Reconstitution of Retrograde Transport from the Golgi to the ER In Vitro

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Abstract. Retrograde transport from the Golgi to the ER is an essential process. Resident ER proteins that escape the ER and proteins that cycle between the Golgi and the ER must be retrieved. The interdependence of anterograde and retrograde vesicle trafficking makes the dissection of both processes difficult in vivo. We have developed an in vitro system that measures the retrieval of a soluble reporter protein, the precursor of the yeast pheromone α-factor fused to a retrieval signal (HDEL) at its COOH terminus (Dean, N., and H.R.B Pelham. 1990. J. Cell Biol. 111:369–377). Retrieval depends on the HDEL sequence; the α-factor precursor, naturally lacking this sequence, is not retrieved. A full cycle of anterograde and retrograde transport requires a simple set of purified cytosolic proteins, including Sec18p, the Lma1p complex, Uso1p, coatomer, and Arf1p. Among the membrane-bound v-SNAP receptor (v-SNARE) proteins, Bos1p is required only for forward transport, Sec22p only for retrograde trafficking, and Bet1p is implicated in both avenues of transport. Putative retrograde carriers (COPI vesicles) generated from Golgi-enriched membranes contain v-SNAREs as well as Emp47p as cargo.

Key words: retrograde transport • Golgi apparatus • endoplasmic reticulum • COPI • SNARE

Proteins designated for secretion are translocated into the endoplasmic reticulum (ER), packaged into transport vesicles, and delivered to the cell surface via the Golgi apparatus. At the same time, endocytosed proteins may travel from the plasma membrane via the Golgi to the ER. Thus, anterograde and retrograde transport occur simultaneously and yet achieve a balance that retains the individual character of each participating membrane.

Transport between different compartments is mediated by coated vesicles. Vesicles involved in early transport steps are either coated with coatomer, a multimeric protein complex of ~700 kD with the small GTPase Arf1p, or COPII consisting of two heterodimers (Sec23/24 and Sec13/31 complex) and the small GTPase Sar1p (Orci et al., 1986; Barlowe et al., 1994). Each coat has been implicated in forward transport; however, retrograde transport seems exclusively to be dependent on coatomer (Cosson and Le tourneur, 1994; Bednarek et al., 1995, 1996; Orci et al., 1997). Coatomer was shown to be enriched on the cis-face of the Golgi apparatus (Duden et al., 1991), whereas a number of conditional mutants in coatomer components display defects in anterograde transport. The effect of coatomer mutations may be explained by an indirect effect in which a failure to retrieve essential SNAP receptor (SNARE) proteins eliminates anterograde transport.

At least a part of the selectivity achieved in vesicle targeting is attributed to the interaction of cognate vesicle or v-SNAREs and target or t-SNAREs. A cycle between the ER and the Golgi could, in principle, be achieved by two v- and two t-SNAREs, one pair directing anterograde targeting and the other directing retrograde targeting. Given that SNAREs are themselves localized by the secretory pathway, one challenge has been to understand how they may be regulated to express targeting activity in the correct location. The same consideration applies to other proteins that shuttle between the ER and the Golgi. In yeast, Golgi proteins such as Emp47p and ER proteins such as Emp24p continuously cycle yet maintain a concentration in one or the other organelle (Schimmöller et al., 1995; Schröder et al., 1995).

Many stable ER proteins undergo slow anterograde transport to the Golgi, from which they are retrieved by a receptor-mediated process. The best known of those re-
Materials and Methods

Yeast Strains, Media, and Yeast Transformation

Yeast strains used in this study are summarized in Table I. Yeast cells were grown in yeast extract, peptone, and dextrose growth medium (YPD). Yeast strains were transformed as described by Schiestl and Gietz (1989).

**Table I. Strains Used in This Paper**

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<th>Strain</th>
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<tbody>
<tr>
<td>RSY445</td>
<td>MATα ura3-52 leu2-3, 112 trp1-289 pep4::URA3 prb1 his4-579</td>
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<td>35S-ufl-1</td>
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<td>MATα his4-619 bet1-1</td>
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<td>MATα ura3-52 leu2-3, 112 his4-619 sec22-2</td>
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For cytosol, yeast cells were grown to early to mid-log phase in YPD. Wild-type and ts-mutant strains were grown at 30 and 23°C, respectively. The cells were harvested by centrifugation and washed twice with water. The cell pellet was resuspended in a minimal volume of buffer B88 (20 mM Hepes, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM Mg(OAc)₂, and pipetted into liquid nitrogen. The cell beads were ground up under liquid nitrogen in a blender (Worthington Biochemical Corp., Freehold, NJ) for large-scale preparations or in a mortar for small-scale preparations. The cell powder was thawed in an ice-water bath, and ATP and DTTO each at a final concentration of 1 mM as well as protease inhibitors were added. The lysate was centrifuged (5 min, 3,000 g; 15 min, 20,000 g; 1 h, 100,000 g) and the 100,000-g supernatant was collected, carefully avoiding the pellet and the liquids which floated to the top.

**Antibodies**

Antibodies directed against c-myc (Evan et al., 1985); Sec21p (Hosobuchi et al., 1992); Sec22p (Bednarek et al., 1995); Sec23p (Barlowe et al., 1994); Bet1p, Bos1p, and coatomer (Rexach et al., 1994); and α-1,6 linked mannose (Baker et al., 1988) have been described. Affinity-purified antibodies against Sec18p and Ykt6p were generous gifts of W. Wickner (Dartmouth Medical School, Hanover, NH) and J. McNew (Sloan-Kettering Cancer Center, New York), respectively.

Monoclonal anti-HA (12CA5) antibodies were purchased from Berkeley Antibody Co. (Richmond, CA). Donkey anti-rabbit and sheep anti-mouse secondary antibodies coupled to hors eradish peroxidase were obtained from Amersham Corp. (Arlington Heights, IL).

Antibodies against Arf1p were raised in rabbits using purified N-myristylated yeast Arf1p. The specificity of the antiserum was tested by immunoblot on total cell lysates using an ECL-kit (Amersham Corp.).

**Purification of Coatomer, COPII, Uso1p, Sec18p, N-myristylated Yeast Arf1p, and N-myristylated Yeast Arf1Q71Lp**

Coatomer was purified according to Hosobuchi et al. (1992). The purification of Sar1p, Sec23/24 complex, and Sec13/31 complex were performed as described by Barlowe et al. (1994) and Salama et al. (1993), respectively. A myc-tagged Uso1p was purified from yeast cytosol according to Barlowe (1997). Bacterially expressed Sec18p-His was purified as previously described (Whiteheart et al., 1994), but after isolation, the peak fractions were pooled and dialyzed against B88 that contained 0.1 mM DTT, 0.1 mM PMSF, 0.1 mM ATP, and 15% glycerol. Escherichia coli BL21(DE3) strains, which coexpress yeast N-myristoyl transferase and either wild-type or dominant-activated (Q71L) yeast Arf1p, were provided by R. Kahn (National Institutes of Health, Bethesda, MD). N-myr-yArf1p and N-myr-yArf1Q71Lp were purified as described previously (Kahn et al., 1995). The Lma1p complex was a generous gift of Zuoyu Xu (Dartmouth Medical School) and was prepared as described (Xu et al., 1997).

**In Vitro Round-Trip Retrieval Assay**

Reagents used for the in vitro round-trip assay were prepared as described by Baker et al. (1988) unless indicated otherwise.

**Stage I: Translocation.** The translocation reaction using [35S]ppp-factor and cop1-35S-factor-myc-HDEL (Dean and Pelham, 1990), [35S]ppp-factor-myc-EDLN (Dean and Pelham, 1990), or [35S]ppp-factor and gels1-1 mutant donor mem-

This study
branes was performed as described by Rexach et al. (1994), except that the scale was increased twofold and after the high-salt treatment, and inclusion of 150 mM KCl, 5 mM Mg(OAc)2, 250 mM sorbitol) for 10 min at 4°C was included. The membranes were then washed twice in B88 and resuspended to 220 μl in B88.

**Stage II: Budding.** To the membranes of the stage I reaction, we added 25 μg/ml Sar1p, 25 μg/ml Sec23/24 complex, 75 μg/ml Sec13/31 complex, 50 μM GDP-MnCl2, 10 mM ATP regeneration system (380 mM B88 before endogenous GDP-MnCl2. Membranes at the interface were diluted to the starting volume with B88 and sedimented for 15 min at 20°C. Membranes in the postnuclear supernatant were centrifuged twice at 15,000 g for 30 min. Washed membranes were then resuspended to 100 μl with B88. Fusion with the acceptor ER was measured by precipitation of populations required for fusion are missing, the COPII vesicles were purified. For the Golgi budding reaction, we incubated the membranes with 0.1 mM GTP, 50 μM GDP-MnCl2, and 15% and 400 μl 10% Nycodenz in B88. The gradients were centrifuged for 16 h at 40,000 rpm (model SW55 rotor; Beckman Instruments). Fractions (300 μl) were collected from the top and TCA-precipitated, and the top 11 fractions were analyzed as described above.

**GDPase Enzyme Assay**

The GDPase assay was performed as described by Yanagisawa et al. (1990). Samples (5 μl) were mixed with 50 μl 20 mM imidazole-HCl, pH 7.4, 2 mM CaCl2, 0.1% Triton X-100, and 5 mM GDP or GDP as control and incubated for 20 min at 30°C. Reactions were terminated by the addition of 10 μl 6.7% SDS. Inorganic phosphate was detected by adding 200 μl of 0.55% ammonium molybdate, 0.15% ascorbic acid in 0.86 N H2SO4. The assay was allowed to develop for 20 min at 42°C and quantified by reading the absorbance at 750 nm in a plate reader (model MR5000; DYNEX Technologies, Inc., Chantilly, VA).

**Results**

**Features of the In Vitro Round-Trip Assay**

We established an in vitro system that allowed us to follow the retrieval of the precursor of the phenome Α-factor to which an HDEL sequence had been fused (536SGpαF-HDEL; Dean and Pelham, 1990). This reporter molecule was chosen because the precursor of Α-factor is a useful tool in monitoring early steps in transport, such as translocation into the ER, budding from the ER, and fusion with the Golgi apparatus. The assay is divided into four stages (Fig. 1A). In the first step [536SGpαF-HDEL is translocated into the lumen of the ER, where it is N-glycosylated. In the second stage, ER-enclosed [536SGpαF-HDEL is packaged into COPI vesicles using purified proteins. Because proteins required for fusion are missing, the COPI vesicles do not fuse with the Golgi apparatus. Transport vesicles are separated from the donor membranes by centrifugation. In the third step, purified Sec18p, the LmA1p complex, and Uso1p as well as acceptor membranes are added to the vesicle fraction. These proteins are necessary and sufficient to allow the fusion of COPII vesicles with the cis-Golgi membrane (Barlowe, 1997). Finally, cytosol is added to the reaction, providing the proteins necessary for retrograde transport. Retrieval depends upon the recognition of the HDEL sequence of [536SGpαF-HDEL by Erd2p, the HDEL receptor. This recognition may either occur in the cis-Golgi or in a compartment formed by the fusion of COPII vesicles containing at least Erd2p, possibly the yeast equivalent of an intermediate compartment. However, since an intermediate compartment has not yet been identified in yeast, we refer to the -HDEL sorting membrane as Golgi. Retrograde transport vesicles capture...
ing \[^{35}\text{S}]\text{gp}\alpha\text{F-HDEL} bound to \text{Erd2p} must reach their destination and fuse with the acceptor ER to complete the reaction.

To measure the amount of retrieved \[^{35}\text{S}]\text{gp}\alpha\text{F-HDEL}, we took advantage of the SDS-PAGE mobility difference between gp\alpha\text{F} in ER membranes from glucosidase wild-type (\text{GLS1}) and mutant (\text{gls1-1}) cells (Latterich and Schekman, 1994). Glucosidase I initiates trimming of an N-linked oligosaccharide by removing a terminal glucose residue on glycoproteins in the ER (Fig. 1 B). Glucosidase II removes the two remaining glucose residues. \text{gls1-1} prevents the trimming of the terminal sugar residue and thus the action of glucosidase II. This mutation does not affect the transport efficiency of \[^{35}\text{S}]\text{gp}\alpha\text{F-HDEL} (data not shown). We used perforated spheroplasts (semi-intact cells) from a \text{gls1-1} strain as donor membranes. Glucose trimming should occur if \[^{35}\text{S}]\text{gp}\alpha\text{F-HDEL} is retrieved to a \text{GLS1} acceptor ER membrane. Trimming results in a higher electrophoretic mobility species of the reporter molecule. We refer to a complete cycle of anterograde and retrograde transport as a round-trip.

We performed a round-trip in vitro assay using acceptor...
membranes from either a GLS1 or a gbsl-1 strain (Fig. 2 A). As predicted, a higher mobility form of [35S]gpaF-HDEL was detected only when glucosidase I activity was present in the acceptor membranes. The trimmed species was absent when gbsl-1 acceptor membranes were used (Fig. 2 A, compare lanes 2 and 3). A species migrating at the position of untrimmed [35S]gpaF-HDEL was present in all lanes. This form most likely represents [35S]gpaF-HDEL retained in COPII vesicles that were unable to fuse with the Golgi, in the Golgi, or in retrograde transport vesicles derived from the Golgi.

More than 1% of the [35S]gpaF-HDEL present in the COPII vesicle fraction (stage II) was trimmed in the final stage of a typical round-trip reaction. Considering the complexity of the reaction, this low efficiency is not surprising. A final signal of about 1.6% would result if each budding and targeting step in a round-trip assay achieved 25% efficiency.

We were concerned that fragments of the donor ER might contaminate the vesicle fraction such that subsequent homotypic membrane fusion with the acceptor ER could account for the trimmed signal. To rule out this possibility, we used acceptor membranes from a kar2-133 strain. This mutant is deficient in homotypic ER membrane lysis (data not shown). The cytosol and membrane fractions were then used in a stage IV incubation at either 20 or 30°C. The transport defect could not be investigated at 37°C because of membrane lysis (data not shown).

At 20°C, reactions containing wild-type and mutant components promoted significant levels of retrieval (Fig. 3, compare lanes WT 20°C and sec21-3 20°C). Retrieval was enhanced at 30°C in the wild-type but not in the mutant reaction (Fig. 3, compare lanes WT 30°C and sec21-3 30°C). In addition, affinity-purified anti-Sec21p antibodies blocked retrieval at the permissive temperature, whereas affinity-purified anti-Ykt6p antibodies had no effect on retrograde transport (Fig. 3). Ykt6p is a SNARE that is thought to be essential for ER-to-Golgi transport (McNew et al., 1997). Because Sec21p is part of the coatomer complex, we suggest that retrieval is mediated by COPI in vitro.

**The HDEL Sequence at the COOH Terminus Is Necessary and Sufficient for Retrieval In Vitro**

If our round-trip reaction requires retrieval, then glucose trimming should depend upon the HDEL signal. We compared [35S]gpaF and [35S]gpaF-HDEL as substrates and found trimming to be signal dependent (Fig. 2 B, compare lanes 3 and 6). The gpaF-HDEL we used in our studies also contained a myc-epitope tag. To ensure that the tag was not responsible for the trimmed signal, we compared the gpaF-HDEL to a gpaF where the HDEL sequence had been removed, but the myc-tag was still present. This resulted in EDLN at the COOH terminus (gpaF-EDLN; Dean and Pelham, 1990). No retrieval was detected for gpaF-EDLN (data not shown). All three proteins behaved similarly in a forward transport reaction with respect to budding from the ER and fusion with the Golgi apparatus (data not shown). These results demonstrate that the HDEL sequence is necessary and sufficient to retrieve a protein normally destined for secretion back to the ER in vitro.

Another prediction for the assay is that retrieval of the reporter should be dependent on functional Erd2p. To test this, we prepared acceptor membranes from an erd2-B36 mutant strain, in which the retrieval of HDEL-containing proteins is strongly impaired in vivo (Semenza et al., 1990). When acceptor membranes from the erd2-B36 mutant were used in the assay, the amount of trimmed gpaF-HDEL was reduced 3-fold compared with wild-type (Fig. 2 C). Taken together, these results demonstrate that the round-trip in vitro assay is capable of recapitulating the retrieval of soluble HDEL-containing proteins, enabling a biochemical investigation of the mechanisms by which retrieval and retrograde transport are achieved.

**Functional Coatomer Is Required for Retrieval In Vitro**

Recent genetic studies suggest a role for coatomer in retrieval (Letourneur et al., 1994; Lewis and Pelham, 1996; Gaynor and Emr, 1997). However, biochemical data showing that retrograde Golgi to ER vesicles are COPI coated is lacking. Therefore, we used our round-trip assay to detect a role for coatomer. SEC21 encodes the γ-subunit of coatomer (Hosobuchi et al., 1992), and the sec21-3 mutant is defective for retrieval in vivo (Gaynor and Emr, 1997). We prepared cytosol and acceptor membranes from either wild-type or sec21-3 strains grown at a permissive temperature. The cytosol and membrane fractions were then used in a stage IV incubation at either 20 or 30°C. The transport defect could not be investigated at 37°C because of membrane lysis (data not shown).

The assembly and disassembly of coats require the action of small GTPases (for review see Salama and Schekman, 1995). Arf1p is the GTPase necessary for coatomer-dependent budding from the Golgi. Because coatomer and Arf1p are sufficient to promote the formation of COPI

![Figure 3](jcb.rupress.org)
vesicles, we performed a retrieval assay where cytosol was replaced with purified myristylated Arf1p and coatamer (Fig. 4 A). Arf1p or coatamer alone stimulated some retrieval of the gpaF-HDEL (Fig. 4 A, compare lanes 1 to 2 and 3). Although endogenous Arf1p and coatamer were detected in the Golgi fraction, addition of both proteins stimulated retrieval to almost the level obtained with cytosol (Fig. 4 A, compare lanes 1 and 4). In contrast, the addition of COPII resulted in background retrieval of gpaF-HDEL (Fig. 4 A, compare lane 5 to lanes 1 and 4).

To further establish the role of Arf1p in our retrieval system, we substituted Arf1p with a dominant-active mutant form of the protein (Arf1Q71Lp). This mutant protein is predominantly in its GTP-bound form, and the hydrolysis of GTP is diminished (Kahn et al., 1995). Upon addition of Arf1p and coatamer, a slight stimulation of the retrieval signal could be observed compared with the cytosol experiment (Fig. 4 B, compare lanes 2 and 3). Replacement of Arf1p with Arf1Q71Lp resulted in a reduction of trimmed gpaF-HDEL (Fig. 4 B, compare lane 4 to lanes 2 and 3). The residual signal is again most likely due to endogenous Arf1p bound to the Golgi membrane. Alternatively, a reduced level of GTP hydrolysis by mutant Arf1p may allow residual transport.

Brefeldin A (BFA) inhibits nucleotide exchange on Arf1p and blocks COPI vesicle formation (Donaldson et al., 1992). Thus, the addition of brefeldin A to the retrograde transport reaction should inhibit retrieval. Indeed, addition of 100 μM brefeldin A to stage IV of the round-trip reaction significantly reduced the formation of trimmed [35S]gpaF-HDEL (Fig. 4 C, compare lanes 2 and 3). The formation of retrieved gpaF-HDEL was at least partially restored in the presence of BFA in reactions supplemented with Arf1p preequilibrated with GTP (data not shown).

**The Involvement of SNAREs in Retrograde Transport**

It has not been possible to assess directly the role of v-SNAREs in retrograde transport because mutants exhibit a defect in anterograde ER-to-Golgi transport. The round-trip reaction permitted us to examine the role of SNAREs in retrieval. We prepared membranes from different SNARE mutant strains grown at a permissive temperature. Stage IV of the assay was performed at 30°C, a semipermissive temperature, which reduced but did not abolish the formation of trimmed [35S]gpaF-HDEL. Stage IV of the assay was performed at 30°C, a semipermissive temperature, which reduced but did not abolish the formation of trimmed [35S]gpaF-HDEL. Stage IV of the assay was performed at 30°C, a semipermissive temperature, which reduced but did not abolish the formation of trimmed [35S]gpaF-HDEL. Stage IV of the assay was performed at 30°C, a semipermissive temperature, which reduced but did not abolish the formation of trimmed [35S]gpaF-HDEL. 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Likewise, tlg2 null mutant membranes, deficient in trafficking at or after the trans-Golgi (Holthuis et al., 1998), showed no defect in our in vitro reactions (data not shown).

The requirement for three v-SNAREs was also examined. Here the exact roles of these molecules have been difficult to distinguish because of the interdependence of the anterograde and retrograde processes. bos1-1 mutant membranes were defective in ER-to-Golgi transport, but not in ER budding, when incubations were conducted at 30°C (Fig. 5, compare B and C). However, no defect in retrieval was observed with bos1-1 mutant membranes incubated at 20°C in stage III followed by 30°C in stage IV (Fig. 5 A). This result suggests that Bos1p acts specifically in anterograde targeting. The converse was found for sec22-3 mutant membranes, which displayed normal ER-to-Golgi and ER budding at 30°C, but were defective in a stage IV retrieval incubation at this temperature (Fig. 5, compare A with B and C). By this analysis, we suggest that Sec22p serves as a retrograde v-SNARE. In yet another contrast, bet1-1 mutant membranes were defective both in retrieval and in ER-to-Golgi transport at 30°C (Fig. 5, A and B). Thus, Bet1p may serve as a v-SNARE in both directions.

Sec18p Is Required for Vesicle Fusion with the ER

Anterograde vesicle targeting requires the NSF ortholog Sec18p, whereas homotypic ER fusion requires the NSF homologue, Cdc48p. To examine which ATPase is responsible for retrograde vesicle fusion with the ER, we performed a retrieval assay including affinity-purified anti-Sec18p antibodies in stage IV. The production of trimmed [35S]gpaF-HDEL was reduced from 2.3 to 0.4% and was restored to 1.7% when excess recombinant Sec18p (15 μg) was preincubated with the antibody. Antibodies against Sec17p (yeast α-SNAP) also blocked retrograde transport. Furthermore, cdc48-3 mutant membranes displayed no retrieval defect. Thus, at least a part of the targeting machinery for anterograde and retrograde vesicle docking is shared. However, although fusion of anterograde vesicles with the cis-Golgi requires Cdc2p, the retrieval reaction (stage IV) was unaffected by up to 8 mM EGTA (data not shown).

Generation of COPI-coated Vesicles from the Golgi Apparatus In Vitro

Transport of gpaF-HDEL from the Golgi to the ER was stimulated by components of the COPI coat (Fig. 4). To monitor directly the uptake of membrane cargo into COPI vesicles, we developed a vesicle budding assay using enriched Golgi membranes as a donor.

We used myc-tagged Emp47p, a Golgi protein that continuously recycles through the ER, as a marker for the formation of retrograde transport vesicles. To follow Golgi membranes in subsequent experiments, we introduced HA-tagged Och1p (a marker of the yeast cis-Golgi membrane), to create strain YAS31, which contains two copies of OCH1 and EMP47 controlled by their own promoters. This strain had a normal growth rate and no apparent morphological abnormalities at temperatures between 23 and 37°C (data not shown).
Membranes from a postnuclear pellet were incubated with buffer alone, COPII (including Sar1p and GTP-γ-S), or COPI (including Arf1p and GTP-γ-S). Slowly sedimenting vesicles were separated from Golgi membranes by velocity sedimentation on a sucrose density gradient. Fractions of the gradient were analyzed by immunoblot. In the buffer control, some Sec21p, the γ-subunit of coat-omer, was at the top of the gradient. This residual protein may represent endogenous protein associated with Golgi membranes (Fig. 6). Likewise, residual Sec23p, a COPII subunit, was present in the load fractions of the gradient. Golgi membrane sedimentation to the bottom of the gradient was confirmed by measuring the distribution of the marker enzyme, GDPase. Other Golgi marker proteins, such as myc-Emp47p and Och1p-HA, were also largely in the more rapidly sedimenting membrane (Fig. 6). No change in the distribution of these proteins was observed in incubations with COPII proteins, although a notable amount of Sec23p migrated to the bottom of the gradient (Fig. 6). In contrast, COPI liberated a fraction of myc-Emp47p and Och1p-HA, which sedimented more slowly (fractions 6–8). This distribution was coincident with Sec21p, suggesting that fractions 6–8 represented COPI-coated vesicles derived from the Golgi apparatus. These vesicles could represent carriers involved in intra-Golgi transport supported by COPI. Och1p, for example, is known to cycle between cis- and trans-Golgi (Harris and Waters, 1996). Alternatively, the vesicles may represent retrograde vesicles involved in a continuous recycling of cis-Golgi proteins. Of course, the vesicle fraction could contain a mixture of anterograde and retrograde COPI vesicles.

If v-SNAREs involved in anterograde transport are recycled, one would predict that they should be found in the COPI-coated retrograde transport vesicles derived from the Golgi fraction. Most of the Bos1p and Sec22p signal was found in the rapidly sedimenting membrane fraction (Fig. 6). Whereas no vesicular release of Bos1p and Sec22p was observed in the presence of COPII, a fraction of Bos1p and Sec22p sedimented more slowly in incubations containing coatomer, Arf1p, and GTP-γ-S (Fig. 6). This observation supports the assignment of a retrograde role for the COPI vesicles formed in this incubation.

To extend these results, we pooled fractions 6–8 of gradients described above and conducted a second fractionation based on coated membrane buoyant density. Again, Golgi membranes incubated without coat proteins did not give rise to a signal, indicating that no fragmentation of the membranes occurred during the procedures (data not shown). The pool from gradients of incubations with coat-omer, Arf11p, and GTP-γ-S produced vesicles in which SNAREs and coatomer co-fractionated (Fig. 7; COPI, compare Bos1p and Bet1p to Ret1p). Incubations with COPII proteins and Sar1p instead of coatomer and Arf1p resulted in no signal of SNAREs or COPII components (Fig. 7; COPII). The Sec23p signal in the load of the flotation gradient is most likely due to trailing from the top of the velocity gradient; the blot was heavily overexposed to enhance the detection of COPII vesicles. This confirms the formation of retrograde COPI-coated transport vesicles from the Golgi-enriched membranes.

Figure 6. Emp47p, Bos1p, Sec22p, and Och1p are cargo of Golgi-derived COPI vesicles. Golgi-budding assays from postnuclear membranes were performed either without addition of coat proteins (buffer) or in the presence of COPI or COPII. The reaction was loaded on a Ficoll/sucrose gradient (see Materials and Methods for details) and centrifuged for 2 h at 100,000 g. Fractions were collected from the top, separated by SDS-PAGE, and analyzed by immunoblot with antibodies directed against Sec21p, Sec23p, Sec22p, Bos1p, the HA-epitope (OCH1-HA), and the myc-epitope (myc-EMP47). The arrows indicate the direction of the gradient from the top to the bottom.
Figure 7. Enrichment of COPI-coated vesicles derived from the Golgi. Fractions 6–8 of the gradients of a Golgi budding reaction performed in the presence of either COPI or COPII and GTP-γ-S were pooled and floated on a Nycodenz gradient as described in Material and Methods. Fractions were collected from the top, separated by SDS-PAGE, and analyzed by immunoblot with antibodies direct against Ret1p (coatomer), Sec23p, Bos1p, and Bet1p. The arrows indicate the direction of movement of lipid particles within the gradient.

Discussion

Extensive genetic and morphological evidence has implicated COPI in retrograde vesicular traffic from the Golgi apparatus to the ER. Unfortunately, because the processes of anterograde and retrograde transport are intimately linked in a cycle, it has been difficult to assign roles to certain proteins unambiguously to one or the other limb of the cycle. For this reason, we developed a cell-free system that reproduces both events.

Previously, we established a transport assay that measures the vesicular traffic of yeast α-factor precursor from the ER to the Golgi (Baker et al., 1988). Dean and Pelham (1990) showed that the yeast retrieval signal, HDEL, appended to the COOH terminus of the α-factor precursor is sufficient to promote the retrieval of intact, glycosylated precursor (gpαF), to the ER in vivo. We combined our cell-free reaction with the use of an HDEL retrieval signal to produce a convenient and reliable tracer of the round-trip reaction. Completion of retrograde transport was monitored by the conversion of a glucosylated form of the gpαF-HDEL, formed in the ER of a strain deficient in glucose trimming of N-linked glycans, to a trimmed species in the ER of a glucosidase proficient strain. Retrieval in vitro was shown to depend on the HDEL signal and on the receptor for this signal, Erd2p.

To focus on those requirements unique to retrograde transport, we incorporated the observations of Barlowe (1997), who showed that COPII vesicles dock and fuse with a crude Golgi membrane fraction in the presence of Sec18p, the Lma1p complex, and Uso1p. Other requirements for anterograde transport were supplied by the membrane fraction. Once in the Golgi membranes, [35S]gpαF-HDEL was retrieved to an acceptor ER fraction in the presence of cytosol. The requirements for cytosol in the retrograde event were satisfied by pure yeast coatomer and myristylated Arf1p. Other obvious requirements for retrieval, such as the Arf1p nucleotide exchange proteins Gea1p and Gea2p (Chardin et al., 1996; Peyroche et al., 1996), and possibly an Arf1p GTPase-activating protein, most likely are provided as peripheral membrane components of the Golgi fraction. Retrieval was inhibited by BFA, and this inhibition was relieved by including Arf1p-GTP in the incubation. Thus, the requirement for Arf1p nucleotide exchange is recapitulated in the reaction.

Arf1p has been suggested to promote COPI vesicle budding by activating phospholipase D (PLD), which hydrolyzes PC to create phosphatidic acid, an acidic phospholipid that attracts coatomer to synthetic liposomes (Kristakis et al., 1996). However, we saw no evidence of this pathway in our round-trip reaction. Membranes isolated from spo14, a yeast mutant missing the standard PLD, displayed normal retrieval in vitro (Waksman et al., 1996). An independent Ca2+-dependent PLD in yeast (Waksman et al., 1997) is unlikely to serve as an alternative in retrograde transport because the retrieval reaction sustained by spo14 mutant membranes was unaffected by excess EGTA. Other targets of Arf1p include phosphatidylinositol-specific kinases. PIP2 produced by these kinases may enhance the action of PLD (Martin et al., 1996). However, wortmannin, an inhibitor of this class of kinases, had no effect on our retrieval reaction.

We used the round-trip reaction to distinguish roles for three v-SNAREs that have been implicated in ER-to-Golgi traffic: Bos1p, Bet1p, and Sec22p. Our results confirm a role for Bos1p in anterograde targeting, and we suggest that Sec22p serves the equivalent role in retrograde targeting. Surprisingly, Bet1p was required in both directions. Bet1p may potentiate SNARE interactions among anterograde and retrograde partners (Stone et al., 1997) and may replace Sec22p, which is not essential in vivo, on the retrograde limb. In parallel, we have confirmed the roles of Sed5p and Ufe1p as t-SNAREs in anterograde and retrograde traffic, respectively.

Targeting/fusion of retrograde transport vesicles with the ER was blocked by Sec18p antibody. Thus, whereas both directions in the ER-to-Golgi cycle require Sec18p, its homologue, Cdc48p, acts in its place to promote homotypic fusion of ER membranes (Latterich et al., 1995). Surprisingly, although these two ATPases act to promote distinct fusion reactions involving the ER, both use the same t-SNARE, Ufe1p (Patel et al., 1998).

We have shown previously that COPII vesicles package the full set of v-SNAREs required for both directions of targeting in the ER-to-Golgi cycle (Rexach et al., 1994). Although these vesicles in principle have the complete apparatus of targeting/fusion to both the Golgi and the ER membranes, they do so only to the former (Rexach et al., 1994). Clearly, some other element, possibly protein or lipid, contributes to the directionality and specificity of...
this process. Two candidates are Tip20p and Sec20p, proteins that have been shown genetically to interact with Sec22p (Cosson et al., 1997; Lewis et al., 1997). Sec20p is a type II integral membrane protein that contains a luminal HDEL sequence that ensures its recycling to the ER. Tip20p is a peripheral protein that anchors to the membrane in association with Sec20p. Perhaps Sec22p only becomes activated as a v-SNARE when it partners in the Golgi with Sec20p and Tip20p on their way back to the ER. How could this interaction be limited to the Golgi and not occur in the ER from which all three proteins will be recycled into the anterograde path?

Sec22p interacts with Uef1p, whereas Bet1p does not (Lewis et al., 1997). Yet they are both required for retrograde transport. Bet1p and Sec22p may form a cis-SNARE complex in transport vesicles. Upon interaction with Tip20p and Sec20p, this complex may dissociate releasing Sec22p to engage in a trans-SNARE complex with Uef1p. Bet1p may be the chaperone for Sec22p in retrograde transport and interact with Uef1p only in the absence of Sec22p. In the anterograde direction, Bet1p and Bos1p would partner, forming the cis-SNARE complex and engaging in a trans-SNARE complex with Sed5p.

Regulated Ca\(^{2+}\) flux may provide a crucial distinction between targeting/fusion in the anterograde and retrograde directions. Although COPII vesicle targeting/fusion to the Golgi membrane requires Ca\(^{2+}\), the retrograde and homotypic fusion events do not (Rexach et al., 1994; Latterich et al., 1995). Syntaxin, the synaptic plasma membrane t-SNARE, is associated with and may regulate a Ca\(^{2+}\) channel to provide Ca\(^{2+}\) for fusion of synaptic vesicles (Leveque et al., 1994; Bezprozvanny et al., 1995). Likewise, Sec18p-mediated priming and docking of Bos1p and Sed5p may promote Ca\(^{2+}\) flux to activate fusion of COPII vesicles with the cis-Golgi membrane in yeast. Perhaps this Ca\(^{2+}\) flux acts to reconfigure the Sec22p–Sec20p–Tip20p complex in preparation for the packaging of an active, retrograde v-SNARE into COPI-coated retrieval vesicles. On return to the ER, this complex would be consumed and not reactivated until the proteins reappear at the Golgi membrane. This speculation makes obvious predictions that could be tested by comparing Sec22p complexes found in COPII and COPI vesicles formed in the round-trip reaction described here.

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