in living cells has been a major step towards resolving the role of the ERGIC in ER-to-Golgi transport. This has revealed that the

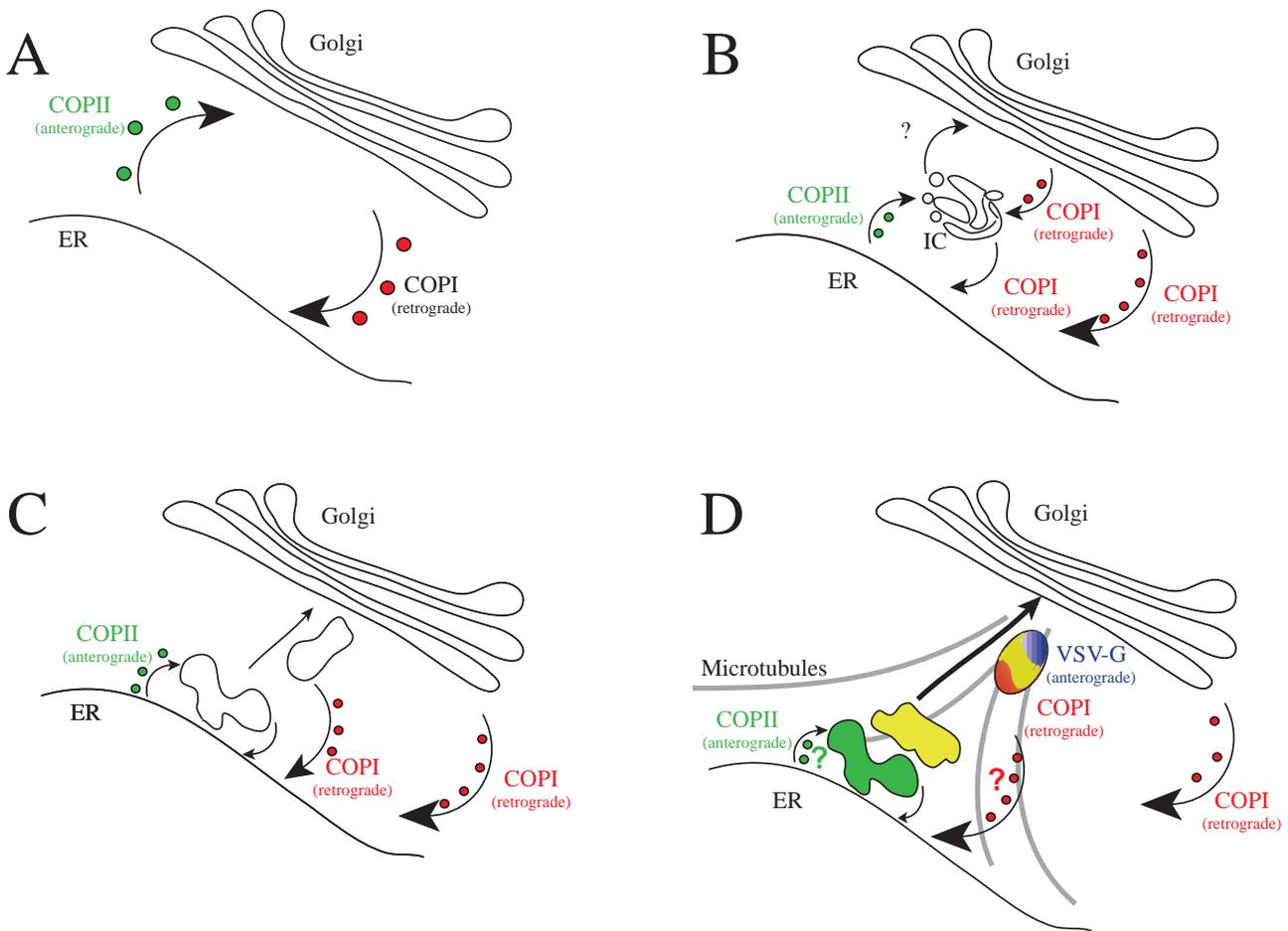


Fig. 1. Different models describing ER-to-Golgi transport. (A) COPII vesicles mediate anterograde, and COPI vesicles mediate retrograde transport between the ER and Golgi complex. (B) The existence of a membrane structure, the intermediate compartment (IC), in mammalian cells raises the questions of how many vesicular transport steps exist between the ER and Golgi complex and of whether further types of vesicle (in addition to COPI and COPII vesicles) exist in this transport step. (C) This model takes into account the data of Presley et al., 1997 and Scales et al., 1997, which demonstrate that the IC (also called VTC) is the long-range ER-to-Golgi transport carrier itself and not a static membrane compartment. COPII vesicles move secretory cargo to VTCs or form de novo VTCs by homotypic fusion. Exchange of COPII for COPI triggers recycling of material to the ER by COPI vesicles and allows VTCs to move along microtubules towards the Golgi complex. This model is consistent with most existing experimental data. (D) A model summarising data from experiments visualising secretory cargo, COPI, COPII and recycling membrane proteins directly in living cells. Neither COPI or COPII vesicles have been observed in these experiments so far. COPI-coated VTCs segregate into anterograde-cargo-rich and COPI/retrograde-cargo-rich domains (Shima et al., 1999). It has not been possible so far to visualise how anterograde and retrograde cargo segregate from each other when VTCs arrive at the Golgi complex.

ERGIC is a mobile membrane structure that itself carries secretory material along microtubules from the ER to the Golgi complex (Presley et al., 1997; Scales et al., 1997; see also examples in Fig. 2). This has led to the introduction of the term transport complex (TC) or vesicular-tubular transport complex (VTC) to describe this organelle better. Based on these findings a model has been proposed (Scales et al., 1997; Lowe and Kreis, 1998; Fig. 1C), in which a VTC receives cargo from COPII vesicles followed by the exchange of COPII for COPI. This was suggested to then give rise to COPI vesicles returning recycling material from the ERGIC to the ER. It was further suggested that, only when retrograde COPI vesicles form from the ERGIC, can it move from its place of origin along microtubules to the Golgi complex (Fig. 1C). Although most aspects of this model still await direct verification, it is consistent with conclusions from yeast genetics, biochemistry and functional studies (Gaynor et al., 1998; Aridor et al., 1995; Rowe et al., 1996; Tisdale et al., 1997). COPII would still be the coat for anterograde transport vesicles forming from the ER, whereas COPI vesicles would act exclusively as retrograde transport carriers.

Subsequent studies using different GFP-tagged membrane markers, including those that are thought to cycle between the ER and Golgi complex, have confirmed the mobile nature of the ERGIC. They have further suggested that VTCs receive material, or possibly form, from a fixed number of stationary ER-exit sites before transport to the Golgi complex (Blum et al., 1999; Chao et al., 1999; Stephens et al., 2000). Our own recent data show directly in living cells that COPII functions predominantly at ER-exit sites and apparently plays no direct role in directed movement of VTCs to the Golgi (Pepperkok et al., 1998; Stephens et al., 2000). VTCs moving in a directed manner towards the Golgi apparatus are coated with COPI but not COPII (Stephens et al., 2000).

Co-visualisation of coat proteins and secretory markers has failed to detect any

COPII vesicles budding from the ER or COPI vesicles budding from moving VTCs (Shima et al., 1999; Stephens et al., 2000; Hammond and Glick, 2000). If COPII vesicles mediate only very short-range transport between the ER and nascent TCs in mammalian cells, they would possibly not be visible at the level of light microscopy. Similarly, COPI-coated vesicles shedding off from moving VTCs might only mediate short-distance retrograde transport to the ER. Therefore, they would quickly lose their coats, which would render them invisible when COPI is used as a marker (Shima et al., 1999). However, imaging of GFP-tagged membrane proteins that cycle between the ER and Golgi and should thus be included in retrograde COPI vesicles has provided no evidence for a net loss of the recycling proteins from VTCs en route to the Golgi by small vesicles (Blum et al., 1999; Shima et al., 1999; Stephens et al., 2000). Instead a progressive and COPI-function-dependent establishment of COPI-rich and anterograde cargo-rich domains was observed in VTCs en route to the Golgi complex (Shima et al., 1999). This is consistent with data from EM analyses demonstrating a role for VTCs in the concentration of soluble secretory proteins by exclusion from COPI-coated buds (Martinez-Menarguez et al., 1999).

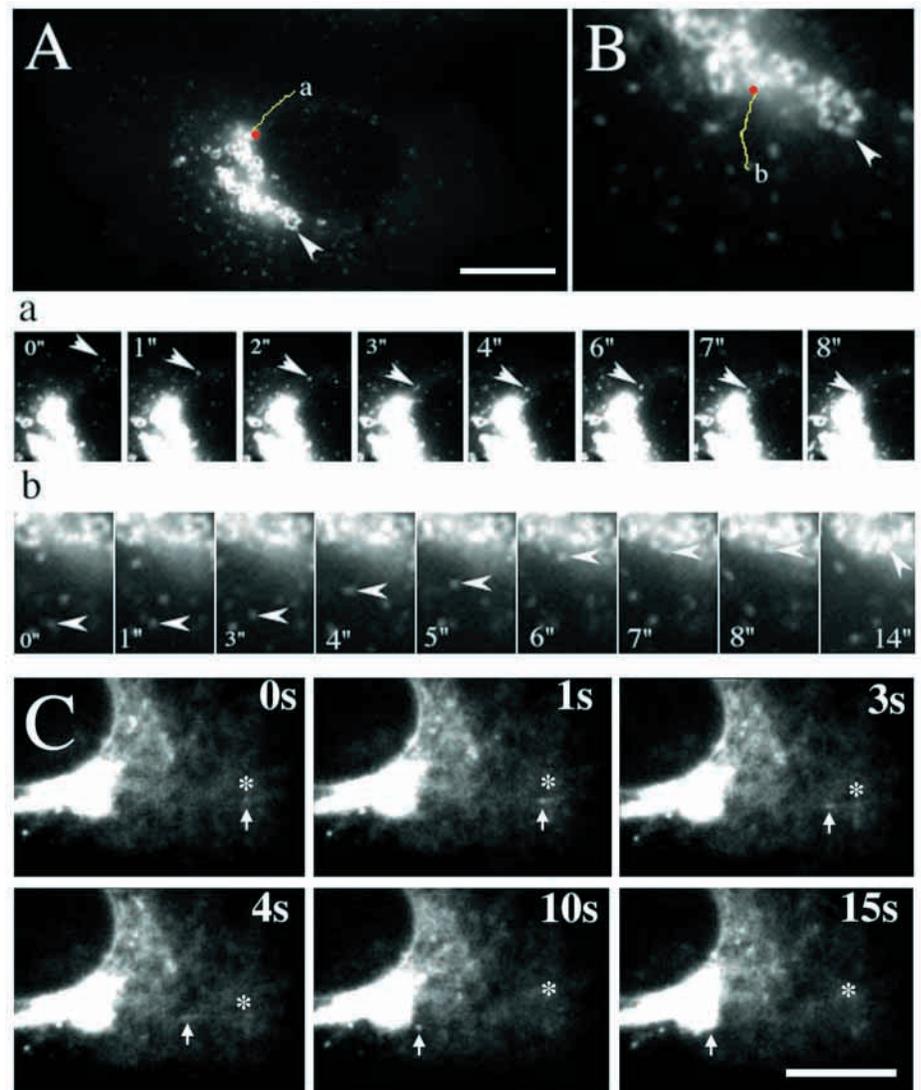


Fig. 2. Examples of COPI-coated VTCs moving secretory cargo between the ER and Golgi complex. (A,B) COPI was fluorescently labelled in living cells by microinjection of an anti-COPI antibody, and the dynamics of COPI coated VTCs were monitored by time-lapse microscopy. Aa and Bb are two examples of VTCs (marked by arrowheads), the trajectories of which are highlighted in A and B. For more details see (Shima et al., 1999). Bar, 20 μm. (C) Visualising ER-to-Golgi transport of GFP tagged ts-O45-G in a living cell by time-lapse microscopy. The arrow indicates a VTC moving from the cell periphery to the Golgi complex. The asterisk indicates the position of the VTC at the beginning of the recording period. Bar, 20 μm.

Although the possibility that a substantial pool of retrograde COPI vesicles is beyond the limit of detection cannot be excluded, these studies clearly suggest that one role of COPI on VTCs is to bind and sequester proteins destined for retrieval to the ER. This interaction results in the observed establishment of retrograde- and anterograde-cargo domains (Shima et al., 1999) within the VTC itself and appears to be coincident with, or possibly required for, directed movement of VTCs into the Golgi complex. Upon arrival at the cis-Golgi, anterograde cargo must physically segregate from retrograde cargo in order to be delivered for further transit through the secretory pathway, whereas retrograde cargo must be returned back to the ER (see Fig. 1D). The mechanism by which this is achieved cannot as yet be revealed by time-lapse microscopy of GFP-tagged markers, owing to the limits of resolution of light microscopy and the lack of appropriate retrograde transport markers.

Retrograde, Golgi-to-ER transport

Lippincott-Schwartz and colleagues analysed GFP-tagged Golgi-resident enzymes in cells treated with the fungal metabolite brefeldin A, which induces relocation of Golgi enzymes to the ER. They proposed that this transport step at least is mediated by long tubular structures, which eventually separate from the Golgi and fuse with the ER (Lippincott-Schwartz et al., 1998). However, treatment of cells with brefeldin A does not represent physiological conditions, and thus the tubular carriers seen in these experiments may not be present in untreated cells but only after BFA-induced relocation of Golgi enzymes to the ER.

At least two functionally independent transport routes from the Golgi to the ER have been proposed to operate under more physiological conditions. One pathway appears to be regulated by COPI and is taken by membrane proteins that possess the motif KKXX, the KDEL-R or luminal proteins ending in KDEL (Jackson et al., 1999; Majoul et al., 1998; Orci et al., 1997; Pelham, 1990). The other, COPI-independent, recycling pathway is used by Shiga toxin or Shiga Toxin B-fragment and is regulated by Rab6 (Girod et al., 1999; White et al., 1999). Expression of a dominant negative Rab6 form (Rab6-GDP) inhibited redistribution of GFP-tagged Golgi-resident enzymes to the ER (Girod et al., 1999), which suggests that these enzymes use this or a closely related Rab6-dependent pathway to gain access to the ER. Co-localisation of Shiga-B fragment and GFP-tagged Rab6 on larger tubular structures that do not contain the KDEL-R supports these functional studies (White et al., 1999). These tubules segregate from the Golgi and are believed to fuse with the ER at the cell periphery. In experiments using GFP-tagged KDEL receptor expressed in HeLa cells, Sciaky et al. showed similar formation of Golgi tubules and subsequent detachment and translocation of these tubules to the cell periphery (Sciaky et al., 1997). Together, these data suggest that tubular rather than vesicular carriers can mediate both COPI-dependent and COPI-independent long-range Golgi-to-ER transport. An important caveat to these observations is that, in every case, the tubules are observed in experiments in which GFP-tagged markers were ectopically expressed. These tubules might therefore be an exaggeration of normal processes resulting from overexpression of the GFP-tagged marker compared with its endogenous counterpart. However,

such tubules have been observed in untransfected cells by both fluorescence microscopy and immunoelectron microscopy (Klumperman et al., 1998).

Post-TGN traffic

Considerable effort has recently focused on analysis of constitutive and regulated membrane trafficking pathways from the trans-Golgi network to the plasma membrane. As in the case of ER-to-Golgi traffic, large, pleiomorphic carriers mediate the majority of cargo transport in the late secretory pathway from the TGN to the plasma membrane in non-polarised cells (Hirschberg et al., 1998; Toomre et al., 1999). These structures undergo highly dynamic morphological changes including fission and fusion during transport. Simultaneous imaging of GFP-tagged cargo (ts-045-G-GFP) and microtubules (labelled using rhodamine-tubulin) showed that these structures moved along microtubule tracks (Toomre et al., 1999). The net movement of these carriers from the perinuclear area to the cell periphery indicates that the movement is along microtubules and therefore involves kinesin family members. Indeed inhibition of kinesin activity by microinjection of blocking antibodies blocks Golgi to plasma membrane transport of tubular carriers containing GFP-tagged p75 neurotrophin receptor (Kreitzer et al., 2000).

Small <250 nm 'vesicle-like structures' operating in post-Golgi transport have also been seen (Wacker et al., 1997; Hirschberg et al., 1998; Toomre et al., 1999). However, quantitative analyses suggest that the larger structures are the major transport carriers for post-Golgi secretory cargo (Hirschberg et al., 1998; Toomre et al., 1999). Nakata et al. have confirmed the presence of these smaller structures as well as larger tubular transport complexes (Nakata et al., 1998). They showed by both time-lapse fluorescence microscopy and electron microscopy that these tubulovesicular structures are also responsible for traffic of a variety of newly synthesised proteins, including synaptophysin, GAP-43, SNAP-25 and the neurotrophin receptor trkA, in axonal transport. Perhaps the most convincing evidence yet that these larger transport carriers mediate TGN to plasma membrane transport comes from a study in which Polishchuk et al. correlated the fluorescent imaging of ts-045-G-GFP with an electron microscopic examination of the same cells (Polishchuk et al., 2000). The occasional presence of γ -adaptin on these structures as seen by immuno-EM suggests that they are maturing membrane systems similar to those observed during the biogenesis of secretory granules (Tooze, 1998) and perhaps also analogous to ER-to-Golgi TCs polarising en route to the Golgi (Shima et al., 1999).

Post-TGN sorting in polarised cells.

The carriers mediating transport from the TGN to the cell membrane in polarised cell systems appear to be morphologically similar to those of non-polarised cells. Simons and co-workers have recently examined post-TGN transport of apical and basolateral cargo molecules simultaneously in cells by using two-colour time-lapse microscopy (Keller et al., 2000). They found that segregation of the markers used (ts-045-G and GPI-GFP) precedes independent transport of the two molecules to the plasma membrane in distinct but morphologically similar transport carriers (TCs). Sorting was found not to be completely

efficient, and in those TCs that contained both cargo molecules there was no further sorting en route to the plasma membrane. This suggests that Golgi to plasma membrane TCs do not sort apical from basolateral cargo. Traffic to the plasma membrane of these TCs was found not to involve an endosomal intermediate. This observation raises the question of the molecular mechanism of this sorting and transport event and suggests a lateral segregation of cargo within the TGN membrane. This might be mediated through intramolecular interactions or through capture into nascent coat assemblies on the cytosolic face of the membrane.

Fusion with the plasma membrane.

Recent advances in light microscopy have enabled the direct visualisation of fusion events at the plasma membrane. Total internal reflection fluorescence microscopy (TIR-FM) enables imaging of only a small (typically 50–200 nm) depth of field directly adjacent to the substratum. This technique has now been applied to both regulated secretion of secretory granules and also to constitutive fusion events (Lang et al., 1997; Steyer and Almers, 1999; Schmoranzler et al., 2000; Toomre et al., 2000). In both cases, cargo-containing structures were observed to migrate towards the plasma membrane, hover in a confined area (as if docked at the membrane) followed by apparent exocytosis. These data indicate that the carriers involved in post-Golgi transport (described above) do not necessarily fuse completely with the plasma membrane. These structures can be seen by both light and electron microscopy to be composed of apparent head and tail domains (Polishchuk et al., 2000; Hirschberg et al., 1998; Toomre et al., 1999; Schmoranzler et al., 2000; Toomre et al., 2000). It is the head domain that appears to undergo fusion with the plasma membrane.

When do we need vesicles?

It is clear from recent *in vivo* studies that for transport between the ER and Golgi and plasma membrane, large, pleiomorphic structures are responsible for the majority of cargo transport in mammalian cells. These findings contrast with biochemical and genetic evidence, in particular from *Saccharomyces cerevisiae*, which supports a vesicular intermediate in these transport steps, notably ER-to-Golgi traffic. It must be remembered, however, that the intracellular membrane organisation of *S. cerevisiae* is considerably different from that of mammalian cells. Notably, VTCs operating in ER-to-Golgi transport have not been definitively identified in *S. cerevisiae*. This may be a direct result of the different organisation of the Golgi in these cells, compared with mammalian cells (Glick, 2000). A scattered Golgi in *S. cerevisiae* may not require large transport intermediates. The more complex, ribbon-like organisation of the mammalian Golgi, coupled with the considerably greater distances required to transport material from ER-exit sites at the cell periphery to the cis-side of the Golgi, may necessitate a more efficient, targeted delivery of cargo. Strong electron microscopy data exist for Golgi-associated vesicles in mammalian cells (e.g. Ladinsky et al., 1999). Thus vesicles might only operate in short-distance transport either to form, for example, a nascent VTC at donor membranes or to release recycling material from it at target membranes. In this model, the VTCs are then responsible exclusively for long-range transport steps along microtubules.

The advantage of such an organisation is that recruitment of motor molecules and their regulators to the membrane-microtubule interface need occur only once for one VTC and not repeatedly for several small vesicles.

One can also envisage, although less likely, a scenario in which the mammalian cell has evolved to eliminate the need for vesicles altogether. Data support a role for 'vesicle' coats, namely COPI and COPII, in cargo selection and functional specialisation of membrane domains. A direct connection between the ER and a nascent TC (which can often indeed be seen by EM (Sesso et al., 1992; Krijnse-Locker et al., 1994; Martinez-Menarguez et al., 1999)) would eliminate the requirement for a budding step at the ER level: the COPII coat would serve as a cargo-selection device and provide a mechanism for the functional specialisation of distinct domains of the ER membrane. The COPI coat might have a similar role on ER-to-Golgi TCs and even at the level of the Golgi membrane. The function of coat complexes therefore may be to select cargo and specialise membrane domains for membrane traffic.

It remains possible that, using current light microscopy techniques, we are simply not able to see small 60–80 nm vesicles in living cells. Also, many of the structures thus far observed could indeed represent vesicle populations, which might even be tethered to one another and therefore appear as a pleiomorphic transport complex. Correlated analyses using light and electron microscopy on the same cell (Polishchuk et al., 2000) could be one way to resolve these questions. Perhaps the most important limitation of the undoubtedly valuable technology of GFP tagging is that, in almost every case, the GFP-tagged molecule is ectopically expressed in a system already containing a background of the untagged form of the protein. This can cause several difficulties. For example, correct functional localisation and dynamics of the TGN membrane protein TGN38 require low level (ideally stable) expression in a homologous system (e.g. rat TGN38 expressed in rat cells (Girotti and Banting, 1996). Non-homologous expression or very high-level homologous overexpression leads to disruption of intracellular morphology and mislocalisation of GFP-TGN38 (Girotti and Banting, 1996). Ideally one would like to examine these GFP-tagged markers by using regulated expression systems in a null background.

In conclusion, our knowledge of the secretory pathway has been greatly enhanced in recent years by the application of GFP technology and the latest advancements in light microscopy. It seems likely that the availability of further spectral variants of GFP will greatly enhance our understanding of these pathways in the near future. This, coupled with developments in light microscopy, such as the recent breaking of the diffraction resolution barrier (Klar et al., 2000), will facilitate the greater use of light microscopy in the analysis of intracellular membrane traffic.

We thank Patrick Keller, Derek Toomre and Kai Simons for communicating results prior to publication and Martin Lowe, David Shima and Jeremy Simpson for critical reading of the manuscript.

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