Ultrastructural characterization of endoplasmic reticulum – Golgi transport containers (EGTC)

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Accepted 21 August 2002
Journal of Cell Science 115, 4263-4273 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00115

Summary

Recent observations made in live cells expressing green fluorescent protein (GFP)-tagged cargo markers have demonstrated the existence of large, mobile transport intermediates linking peripheral ER exit sites (ERES) to the perinuclear Golgi. Using a procedure of rapid ethane freezing, we examined ultrastructurally the intermediates involved in ER-Golgi transport of the vesicular stomatitis virus (VSV) G protein. When released at the permissive temperature of 32°C, VSVG is first found to be concentrated in pleiomorphic, membrane-bound structures (of about 0.4 to 1 μm in diameter) with extensive budding profiles. These structures are devoid of COPII components and Golgi markers, but are enriched in COPI, the retrograde cargo ERGIC53, and the tethering protein p115. The structures appear to be able to undergo fusion with the Golgi stack and are tentatively referred to as ER-Golgi transport containers, or EGTCs. VSVG protein exiting the ERES at 15°C is first found in clusters or strings of COPII-containing small vesicles, and morphological analysis indicates that these clusters and strings of COPII vesicles may coalesce by homotypic fusion to form the EGTCs. Together with the large transport containers mediating transport from the trans-Golgi network to the plasma membrane, EGTCs represents an emerging class of large membranous structures mediating anterograde transport between the major stations of the exocytic pathway.

Key words: COPI, COPII, EGTC, Golgi, VSVG

Introduction

Membrane transport between any two compartments in eukaryotic cells can potentially be mediated by several mechanisms (Mironov et al., 1997). The vesicular transport model describes small (60 to 90 nm) vesicles that bud from the ER and fuse with the cis-Golgi (Rothman and Wieland, 1996). This view has received much experimental support over the years. The observation of early secretory-pathway-associated COPI- (Malhotra et al., 1989; Lowe et al., 1998) and COPII- (Barlowe et al., 1994) coated vesicles left little doubt that transport vesicles exist.

Larger membranous intermediates appear to exist between the ER and Golgi apparatus. These structures are termed ER-Golgi intermediate compartments (ERGICs) (Schweizer et al., 1990; Hauri et al., 2000) or vesicular tubular clusters (VTC) (Bannykh and Balch, 1997). They were first described as pre-Golgi vacuoles – spotty structures marked by the Semliki Forest virus membrane glycoprotein en route to the Golgi (Saraste and Kuismannen, 1984). Several different views of these intermediate structures have since been put forward. The first is that these are distinct, membrane bound entities that are organelles in their own right (Pelham, 1989). They are not connected to the ER and the Golgi membranes, instead they are linked by vesicular transport processes and serve as a midway station for transport from the ER to the Golgi. Another model suggests that these are transient structures formed by homotypic fusion between ER-derived vesicles, which will eventually be consumed by fusion with the first cis-Golgi cisterna (Bannykh et al., 1996; Bannykh and Balch, 1997).

The opposing view, largely based on ultrastructural evidence, is that the membranous intermediates are connected by membranous connections to either the ER or the Golgi. The ERGIC may be a subcompartment of the ER (Krijnse-Locker et al., 1994). It is physically connected to the ER and may serve as the cis-Golgi. Alternatively, the ERGIC may be physically continuous with the first fenestrated cisterna of the Golgi stack and is thus also referred to as the cis-Golgi network (Mellman and Simons, 1992). With all these different models, it is unclear whether vesicular transport is the only mode of traffic between the ER and the Golgi. It is also not known with any certainty if one or two rounds of vesicle budding and fusion are needed. The localization of both COPI and COPII to pre-Golgi structures makes it difficult to dismiss the latter possibility. Both COPI- and COPII-coated vesicles can bud from yeast microsomes (Bednarek et al., 1995). Although COPII components mediate vesicle budding from the ER (Barlowe et al., 1994; Rowe et al., 1996); the apparent requirement for both COPI and COPII for ER-Golgi transport in vitro (Aridor et al., 1995) and in vivo (Scales et al., 1997) further complicates the issue.

On the other hand, nagging concerns over the strict vesicular mode of transport between the ER and Golgi have always existed. One of the most prominent problems is how gigantic molecules that are obviously too large to fit within the dimensions of a typical transport vesicle, such as the collagen

triple helixes, are transported (Bonfanti et al., 1998). Furthermore, a string of recent observations with life cell imaging techniques have cast severe doubts on the notion that vesicles served as the only cargo carriers between the ER and the Golgi. Monitoring the released of temperature-restricted VSVGts045 labeled by fusion to the green fluorescent protein (GFP) (Lippincott-Schwartz et al., 1997; Lippincott-Schwartz et al., 1998), several groups have reported seeing large (0.4 to 1 μm in diameter) structures generated from the ER (Presley et al., 1997; Scales et al., 1997) (for a review, see Stephens and Pepperkok, 2001). These structures move, in a microtubule-dependent manner (Cole and Lippincott-Schwartz, 1995; Lippincott-Schwartz et al., 1997), from peripheral sites of the cell towards the perinuclear region where the Golgi resides. Although varying in size, these cargo-containing structures are obviously too large to be classic transport vesicles. Similar mobile structures were also observed when VSVGts045-GFP accumulated at the trans-Golgi network (TGN) moves to the cell surface (Hirschberg et al., 1998; Toomre et al., 1999; Polishchuk et al., 2000). Thus, it seems that although vesicles do indeed form from the ER, these are not necessarily the final form of transport carrier that ferry cargo from the ER to the Golgi.

The large, VSVGts045-GFP containing carriers have so far been observed only at the resolution of light microscopy. Stereological analysis of serial thin sections of peripheral ERES by Balch and colleagues provided the first insight into these structures at the ultrastructural level (Bannykh et al., 1996). Accordingly, compact pleiomorphic elements, called vesicular-tubular clusters (VTCs), are formed by COPII-mediated budding at specific sites of the ER, referred to as export complexes. VTCs are not continuous with the ER and are highly dynamic and undergo a COPI-dependent maturation process. VTCs that have acquired COPI can be mobilized to the Golgi region along microtubules (Bannykh and Balch, 1997). Striking views of a VTC within an export complex have been revealed by rotary-shadowed replicas using a quick-freeze, deep-etch approach (Bannykh et al., 1996). However, ultrastructural visualization of isolated VTCs in transit to the Golgi has never been reported and represents a major missing link in the evidence for their existence.

We now present, using a procedure of rapid ethane freezing, ultrastructural analysis of cargo-containing large carriers in transit from the ER to the Golgi.

Materials and Methods

Antibodies

The mouse monoclonal anti-VSV-G antibody (P5D4) was purchased from Boehringer Mannheim (Germany). Rabbit polyclonal antibodies against β-COP (Pepperkok et al., 1993; Peter et al., 1998), GS28 (Subramaniam et al., 1996) and Sec13 (Tang et al., 1997) have been described previously. The rabbit polyclonal antibody against p58 (Lahtinen et al., 1992; Lahtinen et al., 1996) was kindly provided by Jakko Sarraste, University of Bergen. Antibodies against p115 were produced by immunizing rabbits with a p115 glutathione S-transferase fusion protein (V. N. Subramaniam and W. Hong, unpublished).

VSVts045 infection

NRK cells maintained in DMEM with 10% fetal calf serum (FCS) were grown to confluence on 50 μm thick sapphire coverslips. Cells were infected with VSVts045 in DMEM without fetal calf serum (FCS) at 32°C for 1 hour. The infected cells were then incubated at the restrictive temperature (40°C) for 2 hours post-infection in DMEM with 10% FCS to accumulate newly synthesized G-protein in the ER. Cells were quickly washed with ice-cold washing buffer (50 mM Hepes, 90 mM KOAC, pH 7.2) and kept on ice. ER-restricted VSVG protein was released by incubation at the permissive temperature (32°C) for various time periods. Cells were washed with ice-cold washing buffer and then processed for transmission electron microscopy as outlined below.

Transmission electron microscopy

Cells grown to about 70% confluence on synthetic sapphire coverslips were quick frozen in liquid ethane at –170°C. The vitrified ice in the samples was substituted with methanol at –85°C over 12 hours and embedded in Lowicryl HM-20 as described previously (Parson et al., 1995; Steyer et al., 1997) with minor alterations. Embedding was carried out in specially designed coverslip holders (FH-Cryotec; Singapore). Ultrathin Lowicryl sections were then processed for immunogold labeling with various antibodies followed by staining with uranyl acetate and lead citrate. Transmission electron microscopy was carried out with a Joel 1220 electron microscope at 80kV.

Alternatively, for the enhancement of membrane morphology, substitution was performed in acetone supplemented with 0.5% uranyl acetate, 2% osmium tetroxide and 0.25% glutaraldehyde at –85°C over 12 hours. The temperature was subsequently raised to 4°C and the sample was infiltrated and embedded in Spurr's resin. Ultrathin sections obtained were stained with lead citrate and transmission electron microscopy was carried out as above.

Results

A sample preparation procedure by rapid freezing in liquid ethane

A recently described procedure involving a rapid ethane-freezing technique was found to preserve membrane structure as well as protein antigenicity for analysis using both fluorescence microscopy and electron microscopy (Steyer et al., 1997). Since the cellular settings are preserved in situ almost instantaneously, this procedure allows a snapshot of the cell and its internal structures at the ultrastructural level. We applied this technique, with some modifications, to our analysis of the transport of temperature-restricted VSVG from the ER to the Golgi. As observed by indirect immunofluorescence (data not shown), the temperature-sensitive VSVG misfolds at the non-permissive temperature (40°C) and fails to exit the ER. Upon transferring cells to the permissive temperature of 32°C, VSVG folds, exits the ER and is transported to the Golgi through large intermediates in a synchronized wave as previously described (Tang et al., 1997; Tang et al., 2001; Zhang et al., 1997; Zhang et al., 1999).

Association of VSVGts045 with large pleiomorphic structures with extensive bud-forming profiles

Ten minutes after release at 32°C from the non-permissive temperature (40°C), we observed that the VSVG appears to accumulate in pleiomorphic, membrane-bound structures with some kind of matrix surrounded by a limiting membrane with a distinct, extensive bud-forming profile at its edges (Fig. 1A). Interestingly, most of the immunogold labeling of VSVG appears to be enriched at the interior matrix portion of the structure. These structures are most frequently found at the

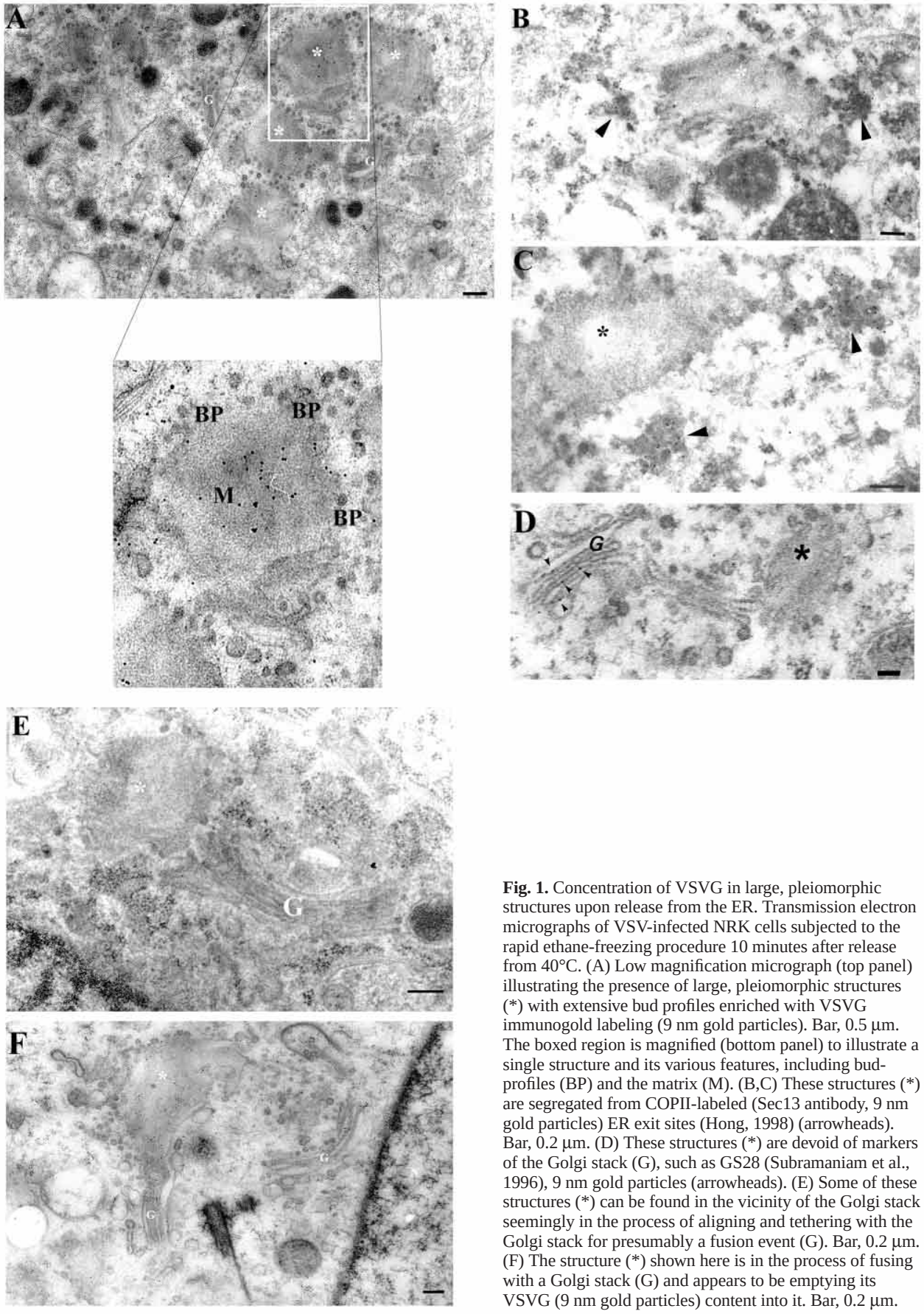

Fig. 1. Concentration of VSVG in large, pleiomorphic structures upon release from the ER. Transmission electron micrographs of VSV-infected NRK cells subjected to the rapid ethane-freezing procedure 10 minutes after release from 40°C. (A) Low magnification micrograph (top panel) illustrating the presence of large, pleiomorphic structures (*) with extensive bud profiles enriched with VSVG immunogold labeling (9 nm gold particles). Bar, 0.5 μm. The boxed region is magnified (bottom panel) to illustrate a single structure and its various features, including bud-profiles (BP) and the matrix (M). (B,C) These structures (*) are segregated from COPII-labeled (Sec13 antibody, 9 nm gold particles) ER exit sites (Hong, 1998) (arrowheads). Bar, 0.2 μm. (D) These structures (*) are devoid of markers of the Golgi stack (G), such as GS28 (Subramaniam et al., 1996), 9 nm gold particles (arrowheads). (E) Some of these structures (*) can be found in the vicinity of the Golgi stack seemingly in the process of aligning and tethering with the Golgi stack for presumably a fusion event (G). Bar, 0.2 μm. (F) The structure (*) shown here is in the process of fusing with a Golgi stack (G) and appears to be emptying its VSVG (9 nm gold particles) content into it. Bar, 0.2 μm.

perinuclear region but can also be found at places remote from the nucleus. These structures are also present in uninfected cells, ruling out the possibility that they represent artifactual membrane profiles generated by VSV infection. We have also observed similar structures in other cell lines such as HeLa, Att20 and HepG2 (data not shown).

To ascertain the relationship between these structures and the ERES, we performed immunogld labeling with antibodies against the COPII component Sec13 (Tang et al., 1997; Tang et al., 2000; Tang et al., 2001). As shown in Fig. 1B,C, these structures appear to be segregated from the ERES marked by Sec13. They are devoid of Golgi stack markers such as GS28 (Fig. 1D). These structures can often be found in the vicinity of the Golgi apparatus (Fig. 1E). Fig. 1F shows such a structure, which appears to be undergoing direct fusion with the Golgi stack. In doing so, the VSVG cargo from the structure seems to flow into the Golgi cisternae.

Membrane structures with such unique morphological features have not been previously described in the literature using conventional fixation procedures. Since VSVG released from the ER can be found in these structures within the short time frame of 10 minuted, and they appear to play a direct role in the delivery of VSVG to the Golgi, they are likely to be transport carriers operating between the ER and the Golgi apparatus. Our observations suggest that these may indeed be similar in nature to the large, mobile transport carriers that are labeled by VSVGts045-GFP documented in several recent studies employing live cell imaging techniques. We have thus tentatively named these structures ER-Golgi transport containers (EGTCs).

An EGTC is an isolated membrane-bound entity

The observation that VSVGs are found within EGTCs fairly soon after exit from the ER suggests that EGTCs may be derived from the ER. One way this may occur is for pieces of membranes to be pinched off from subregions of the ER. Membranous continuity between EGTCs and the ER may therefore be observed prior to their physical separation. Serial thin sections of NRK cells were subjected to the procedure of ethane freezing, and a typical serial 70 nm section is shown in Fig. 2A. In none of these serial sections did we observe any membranous continuity between an EGTC and the ER or Golgi. A 3D reconstruction of the EGTC (Fig. 2B) reveals an irregularly shaped structure surrounded at its edges by 80 nm vesicles. Similar observations were made in other serial sectioning experiments. These observations suggest that EGTCs are structures distinct from the ER and are probably not derived from the ER by a simple membrane fission event.

Segregation of anterograde and retrograde cargo occurs in the EGTC

The concentration of an anterograde cargo (VSVG) in EGTCs suggests that the EGTC is an intermediate for ferrying ER-derived cargo to the Golgi. The extensive bud-forming profile of EGTCs suggests that EGTCs themselves are undergoing active sorting by vesicle budding. This prompted us to examine the nature of the coat that is associated with the buds/vesicles. It has been previously shown that a COPII/COPI-coupled exchange occurs during VTC maturation (Aridor et al., 1995), and isolated COPII vesicles can recruit COPI (Rowe et al., 1996). In addition, COPI has been shown to be enriched in transport intermediates between the ER and the Golgi. We have shown above that EGTCs are devoid of COPII labeling. However, perhaps not surprisingly, we found that EGTCs are fairly enriched in COPI using immunogold labeling with an antibody against β-COP (Fig. 3A). Furthermore, double immunogold labeling against VSVG and β-COP revealed that the anterograde cargo is in fact very well segregated from the COPI protein within an EGTC. VSVG is mostly found in the interior matrix of the EGTC, whereas β-COP is found largely near the bud profiles (Fig. 3A,B). The role of the COPI coat in Golgi-ER retrograde transport is well known (Stephens and Pepperkok, 2000; Stephens and Pepperkok, 2001), and the presence of COPI coat in the EGTC indicates that a COPI-mediated retrograde transport process may be occurring within the structure itself. The observation that there is a clear physical segregation between the anterograde cargo (VSVG) and the retrograde transport machinery (COPI) indicates that the EGTC may serve a function sorting anterograde from retrograde cargo in protein trafficking between the ER and the Golgi.

To further investigate this point, we examined the distribution of p58 (or the rodent orthologue of primate ERGIC53) (Lahtinen et al., 1992; Lahtinen et al., 1996), a lectin molecule that bears an ER retrieval signal and is a major retrograde cargo in post-ER structures (Nilsson and Warren, 1994; Hauri et al., 2000). We have previously shown that ERGIC53 and p58 are segregated from the anterograde traffic in ER-Golgi transport intermediates (Tang et al., 1995). Immunogold labeling with an antibody against p58 shows that the protein is also found in EGTCs (Fig. 3C) but not in the COPII-positive vesicles cluster observed at 15°C (data not shown). Furthermore, the localization pattern of p58 showed a much higher degree of similarity to that of COPI than VSVG, being also enriched in the bud profiles rather than the interior of the structure. EGTCs therefore contains both anterograde and retrograde cargo, and their extensive bud-forming profile probably represents the process of COPI-mediated recycling of retrograde cargo (such as ERGIC53/p58) for transport back to the ER.

There has never been a confirmed observation of vesicle fusion to target membrane at the early secretory pathway at the ultrastructural level. This is understandable as it would be difficult to distinguish small vesicles in the act of fusing to membranes from those budding from the same compartment. As shown above in Fig. 1E, the large size of the EGTC has facilitated the observation of its fusion with the Golgi. We further confirm that the EGTCs are fusion competent in terms of the membrane-tethering machinery they carry. Transport carriers are tethered to the target membrane via tethering proteins to facilitate the subsequent docking and fusion process (Waters and Pfeffer, 1999). One such tethering protein that has been shown to be required for ER-Golgi transport is the yeast Uso1p (Sapperstein et al., 1996) and its mammalian homologue p115 (Alvarez et al., 1999). p115 has been recently shown to be recruited onto COPII vesicles by Rab1 and persists in a subsequent transport intermediate (Allan et al., 2000). As shown in Fig. 3D, there is a significant amount of p115 on the EGTCs, confirming that these structures are equipped with components of the tethering machinery.

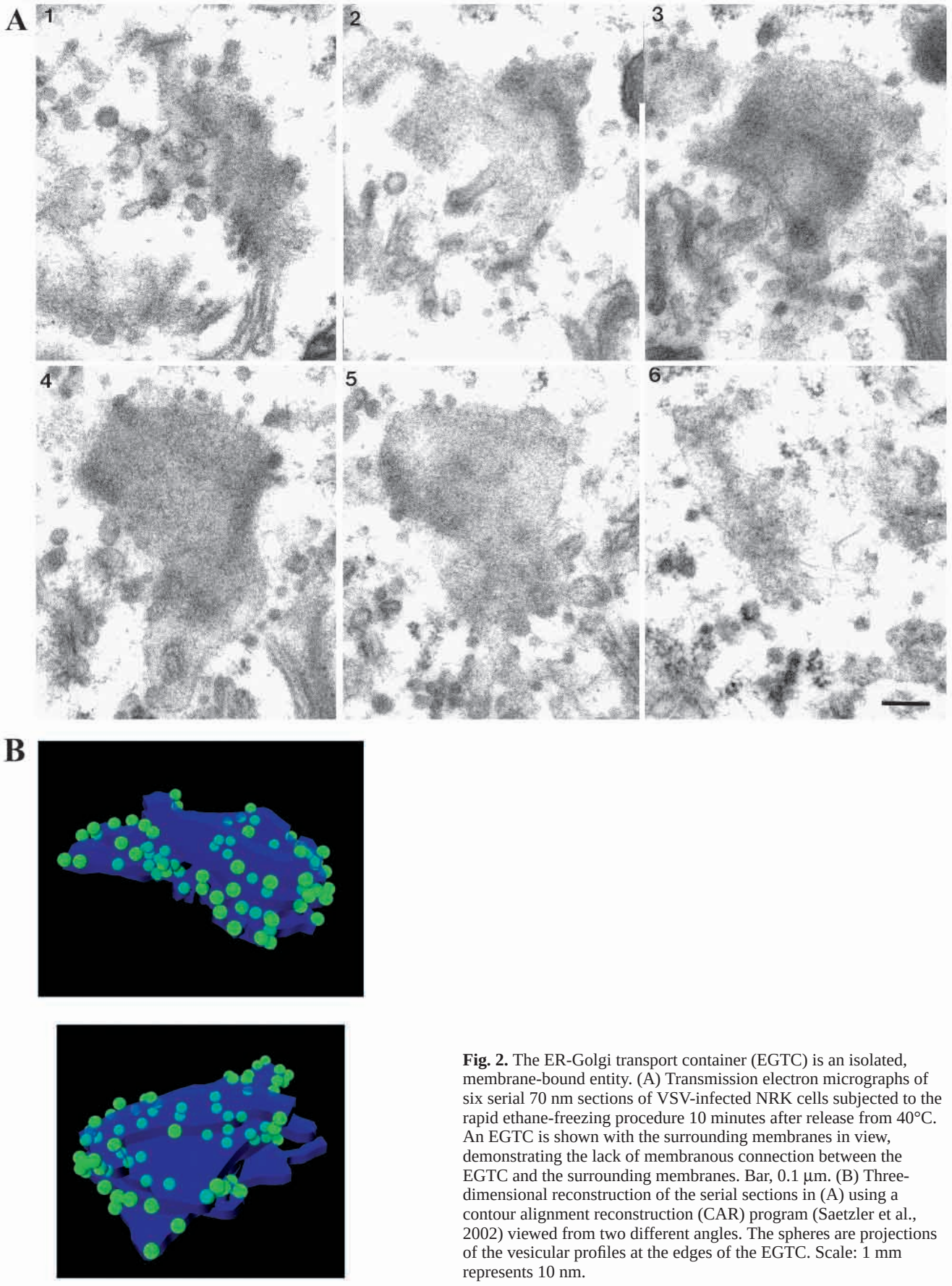

Fig. 2. The ER-Golgi transport container (EGTC) is an isolated, membrane-bound entity. (A) Transmission electron micrographs of six serial 70 nm sections of VSV-infected NRK cells subjected to the rapid ethane-freezing procedure 10 minutes after release from 40°C. An EGTC is shown with the surrounding membranes in view, demonstrating the lack of membranous connection between the EGTC and the surrounding membranes. Bar, 0.1 μm. (B) Three-dimensional reconstruction of the serial sections in (A) using a contour alignment reconstruction (CAR) program (Saetzler et al., 2002) viewed from two different angles. The spheres are projections of the vesicular profiles at the edges of the EGTC. Scale: 1 mm represents 10 nm.

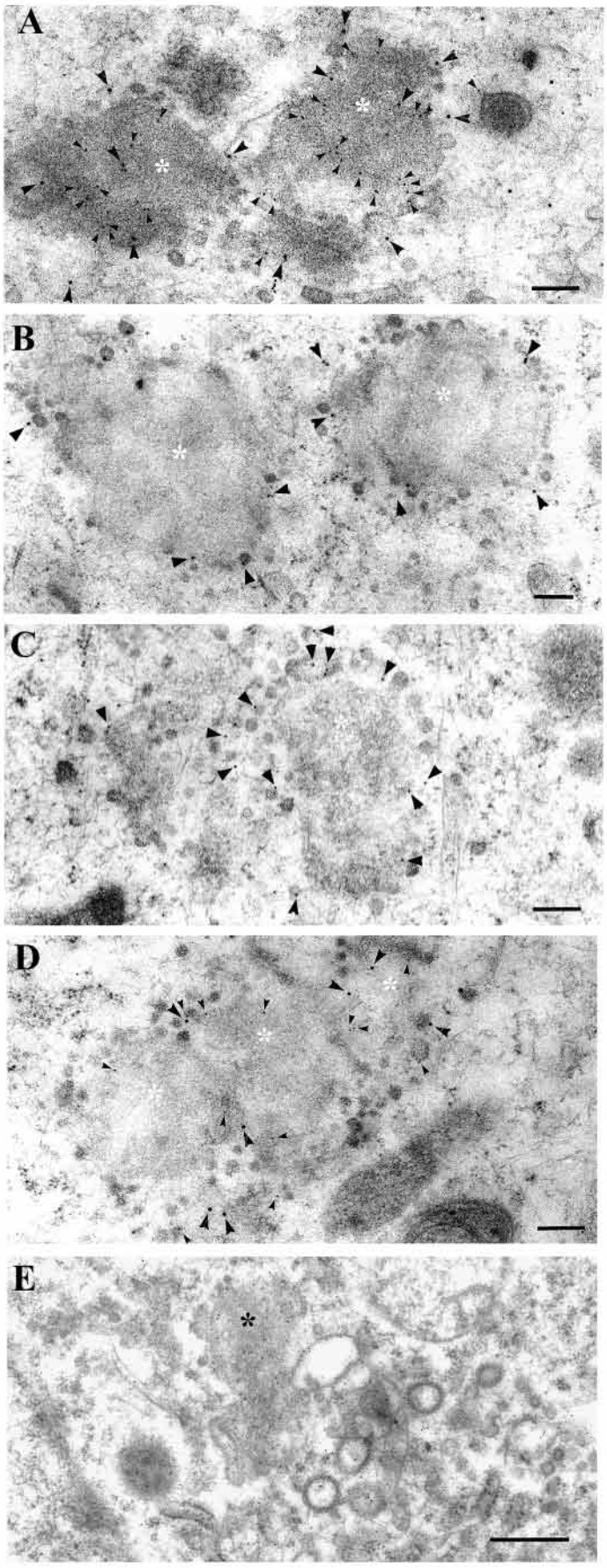

Fig. 3. The EGTC is enriched in the markers expected for an ER-Golgi transport intermediate. Transmission electron micrographs of VSV-infected NRK cells subjected to the rapid ethane freezing procedure 10 minutes after release from 40°C. (A) Double immunogold labeling of VSVG (9 nm gold particles, small arrowheads) and COPI (β-COP antibody, 14 nm gold particles, large arrowheads). Although found within the same structures (*), VSVG labeling is more concentrated at the central matrix whereas β-COP labeling (14 nm gold particles, arrowheads) is mostly associated with the bud profiles at the periphery. This is also illustrated in the single β-COP labeling (10 nm gold particles, arrowheads) in B. This is also the case for p58 (p58 antibody, 10 nm gold particles, arrowheads) labeling as shown in C. Bars, 0.2 μm. (D) Double immunogold labeling for β-COP (14 nm gold particles, large arrowheads) and the tether protein p115 (p115 antibody, 9 nm gold particles, small arrowheads), demonstrating that p115 is also found at a significant level in the EGTC. (E) Immunogold labeling for the soluble cargo albumin (albumin antibody, 10 nm gold particles) in HepG2 cells. Bars, 0.2 μm.

A true cargo carrier should also carry within its lumen soluble cargo destined for secretion. We examined the EGTC of HepG2 cells to see if it is enriched in the lumenal cargo albumin. As shown in Fig. 3E, immunogold labeling for albumin is indeed found at significant levels in EGTCs of HepG2 cells. The above observations confirm that the EGTCs are authentic ER-Golgi transport carriers as they appear to carry both membrane and soluble cargoes, are equipped with components of the ER-Golgi membrane transport machinery and are apparently endowed with the ability to segregate retrograde cargoes by COPI-mediated vesicle budding.

EGTCs are probably derived from coalesced COPII vesicles

The current dogma states that COPII-coated vesicles mediate the first step of exit from the ER (Barlowe et al., 1994). EGTCs are, however, devoid of COPII labelling. Since an origin of EGTCs via tubulation or pinching off of the ER membrane is not supported by the of lack membranous continuity with the ER, the other obvious possibility is that they are derived from COPII vesicles that bud from the ER by homotypic fusion (Bannykh and Balch, 1997; Allan and Balch, 1999). The formation of COPII vesicles containing VSVG may therefore take place prior to the biogenesis EGTCs. However, we were not able to clearly observe the formation of VSVG-containing COPII-coated vesicles upon VSVG exit from the ER. It is conceivable that the structural intermediates involved might have been too transient for detection owing to its rapid conversion into EGTCs. We therefore examined the release of VSVG at 15°C, a temperature that has been shown by previous studies to block ER-to-Golgi transport at the level of the ERES and/or ERGIC (Schweizer et al., 1990; Hauri et al., 2000) or the VTC (Bannykh and Balch, 1997). In VSV-infected cells transferred from 40°C to 15°C, we were indeed able to clearly discern COPII-coated, VSVG-enriched vesicles. These are usually found bunched together as clusters. Such COPII vesicle clusters are not observed in VSV-infected cells transferred from 40°C to 32°C.

To see if these clusters of COPII vesicles are indeed free structures that are distinct from the ER, serial sections of VSV-infected cells transferred from 40°C to 15°C were double-

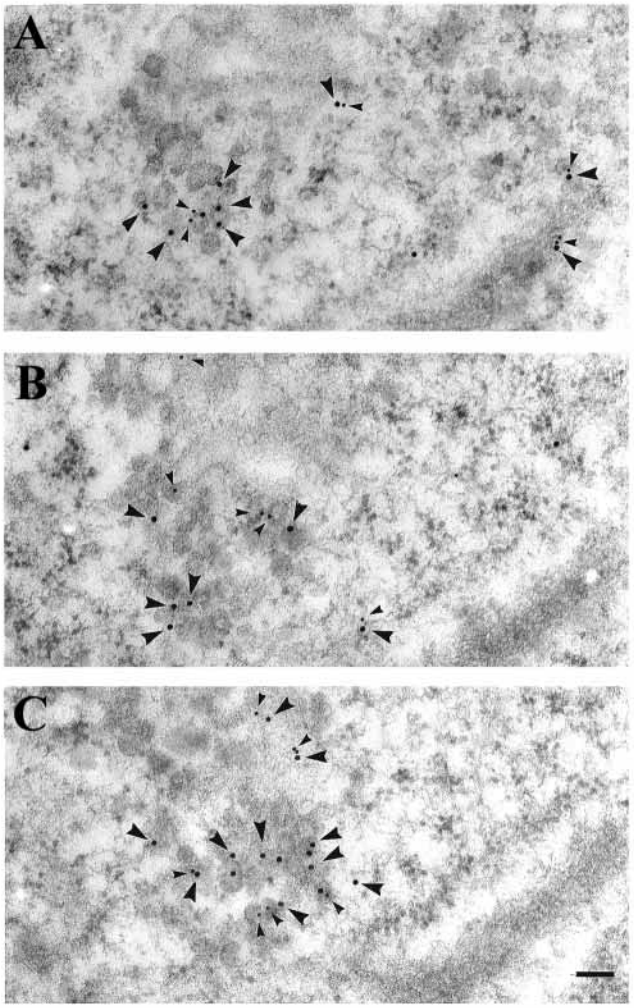

Fig. 4. VSVG is first found in COPII-positive vesicle chains/clusters upon release from the ER at 15°C. Transmission electron micrographs of three 70 nm serial sections (A, B and C) of VSV-infected NRK cells subjected to the rapid ethane freezing procedure for 3 hours upon transfer from 40°C to 15°C. The sections were double-labeled for VSVG (9 nm gold particles, small arrowheads) and Sec13 (14 nm gold particles, large arrowheads). Bar, 0.1 μm.

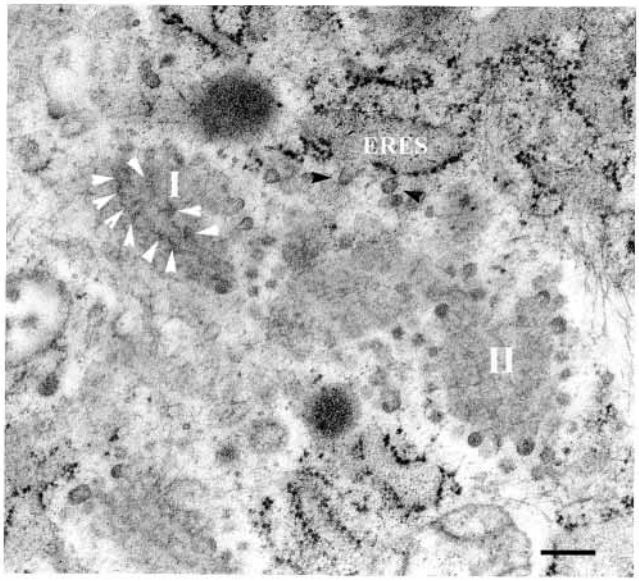

Fig. 5. EGTCs have distinct morphological profiles that may correspond to different chronological stages of its biogenesis. Transmission electron micrographs of uninfected NRK cells subjected to the rapid ethane freezing procedure followed by osmium tetroxide treatment to enhance the morphology of membrane structures. Two morphologically distinct EGTCs are on view here. The upper left structure (I) appears to have extensive electron-dense membrane structures within it (white arrowheads) that are not found in the lower right structure. These membrane structures may reflect COPII coats that are frequently seen to line budding profiles and vesicles (black arrowheads) associated with the ERES. Bar, 0.2 μm.

labeled for Sec13 and VSVG. It is clear from the serial sections (Fig. 4) that these COPII vesicle clusters are free from any membranous continuity with the ER or the Golgi and therefore represents free vesicles that have come together to form clusters. It follows that EGTCs may be derived from these structures, and the basis of the 15°C block observed for years may now be explained in terms of arrested or delayed formation of EGTCs from COPII vesicles. This notion is consistent with our observation that the number of EGTCs observed is much lower in VSV-infected cells kept at 40°C, or transferred from 40°C to 15°C, compared with cells transferred from 40°C to 32°C (data not shown).

Our observations at 15°C suggest that COPII vesicle formation does indeed occur and there is an inhibition or delay in the consumption of vesicles at low temperature. However, our method of sample processing have thus far not been of sufficient resolution and contrast in terms of membrane morphology to provide a clear picture as to how exactly EGTCs were derived from COPII vesicles. Multiple COPII vesicle clusters may eventually fuse with each other to form a nascent EGTC de novo. Alternatively, a COPII vesicle cluster may also fuse with a preformed EGTC, which may be a long living, ever-present structure. To increase the resolution of our analysis in the hope of providing a clue to the origin of EGTCs, we performed free substitution after ethane freezing in acetone followed by an osmium enhancement step. This enhancement resulted in a higher contrast in terms of membrane structures, but we were now unable to perform further immunogold labeling with these samples.

With the enhanced contrast of membrane structures, we observed that some EGTCs have distinct morphological profiles that may correspond to different chronological stages of its biogenesis. As shown in Fig. 5, some EGTCs appear to contain extensive electron dense membrane structures. This electron-dense membrane structures may represent strings of COPII vesicles that are in the process of forming a nascent EGTC by homotypic fusion. Furthermore, electron-dense coats are frequently found on budding profiles and chains and clusters associated with the ERES (Fig. 6A-C). These strings of vesicles bear morphological similarities to those formed by COPII coat proteins on liposomes (Antonny et al., 2001). It is conceivable that the initial fusion of these chains and clusters of COPII vesicles derived from the ERES results in a nascent EGTC. EGTCs may therefore be derived from COPII vesicles that coalesce through a homotypic fusion process.

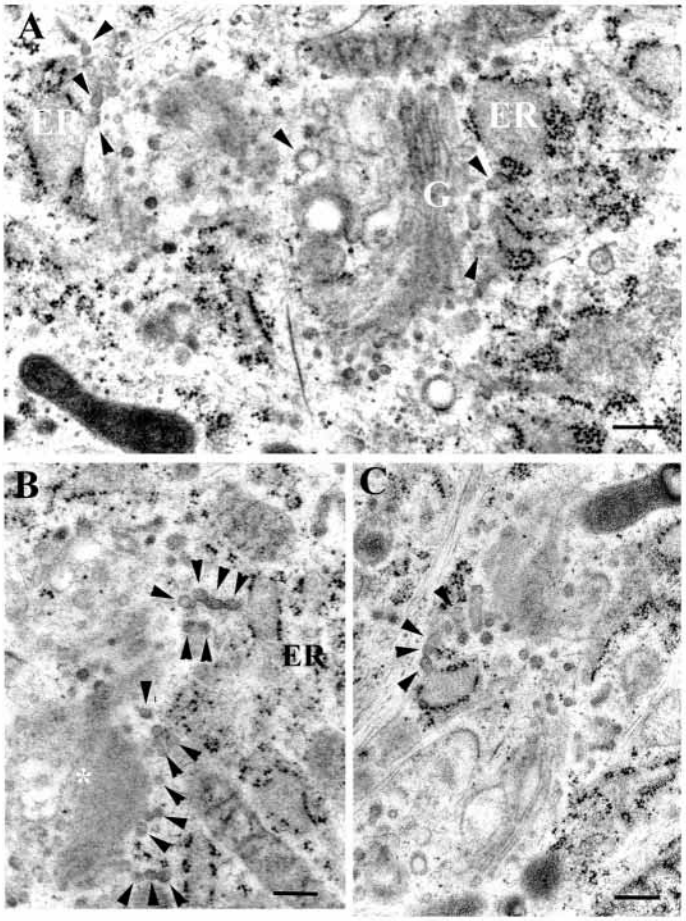

Fig. 6. EGTCs may be derived from COPII vesicle cluster/chains that coalesce by homotypic fusion processes. Transmission electron micrographs of uninfected NRK cells subjected to the rapid ethane-freezing procedure followed by osmium enhancement as in Fig. 5. (A) Electron-dense material, probably corresponding to COPII, can be found on vesicles and bud profiles associated with the ER transitional elements (arrowheads). (B,C) Vesicles with this electron-dense coat are frequently found in chains or clusters (arrowheads), and these chains and clusters may well be fusing with a nearby, existing EGTC (*).

Discussion

Ultrastructural visualization of large ER-Golgi transport carriers

The ethane-freezing procedure achieved preservation of cellular ultrastructure in the timeframe of milliseconds, rather than the seconds or minutes needed for chemical fixation. More importantly, it circumvents the problem of a loss in antigenicity owing to steric hindrance of the accessibility of antibody to epitope that may result from protein crosslinking by aldehyde fixatives. The ethane-freezing procedure allowed us to observe and analyze EGTCs in transit from the ER to the Golgi at the ultrastructural level. Several lines of evidence suggest that these structures are indeed ER-Golgi transport carriers.

The first evidence is the observation that the cargo protein VSVGts045, which is temperature-restricted in the ER, became concentrated in the EGTCs approximately 10 minutes after transfer to the permissive temperature. In this regard, two points are particularly noteworthy. In agreement with previous observations made on export complexes (Bannykh et al., 1996), EGTCs are not continuous with the ER. Importantly, EGTCs are also devoid of COPII labeling. They are therefore not synonymous with transitional elements (Palade, 1975) or the part of the export complex that are smooth regions of the ER where COPII budding occurs (Bannykh and Balch, 1997). On the other hand, when transport is arrested at 15°C upon VSVG being released from the ER, we observed VSVG-containing vesicular clusters with COPII labeling. These structures are distinct from the EGTCs. The above observations, taken together, suggest that the EGTCs are derived from strings and clusters of COPII vesicles and have lost their COPII coat.

The second line of evidence is the fact that the EGTCs are also selectively enriched in COPI. The extensive bud-forming profiles at the edges of these structures are indicative of active ongoing vesicle budding processes. Previous analyses of VTCs have implied that a coupled COPII-COPI exchange process takes place after COPII vesicle formation (Aridor et al., 1995). Imaging of VSVGts045-GFP-labeled structures in vivo also suggests that the COPII coat on these is replaced by COPI as the structures migrates from the peripheral to a perinuclear region (Scales et al., 1997). Our observations of distinct COPII-labeled vesicular clusters and COPI-labeled EGTCs are therefore in agreement with the above. Furthermore, the retrograde cargo p58 [or ERGIC53 in primate cells (Hauri et al., 2000)] is found in the COPI-positive budding profiles of EGTCs. Within the EGTCs themselves, p58 and COPI appear to be associated with the bud profiles, whereas VSVGs are found at the interior matrix of the structure, which may be filled with membranes. We have therefore shown ultrastructurally, as predicted by previous studies at the light microscopy level, that there is a segregation of anterograde and retrograde cargo at the EGTC (Tang et al., 1995; Shima et al., 1999; Stephens et al., 2000) and that COPI is responsible for segregating retrograde cargo by vesicle budding.

VSVG is not the only cargo protein that can be found in the EGTCs. Klumperman and colleagues have recently demonstrated that two lumenal secretory proteins, amylase and chymotrypsin, exhibit a first concentration step in VTC structures, which could be equivalent to the EGTCs described here (Martinez-Menarguez et al., 1999). In agreement with that, we showed here that another lumenal protein, albumin, is also concentrated in EGTCs. It is known that ‘empty’ COPI and COPII vesicles can be formed in vitro without carrying any cargo proteins (Matsuoka et al., 1998; Spang et al., 1998). The EGTCs, however, appear to be authentic carriers of both membrane and soluble cargoes.

It has been exceedingly difficult to observe membrane fusion of transport carriers of any type with the target membrane (in this case the Golgi) at the ultrastructural level. The fusion competence of COPI and COPII vesicles were assessed previously by biochemical assays. We showed here that the EGTCs contain the tethering protein p115 and could indeed be seen, albeit rarely, to physically fuse with the Golgi stack and in doing so transfer its membrane content (VSVG) to the Golgi. From COPI association, cargo concentration and segregation to fusion with the Golgi, the EGTCs observed here appeared to have all the properties that are expected of a membrane carrier functioning in ER-Golgi transport.

Where do EGTCs come from? In a model proposed by

Bannykh and Balch, VTCs are derived from COPII vesicles that have rapidly lost their coats (Bannykh and Balch, 1997). If the VTCs and the EGTCs described in this report are similar or identical structures, the EGTCs are probably also derived from COPII vesicles. Indeed, although not apparent at 32°C, VSVG-containing vesicle clusters with a distinct COPII coat can be easily observed at 15°C. Correspondingly, the number or abundance of EGTCs is diminished at 15°C compared with 32°C. There appears to be an inhibition or a slowing down in the process of COPII vesicle conversion into EGTCs.

Another important question, which we are not able to adequately address with the current experimental system, is whether nascent EGTCs can be created de novo via homotypic fusion of COPII vesicles. The obvious morphological differences amongst the EGTCs may reflect different temporal stages in the biogenesis and maturation of the structures. Nascent or immature EGTCs may have more distinct electron-dense membrane profiles corresponding to newly acquired vesicles. Another non-quantitative observation is that an 'old' EGTC that is closer to the stage of fusion with the Golgi apparatus appears to have much fewer bud-forming profiles compared with 'younger' EGTCs that are actively involved in segregating and directing retrograde transport via a COPI-mediated process. On the other hand, instead of formation of nascent EGTCs, it is also possible to envisage the scenario whereby COPII vesicles are assimilated into preformed EGTCs. A significant advance in the technique of ultrastructural analysis is necessary before this question can be answered satisfactorily.

On the basis of our observations and current knowledge, the process of EGTC-mediated ER-Golgi transport can be modeled schematically as outlined in Fig. 7. COPII-mediated cargo export from the ERES takes the form of chains or clusters of vesicles that quickly coalesce to form a nascent EGTC. Consistent with this possibility, Sar1 has the ability to induce the formation of cargo-containing membrane tubules from the ER, which can be converted into strings of vesicles by other COPII subunits (Aridor et al., 2001). This process is apparently inhibited or slowed at 15°C, and as a result COPII positive VSVG-enriched vesicle clusters become readily observable. Within the EGTC itself, anterograde cargo is segregated into interior membrane domains from retrograde cargo, and the latter are actively removed via the action of COPI. The EGTC thus 'matures' by COPI-vesicle-mediated retrograde transport of its components back to the ER. It is not absolutely clear how this occurs, but it may simply involve diffusion of COPI vesicles to the ER. As indicated by live cell observations in light microscopy, a mobile transport intermediate containing GFP-VSVG would hover near the cell periphery until COPII-COPI exchange is complete before its starts to move (Scales et al., 1997). As EGTCs 'mature', those at the cell periphery would move to the perinuclear region via microtubule tracks where it would eventually fuse with the Golgi (III).

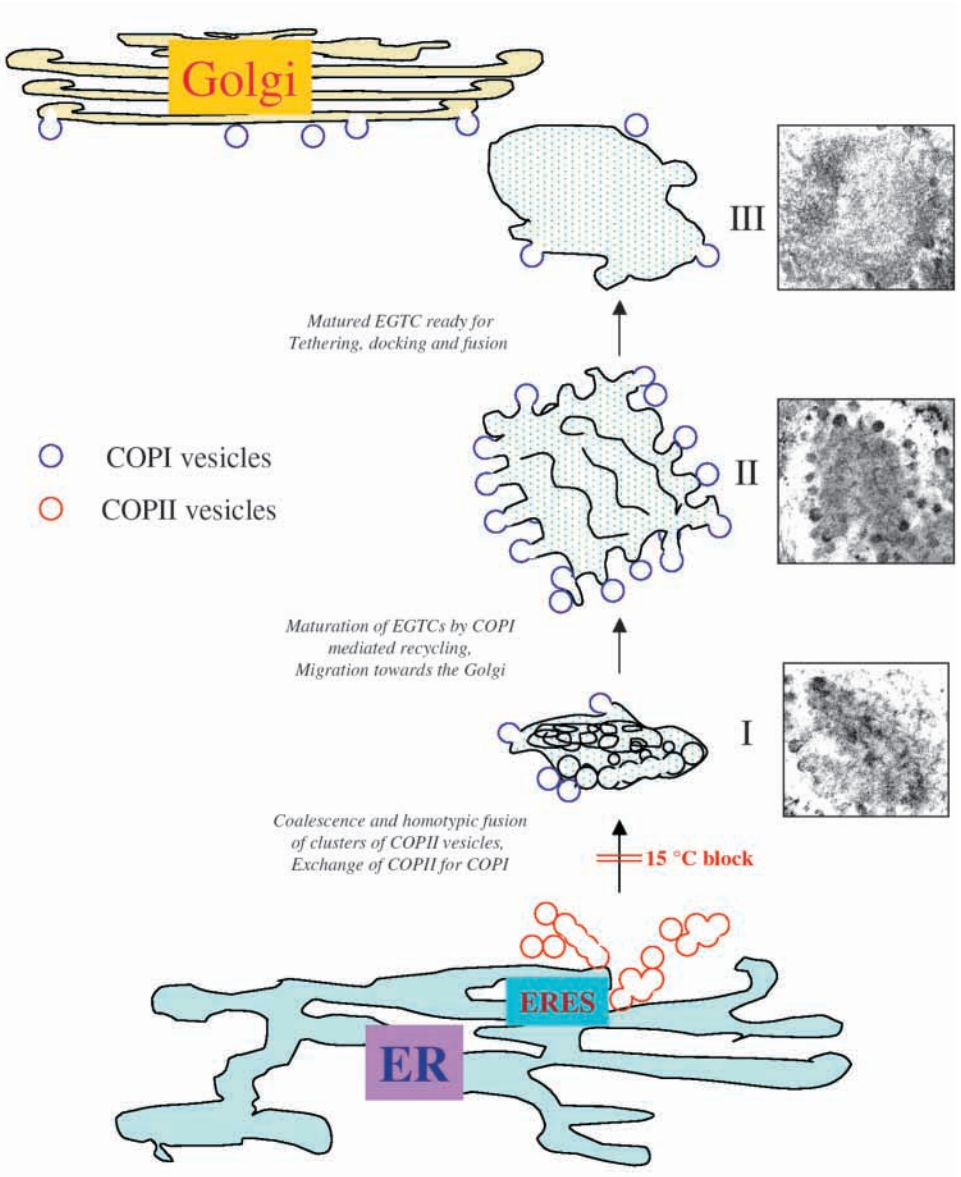

Fig. 7. (A) Schematic model of ER-Golgi transport mediated by EGTC. COPII vesicles that bud from the ERES quickly coalesce to form clusters. These could either fuse with an existing EGTC or aggregate with each other to form a nascent EGTC (I). This process is apparently inhibited or slowed at 15°C. Within the EGTC, anterograde cargo is segregated from retrograde cargo via the action of COPI, and the EGTC 'matures' by COPI-mediated retrograde transport of its components back to the ER (II). 'Mature' EGTCs travel to the perinuclear region via microtubule tracks where it eventually fuses with the Golgi (III).

EGTCs and GPCs – a new perspective in membrane transport

Using correlative light-electron microscopy, Mironov and colleagues were able to monitor the life cycle of VSVG-GFP-containing Golgi-to-plasma membrane carriers (GPCs) and take a snapshot of their ultrastructure at any point during transit (Polischuk et al., 2000). The GPCs are similar in dimensions to the EGTCs (ranging from 0.3 to 1.7 μm). It is not known for certain how they were formed in the first place, although the authors favor a maturation process involving the controlled drawing out and then progressive breakdown of TGN material from the Golgi body. Interestingly, the API coat could be detected on some GPCs, and it may serve a function analogous to COPI on EGTCs. GPCs were also found to fuse with the plasma membrane directly and en bloc, without any dramatic shape change or breakdown into small fragments. The mode of membrane fusion of EGTCs therefore resembles that of GPCs.

The exact mechanism of how vesicles may be directed to and from the Golgi has remained largely speculative. With regards to ER to Golgi transport, consolidating a large number of vesicles into a few large transport containers would simplify trafficking, both in terms of availability of motor proteins for mobility and the regulation of their association with the carrier. A large membrane container would also provide the dimension or structural basis necessary for effective segregation of retrograde cargo from anterograde cargo into subdomains. Likewise, a stochastic process of small vesicle budding from the TGN would make directed transport to the plasma membrane difficult and inefficient. The demonstration of the presence of EGTCs and GTCs at the ultrastructural level puts into perspective the notion that large, membranous cargo carriers mediate the anterograde transport between the major stations of the exocytic pathway. However, this does not appear to be the end of the story. Recent reports suggest that both the TGN and the ER are able to form tubular structures (Liljedahl et al., 2001; Aridor et al., 2001). Although the relationship between these tubular structures and the EGTCs or GTCs is uncertain, there is excellent evidence that these tubules are also functional transport intermediates. Therefore, instead of small 60-100 nm vesicles, we now have to revise our various models of membrane biogenesis, movement, docking and fusion with these new perspectives in mind. Future efforts would be directed towards the understanding of the mechanisms responsible for their formation, their interaction with microtubules, the mechanistic basis of their mobility and the details of the processes that lead to their docking and fusion with target membranes

We are grateful to Jakko Saraste and Rainer Pepperkok for their gifts of antibodies, Kathryn Howell for advice and discussions and Brian Storrie for valuable comments. B.L.T. is an appointee of the Office of Life Science, National University of Singapore and is grateful to Ya Wang for technical assistance. W.H. is also a faculty member of the Department of Biochemistry, National University of Singapore. W.H. and B.L.T. are supported by grants from the Agency for Science, Technology and Research of Singapore to the Institute of Molecular and Cell Biology.

References

Allan, B. B. and Balch, W. E. (1999). Protein sorting by directed maturation of Golgi compartments. *Science* **285**, 63-66.

Allan, B. B., Moyer, B. D. and Balch, W. E. (2000). Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. *Science* **289**, 444-448.

Alvarez, C., Fujita, H., Hubbard, A. and Sztul, E. (1999). ER to Golgi transport: Requirement for p115 at a pre-Golgi VTC stage. *J. Cell Biol.* **147**, 1205-1222.

Antonny, B., Madden, D., Hamamoto, S., Orci, L. and Schekman, R. (2001). Dynamics of COPII coat with GTP and stable analogues. *Nat. Cell Biol.* **3**, 531-537.

Aridor, M., Bannykh, S. I., Rowe, T. and Balch, W. E. (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. *J. Cell Biol.* **131**, 875-893.

Aridor, M., Fish, K. N., Bannykh, S., Weissman, J., Roberts, T. H., Lippincott-Schwartz, J. and Balch, W. E. (2001). The Sar1 GTPase coordinates biosynthetic cargo selection with endoplasmic reticulum export site assembly. *J. Cell Biol.* **52**, 213-229.

Bannykh, S. I. and Balch, W. E. (1997). Membrane dynamics at the endoplasmic reticulum-Golgi interface. *J. Cell Biol.* **138**, 1-4.

Bannykh, S. I., Rowe, T. and Balch, W. E. (1996). Organization of endoplasmic reticulum export complexes. *J. Cell Biol.* **135**, 19-35.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, H., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M. and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum *Cell* **77**, 895-907.

Bednarek, S. Y, Ravazzola, M., Hosobuchi, M., Amherdt, M., Perrelet, A., Schekman, R. and Orci, L. (1995). COPI- and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. *Cell.* **83**, 1183-1196.

Bonfanti, L., Mironov, A. A., Jr, Martinez-Menarguez, J. A., Martella, O., Fusella, A., Baldassarre, M., Buccione, R., Geuze, H. J., Mironov, A. A. and Luini, A. (1998). Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. *Cell.* **95**, 993-1003.

Cole, N. B. and Lippincott-Schwartz, J. (1995). Organization of organelles and membrane traffic by microtubules. *Curr. Opin. Cell Biol.* **7**, 55-64.

Hauri, H.-P., Kappeler, F., Andersson, H. and Appenzeller, C. (2000). ERGIC-53 and traffic in the secretory pathway. *J. Cell Sci.* **113**, 587-596.

Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. F., Siggia, E. D., Phair, R. D. and Lippincott-Schwartz, J. (1998). Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. *J. Cell Biol.* **143**, 1485-1503.

Hong, W. (1998). Protein transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Sci.* **111**, 2831-2839.

Krijnse-Locker, J., Ericsson, M., Rottier, P. J. and Griffiths, G. (1994). Characterization of the budding compartment of mouse hepatitis virus: evidence that transport from the RER to the Golgi complex requires only one vesicular transport step. *J. Cell Biol.* **124**, 55-70.

Lahtinen, U., Dahllof, B. and Saraste, J. (1992). Characterization of a 58 kDa cis Golgi protein in pancreatic exocrine cells. *J. Cell Sci.* **103**, 321-333.

Lahtinen, U., Hellman, U., Wernstedt, C., Saraste, J. and Pettersson, R. F. (1996). Molecular cloning and expression of a 58-kDa cis-Golgi and intermediate compartment protein. *J. Biol. Chem.* **271**, 4031-4037.

Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., van Lint, J. and Malhotra, V. (2001). Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. *Cell* **104**, 409-420.

Lippincott-Schwartz, J. and Smith, C. L. (1997). Insights into secretory and endocytic membrane traffic using green fluorescent protein chimeras. *Curr. Opin. Neurobiol.* **7**, 631-639.

Lippincott-Schwartz, J., Cole, N. and Presley, J. (1998). Unravelling Golgi membrane traffic with green fluorescent protein chimeras. *Trends Cell Biol.* **8**, 16-20.

Lowe, M. and Kreis, T. E. (1998). Regulation of membrane traffic in animal cells by COPI. *Biochim. Biophys. Acta* **14**, 53-66.

Malhotra, V., Serafini, T., Orci, L., Shepherd, J. C. and Rothman, J. E. (1989). Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* **58**, 329-336.

Martinez-Menarguez, J. A., Geuze, H. J., Slot, J. W. and Klumperman, J. (1999). Vesicular tubular clusters between the ER and the Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell* **98**, 81-90.

Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Schekman, R. and Yeung., T. (1998). COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. *Cell* **93**, 263-275.

Mellman, I. and Simons, K. (1992). The Golgi complex: In vitro veritas? *Cell* **68**, 829-840.

Mironov, A. A., Weidman, P. and Luini, A. (1997). Variations on the intracellular transport theme: maturing cisternae and trafficking tubules. *J. Cell Biol.* **138**, 481-484.

Nilsson, T. and Warren, G. (1994). Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Curr. Opin. Cell Biol.* **6**, 517-521.

Palade, G. E. (1975). Intracellular aspects of the process of protein transport. *Science* **189**, 347-354.

Parsons, T. D., Coorssen, J. R., Horstmann, H. and Almers, W. (1995). Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. *Neuron* **15**, 1085-1096.

Pelham, H. R. B. (1989). Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5**, 1-23.

Pepperkok, R., Scheel, J., Horstmann, H., Hauri, H-P., Griffiths, G. and Kreis, T. E. (1993). β-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. *Cell* **74**, 71-82.

Peter, F., Wong, S. H., Subramaniam, V. N., Tang, B. L. and Hong, W. (1998). α-SNAP but not γ-SNAP is required for ER-Golgi transport after vesicle budding and the Rab1-requiring step but before the EGTA-sensitive step. *J. Cell Sci.* **111**, 2625-2633.

Polishchuk, R. S., Polishchuk, E. V., Marra, P., Alberti, S., Buccione, R., Luini, A. and Mironov, A. A. (2000). Correlative light-electron microscopy reveals the tubular-saccular ultrastructure of carriers operating between Golgi apparatus and plasma membrane. *J. Cell Biol.* **148**, 45-58.

Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J. and Lippincott-Schwartz, J. (1997). ER-to-Golgi transport visualized in living cells. *Nature* **389**, 81-85.

Rothman, J. E. and Wieland, F. T. (1996). Protein sorting by transport vesicles. *Science* **272**, 227-234.

Rowe, T., Aridor, M., McCaffery, M., Plutner, H., Nuoffer, C. and Balch, W. E. (1996). COPII vesicles derived from mammalian endoplasmic reticulum microsomes recruit COPI. *J. Cell Biol.* **135**, 895-911.

Saetzler, K., Soehl, L. F., Bollmann, J. H., Borst, J. G., Frotscher, M., Sakmann, B. and Luebke, H. R. (2002). Three dimensional reconstruction of a calyx of Held and its postsynaptic principal neuron in the MNTB. *J. Neurosci.* (in press).

Sapperstein, S. K., Lupashin, V. V., Schmitt, H. D. and Waters, M. G. (1996). Assembly of the ER to Golgi SNARE complex requires Uso1p. *J. Cell Biol.* **132**, 755-767.

Saraste, J. and Kuismanen, E. (1984). Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell* **38**, 535-549.

Scales, S. J., Pepperkok, R. and Kreis, T. E. (1997). Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* **90**, 1137-1148.

Schweizer, A., Fransen, J. A., Matter, K., Kreis, T. E., Ginsel, L. and Hauri, H.-P. (1990). Identification of an intermediate compartment involved in protein ransport from endoplasmic reticulum to Golgi apparatus. *Eur. J. Cell Biol.* **53**, 185-196.

Shima, D. T., Scales, S. J., Kreis, T. E. and Pepperkok, R. (1999). Segregation of COPI-rich and anterograde-cargo-rich domains in endoplasmic-reticulum-to-Golgi transport complexes. *Curr Biol.* **9**, 821-824.

Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R. and Orci, L. (1998). Coatomer, Arf1p, and nucleotide are required to bud coat protein complex I-coated vesicles from large synthetic liposomes. *Proc. Natl. Acad. Sci. USA* **95**, 11199-11204.

Stephens, D. J. and Pepperkok, R. (2001). Illuminating the secretory pathway: when do we need vesicles? *J. Cell Sci.* **114**, 1053-1059.

Stephens, D. J., Lin-Marq, N., Pagano, A., Pepperkok, R. and Paccaud, J. P. (2000). COPI-coated ER-to-Golgi transport complexes segregate from COPII in close proximity to ER exit sites. *J. Cell Sci.* **113**, 2177-2185.

Steyer, J. A., Horstmann, H. and Almers, W. (1997). Transport, docking and exocytosis of single secretory granules in live chromaffin cells. *Nature* **388**, 474-478.

Subramaniam, V. N., Peter, F., Philp, R., Wong, S. H. and Hong, W. (1996). GS28, a 28-kilodalton Golgi SNARE that participates in ER-Golgi transport. *Science* **272**, 1161-1163.

Tang, B. L., Low, S. H., Hauri, H.-P. and Hong, W. (1995). Segregation of ERGIC53 and the mammalian KDEL receptor upon exit from the 15 degrees C compartment. *Eur. J. Cell Biol.* **68**, 398-410.

Tang, B. L., Peter, F., Krijnse-Locker, J., Low, S. H., Griffiths, G. and Hong, W. (1997). The mammalian homolog of yeast Sec13p is enriched in the intermediate compartment and is essential for protein transport from the endoplasmic reticulum to the Golgi apparatus. *Mol. Cell. Biol.* **17**, 256-266.

Tang, B. L., Zhang, T., Low, D. Y. H., Wong, E. T., Horstmann, H. and Hong, W. (2000). Mammalian homologues of yeast Sec31p: an ubiquitously expressed form is localized to ER exit sites and is essential for ER-Golgi transport. *J. Biol. Chem.* **275**, 13597-13604.

Tang, B. L., Ong, Y. S., Huang, B., Wong, E. T., Qi, R., Horstmann, H. and Hong, W. (2001). A membrane protein enriched in ER exit sites interacts with COPII. *J. Biol. Chem.* **276**, 40008-40017.

Toomre, D., Keller, P., White, J., Olivo, J. C. and Simons, K. (1999). Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells. *J. Cell Sci.* **112**, 21-33.

Waters, M. G. and Pfeffer, S. R. (1999). Membrane tethering in intracellular transport. *Curr. Opin. Cell Biol.* **11**, 453-459.

Zhang, T., Wong, S. H., Tang, B. L., Xu, Y., Peter, F., Subramaniam, V. N. and Hong, W. (1997). The mammalian protein (rbet1) homologous to yeast Bet1p is primarily associated with the pre-Golgi intermediate compartment and is involved in vesicular transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* **139**, 1157-1168.

Zhang, T., Wong, S. H., Tang, B. L., Xu, Y. and Hong, W. (1999). Morphological and functional association of Sec22b/ERS-24 with the pre-Golgi intermediate compartment. *Mol. Biol. Cell.* **10**, 435-453.