# Imaging of procollagen transport reveals COPI-dependent cargo sorting during ER-to-Golgi transport in mammalian cells

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## Summary

**We have examined the ER-to-Golgi transport of procollagen, which, when assembled in the lumen of the ER, is thought to be physically too large to fit in classically described 60-80 nm COPI- and COPII-coated transport vesicles. We found that procollagen exits the ER via COPII-coated ER exit sites and is transported to the Golgi along microtubules in defined transport complexes. These procollagen-containing transport complexes are, however, distinct from those containing other cargo proteins like ERGIC-53 and ts-045-G. Furthermore, they do not label for the COPI coat complex in contrast to those containing ts-045-G. Inhibition of COPII or COPI function before addition of ascorbate, which is required for the folding of procollagen, inhibits export of procollagen from the ER. Inactivation of COPI coat function after addition of ascorbate results in the localisation of procollagen to transport complexes that now also contain ERGIC-53 and are inhibited in their transport to the Golgi complex. These data reveal the existence of an early COPI-dependent, pre-Golgi cargo sorting step in mammalian cells.**

Movies available on-line

Key words: Procollagen, ER, COPII, Golgi, sorting.

## Introduction

Transport of material through the secretory pathway can be subdivided into at least three distinct steps. First, ER export and subsequent delivery to the Golgi complex; second, intra-Golgi transport; and third, post-Golgi trafficking. ER-to-Golgi and intra-Golgi transport is accompanied by a cargo concentration step either within the ER lumen or immediately following exit and is thought to predominantly serve the quality control of secretory cargo. In contrast to ER exit, which has so far been thought to occur similarly for all secretory cargo in mammalian cells, cargo sorting into cargo-specific transport carriers occurs at the trans Golgi/trans Golgi network and is a prerequisite for proper delivery of cargo to its destination (Griffiths and Simons, 1986; Müsch et al., 1996; Keller and Simons, 1997; Keller et al., 2001).

Transport between the ER and the Golgi complex in mammalian cells is thought to occur in small 60-80 nm transport vesicles that transport cargo directly to the Golgi and/or by coalescence to form larger tubular-vesicular transport complexes (TCs) mediating long-range transport along microtubules (Lippincott-Schwartz et al., 2000; Stephens and Pepperkok, 2001). The vesicular coat complexes COPII and COPI are involved in this transport step (Rothman and Wieland, 1996; Sheckman and Orci, 1996). COPII mediates the selection of cargo within the ER and the budding of vesicular or vesicular tubular intermediates that form a nascent transport complex (TC). These subsequently become coated with the COPI complex prior to transport towards the Golgi apparatus in larger COPI-coated transport complexes (Aridor et al., 1995; Shima et al., 1999; Stephens et al., 2000). COPI mediates the retrieval of ER-resident and other recycling proteins from post-ER membranes back to the ER (Letourner et al., 1994; Bannykh and Balch, 1998).

The majority of work to date addressing export of secretory cargo from the ER has focused on the sorting of transmembrane proteins that are believed to directly engage coat proteins on their cytosolic face of the membrane for selection into, and concentration within, newly forming transport carriers. In particular, ts-045-G has been widely used as a model protein for these studies, as it can be accumulated within the ER at 39.5°C and released as a relatively synchronous wave of transport at the permissive temperature 31°C (Lippincott-Schwartz et al., 2000; Stephens et al., 2000; Scales et al., 1997; Presley et al., 1997). Although this system is undoubtedly of much use in elucidating the mechanisms and principles behind cargo exit from the ER, it only represents one class of cargo, which is not a naturally occurring mammalian protein. When one considers soluble cargo molecules like enzymes or growth factors, it immediately becomes apparent that they cannot directly engage COPII coat complexes on the ER membrane in the same way that transmembrane proteins can, that is by virtue of sorting motifs in their cytosolic parts (Nishimura and Balch, 1997; Sevier et al., 1999). This raises the question of how these molecules are efficiently exported from the ER. Is there a membrane receptor for each and every soluble cargo molecule or do generic receptors exist for different classes of cargo?

We have previously shown that small soluble secretory cargo in the form of lumGFP [GFP translocated into the ER by virtue of a cleavable signal sequence (Blum et al., 2000)] fills tubules that emanate from the ER and translocate to the Golgi upon shifting cells from a 15°C temperature block to 37°C.

Importantly, we found that ts-045-G was sequestered, apparently within these tubules, into distinct domains that were COPI coated (Blum et al., 2000). This provided us with the first hint of possible cargo segregation during ER-to-Golgi transport.

Quantitative EM data suggest that small soluble cargoes such as amylase and chymotrypsinogen are not actually concentrated upon exit from the ER but at a later stage during transport to the Golgi (Martinez-Menarguez et al., 1999). The simplest explanation for this is that they enter nascent COPII transport vesicles passively and are not actively concentrated into them. One would therefore expect these cargoes to be contained in the majority of transport carriers exiting the ER. One must also consider extremely large macromolecular cargoes. Do such large cargo complexes utilise the same mechanisms for ER-to-Golgi transport as smaller soluble cargoes or distinct ones? For example, newly synthesised monomeric polypeptide chains of type I procollagen, pro α1 (I) are 1464 amino acids in length and are believed to assemble into a continuous triple helical molecule of >300 nm length (Bächinger et al., 1982; Tromp et al., 1988; Brodsky and Ramshaw, 1997; Lamandé and Bateman, 1999). This rigid rod-like structure would clearly be too large to fit into conventional 60-80 nm vesicles budding from the ER (Rothman and Wieland, 1996; Schekman and Orci, 1996). It remains therefore unclear how such cargo is packaged into transport carriers and how it is subsequently transport to and through the Golgi complex.

In order to address this question we have tagged procollagen with spectral variants of GFP and followed its transport from the ER to the Golgi complex in living cells. We show that procollagen is segregated at ER exit sites into transport complexes distinct from those carrying ts-O45-G and ERGIC53. Our data show that this segregation requires COPI function and demonstrate for the first time in mammalian cells a COPI-dependent pre-Golgi step.

## Materials and Methods

All chemical reagents were purchased from Sigma (Taufkirchen, Germany) and all restriction and modifying enzymes were from Roche (Mannheim, Germany) or New England Biolabs (Frankfurt, Germany) unless stated otherwise.

### Cloning of PC-FP

A cDNA encoding human procollagen type I (α1) (pro α1 (I)) was obtained from the ATCC (ATCC number 95498) and cloned into pEGFPN2 (Clontech, Heidelberg, Germany) using the unique *Eco*RI-*Eco*NI sites within the coding region. The remaining coding sequence of pro α1 (I) was amplified by PCR and cloned into the *Eco*NI-*Sal*I sites of the above clone to generate a full-length coding region as a GFP fusion (PC-FP). This was subsequently subcloned using *Bgl*II-*Pst*I (using a *Pst*I site that was included in the PCR primer) to pECFPN1 (Clontech, Heidelberg, Germany) to generate PC-CFP. The sequence amplified by PCR was confirmed by sequencing. Wherever possible PC-GFP was used for imaging; multi-label experiments were performed using PC-CFP. No difference between the two was found in in any experiment. We therefore use the notation PC-FP (procollagen-fluorescent protein) to describe these fusions interchangeably.

### Tissue culture

Vero cells (ATCC CCL81) were grown in MEM containing 10% foetal calf serum; HeLa cells (provided by Michael Way, EMBL-Heidelberg) were grown in DMEM (Life Technologies, Karlsruhe, Germany) containing 10% foetal calf serum. The HeLa and Vero cells used in this study express also endogenous procollagen (pro α1 (I)) as detected using specific antibodies) (Fisher et al., 1989; Fisher et al., 1995). Similar results were obtained using either cell line. Cells were plated 24-48 hours prior to injection on live cell dishes (MatTek, Ashland, MA, USA). Cells were injected and subsequently imaged in MEM without phenol red, supplemented with 30 mM HEPES, pH 7.4 and 0.5 g/l sodium bicarbonate. Microinjection was performed as previously described (Shima et al., 1999; Stephens et al., 2000; Pepperkok et al., 1993). HeLa cells were transfected using Fugene6 (Roche, Mannheim, Germany) according to the manufacturers instructions.

### Expression of markers

PC-FP was injected at a concentration of 50 μg/ml into the nucleus of either HeLa or Vero cells. 120-180 minutes after incubation at 37°C, cycloheximide was added (100 μg/ml final concentration) and cells were imaged with or without the addition of ascorbate-2-phosphate to a final concentration of 50 μg/ml. This concentration was found to give the most reproducible results with regard to the formation of PC-FP containing TCs and rapid, synchronous transport to the Golgi and was not found to be detrimental to cells during the time course of these experiments. Cells were also seen to continue to divide when cultured in 50 μg/ml of ascorbate overnight, and furthermore, this concentration has also previously been shown not to affect cell proliferation, protein synthesis or carbohydrate synthesis (Levene and Bates, 1975). All experiments that did not involve PC-FP were also carried out in the presence of the same concentration of ascorbate with no noticeable effects. Genuine pre-Golgi TCs containing PC-FP were identified in all experiments (both using live and fixed cells) by close inspection of cells expressing appropriately low levels of PC-FP. This, coupled with the early time points following ascorbate addition (10 minutes) that were used in the experiments described here, enabled us to unequivocally identify pre- as opposed to post-Golgi TCs.

Localisation of PC-FP and ts-045-G containing TCs was performed by transfection of both markers (Stephens et al., 2000), incubating cells at 39.5°C for 16 hours in the absence of ascorbate followed by 30 minutes at 39.5°C in the presence of ascorbate. Cells were subsequently incubated for 5 minutes at 32°C prior to fixation and processing for immunofluorescence. To determine the effect of anti-EAGE injection this procedure was reproduced with the exception that ascorbate was added concomitantly with reduction of the temperature to 32°C. After 5 minutes at 32°C, cells were injected with anti-EAGE (Pepperkok et al., 1993) at a concentration of 1.5 mg/ml. This enabled us to determine the effect of COPI inhibition on TC dynamics as well as formation.

15°C temperature blocks were generated by incubating cells in a water bath at 15°C in growth medium supplemented with 30 mM HEPES pH 7.4. Expression of $ARF1^{(Q71L)}$ and $SAR1a^{(H79G)}$ mutants was achieved by co-injection of plasmid DNA encoding the respective mutants with DNA encoding the marker of interest. Anti-EAGE (Pepperkok et al., 1993) was injected at a concentration of 1.5 mg ml into the cytoplasm of cells previously injected with plasmid DNA encoding PC-GFP or other markers.

### Immunofluorescent labelling and microscopy

For immunofluorescence, cells were fixed using 3.5% paraformaldehyde, permeabilised with 0.1% Triton X-100 and immunostained as described previously (Stephens et al., 2000). The antibodies used were as follows: anti-ERGIC-53 used at 1:50 (Schweizer et al., 1988); anti-BSTR (β′ COP) (Pepperkok et al., 2000) 1:500; CM1A10 (β′ COP) (Palmer et al., 1993), 1:1000. Primary antibodies were detected using anti-mouse or anti-rabbit secondary

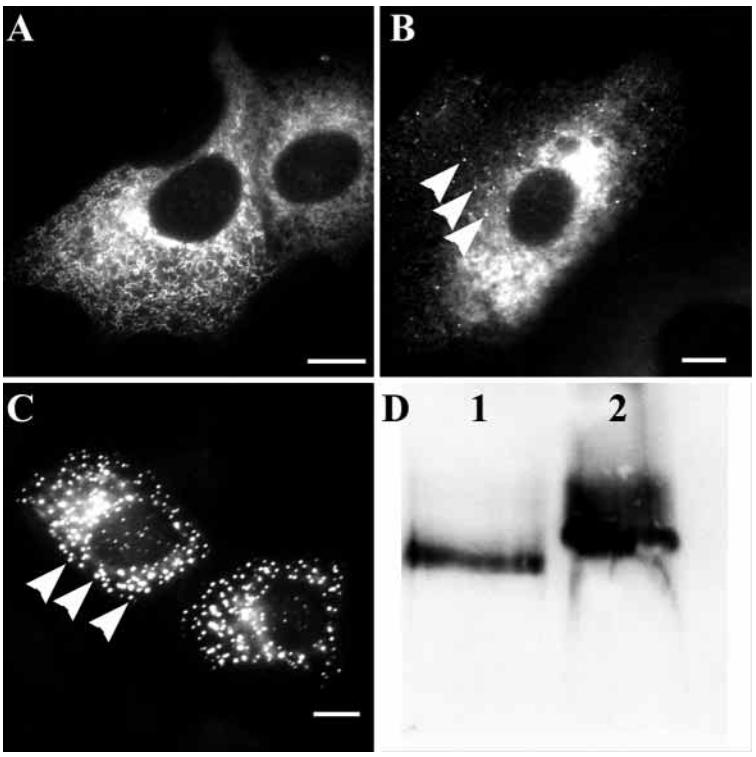


**Fig. 1.** Expression-level-dependent and ascorbate-dependent localisation of PC-GFP. Cells expressing low levels of PC-GFP, 3 hours after microinjection of plasmid DNA, localise the protein to the ER in the absence of ascorbate (A), but 10 minutes after the addition of ascorbate, PC-FP localises to punctate TCs that traffic to the Golgi (B). Time lapse movies showing the dynamics of procollagen in the absence (Movie 1) and presence (movie 2) of ascorbate are available at jcs.biologists.com/supplemental. In contrast cells expressing high levels of PC-GFP, 6 hours after microinjection of plasmid DNA, localise the protein, in the absence of ascorbate, to aggregates located within the ER (C). The exposure times for (B) and (C) are the same; arrowheads are ndicated TCs in (B) and aggregates in (C). Cells expressing such high levels of PC-FP were not used during any of the experiments described here. PC-GFP is intact when expressed in cells. Bars=5 μm. (D) shows an immunoblot with the anti-procollagen antibody LF-39 of mock-transfected HeLa cells (lane 1) or HeLa cells transfected with PC-GFP (lane 2). The mobility shift in lane 2 is caused by the presence of the GFP moiety and partial glycosylation.

antibodies labelled with Cy3 or Cy5 as required. Living and fixed cells were imaged using either an Olympus/TILL Photonics time-lapse microscope (Stephens et al., 2000) or Leica DM/IRBE inverted microscope with a 63×, N.A. 1.4PL Apo objective and individual custom filters from Chroma (Brattleboro, VT, USA) for GFP, CFP, YFP, Cy3 and Cy5. Images were captured with a Hamamatsu CCD camera (ORCA-1) using Openlab software (Improvision, Coventry, UK). Following acquisition, images were converted to an image depth of 8 bit and processed using NIH Image, Adobe Photoshop v6.0. QuickTime movies were generated using NIH Image, QuickTime Pro and Adobe Premiere v5.1. Trajectories of particles and velocities were determined using a macro written for NIH Image by Jens Rietdorf (ALMF, EMBL, Heidelberg).

## Results

### GFP-tagged procollagen provides a regulated ER-to Golgi transport system

Full-length cDNA for human type I, alpha 1 procollagen (pro α1 (I)) was cloned in frame with EGFP or ECFP to generate PC-GFP and PC-CFP (referred to in the following interchangeably as PC-FP). The fluorescent proteins were fused after the C-terminal propeptide of pro α1 (I), which is cleaved following secretion of procollagen from cells but prior to the assembly of procollagen supramolecular arrays (Brodsky and Ramshaw, 1997; Lamandé and Bateman, 1999; Prockop and Kivirikko, 1995). Thus, GFP should remain covalently attached to procollagen throughout its transport through the secretory pathway. The PC-FP expressed in cells migrated slower on SDS-PAGE than endogenous procollagen (Fig. 1D), owing to the presence of the GFP moiety, and no apparent breakdown products of PC-FP could be detected, suggesting stability of the molecule (Fig. 1D). Consistent with this, PC-GFP fluorescence overlapped with the immunostaining of antibodies directed against both the N- and C-terminal pro-peptides of pro α1 (I) (LF-39 and LF-67 respectively (Fisher et al., 1989; Fisher et al., 1995). Low-level expression of PC-FP, obtained 2 to 3 hours after microinjection of respective cDNAs, resulted in a uniform distribution of PC-FP within the ER (Fig. 1A, Fig. 2A; see Movie 1 at jcs.biologists.org/supplemental).

It is important to note that cells expressing only low levels of procollagen, 2 to 3 hours after microinjection of DNA, were used in the experiments described here. At this point in each experiment, cycloheximide was added to a final concentration of 100 μg/ml to prevent further protein synthesis. After the addition of ascorbate, which is an essential co-factor of prolyl hydroxylase that is required during the folding and assembly of PC (Brodsky and Ramshaw, 1997; Lamandé and Bateman, 1999; Prockop and Kivirikko, 1995), PC-FP redistributed (within 10 minutes) to small spherical structures dispersed throughout the cytoplasm (Fig. 1B, Fig. 2B; arrowheads**)**. Time-lapse imaging revealed fast, directed transport of these structures along curvi-linear tracks to the juxtanuclear region of the Golgi complex (Fig. 1B, Fig. 2B; see Movie 2 at jcs.biologists.org/supplemental). Their speed ranged between 0.6 and 1.3 μm/s, and movement was blocked by the microtubule disrupting drug nocodazole and overexpression of p50 (dynamitin, data not shown), which disrupts dynein-mediated transport (Presley et al., 1997; Echeverri et al., 1996), suggesting that transport of PC-FP occurs along microtubules in a dynein–dynactin-dependent manner. 10 minutes after the addition of ascorbate, PC-FP accumulated in the juxtanuclear Golgi region of the cells (particularly apparent towards the end of Movie 2). At this time point, all PC-FP TCs are seen to move towards the Golgi apparatus from the periphery (see Movie 2) as would be expected for ER-to-Golgi transport intermediates. Structures emanating from the Golgi moving towards the cell periphery were first observed 30 minutes after the addition of

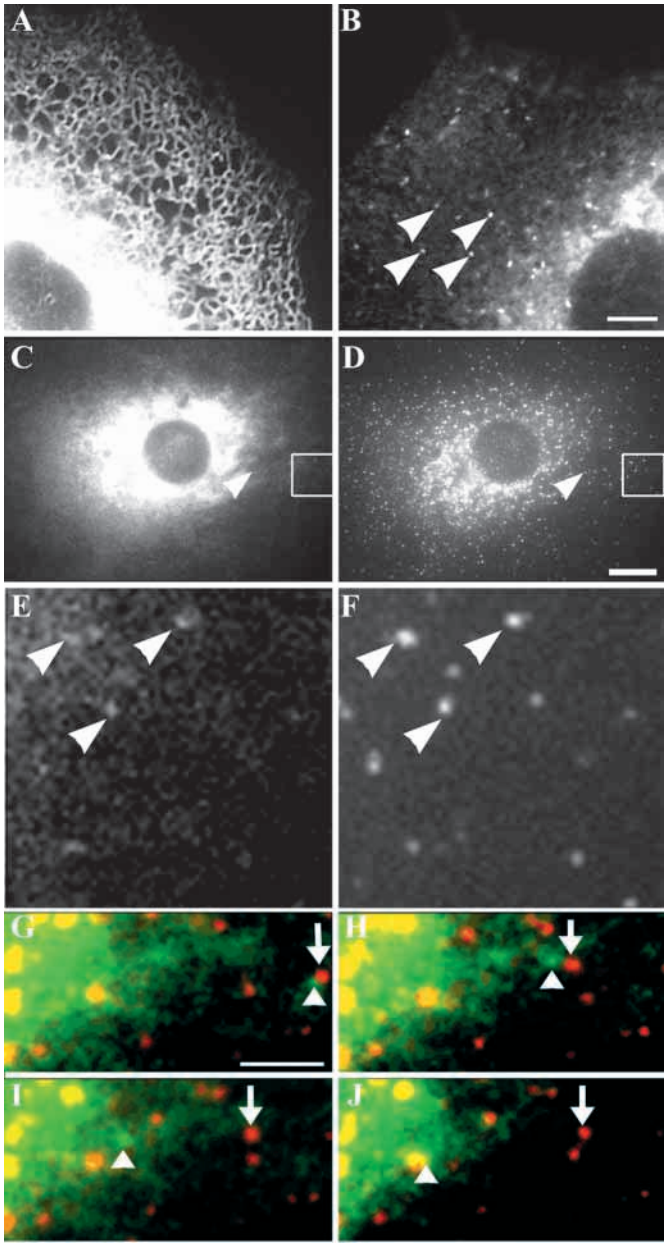


**Fig. 2.** Ascorbate-dependent trafficking of PC-FP in living cells. (A) Low level expression of PC-FP in Vero cells in the absence of ascorbate. (B) Localisation of PC-FP 10 minutes after the addition of ascorbate. (C) and (D) show colocalisation of PC-CFP (C) and SEC24Dp-YFP (D) in living cells 2 minutes after the addition of ascorbate. (E) and (F) show an enlargement of the area bounded by the white box (width 5 μm) in (C) and (D), respectively. Examples of colocalisation of PC-FP with SEC24Dp-YFP are marked with arrowheads. (G-J) PC-FP exits the ER at or in close proximity to COPII-labelled ER exit sites. Still images from a time lapse series are shown, taken 5 minutes after the addition of ascorbate, showing part of a cell expressing PC-CFP (green) and YFP-SEC24Dp (red). Note the movement of SEC24Dp and PC-CFP together (H versus G) followed by segregation of PC-CFP (arrowhead) from YFP-SEC24Dp (arrow). The PC-CFP-labelled structure passes close by another YFP-SEC24Dp structure (J) before becoming lost amongst the large amount of label in the Golgi region. Images were taken 4 seconds apart. Bars=5 μm. Time lapse movies showing the dynamics of procollagen in the absence (Movie 1) and presence (Movie 2) of ascorbate and of the series of images shown in C-F (Movie 3) are available at jcs.biologists.org/supplemental.

ascorbate (not shown). At later time points, 1-3 hours after ascorbate addition, PC-FP fluorescence completely disappeared, consistent with its secretion from cells. The experiments described here were focused upon characterisation of ER-to-Golgi transport of procollagen, and therefore we undertook all experiments, unless otherwise stated, 10 minutes after the addition of ascorbate to the culture medium. In addition, all cells that were fixed and subsequently processed were first inspected by microscopy to confirm trafficking of procollagen following ascorbate addition. Thus, analysis of cells at time intervals of between 10 and 20 minutes after the addition of ascorbate provides us with a means to identify bona fide ER-to-Golgi intermediates (TCs) and not post-Golgi carriers. Note that the ER localisation of PC-FP is such that one sees a large amount of fluorescence in the perinuclear area of the cell (Fig. 1A, Fig. 2A, Fig. 6). This is necessary in order to obtain good contrast of the peripheral ER network and does not represent Golgi staining. Indeed, there is a considerable amount of ER membrane in this area of these cells as evidenced by immunolabelling with antibodies against well characterised ER markers such as PDI and also by live cell imaging with ER-CFP (Stephens et al., 2000).

At higher levels of expression (>6 hours after injection), in the absence of ascorbate, PC-FP was seen to aggregate into large spherical structures still apparently bounded by the ER membrane (Fig. 1C). These aggregates completely disappear upon incubation with ascorbate for 3 hours but in a non-synchronous manner. Compared to TCs identified in cells expressing low levels of PC-FP, these large structures contained at least 10-15 times more PC-FP as adjudged by quantitation of fluorescence. Furthermore, these structures were relatively immobile in cells, and long-range transport (greater than 2 μm) was not observed. Therefore, these aggregate structures could be clearly distinguished from PC-FP TCs. It is likely that these aggregates represent previously described higher order structures of procollagen and PDI, which form within the lumen of the ER (Kellokumpu et al., 1997). Indeed, these aggregate structures, unlike ER-to-Golgi TCs, were seen to label with anti-PDI antibodies (data not shown), which is consistent with being part of the ER. Cells expressing such high levels of PC-FP were not used in any of the experiments described here.

In summary, these data show that PC-FP, when expressed at low levels, is secreted from cells in an ascorbate-dependent manner and can thus be used as a regulated ER-to-Golgi transport marker in living cells. Furthermore, it behaves similarly, if not identically, to endogenous PC and is transported from the ER to the Golgi complex in transport complexes (TCs) similar to those described earlier for various membrane proteins (Scales et al., 1997; Presley et al., 1997; Chao et al., 1999) and the small soluble secretory cargo lumenal GFP (Blum et al., 2000).

## TCs containing PC-FP segregate from COPI-coated TCs carrying ts-O45-G and ERGIC-53

Previous work has shown that COPII does not remain

**Table 1. Colocalisation of TCs with different markers**

| | ts-045-G | PC-FP | COPI | ERGIC53 |
|---|---|---|---|---|
| **37/32°C**† | | | | |
| PC-FP TCs | 4.3% (2-10%)* | - | 3.1% (0-5%) | 2.0% (0-5%) |
| ts-045-G TCs | - | 1.8% (0-5%) | 90.5% (85-95%) | 56.0% (50-60%) |
| **15°C** | | | | |
| PC-FP TCs | n.d. | - | 2.9% (0-5%) | 4.1% (3-5%) |
| COPII‡ | | | | 87.3% (85-95%) |
| COPI‡ | | | - | 96.7% (95-98%) |
| **Anti-EAGE antibodies**§ | | | | |
| PC-FP TCs | | | | 77.5% (75-90%) |

*The mean values, for a total of at least 100 TCs scored per experiment, is shown. The spread of values obtained from analysis of individual cells is shown in parenthesis; i.e. several cells in which PC-FP TCs were observed contained no co-localising COPI label (0%) whereas others contained as much as 5%.
†Cells were incubated at 37 or 32°C in the case of procollagen or ts-O45-G as transport marker, respectively.
‡Distinct COPII or COPI coated structures distributed throughout the cytoplasm at 15°C were scored.
§Anti-EAGE antibodies inhibiting COPI function were injected as described in experimental procedures.

associated with TCs in transit to the Golgi apparatus (Stephens et al., 2000) but is instead only detected in close proximity to the ER membrane (Stephens et al., 2000; Hammond and Glick, 2000). Since the behaviour of PC-FP TCs was similar to those described earlier for ts-O45-G, we asked whether PC-FP also exits the ER via COPII-coated ER exit sites and loses its COPII coat prior to transport to the Golgi. Analysis of double colour time-lapse image series (Fig. 2C-J) showed that, already 2 minutes after the addition of ascorbate, newly forming TCs containing PC-FP could be observed forming from the ER network (Fig. 2C,D, arrowheads). These structures precisely coincide with YFP-SEC24Dp (Fig. 2D, which is particularly apparent in panels E and F, which show enlarged views of the region bounded by the white box in C and D respectively), suggesting that newly forming PC-FP TCs emerge from sites labelled with SEC24Dp-YFP (Fig. 2G-J; SEC24Dp in red, marked with an arrow; see also Movie 3 at jcs.biologists.org/supplemental). Subsequently (5 minutes after the addition of ascorbate), SEC24Dp-YFP and PC-CFP segregated, and PC-CFP was transported to the Golgi independent of COPII (Fig. 2G-J, arrowhead) as previously described for ts-045-G (Stephens et al., 2000).

Immunostaining of fixed cells 10 minutes after the addition of ascorbate, and identification of moving TCs by visual inspection, showed that the majority of PC-FP TCs, which initially all moved towards the Golgi at this time point, did not positively immunolabel with antibodies against either COPI (Fig. 3A,B) or ERGIC-53 (Fig. 3C,D). This observation was striking since it has previously been reported that the majority of ts-045-G containing TCs en route to the Golgi complex do label for β-COP (Shima et al., 1999; Griffiths et al., 1995; Pepperkok et al., 1998) and contain ERGIC-53 (Pepperkok et al., 1998), results which were confirmed in this study (Fig. 4G-L; Table 1). Quantitative analysis showed that on average only 2% and 3.1% of the PC-FP TCs contained ERGIC-53 or COPI, respectively (Table 1). In contrast, at least 50% of ERGIC-53

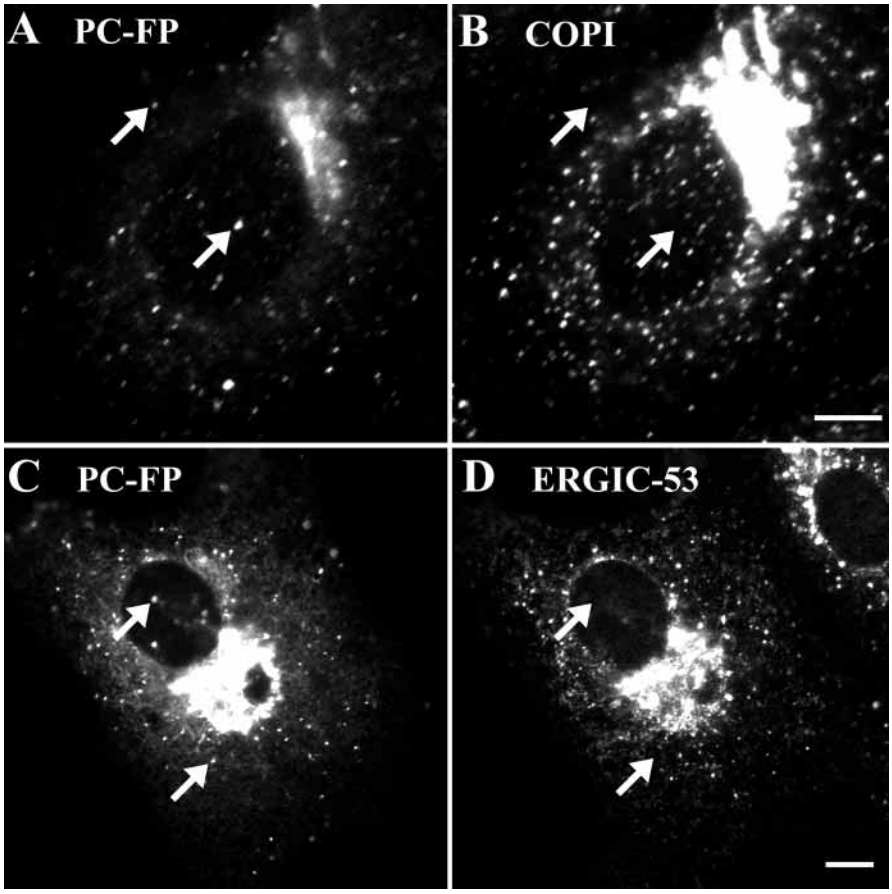


**Fig. 3.** PC-GFP transport complexes do not label for ERGIC-53 or COPI during transport to the Golgi. Cells expressing PC-GFP in the presence of ascorbic acid were fixed 10 minutes after the addition of ascorbate and processed for immunofluorescence with antibodies directed against COPI (B) or ERGIC-53 (D). Note that PC-FP structures (A) do not label for COPI (B) and similarly PC-GFP TCs (C) do not label for ERGIC-53 (D, arrows). Bars=5 μm.

structures contained COPI and vice versa (data not shown) (Griffiths et al., 1995).

We also analysed the localisation of PC-FP and ts-045-G following release of the two markers from the ER. Cells were transfected with plasmids encoding both markers and were incubated at 39.5°C overnight to accumulate ts-045-G-YFP in the ER. After 16 hours at 39.5°C, ascorbate and cycloheximide were added to the culture medium for 30 minutes. Cells were then incubated for 6 minutes at 32°C and fixed with paraformaldehyde. Under these conditions, PC-FP was not efficiently transported out of the ER, and TCs were not observed moving to the Golgi at 39.5°C. Only after shifting the temperature to 32°C were TCs clearly identifiable and all moved towards the Golgi showing that they are bona fide ER-to-Golgi transport intermediates. Analysis of cells co-expressing PC-FP and ts-045-G-YFP showed that only 4.3% of the PC-FP TCs contained ts-045-G-YFP during ER-to-Golgi transport (Table 1). These TCs were also positive, by immunolabelling, for COPI (Fig. 4A-F). Control experiments confirmed previously published reports (Scales et al., 1997; Pepperkok et al., 1998) that ts-045-G-labelled TCs also label for COPI (90.5%) and ERGIC-53 (56%) (Fig. 4G-L; Table 1). These results show that PC-FP is transported from the ER to the Golgi complex in TCs that are distinct from COPI-coated ER-to-Golgi TCs carrying ts-O45-G, ERGIC 53, lumenal GFP (D.S. and R.P., unpublished) (see Blum et al. (2000)). This suggests that mechanisms must exist to segregate PC-FP from ts-O45-G and other membrane proteins. In order to address this problem we first asked where and when this segregation takes place during ER-to-Golgi transport.

To examine the point of segregation of PC-FP from ERGIC-53, we incubated cells at 15°C. At this temperature, ER-to-Golgi transport is arrested at a very early stage following ER exit analogous to newly formed TCs (Kuismanen et al., 1992; Blum et al., 2000). Cells expressing PC-FP were incubated at 15°C for 60 minutes in the presence of cycloheximide followed by the addition of ascorbate and a further incubation at 15°C for 90 minutes. As can be seen in Fig. 5, PC-FP (Fig. 5A; arrows) did not significantly overlap with ERGIC-53 (Fig. 5B; arrows) under these conditions. Similar results were obtained when cells were incubated with ascorbate, coincident with the shift to 15°C, and when cells were first incubated in the presence of ascorbate at 37°C for 10 minutes prior to shifting to 15°C (both not shown). Thus, PC-FP and ERGIC-53 are already segregated from one another when transport is blocked at this early stage of the secretory pathway. Quantitation of these experiments showed that only 4.1% and 2.9% of PC-FP containing structures at 15°C also contained ERGIC-53 or COPI respectively (Fig. 5; Table 1). In contrast, consistent with previous observations (Griffiths et al., 1995), 96.7% of COPI structures also contained ERGIC-53. We believe that these results represent segregation of procollagen from ERGIC-53 upon or very shortly after ER exit. Interestingly, a small but

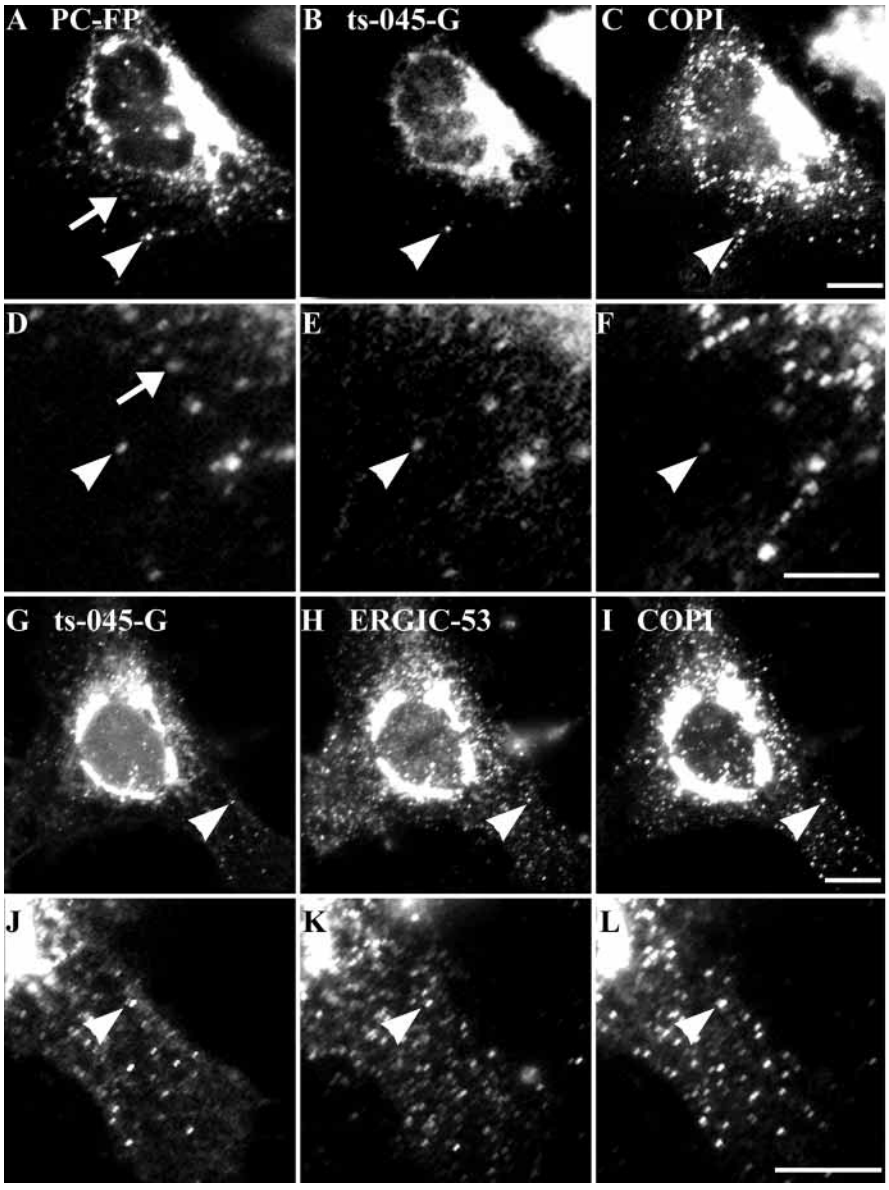


**Fig. 4.** PC-GFP transport complexes do not label for ts-045-G during transport to the Golgi. Those that do label for COPI contain both PC-FP and ts-045-G. Cells expressing PC-FP and ts-045-G-FP were incubated for 16 hours at 39.5°C, after which ascorbate and cycloheximide were added to the culture medium for 30 minutes. Cells were then incubated for 6 minutes at 32°C, fixed with paraformaldehyde and processed for immunofluorescence. PC-CFP TCs (A,D) largely exclude ts-045-G (B,E; arrow) and COPI (C,F). (D-F) An enlargement of the corresponding regions in (A-C), respectively. Those TCs that do label for both PC-CFP (A,D; arrowhead) and ts-045-G-YFP (B,E; arrowhead) also label for COPI (C,F; arrowhead). In contrast, TCs containing ts-045-G-YFP (G; arrowhead) also contain ERGIC-53 (H; arrowhead) and COPI (I; arrowhead). Bars=5 μm.

significant population of COPII structures (on the average 12.7%) (Table 1) that did not label for ERGIC 53 at 15°C was consistently found, suggesting that they represent a population of specialised COPII-coated ER exit sites preferentially used by cargoes such as procollagen.

### Anti-COPI antibodies inhibit transport of PC-FP and segregation from ts-O45-G at ER exit sites

To investigate further at which level segregation of PC-FP from ts-O45-G and other membrane proteins occurs we next investigated how transport of PC-FP was dependent on the function of COPII or COPI. Co-expression of PC-FP with a SAR1, a dominant-negative mutant (SAR1a$^{(H79G)}$) that cannot hydrolyse GTP (Aridor et al., 1995; Kuge et al., 1994), followed by incubation with ascorbate, led to a complete arrest of PC-FP within the ER (Fig. 6B) in contrast to the punctate distribution of PC-FP in TCs from the same dish but injected with control IgG (Fig. 6A, arrowheads). This shows that export of PC-FP from the ER and the subsequent formation of PC-FP TCs involves the function of the COPII complex.

COPI function was inhibited by expression of dominant-negative form of ARF1 (ARF1$^{(Q71L)}$), the small GTP-binding protein responsible for the recruitment of COPI to membranes (Zhang et al., 1994; Dascher and Balch, 1994). Expression of ARF1$^{(Q71L)}$ blocked PC-FP transport (Fig. 6D). Interestingly, this block also apparently occurred at the level of exit from the ER since PC-FP was seen to retain its ER localisation after ascorbate treatment without any significant formation of PC-FP TCs (Fig. 6D). This result was surprising, as previous work using ts-O45-G as a transport marker showed that COPI was not directly involved in ER exit and appearance of TCs (Shima et al., 1999; Scales et al., 1997; Pepperkok et al., 1998). A likely explanation for our results might be that the inhibition of ER exit by ARF1$^{(Q71L)}$ was caused indirectly, through inhibition of recycling of machinery back to the ER for example, because ARF1$^{(Q71L)}$ was expressed 4-6 hours before treatment of cells with ascorbate.

To address this problem, COPI function was inhibited by microinjection of monovalent $F_{ab}$ fragments of an antibody that blocks COPI function (anti-EAGE (Pepperkok et al., 1993)). Anti-EAGE was microinjected either before or after addition of ascorbate to the cells. Control experiments confirmed the efficacy of anti-EAGE injection by blocking transport of ts-045-G-GFP (not shown). Injection of anti-EAGE prior to the addition of ascorbate arrested PC-FP within the lumen of the ER (Fig. 6F; Movie 4 at jcs.biologists.org/supplemental), similar to the effect of expression of ARF1$^{(Q71L)}$ (Fig. 6D). This suggests a function for COPI in the accumulation of PC-FP in nascent TCs. However, injection 10 minutes after the addition of ascorbate led to a progressive but not immediate inhibition of PC-FP transport (Fig. 7A) (Movie 5 at jcs.biologists.org/supplemental). Only about 5% of the pre-existing PC-FP TCs were seen to be moving immediately following injection of anti-EAGE. The remaining TCs were immobile and apparently stable (Fig. 7A) (Movie 3, showing 3 minutes of imaging). In contrast, in cells microinjected with control antibodies, 40-50% of PC-FP TCs were mobile during the time frame of the imaging (3 minutes) and indistinguishable in their behaviour from TCs in non-injected cells (not shown). Indistinguishable results were obtained with ts-O45-G –GFP as the transport marker (not shown). At later time points, 20-30 minutes after injection of anti-EAGE, all of the pre-existing, PC-FP-containing TCs were seen to be immobile (Fig. 7B), and the number of PC-FP TCs compared with the control injected cells was significantly decreased (Fig. 7B), suggesting that anti-EAGE injection inhibits their

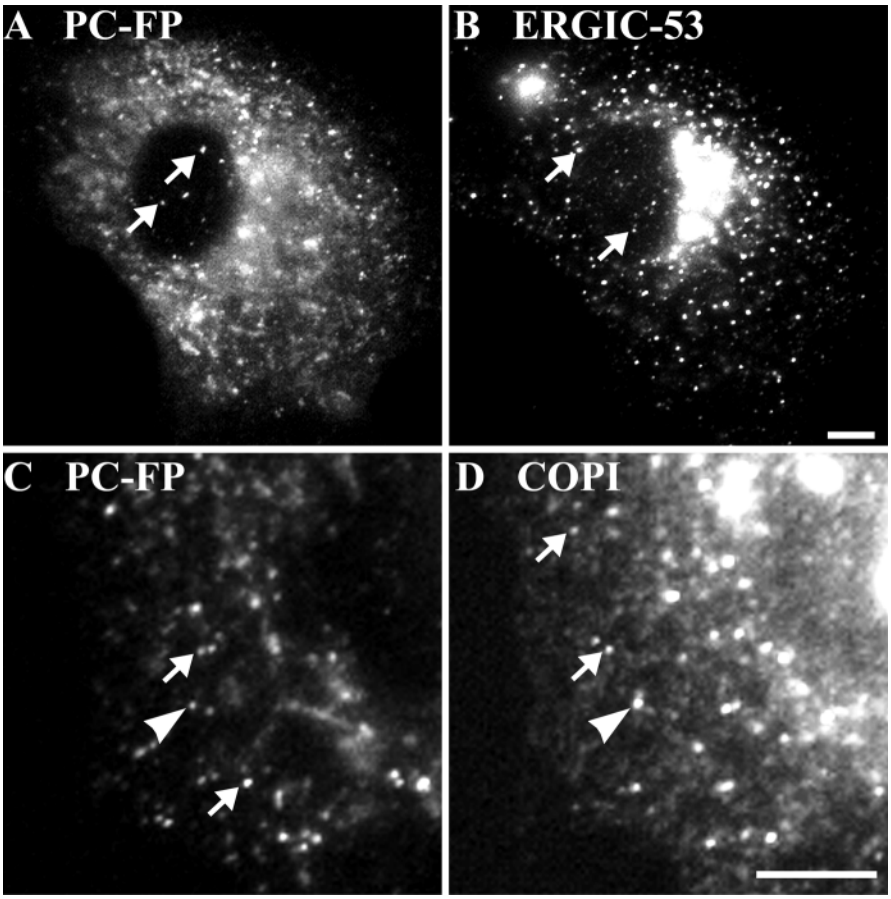


**Fig. 5.** Segregation of PC from ERGIC-53 occurs at the level of exit from the ER. Cells expressing PC-FP were incubated, in the presence of cycloheximide, at 15°C for 60 minutes prior to addition of ascorbate and further incubation at 15°C for 90 minutes. Cell were then fixed with paraformaldehyde at 15°C and processed for immunofluorescence with anti-ERGIC-53. The majority of PC-FP containing structures (A; arrows) do not colocalise with ERGIC-53 (B; arrows). Similarly, the majority of PC-FP TCs (C; arrows) do not colocalise with COPI labelled TCs (D; arrows). Occasional examples of COPI-labelled PC-FP containing TCs are seen (C,D; arrowhead). For quantitation of results see Table 1. Bars=5 μm.

formation. This is consistent with the arrest of PC-FP in the ER when injection was performed before addition of ascorbate (Fig. 6F).

These data show that COPI controls transport of both ts-O45-G and PC-FP at the ER exit level. This appears to be in contrast to our finding that PC-FP containing TCs are devoid of COPI en route to the Golgi complex. A simple explanation for this is that COPI is involved in a step that occurs shortly after COPII-dependent export from the ER, where COPI mediates the segregation of the two transport markers into distinct TCs at, or directly adjacent to, the ER exit site. If this was the case then injection of anti-EAGE should inhibit the segregation of PC-FP from ERGIC-53 at the ER exit level. Cells expressing PC-FP were incubated in the presence of cycloheximide and ascorbate. The total incubation time of the cells in ascorbate before injection of anti-EAGE was approximately 30 minutes, including transfer to the microscope, identification of cells expressing low levels of PC-FP in which Golgi-directed TCs could be identified and microinjection of anti-EAGE. Cells were then fixed and processed for immunofluorescence. In cells microinjected with anti-EAGE (Fig. 8C,D) segregation of PC-FP from ERGIC-53 was no longer apparent; 77.5% of PC-FP-labelled structures contained ERGIC-53. Overlap of PC-FP and ERGIC-53 is particularly evident in the insets to Fig. 8C and D (arrowheads) showing colocalisation of the markers within TCs lying directly adjacent to ER membranes. Cells injected with control IgG showed the same segregation of PC-FP TCs from ERGIC-53 immunostaining (Fig. 8A,B; arrows).

## Discussion

We have established a regulated system for the analysis of ER-to-Golgi transport of procollagen in living cells. We show that tagging of the pro α1 (I) form of procollagen with spectral variants of GFP generates transport-competent molecules that are secreted from cells. PC-FP undergoes ascorbate-dependent export from the ER, consistent with the known mechanism of procollagen assembly in the ER and subsequent secretion (Brodsky and Ramshaw, 1997; Lamandé and Bateman, 1999; Prockop and Kivirikko, 1995). The pro α1 (I) form of procollagen was chosen since, unlike pro α2 (I) for example, it can form homotrimers in vivo (Jiminez et al., 1977) and therefore upon overexpression would generate a naturally occurring, functionally relevant form of the molecule. The ER-to-Golgi transport carriers of PC-FP are small punctate structures (transport complexes) similar to those described earlier for other secretory markers (Scales et al., 1997; Presley et al., 1997; Chao et al., 1999). Their formation requires COPII function and they move in a directed manner to the Golgi, with virtually all PC-FP TCs labelled with antibodies directed against both N- and C-terminal propeptides of pro α1 (I) as well as antibodies directed against GFP.

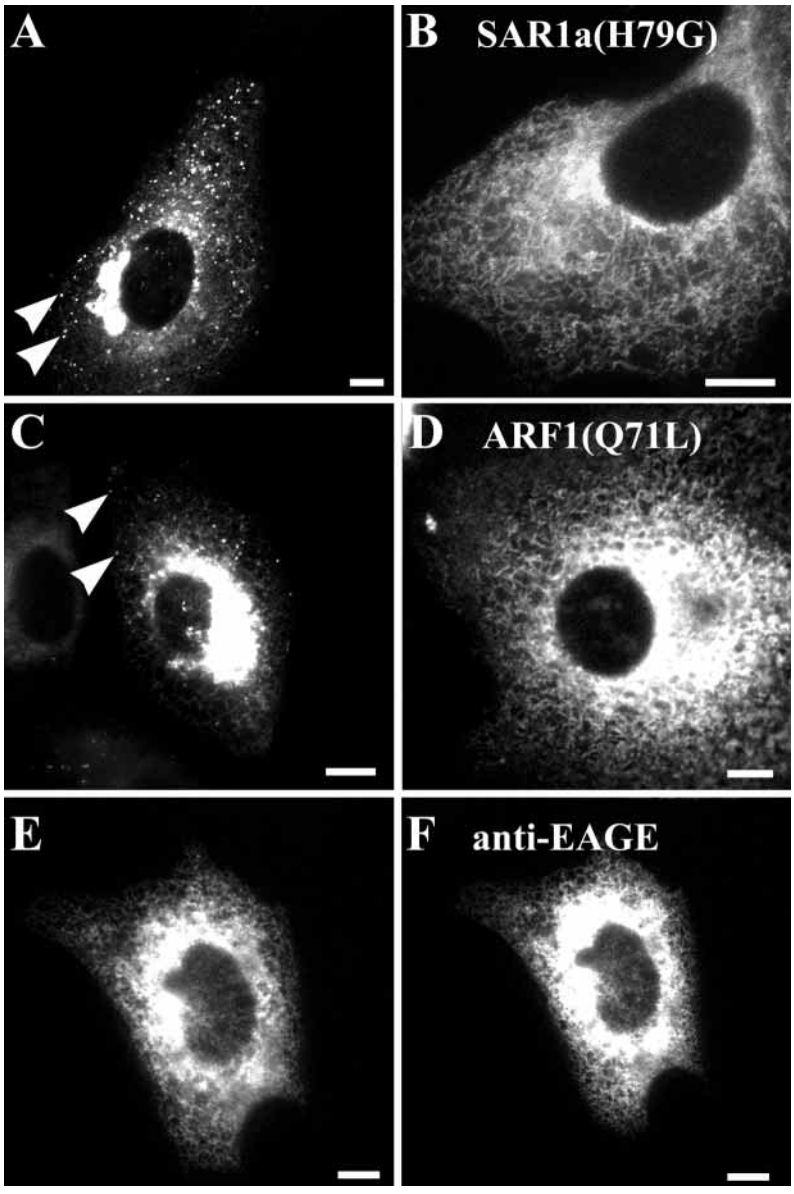


**Fig. 6.** Transport of PC-FP through the early secretory pathway is dependent on the function of the COPI and COPII coat complexes. (A,B) Cells expressing PC-FP were microinjected with a control plasmid (pcDNA3.1 (A); arrowheads indicate examples of TCs) or plasmid expressing SAR1a$^{(H79G)}$ (B) and incubated at 37°C for 2 hours followed by addition of ascorbate. Images were taken 30 minutes after ascorbate addition. Efficacy of injected SAR1a$^{(H79G)}$ expression was confirmed by anti-ERGIC-53 labelling (not shown). Control cells (A) were located on the same dish as SAR1a $^{(H79G)}$ expressing cells (B) for these experiments. (C,D) Cells expressing PC-FP were microinjected with a control plasmid (C; arrowheads indicate examples of TCs) or plasmid expressing pARF1$^{(Q71L)}$ (D). Ascorbate was added to the medium 4 hours after microinjection. Cells were imaged 30 minutes after the addition of ascorbate. (E,F) Microinjection of anti-EAGE blocks transport of PC-FP through the early secretory pathway. Cells expressing PC-CFP were injected with anti-EAGE in the absence of ascorbate and subsequently incubated with ascorbate for 1 hour following microinjection of anti-EAGE. (E) shows the cell before injection, (F) shows the same cell 1 hour after antibody injection. Note the differences in the ER labelling pattern showing the viability of the cell after injection. Bars=5 μm.

Altogether this suggests that PC-FP behaves similar, if not identically, to wild-type procollagen and therefore represents a faithful fluorescent marker to study PC transport in living cells.

The rapidity with which we observe transformation of PC-FP from a faint ER localisation to bright, uniform punctate TCs trafficking towards the Golgi complex suggests that active concentration processes exist to accumulate PC-FP prior to export from the ER. Such a selective recruitment into ER export sites could conceivably occur through membrane-anchored receptors or independently by some mechanism coupled to the assembly of the trimeric PC molecule. It seems logical that the most likely way in which PC-FP could engage the cytosolic COPII complex for concentration into export sites is by a membrane receptor. This receptor should interact with transport-competent PC-FP in the lumen of the ER and via its cytosolic tail with COPII units in the cytoplasm.

Type I procollagen is believed to assemble into a large rod-like structure of approximately 330 nm (Bächinger et al., 1982). This directly implies that the fully assembled PC molecule would be too large to fit inside conventional 60-80 nm diameter COPII transport vesicles (Rothman and Wieland, 1997; Sheckman and Orci, 1997). The data presented here are, however, entirely consistent with COPII-dependent exit of procollagen from the ER. We not only visualise PC-FP transport complexes emerging from COPII-labelled ER exit sites but also find that inhibition of COPII function arrests procollagen in the ER. Thus we propose that COPII function is also required for the secretion of large soluble cargo such as procollagen. Our data are consistent with proposed models of COPII function in terms of cargo selection and membrane deformation but suggest now that intermediates other than 60-80 nm vesicles may also be generated by the action of the COPII complex on the ER membrane. An alternative explanation may be that full assembly of type I procollagen occurs not in the ER but on the level of post-ER transport complexes. In this case COPII vesicles would be involved in ER exit of the unassembled PC, which subsequently gives rise to the formation of PC-FP-containing TCs, where full assembly would take place. The latter explanation we consider less likely because the consensus within the literature tends towards a complete folding of procollagen prior to ER exit (Brodsky and Ramshaw, 1997; Lamandé and Bateman, 1999; Prockop and Kivirikko, 1995; Walmsley et al., 1999). This hypothesis is also entirely consistent with the ascorbate-dependent exit of procollagen from the ER and the fact that unassembled procollagen chains are degraded by the proteasome and not by lysosomal enzymes (Fitzgerald et al., 1999).

The most striking result reported here is that procollagen is transported from the ER to the Golgi in transport complexes distinct from those containing ts-O45-G and ERGIC-53. These observations cannot be explained by different ER export kinetics alone for two reasons. Firstly, PC-FP-containing TCs do not contain ERGIC-53. ERGIC-53 is an itinerant ER-to-Golgi recycling protein and therefore should be present within all ER-to-Golgi TCs at steady state regardless of the kinetics of formation of these TCs. Secondly, PC-FP TCs are also devoid of cytosolic, vesicular coat complex COPI, in contrast to ts-O45-G containing TCs. Therefore, we propose the existence of at least two cargo transport pathways from the ER to the Golgi, one taken by PC-FP and one by ts-045-G and ERGIC-53. It appears that ts-O45-G and PC-FP are concentrated in the same or close by ER exit sites and segregation of the two markers occurs subsequently. This then immediately raises the question of where exactly and how does segregation of the two pathways take place.

Our data show that COPI is directly or indirectly involved at an early step close to ER exit. Injection of anti-COPI

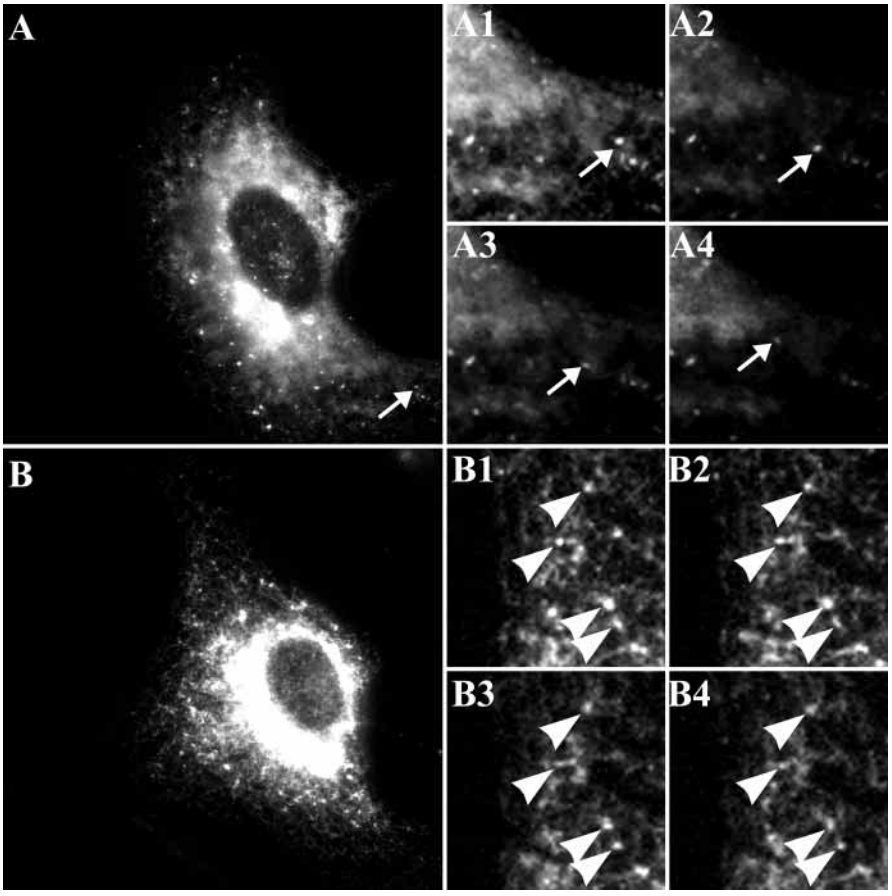


**Fig. 7.** PC-GFP dynamics following injection of anti-EAGE. Cells expressing PC-FP were incubated in the presence of ascorbate for 10 minutes prior to microinjection of anti-EAGE. When imaged immediately after injection, most PC-GFP-labelled structures were immobile (A; arrowhead). A small proportion (1-5 per cell) remained motile and trafficked to the Golgi (A; arrow) and (A1-A4, which shows time lapse images of the cell in A taken 3 seconds apart) (see also Movie 4 at jcs.biologists.org/supplemental). At later time periods after injection (30 minutes in the continued presence of ascorbate) few punctate structures remain and significant ER labelling is seen in the cells but those that are still visible are immobile (B1-B4, showing images 3s apart, arrowheads mark static structures) (see also Movie 5 at jcs.biologists.org/supplemental). Bars=5 μm.

antibodies results in a progressive inhibition of both PC- and ts-O45-G-labelled TC movement and the appearance of new TCs at ER exit sites. Coincident with this, colocalisation of PC-FP with ERGIC-53 at ER exit sites is enhanced. Furthermore, injection of antibodies before addition of ascorbate or expression of constitutively active ARF1 mutant results in arrest of procollagen in the ER. These findings appear to be in contrast to our observations that COPI is absent from PC-FP TCs en route to the Golgi complex. However, a simple explanation for this is that cargo is sorted in a COPI-dependent manner at the level of TC formation adjacent to the ER membrane. Thus, inactivation of COPI would inhibit segregation of PC from ts-O45-G and other markers as we observed it here. This hypothesis is also consistent with earlier findings suggesting that COPI has a direct and early function in the biogenesis of nascent TCs (Aridor et al., 1995; Lavoie et al., 1999). An alternative hypothesis would be that inhibition of COPI function allows fusion of previously distinct carriers containing either procollagen or ERGIC-53.

Another mechanism of segregation, although less likely, could be one in which other secretory cargo are segregated from procollagen en route to the Golgi, analogous to the formation of secretory granules in which components of immature secretory granules are removed in a clathrin-dependent process during maturation (Tooze, 1998). Observations along this line have been made earlier demonstrating that ts-O45-G-containing TCs establish an anterograde-cargo-rich (ts-O45-G) and retrograde-cargo-rich (ERGIC-53, and COPI) domain en route to the Golgi in a COPI-dependent manner (Shima et al., 1999). Therefore, one could speculate that PC-FP and ts-O45-G were segregated on moving TCs by a distinct but similar COPI-dependent mechanism. However, the absence of dual labelled structures (PC-FP and ts-O45-G), described here for all time points upon separation of respective TCs from the ER, argues against this. Furthermore, when ER-to-Golgi transport was arrested at 15°C, segregation of ERGIC-53- and PC-FP-containing TCs was already complete, in contrast to the observations made by Shima et al. (Shima et al., 1999) where domain segregation occurred after the 15°C transport block. Furthermore, the segregation of cargoes observed here is unlikely to be a result of differential localisation within a single structure owing to the large distances (2-5 μm) typically observed between PC-FP and ERGIC-53 containing TCs (Fig. 3).

Our observations here are entirely consistent with recent data obtained from experiments using the yeast *Saccharomyces cerevisiae* showing that different cargo molecules can be sorted into different COPII vesicle populations following exit from the endoplasmic reticulum (Muñiz et al., 2001). Whether the process described by Muñiz et al. (Muñiz et al., 2001) represents COPII-mediated sorting of components as opposed to lateral segregation of GPI-anchored proteins from others within the lipid bilayer remains unclear. A further important point is that the entire ER in *S. cerevisiae* appears to act as transitional ER facilitating the generation of COPII-coated vesicles (Rossanese et al., 1999). In contrast, the transitional ER of mammalian cells is organised into discrete ER export sites (Stephens et al., 2000; Hammond and Glick, 2000). This functional distribution would make it easier to have a simple partitioning of GPI-anchored proteins from others within the ER membrane in *S. cerevisiae* followed by COPII-mediated budding. Our data here suggest that there is a COPI-mediated sorting event that occurs during or shortly after exit from the ER. Together this suggests there may be more than one mechanism for pre-Golgi protein sorting in operation.

The presence of distinct transport complexes containing ts-O45-G or procollagen en route to the Golgi complex provides evidence for pre-Golgi sorting in mammalian cells. It is presently unclear to what extent the ts-O45-G- and procollagen-containing transport complexes are also different in their morphology at the ultrastructural level. More work combining the light microscopy approach used here with electron microscopy techniques will be necessary to address

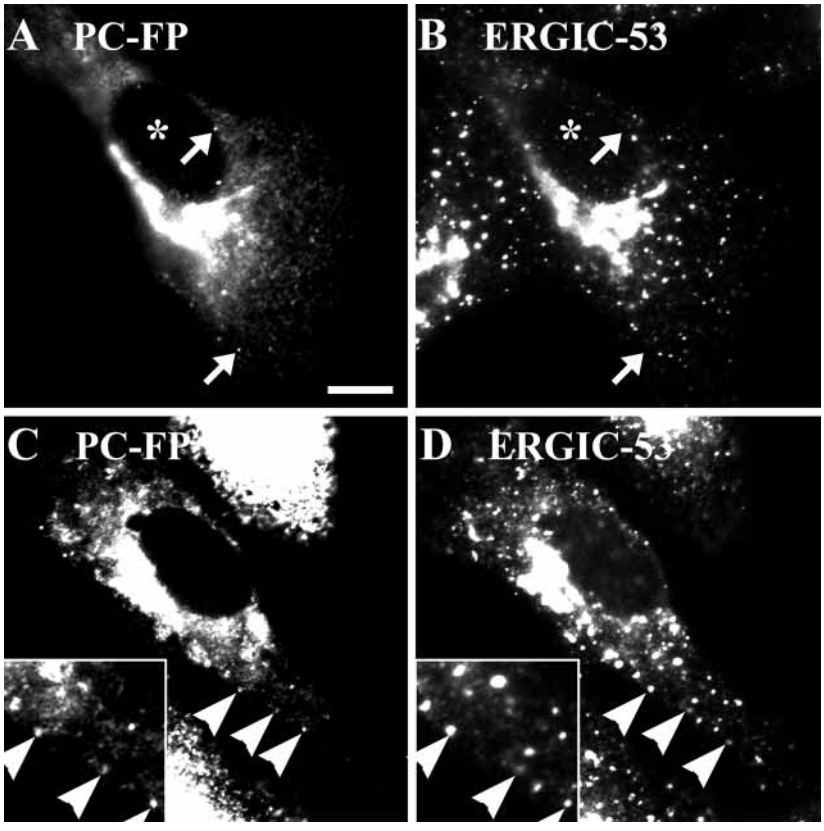


**Fig. 8.** Inhibition of COPI function by microinjection of anti-EAGE results in colocalisation of PC-FP with ERGIC-53. (A) and (B) show control cells expressing PC-FP (A) to which ascorbate was added 30 minutes before microinjection of control IgG. After a further 30 minutes at 37°C, cells were fixed and immunolabelled with anti-ERGIC-53 (B). Note structures containing PC-FP in (A; arrows) that do not label for ERGIC-53 in (B; arrows). (C,D) show cells expressing PC-FP (C) that, 30 minutes after the addition of ascorbate, were microinjected for anti-EAGE, incubated for a further 30 minutes at 37°C, fixed and immunolabelled for anti-ERGIC-53 (D). Note structures containing PC-FP in (C; arrowheads) that also label for ERGIC-53 in (D; arrowheads). The insets in (C) and (D) show zoomed areas from the highlighted regions. Bar=5 μm (applies to all panels).

this point (e.g. Mironov et al., 2000). It is also unclear why pre-Golgi sorting must occur. It is possible that segregation within the Golgi provides a means for the differential glycosylation of proteins that might otherwise receive identical modifications. Three-dimensional reconstruction of Golgi structure shows that there exist regions of cisternal Golgi interconnected with bridging tubules (Ladinsky et al., 1999), providing a basis for continued segregation of proteins once TCs have reached the Golgi. The hypothesis presented by Ladinsky et al. (Ladinsky et al., 1999) that TCs fuse homotypically to form the first Golgi cisternae may provide a mechanism by which this segregation is maintained. One possibility is that an alternative ER-to-Golgi pathway exists for large protein complexes only. Pre-Golgi sorting could occur for some cargo molecules like procollagen, algal scale structures and aggregated protein complexes, which are too big to enter small transport vesicles, or that need to take special routes through the Golgi complex (Melkonian et al., 1991; Bonfanti et al., 1999; Volchuk et al., 2000). Alternatively, pre-Golgi sorting may represent the first step in functional segregation and compartmentalisation of proteins within the cell. In this context it will be important to determine whether the sorting of different secretory proteins from one another before they reach the Golgi is maintained during transport through the Golgi itself. Careful examination of the distribution and lateral mobility of secretory cargo proteins within the Golgi may enable us to address this question in the future.

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