Differential effects of a GTP-restricted mutant of Sar1p on segregation of cargo during export from the endoplasmic reticulum

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Summary

Export of cargo from the endoplasmic reticulum (ER) is the first membrane trafficking step in the secretory pathway. To date, all cargo proteins appear to use a common set of machinery for the initial stages of export, namely the COPII coat complex. Recent data from both yeast and mammalian systems have emerged suggesting that specific cargoes could be sorted from one another at the point of exit from the endoplasmic reticulum or immediately afterwards. Here, we have examined the mechanisms used for export of different types of cargo molecule from the endoplasmic reticulum. All cargoes examined utilise the COPII machinery, but specific differences are seen in the accumulation of cargo into ER-derived pre-budding complexes following expression of a GTP-restricted mutant of the Sar1p GTPase. Glycosylphosphatidylinositol (GPI)-anchored GFP is seen to be restricted to the ER under these conditions whereas other cargoes, including ts045-G and lumFP accumulate in pre-budding complexes. Following exit, GPI-FP, lumFP and ts045-G-FP all travel to the Golgi in the same vesicular tubular clusters (VTCs). These data show a differential requirement for efficient GTP hydrolysis by the Sar1p GTPase in export of cargo from the ER.

Key words: GPI anchor, ER, COPII, Cargo sorting, GTP hydrolysis

Introduction

Transport of proteins from the endoplasmic reticulum (ER) to the Golgi is the first transport step in the secretory pathway (Barlowe, 2002; Klumperman, 2000). Data have accumulated into a model in which transport is mediated by the sequential action of the coat protein complexes II and I (COPII and COPI) (Aridor et al., 1995; Scales et al., 1997; Stephens et al., 2000). Export of cargo from the ER is coordinated by assembly of the COPII coat (Barlowe, 2002; Barlowe et al., 1994) at discrete sites on the ER membrane known as ER export sites (ERES) or transitional ER (Orci et al., 1991). In yeast and mammalian cells assembly of the COPII coat is directed by Sec12p-dependent GDP-GTP exchange on the small GTPase, Sar1p (Nakano et al., 1989; Weissman et al., 2001). Sar1p activation by Sec12p results in recruitment of the Sec23p-Sec24p complex, and subsequently the Sec13p-Sec31p complex, to these cargo exit sites.

Transmembrane proteins are exported from the ER in a signal-mediated manner (Barlowe, 2003) often through direct coupling to the COPII coat (Miller et al., 2003; Mossessova et al., 2003). Many soluble cargoes couple to the COPII machinery by engaging with receptors that physically link soluble cargo within the lumen of the ER with the cytosolic vesicle budding machinery. Examples of this include ERGIC-53 which acts as a lectin for the export of blood coagulation factors V and VIII (Nichols et al., 1998) and Erv29p which packages glycosylated pro α-factor in yeast (Belden and Barlowe, 2001). Data from experiments in yeast have shown that different cargoes can be sorted into different populations of COPII coated vesicles (Muniz et al., 2001). Specifically, the glycosylphosphatidylinositol (GPI)-anchored cargo Gas1p, which cannot couple to cytosolic machinery, was found in a distinct population of vesicles to the transmembrane cargo Gap1p. This process appears to be mediated by the action of the small GTPase Ypt1p, by tethering factors and SNARE proteins (Morsomme et al., 2003; Morsomme and Riezman, 2002). In addition, we have previously shown that procollagen (PC), is transported in pre-Golgi carriers that are distinct from those containing other cargoes (Stephens and Pepperkok, 2002).

Introduction of a mutation (H79G) into Sar1p results in a protein with a significantly reduced ability to hydrolyse GTP. Sar1p$^{(H79G)}$ supports the budding of COPII vesicles during in vitro reactions (Nakano et al., 1994; Saito et al., 1998) but does not support productive transport to the Golgi (Aridor et al., 1995; Oka and Nakano, 1994; Saito et al., 1998). Incubation of permeabilised mammalian cells with Sar1p$^{(H79G)}$ results in the formation of tubular structures emanating from cargo export sites on the ER membrane (Aridor et al., 2001). Interestingly, these tubules label for Sar1p$^{(H79G)}$ along their length and contain ts045-G (Aridor et al., 2001). Sequestration of ts045-G into these structures was found to be a result of direct interaction between its cytosolic domain and Sar1p (Aridor et al., 2001), which led to the conclusion that Sar1p coordinates cargo selection with export site assembly.

Here, we have investigated the molecular mechanism by which different classes of cargo are exported from the ER. Secretory transport has been typically studied using ts045-G, a temperature sensitive transmembrane glycoprotein from the vesicular stomatitis virus (Balch and Keller, 1986; Kreis, 1986; Presley et al., 1997; Scales et al., 1997; Stephens et al., 2000). Here, we have investigated the transport requirements of different classes of cargo proteins tagged with spectral variants of green fluorescent protein (GFP): transmembrane cargo [ts045-G-FP (Scales et al., 1997)], small soluble cargo [lumFP (Blum et al., 2000)], large macromolecular soluble cargo [procollagen-FP (Stephens and Pepperkok, 2002)] and lipid anchored cargo [GPI-FP (Keller et al., 2001)]. Notably, lumFP, PC-FP and GPI-FP would all, including Sar1p, be incapable of interacting with the cytosolic COPII machinery directly. We show that all cargoes with the exception of procollagen are transported to the Golgi in the same vesicular tubular clusters (VTCs). Efficient transport of all cargoes requires the function of COPI, COPII, Rab proteins and the dynein-dynactin complex. GPI-anchored proteins are transported to the Golgi in structures that also contain ERGIC-53 and ts045-G. We also show that, in contrast to lumFP and ts045-G, neither procollagen nor GPI-anchored cargo is effectively packaged into nascent COPII-coated structures in the presence of GTP-γ-S or on expression of a GTP-restricted mutant of Sar1p. Our results reveal a selective requirement for efficient GTP hydrolysis by Sar1p to effectively sort a subset of cargo into COPII coated ER export sites.

Materials and Methods

All chemical reagents were purchased from Sigma (Poole, UK). unless stated otherwise.

Cell culture and expression of markers

HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) (Invitrogen, Glasgow, UK). In all cases, identical results were obtained using African green monkey kidney (Vero) cells (ATCC CCL81). Cells were plated 24-48 hours prior to injection on live-cell dishes (MatTek, Ashland, MA, USA) and injected using an Eppendorf Femtojet/Injectman system (Eppendorf, Cambridge, UK). Live cells were imaged in imaging medium [modified Eagle's medium (MEM) without phenol red, supplemented with 30 mM HEPES, pH 7.4 and 0.5 g l^{-1} sodium bicarbonate]. Details of plasmids encoding cargo proteins are detailed in the appropriate references: FP-Sec23A (Stephens, 2003), PC-FP (Stephens and Pepperkok, 2002), lumFP (Blum et al., 2000; Stephens et al., 2000), ts045-G-FP (Scales et al., 1997; Toomre et al., 1999) and GPI-FP (Keller et al., 2001). Spectral variants of GFP, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are referred to interchangeably in the text as FP. Temperature blocks of 15°C were generated by incubating cells in a water bath at 15°C in imaging medium containing 10% FCS. Expression of $ARF1^{(Q71L)}$ (Dascher and Balch, 1994) and $Sar1p^{(H79G)}$ (Kuge et al., 1994) mutants, $p50^{dynamitin}$ (Echeverri et al., 1996) and Rab-GDIβ (Yang et al., 1994) was achieved by co-injection of plasmid DNA encoding the respective proteins with DNA encoding the marker of interest. Plasmids were purified using Qiagen plasmid Midi kits (Qiagen, Crawley, UK) and centrifuged at 25,000 ***g*** before use. In each case, plasmids encoding cargo proteins were injected at 10 ng μl^{-1} with plasmids encoding inhibitor proteins at 50 ng μl^{-1}. BFA was used at a final concentration of 5 μg ml^{-1}, GTP and GTP-γ-S at 50 μM.

YFP-Sec23Ap was characterised as previously described for GFP-Sec24Dp (Stephens et al., 2000). Briefly, when expressed in either HeLa or Vero cells, YFP-Sec23Ap colocalises with Sec24p, Sec13p and Sec31p, it does not inhibit transport when expressed at low level and functionally interacts with Sar1p in vitro and in vivo (data not shown). For further details see (Stephens, 2003).

Wide-field and confocal microscopy

For immunofluorescence, cells were fixed using 3.5% paraformaldehyde, permeabilised with 0.1% Triton X-100 and immunostained as described (Stephens et al., 2000). Primary antibodies were detected using anti-mouse or anti-rabbit secondary antibodies labelled with Alexa-488, Alexa-568 or Alexa-647 as required. Living and fixed cells were imaged using an Olympus/TILL Photonics imaging system as described previously (Stephens, 2003). Live cells were imaged at 37°C with the microscope enclosed in a heated Perspex box (Solent Scientific, Portsmouth, UK).

Fluorescence recovery after photobleaching (FRAP) experiments were performed on a Leica TCS SP2 AOBS confocal microscope attached to a Leica DM IRE2 inverted microscope using a 63× PLAPO BL N.A. 1.4 oil immersion lens. GFP fusion proteins were excited and bleached using the 488 nm line of an argon laser. Cells were grown as described above and maintained at 37°C using a temperature-controlled heating-perfusion system (Digitimer, Welwyn Garden City, UK) combined with Perspex incubation chamber (Solent Scientific). Pre- and post-bleach images were acquired at low laser power (attenuated to 10% using an acousto-optical tuneable filter) with the pinhole set at 1 Airy disk and two-fold line averaging. FRAP within a specific region of interest was achieved using the FRAP macro function within the Leica LCS software. Fluorescence intensity within the entire bleached region was quantified using ImageJ and presented graphically using Microsoft Excel and Adobe Illustrator.

Results

We have studied ER-to-Golgi transport of four 'marker' cargoes: (1) ts045-G-FP (Scales et al., 1997; Toomre et al., 1999), a transmembrane cargo protein derived from the vesicular stomatitis virus fused to green fluorescent protein (GFP) (spectral variants of which are from here on described as FPs), (2) lumFP (Blum et al., 2000), a small soluble cargo protein with a cleavable signal sequence and which contains no known sorting signals; (3) GPI-FP (Keller et al., 2001), a GPI-anchored form of GFP, previously shown to reflect transport pathways of GPI-anchored proteins, and (4) PC-FP, a marker for the transport of procollagen (Stephens and Pepperkok, 2002).

We have previously shown that procollagen can be sorted from other cargoes at or shortly after the point of ER export (Stephens and Pepperkok, 2002). ER-to-Golgi transport of small soluble cargo (lumFP) following release of a 15°C temperature block has also been examined and found to occur in tubular structures, distinct from punctate VTCs carrying ts045-G-FP (Blum et al., 2000). More recent data have suggested that export may indeed be mediated by tubular carriers emerging from the ER at sites directly adjacent to those labelled with COPII (Mironov et al., 2003). Evidence has also been presented for pre-Golgi sorting of GPI-anchored proteins from other cargoes (Muniz et al., 2001). These studies covered a wide range of proteins in diverse systems and so we chose to investigate the transport of different classes of cargo out of the ER in a comparative study. We initially asked whether GPI-anchored cargoes are transported to the Golgi in mammalian cells using distinct carriers to those containing other protein

cargoes, as is the case for *Saccharomyces cerevisiae* (Muniz et al., 2001). When cells are incubated at 15°C, transport is inhibited at a point very shortly after exit of proteins from the ER (Blum et al., 2000; Klumperman et al., 1998; Kuismanen et al., 1992). Under these conditions, ts045-G-FP and lumFP colocalise directly adjacent to ER exit sites (Blum et al., 2000) (Fig. 1A-C, arrowheads). Subsequent transport of lumFP from the 15°C compartment to the Golgi occurs in punctate structures that colocalise with ts045-G-FP (Blum et al., 2000). In addition to these punctate structures, lumFP-containing tubules were seen emanating from the ER upon warming from 15°C to 32°C that exclude ts045-G-FP (Blum et al., 2000). Here we describe these two cargoes as colocalising at essentially all points after exit from the ER. The key difference between these experiments is the use of a temperature-block and re-warming protocol in (Blum et al., 2000). Tubules containing lumFP are only seen when this protocol is employed and not under the 'steady state' conditions used in the current experiments. However, at 15°C, these carriers are distinct from those containing PC-FP (Fig. 1D-F, arrows) as they are during subsequent transport to the Golgi (Stephens and Pepperkok, 2002). Significantly, co-expression of GPI-FP with either ts045-G-FP or lumFP (Fig. 1G-I and J-L, respectively), followed by inhibition of transport using a 15°C temperature block leads to the presence of those molecules in the same carriers (Fig. 1E-H, arrowheads). GPI-FP and lumFP also localise to the same Golgi-directed VTCs when imaged at early time points (30-60 minutes) after microinjection of encoding plasmids (not shown), consistent with equivalent rates of synthesis and secretion. These '15°C-structures' that contain lumFP also localise at, or directly adjacent to, COPII-coated structures (labelled with an anti-Sec13p antibody) (Fig. 2A-C) and the itinerant ER-to-Golgi recycling protein ERGIC-53 (Fig. 2D-F).

To address the general molecular requirements for the transport of different classes of cargo from the ER to the Golgi in mammalian cells, we analysed the effect of targeted inhibition of traffic through the early secretory pathway upon the transport of different classes of cargo molecule in living cells. Live-cell imaging was used to avoid differences in localisation induced by chemical fixation. When expressed in cells, lumFP is barely detectable owing to its rapid rate of secretion (Blum et al., 2000) (Fig. 3A). Consequently, analysis of the early stages of secretion of lumFP is simply achieved by visualising the protein retained within the cell following inhibition of ER export or subsequent ER-to-Golgi transport. In unperturbed cells, GPI-

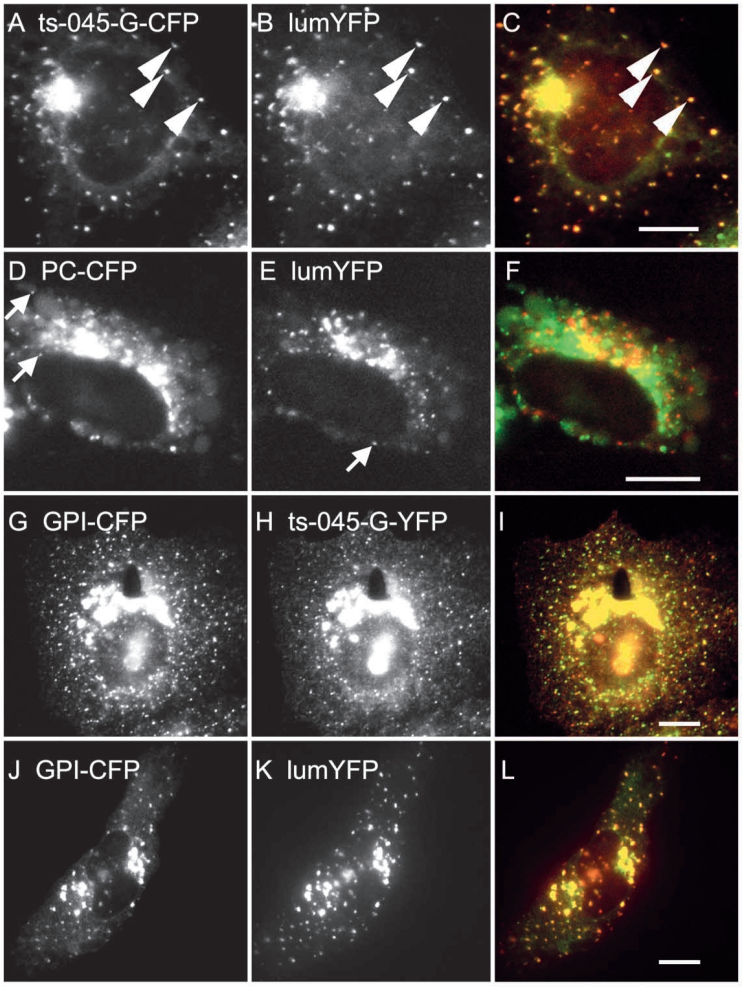

Fig. 1. Inhibition of transport at 15°C. Cells were transfected with plasmid DNA encoding cargo proteins and incubated at 15°C for 2 hours prior to imaging. ts045-G-CFP (A) colocalises with lumYFP (B) (arrowheads) whereas procollagen-CFP (D) is localised to different discrete structures when compared with lumYFP (arrows) (E). GPI-CFP (G and J) colocalises with both ts045-G-YFP (H) and lumYFP (K). C,F,I,L are merged images of the respective images in the same row. Bars, 10 μm.

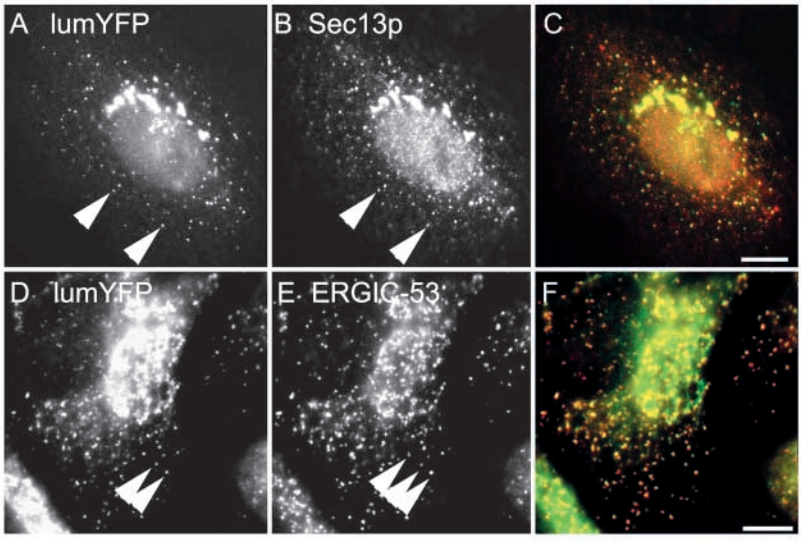

Fig. 2. Colocalisation of lumFP, COPII and ERGIC-53 at 15°C. Cells were transfected with plasmid DNA encoding lumYFP and incubated at 15°C for 2 hours prior to fixation, processing for immunofluorescence and imaging. lumYFP (A,D) colocalises with COPII (Sec13p) (B) and ERGIC-53 (E) (arrowheads). C and F are merged images of the respective images in the same row. Bars, 10 μm.

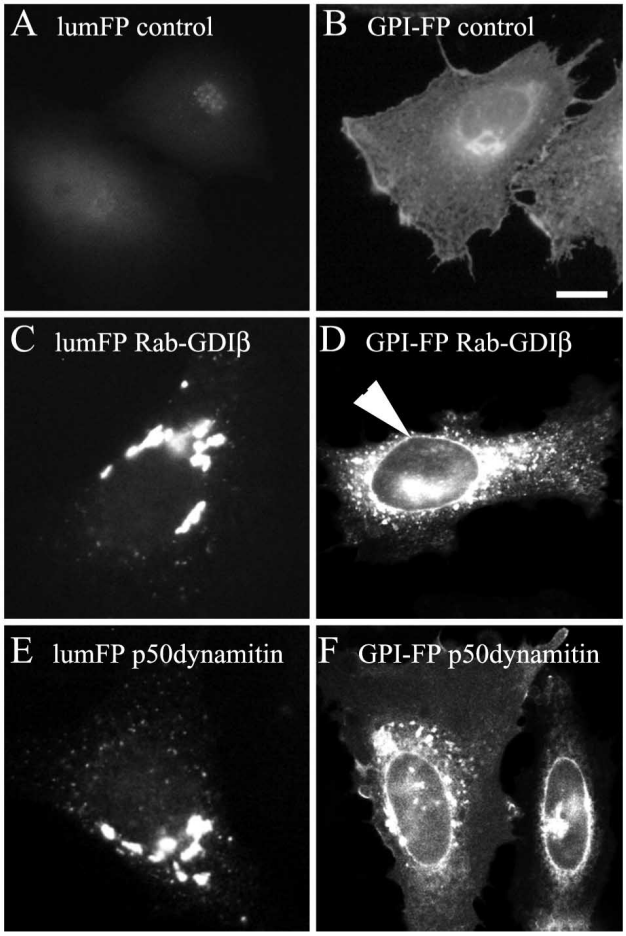

Fig. 3. General dependence of secretory cargo exit on functional Rab proteins and dynactin complex. Cells were co-injected with plasmid DNA encoding either lumYFP (A,C,E) or GPI-YFP (B,D,F) and CFP (A,B), Rab-GDIβ (C,D) or p50^{dynamitin} (E,F). LumYFP and GPI-YFP plasmids were injected at a concentration of 10 ng μl^{-1} with inhibitor encoding plasmids at a concentration of 50 ng μl^{-1}. Panels show the localisation of lumFP or GPI-FP in living cells, imaged at 37°C after 3 hours of expression. Arrowhead in D shows nuclear envelope. Bars, 10 μm.

FP is rapidly transported to the plasma membrane when expressed (Keller et al., 2001) (Fig. 3B). Fig. 3 and Table 1 show that effective transport of small soluble cargo and GPI-anchored cargo through the early secretory pathway is blocked by inhibition of Rab protein function by overexpression of Rab-GDIβ (Fig. 3C and D, notice the staining of the nuclear envelope in Fig. 3D, arrowhead) and inhibition of dynein-mediated transport through expression of the p50^{dynamitin} subunit of the dynactin complex (Fig. 3E and Fig. 2F). By contrast, microinjection of an "empty" expression vector shows no inhibition of transport. In these experiments, overexpression of p50^{dynamitin} or Rab-GDIβ does not block the early secretory pathway as effectively as the inhibition of the COPI and COPII coat complexes does, shown by transport of GPI-FP to the plasma membrane (see Fig. 3D and F). This is probably a reflection of the fact that microtubule-based transport is not essential for secretion but greatly enhances its efficiency (Cole and Lippincott-Schwartz, 1995; Mizuno and Singer, 1994). Secretion of ts045-G-FP is controlled by virtue of it being a temperature-sensitive mutant that accumulates in the ER at 39.5°C but rapidly exits and is transported to the Golgi at 32°C (Balch and Keller, 1986; Kreis, 1986). Exit of PC-FP from the ER is regulated by the addition of ascorbate, which is required for the folding of procollagen (Prockop and Kivirikko, 1995; Stephens and Pepperkok, 2002). In the absence of ascorbate, PC-FP remains within the ER and upon ascorbate addition, is transported through the secretory pathway. We show that transport of all classes of cargo examined – transmembrane (ts045-G-FP), small soluble (lumFP), large soluble (PC-FP) and GPI-anchored (GPI-FP) are dependent upon the COPI complex – by overexpression of a GTP-restricted ARF1p$^{(Q79L)}$ mutant and also by microinjection of a COPI-inhibiting antibody anti-EAGE (Pepperkok et al., 1993) (Table 1).

To investigate the requirements for export of different classes of cargo from the ER, we first studied the effect of expression of Sar1p$^{(H79G)}$ [a GTP-restricted mutant of Sar1p (Aridor et al., 1995; Oka and Nakano, 1994; Saito et al., 1998)] on cargo export. Co-expression of lumFP and Sar1p$^{(H79G)}$ results in accumulation of lumFP in intracellular structures

Table 1. Localisation of secretory proteins on inhibition of ER-to-Golgi transport

	Sar1p$^{(H79G)}$ [COPII function]	Anti-EAGE [COPI function]	ARF1$^{(Q71L)}$ [COPI function]	p50^{dynamitin} [dynein-dynactin]	Rab-GDIβ [Rab1 and Rab2]	Control [pcDNA3.1–]
PC-FP	ER	ER	ER	Punctate	Punctate	Secreted
ts045-G-FP	Punctate (juxtanuclear)	Punctate (juxtanuclear)	Punctate (juxtanuclear)	Punctate and plasma membrane	Punctate	Plasma membrane
LumFP	Punctate (juxtanuclear)	Punctate (juxtanuclear)	Punctate (juxtanuclear)	Punctate	Punctate	Secreted
GPI-FP	ER	ER	ER	ER, punctate and plasma membrane	ER and punctate	Plasma membrane

BFA treated cells were co-injected with plasmids encoding the respective marker along with a five-fold concentration excess of plasmids encoding the respective inhibitor in combinations shown in the table. Anti-EAGE was microinjected into cells expressing cargo in the presence of BFA. BFA was washed out and cells incubated for a further 2 hours. Inhibition of ER-to-Golgi transport was determined as described in the text by imaging live cells and after immunostaining with antibodies directed against ERGIC-53 to identify ER-to-Golgi TCs. Following the inhibition by Sar1p$^{(H79G)}$, anti-EAGE or ARF1$^{(Q71L)}$, 90% (±5%) of punctate structures were ERGIC-53 positive. For p50^{dynamitin} or Rab-GDIβ mediated inhibition, 60% (±10%) of structures were ERGIC-53 positive probably reflecting the lower efficacy of these inhibitors in blocking ER-to-Golgi transport. These experiments represent 'end-point' localizations for each of the cargo proteins. Identical results were obtained following expression of inhibitors for 8 or 16 hours. Between 30 and 50 cells were examined for each experiment and the tabulated results reflect the localization in >90% of these cells.

localised to the juxtanuclear region (Fig. 4A). These structures also contain ts045-G-FP (Fig. 5) and ERGIC-53 (not shown). By contrast, when GPI-FP and Sar1p$^{(H79G)}$ are expressed together in the same cell, GPI-FP localises predominantly to the ER without any obvious labelling of post-ER structures (Fig. 4B, notice nuclear envelope staining, arrowhead).

The fungal metabolite brefeldin A (BFA) results in the redistribution of Golgi proteins into the ER (Lippincott-Schwartz et al., 1989) and can be used to reversibly retain cargo proteins within the ER. Wash-out of brefeldin A allows for rapid export of proteins from the ER and subsequent transport to the Golgi. Following incubation of cells with BFA for 2 hours, both lumFP and GPI-FP were localised to the ER (Fig. 4C and D, respectively). Notably, in addition to clear labelling of the ER, lumFP consistently showed accumulation into punctate structures distributed throughout the cells but concentrated in the juxtanuclear region (enlargement to Fig. 4C). Cells were subsequently microinjected with 50 μM GTP-γ-S, BFA removed by extensive wash out, and cells incubated for a further 60 minutes. Under these conditions, GPI-FP

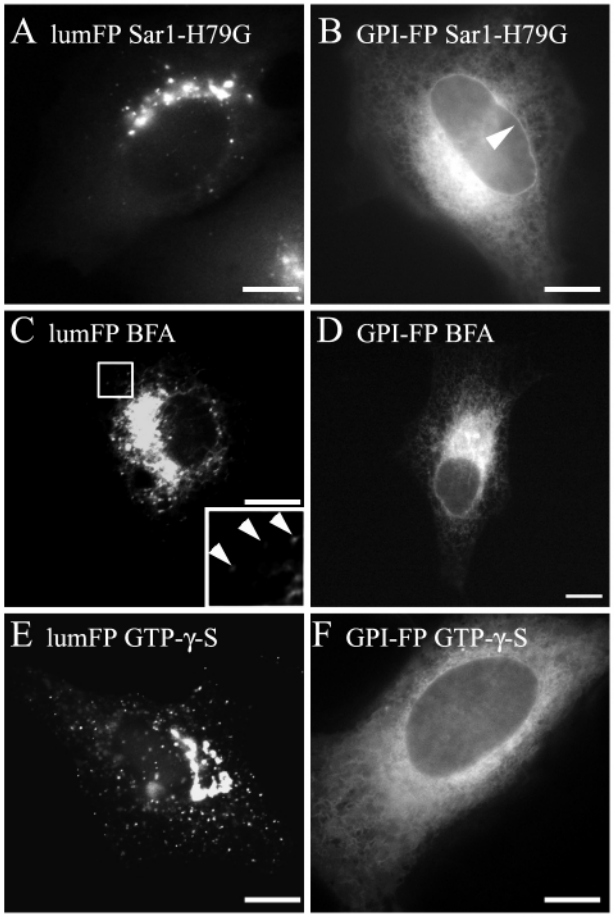

Fig. 4. Export of GPI-FP from the ER requires GTP hydrolysis by the Sar1p GTPase. (A,B) Cells co-injected with plasmids encoding Sar1p$^{(H79G)}$ and either lumFP (A) or GPI-FP (B). Arrowhead shows localization to the nuclear envelope. (C,D) Cells expressing lumFP (C) or GPI-FP (D) were incubated in BFA for 2 hours. Subsequently, cells were microinjected with GTP-γ-S, and BFA was washed-out for 1 hour (E,F). LumFP exits the ER in the absence of GTP hydrolysis by Sar1p (compare A and E) but GPI-FP remains exclusively within the ER (compare B and F). In addition to clear labelling of the ER, lumFP showed accumulation into punctate structures distributed throughout the cells but concentrated in the juxtanuclear region (C, enlargement, arrowheads). Bars, 10 μm.

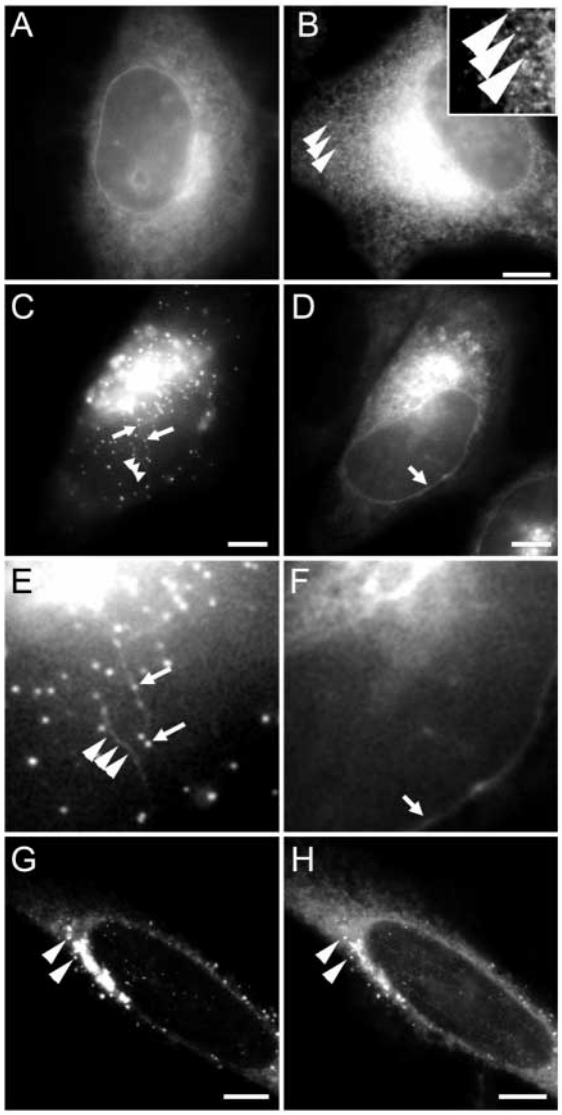

Fig. 5. GPI-FP transiently localises to ER export sites but is not incorporated into nascent pre-budding complexes. (A,B) Cells were microinjected with plasmids encoding GPI-FP with Sar1p$^{(H79G)}$, incubated at 37°C in the presence of 5 μg ml^{-1} BFA for 2 hours and subsequently washed in growth medium and incubated at 37°C for 1 hour (A), or 5 minutes (B) before imaging of living cells. Inset to panel B shows deliberately enhanced contrast to clearly show punctate structures (arrowheads). (C-F) Cells injected with plasmids encoding YFP-Sec23Ap, GPI-CFP and Sar1p$^{(H79G)}$ and incubated in the presence of brefeldin A for 2 hours; cells were then washed and imaged at 37°C. (E,F) Enlarged areas of C and D. Thirty minutes after BFA wash-out, YFP-Sec23Ap (C, enlarged in E) is localised to tubules (arrowheads) that are decorated with punctate structures (arrows). Other punctate structures are clustered around, the perinuclear area. By contrast, GPI-CFP (D) (enlarged in F) remains exclusively localised within the ER (arrow highlights nuclear membrane staining). (G,H) In cells expressing Sar1p$^{(H79G)}$, BFA wash-out results in accumulation of YFP-Sec23Ap (G) in punctate structures clustered in the perinuclear area (arrowheads) that also contain ts045-G-FP (H) (arrowheads). Bars, 5 μm.

remains within the ER without obvious labelling of post-ER structures (Fig. 4F), while for lumFP, the ER was completely emptied with the marker becoming accumulated in punctate structures throughout the cytoplasm and accumulates in the juxtanuclear area (Fig. 4E). These structures appear functionally identical to those generated by expression of Sar1p$^{(H79G)}$ in that they also contain ts045-G-FP (when expressed) and ERGIC-53 (not shown). Injection of GTP instead of GTP-γ-S had no effect on transport of these cargoes; lumFP was efficiently secreted from cells while GPI-FP was transported to the plasma membrane (not shown). Although lumFP accumulates in punctate structures as well as ER membranes in the presence of BFA (Fig. 4C), microinjection of GTP-γ-S or Sar1p$^{(H79G)}$ results in an exclusive localization to punctate structures (Fig. 4A and E, respectively) with no detectable presence of the protein in the ER. By contrast, GPI-FP is exclusively present within the ER in these experiments (Fig. 4B and F).

We also used BFA to hold cargo within the ER during expression of Sar1p$^{(H79G)}$. Wash-out of BFA and live-cell imaging allows visualisation of the immediate export of cargo from the ER in the presence of Sar1p$^{(H79G)}$. Under these conditions, GPI-FP remains predominantly localised to the ER even after extensive wash-out (1 hour) of BFA with a slight accumulation in the Golgi area (Fig. 5A). Juxtanuclear labelling in this case may indeed reflect the large amount of ER membrane in this area, although we cannot exclude some transport to the Golgi in these experiments. We also noticed that at short time points after wash-out of BFA (5 minutes), punctate structures coincidently formed with the ER membrane (Fig. 5B, arrowheads, particularly evident in the contrast-enhanced enlargement within panel B). In contrast to the appearance of these structures when imaging other cargoes such as ts045-G (Stephens et al., 2000), these GPI-FP containing structures are very short-lived, disappearing within 1-2 minutes of their appearance. Time-lapse imaging revealed the appearance and disappearance of these structures to be highly synchronous, with no evidence of budding-off and moving to the Golgi.

We found that, using the BFA wash-out protocol, Sar1p$^{(H79G)}$ expression resulted in the formation of tubules in living cells that were labelled with YFP-Sec23Ap (Fig. 5C, enlarged in 5E, one such tubule is traced by arrowheads). By contrast, GPI-FP was excluded from these structures and remained predominantly localised to the ER in these same cells (Fig. 5D, showing the same cell as in 5C, enlarged in 5F). These tubules are very difficult to image and we have not been able to retain them following either methanol or paraformaldehyde fixation of cells. We commonly observed that these tubules were decorated with concentrations of YFP-Sec23Ap along their length (see Fig. 5C and E, arrows) and these punctate structures (which are preserved following fixation) also label with antibodies directed against human Sec31p (data not shown). The structures visualised here in intact cells, appear to be functionally equivalent to pre-budding complexes generated by addition of Sar1p$^{(H79G)}$ to permeabilised cells (Aridor et al., 2001). Consistent with this, when cells are simultaneously microinjected with cDNAs encoding ts045-G-FP, YFP-Sec23Ap and Sar1p$^{(H79G)}$ and incubated for 3 hours at 32°C, ts045-G-FP colocalises with Sec23Ap-YFP in the punctate structures (Fig. 5G and H, arrowheads).

We then wished to determine whether these ts045-G-FP-positive structures, seen following co-expression with Sar1p$^{(H79G)}$, remained continuous within the ER. FRAP experiments showed that when ts045-G-FP-containing, post-ER structures are bleached, a time-dependent increase in fluorescence intensity of the same structures is observed (Fig. 6A and B, ○). Under the same conditions of Sar1$^{(H79G)}$ expression, GPI-FP shows recovery with almost identical kinetics (Fig. 6B, ■) and is indistinguishable from recovery kinetics of ts045-G-FP retained within the ER at 39.5°C (Fig. 6B, ●) or within the ER following incubation with brefeldin A (not shown). By contrast, photobleaching of ts045-G-FP containing VTCs in transit to the Golgi (at 32°C in unperturbed cells not expressing Sar1p$^{(H79G)}$, see Fig. 6B, ▲) showed only limited recovery of fluorescence (Fig. 6B). In these experiments a large area of the cell was bleached to ensure that VTCs would not move out of the bleach frame between bleaching and post-bleach imaging. Inspection of the individual frames from these sequences reveals that fluorescence recovery occurred almost exclusively in ER membranes (Fig. 6C) and not in the majority of large, punctate, post-ER structures (Fig. 6C, arrowheads with asterisk in pre-bleach image). Some structures that were bleached show very limited recovery (small arrowhead in the example shown in Fig. 6C). In five separate bleach experiments, 10% of structures (six out of 60) showed any detectable recovery but this was never more than 20% of the initial fluorescence intensity. Importantly, the structures that do show recovery are stationary and do not move during the 100 seconds of post-bleach imaging shown. These are likely to be VTCs that are emerging from the ER and not yet fully formed as independent structures. Some structures are seen to move from outside to within the bleached region (Fig. 6C, +50.7 seconds and +99.7 seconds, arrows). Fluorescence loss in photobleaching (FLIP) was also used to confirm these observations (Fig. 6C). Cells expressing lumFP and Sar1p$^{(H79G)}$ were alternately bleached (within the boxed area) at high laser power and imaged at low laser power [Fig. 6D, top (FLIP)]. Fluorescence intensity outside of the bleached area progressively decreased, consistent with a mobile pool of fluorophore. In control experiments [Fig. 6D, bottom (control)], in which the bleach area selected was outside of the imaged cell, no significant decrease in fluorescence intensity was observed. Identical results were obtained for ts045-G-FP (not shown). In summary, these data show that punctate structures that accumulate in the presence of Sar1p$^{(H79G)}$ are continuous with the ER.

Discussion

In yeast (Belden and Barlowe, 2001), animal (Martinez-Menarguez et al., 1999) and plant systems (Phillipson et al., 2001), data now exist which show that soluble cargo within the lumen of the ER is exported for transport to the Golgi using the same COPII machinery as employed by membrane proteins. Indeed, considerable evidence is now accumulating that soluble cargo molecules are coupled to this machinery by transmembrane ‘cargo-receptors’ such as ERGIC-53 (Appenzeller et al., 1999) and Erv29p (Belden and Barlowe, 2001). Combined with ours, these data suggest the existence of a common core machinery, and by inference a common mechanism for transport, for all classes of cargo from the ER

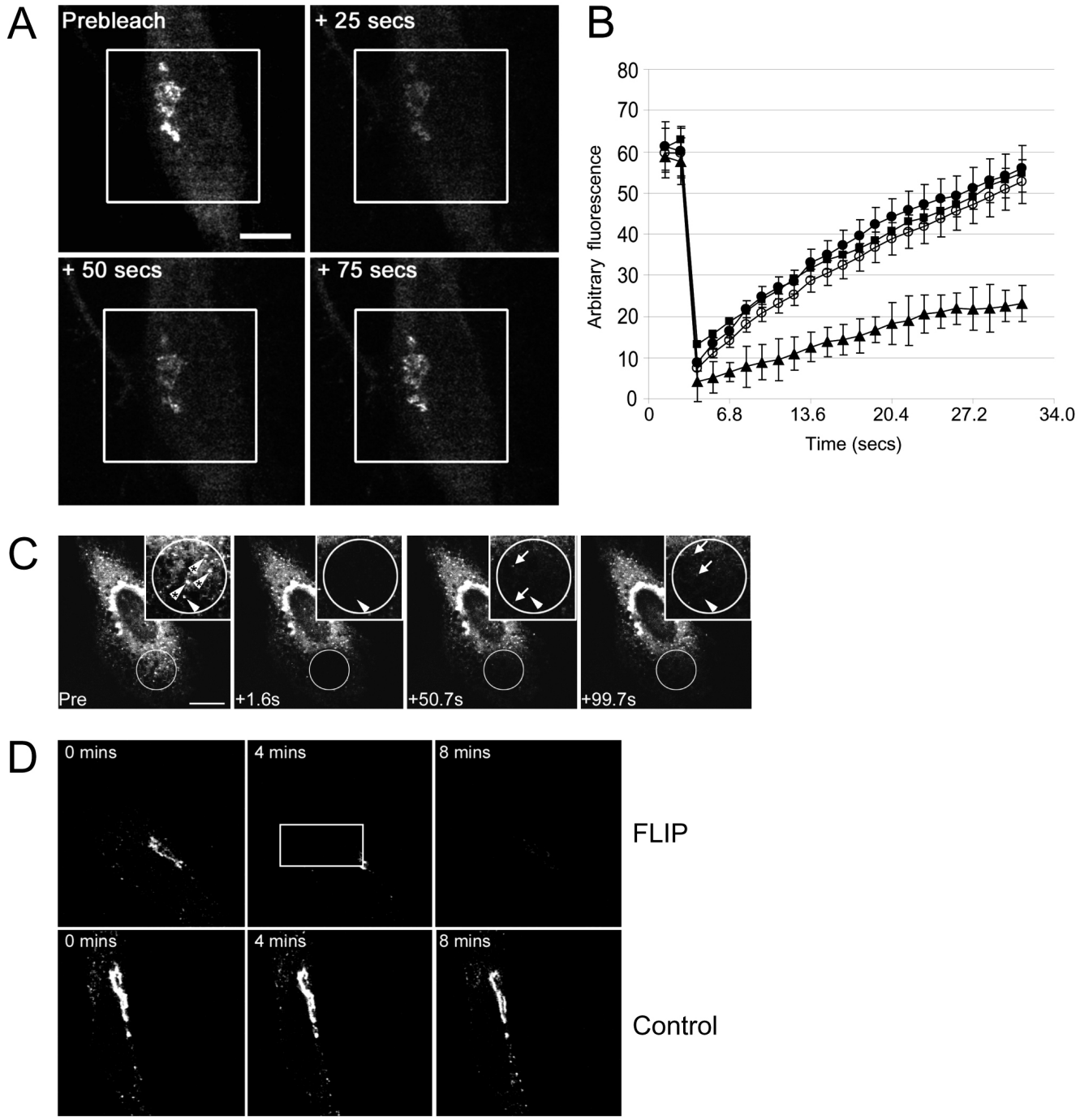

Fig. 6. Diffusion-mobility of ts045-G-FP in pre-budding complexes and GPI-FP in the ER membrane. (A) Cells expressing ts045-G and Sar1p$^{(H79G)}$ were imaged before (pre-bleach) and after photobleaching of the central boxed area. Cells were imaged for five frames before bleaching a central region of the cell and monitoring recovery by measuring fluorescence intensity (arbitrary units) within the bleached area (the first post-bleach frame is defined as time zero on the *x*-axis). Frames were 0.677 seconds apart; bleaching was performed using four scans of a central region of interest with maximum laser power. The first post-bleach image was acquired approximately 4 seconds after the end of the bleaching step. (B) Cells expressing ts045-G-GFP and Sar1p$^{(H79G)}$ at 32°C (○), GPI-GFP and Sar1p$^{(H79G)}$ at 32°C (■), ts045-G-GFP at 39.5°C (●), or ts045-G-GFP at 32°C (▲) were analysed using fluorescence recovery after photobleaching. Data shown is the average from six cells of three independent experiments. Error bars showthe standard error of the mean (s.e.m.). (C) Cells expressing ts045-G-FP were temperature-shifted from 39.5°C to 32°C for 5 minutes and imaged by using FRAP. The cell was bleached within the boxed region and 18 seconds later imaged every 1.3 seconds. The top right hand corner of each panel shows a 2× zoom of the bleached region. Arrowheads with asterisks highlight structures that do not show fluorescence recovery, arrowhead shows a structure that shows limited recover. Arrows highlight structures that move into the bleached region during the post-bleach period. (D) Cells expressing lumFP and Sar1p$^{(H79G)}$ were sequentially imaged at low laser intensity and high laser intensity (FLIP) (within the boxed region only). Fluorescence loss occurs outside the bleached region. In control cells, in which a bleach region was selected away from any cells, no significant loss of fluorescence occurs.

to the Golgi. A common set of machinery would serve to enhance the efficiency and reliability of transport through the early secretory pathway.

Despite a common set of transport machinery and traffic in common VTCs, we have shown that the mechanism of export of GPI-anchored cargo and procollagen is distinct from that of, for example, ts045-G. Upon synchronised release of cargo from the ER, we have observed a very transient accumulation of GPI-FP into structures that closely resemble ER exit sites. Other work has previously shown that ts045-G can be exported from the ER into nascent VTCs in the presence of GTP-γ-S (Pepperkok et al., 1998). Furthermore, incubation of permeabilised cells with Sar1p$^{(H79G)}$ protein results in the generation of multiple tubules emanating from ER exit sites, that contain ts045-G and are coated with COPII components (Aridor et al., 2001). Our results here are entirely consistent with these observations. The punctate structures containing GPI-FP that form on BFA wash-out in the presence of Sar1p$^{(H79G)}$, are highly reminiscent of the early stages of the formation of these tubules (Aridor et al., 2001), and are approximately equal in number to COPII-coated ER exit sites in these cells suggesting that they are ER exit sites (Stephens et al., 2000). Thus, GPI-FP is able to transiently enter ER cargo export sites on BFA wash-out, but, in the presence of Sar1p$^{(H79G)}$, is not efficiently retained within them and returns to the ER. Like ts045-G, small freely diffusible cargo (lumFP) can enter these sites and subsequently become efficiently exported from the ER into pre-budding complexes (this study) (Aridor et al., 2001). It is not immediately clear why lumFP should become concentrated in these structures above the level present in the ER membrane. One possibility is that this cargo is not in fact 'transport-neutral' but actually associates with endogenous proteins that are themselves concentrated in these structures but do not contain ts045-G-FP. An alternative explanation could be that it becomes occluded into fully budded structures that are discontinuous with the ER. We have also previously shown that procollagen is not efficiently exported from the ER under these conditions (Stephens and Pepperkok, 2002) but we have never observed the same transient accumulation into ER export sites. It seems probable that the mechanistic difference between GPI-FP and ts045-G export lies in the ability of these proteins to be retained within these structures; ts045-G can directly couple to the COPII machinery by interaction with Sar1p (Aridor et al., 2001) whereas interaction of GPI-FP with cytosolic COPII components could only be mediated through a separate receptor.

Disruption of dynactin or Rab protein function inhibits export of all cargoes examined. No significant difference was observed in the export of cargo from the ER, with the exception of GPI-FP which showed some ER localization following expression of either Rab-GDIβ or p50^{dynamitin}. This might reflect different requirements for these factors in export of GPI-FP from the ER or, alternatively, these experiments might highlight slightly different rates of export of GPI-FP and other cargoes. We find that overall rates of export are very similar but we cannot rule out subtle differences highlighted only by specific protocols.

In agreement with previously published data that show the possibility for COPII components to recycle on and off the membrane in the presence of Sar1$^{(H79G)}$ (Ward et al., 2001), ts045-G-GFP and lumFP show dynamic behaviour when localised to Sar1$^{(H79G)}$-induced pre-budding complexes. After bleaching, ts045-G-FP fluorescence recovers within the bleached area. We also observe 100% recovery of fluorescence within the bleached region which suggests a fully mobile population of fluorophore and is consistent with previous observations of the behaviour of ts045-G-GFP (Nehls et al., 2000). FLIP experiments confirm the continuity of these structures with the ER. These data suggest that either pre-budding complexes are continuous with the ER network and that there is free diffusion of cargo in to these structures, or that there is ongoing traffic to these structures. Significantly, the recovery of GPI-FP localised within the ER under these conditions shows almost identical recovery kinetics. This raises the possibility of a direct connection between pre-budding complexes and the ER in live cells. One would expect free diffusion within the ER lumen (as seen for GPI-FP under these conditions) to exactly match diffusion into pre-budding complexes if they both were directly connected.

Recent studies using correlative video and electron microscopy have led to the suggestion that continuities between ER, ER-to-Golgi carriers and perhaps even the Golgi exist (Mironov et al., 2003). At the low level of ts045-G-FP expression used here, we find that VTCs translocating to the Golgi in transfected but otherwise unperturbed cells, do not show recovery of fluorescence in FRAP experiments. It is therefore probable that the punctate structures we observed in these experiments are completely detached from the ER. The work of Mironov and colleagues (Mironov et al., 2003) suggests that ts045-G-FP accumulates into two forms of carriers, VTCs and tubules continuous with the ER. We have examined both translocating and static structures by using FRAP and have not seen significant recovery of any of them. Some punctate structures show very limited recovery, to a similar level to that of the surrounding ER. It is possible that these accumulations of cargo (ts045-G-FP) within tubules are sequestered from, and thus independent of, the freely diffusible pool of cargo in the ER. Alternatively, there might be insufficient levels ts045-G-FP remaining in the ER to diffuse into these carriers. Perhaps the simplest explanation would be that, in HeLa cells both segregated VTCs and continuities co-exist, but our quantitative photobleaching data suggest that, at least in HeLa cells, the continuities are in the minority.

A simple explanation for our data is that positive selection of GPI-FP and procollagen into nascent COPII-coated pre-budding complexes (Aridor et al., 2001) requires efficient hydrolysis of GTP by the Sar1p GTPase. The equivalent mutant of Sar1 in yeast (H77L) (Saito et al., 1998) shows the same basal rate of GTP hydrolysis as wild-type Sar1p. The effect of this mutation is a greatly reduced Sec23p-dependent acceleration of GTPase activity (Saito et al., 1998) by Sar1p. This suggests that the specific effect we observed is linked to the coupling of the Sar1p GTPase to its GTPase activating protein Sec23p and the associated increase in GTP hydrolysis. Assays that measure packaging of GPI-anchored proteins from the ER were performed using GTP and not GTP-γ-S (Doering and Schekman, 1996; Muniz et al., 2001). Therefore, these assays would permit the incorporation of GPI-anchored cargo into nascent COPII vesicles. Recent data from Sato and Nakano suggest that GTP hydrolysis by Sar1p can be linked to the specificity of cargo incorporation into budding structures

(Sato and Nakano, 2004). Specifically, assembled cargo-receptor complexes were found to accelerate the rate of GTP hydrolysis on Sar1p and consequently increase the fidelity of their incorporation into budding vesicles. Our data support these observations and suggest a wider role for the control of cargo export through the regulation of Sar1p-mediated GTP hydrolysis. Efficient sorting of certain cargo proteins into COPII structures following GTP hydrolysis by Sar1p might represent a mechanism similar to that by which Arf1p mediates selective incorporation of cargo into COPI vesicles (Lanoix et al., 1999; Nickel et al., 1998; Pepperkok et al., 2000). Alternatively, it is possible that these data reflect different affinities of cargoes and/or cargo receptors for Sar1. When 'locked' in the GTP bound state, some cargo might have higher affinity for COPII complexes than others. We think this unlikely because we observe the same effects across a range of $Sar1p^{(H79G)}$ concentrations. Furthermore, we observe the same results following microinjection of GTP-γ-S as following expression of $Sar1p^{(H79G)}$, and consequently we believe that the data support a role for GTP hydrolysis by Sar1p in cargo selection and retention in COPII budding complexes.

In showing distinct mechanisms for export of cargo from the ER, our data is consistent with the identification of distinct classes of COPII vesicles, one containing transmembrane proteins and the other GPI-anchored proteins (Muniz et al., 2001). It is possible that subtle kinetic differences resulting from alternative mechanisms of coupling to the export machinery, result in segregation of cargo into distinct COPII-coated vesicles in mammalian cells, as is seen in yeast (Muniz et al., 2001). These vesicles might then coalesce to form a VTC, resulting in mixing of the contents of distinct COPII-derived vesicles and transport of these cargoes to the Golgi in the same VTC. Incorporation of specific tethering- and fusion molecules (Morsomme et al., 2003; Morsomme and Riezman, 2002) into these classes of vesicle would provide a means to control the formation of VTCs to maintain or eliminate segregation of cargo following ER export. Alternatively, it is conceivable that these mechanisms serve to segregate cargo into different membrane domains in the vicinity of ERES that are subsequently and independently of one another protruded from the ER. Different homologues (Pagano et al., 1999) or splice forms of COPII proteins such as Sec24p could be used to facilitate any mechanistic differences in export and we are currently investigating this possibility in more detail. In summary, these results suggest that either GTP hydrolysis by Sar1p is required for functional coupling of certain soluble cargo proteins to export receptors, or that it is needed for the generation of specialised membrane domains for export. In either case, these data provide a potential mechanism for segregating cargoes from one another at the point of exit from the ER.

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