Characteristics of Endoplasmic Reticulum-derived Transport Vesicles

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Abstract. We have isolated vesicles that mediate protein transport from the ER to Golgi membranes in perforated yeast. These vesicles, which form de novo during in vitro incubations, carry lumenal and membrane proteins that include core-glycosylated pro-α-factor, Betl, Sec22, and Bosl, but not ER-resident Kar2 or Sec61 proteins. Thus, lumenal and membrane proteins in the ER are sorted prior to transport vesicle scission. Inhibition of Yptlp-function, which prevents newly formed vesicles from docking to cis-Golgi membranes, was used to block transport. Vesicles that accumulate are competent for fusion with cis-Golgi membranes, but not with ER membranes, and thus are functionally committed to vectorial transport. A 900-fold enrichment was developed using differential centrifugation and a series of velocity and equilibrium density gradients. Electron microscopic analysis shows a uniform population of 60 nm vesicles that lack peripheral protein coats. Quantitative Western blot analysis indicates that protein markers of cytosol and cellular membranes are depleted throughout the purification, whereas the synaptobrevin-like Betl, Sec22, and Bosl proteins are highly enriched. Uncoated ER-derived transport vesicles (ERV) contain twelve major proteins that associate tightly with the membrane. The ERV proteins may represent abundant cargo and additional targeting molecules.

Morphological analysis of eukaryotic cells led to the hypothesis that small vesicles mediate the directional transport of proteins between successive organelles of the secretory pathway (Jamieson and Palade, 1967; Palade, 1975). The machinery that catalyzes vesicular transport is now being studied at the molecular level using in vitro assays that reconstitute intercompartmental protein transport in perforated cells and membrane extracts (Pryer et al., 1992). The characterization of vesicles that mediate intercompartmental protein transport in vitro will help address the role of integral membrane proteins that function in vesicular traffic.

Although most transport vesicles are transient intermediates, they can be forced to accumulate as stable intermediates when a component of the vesicle targeting machinery is inactivated. Most transport vesicles are 50-100 nm in size and may be coated with a peripheral protein lattice. In coated transport vesicles, the subunits of the coat are the most abundant proteins in pure vesicle preparations. Clathrin complexes coat vesicles derived from the trans-Golgi and plasma membranes (Brodsky, 1988). Assembly of clathrin lattices onto the donor membrane is guided by adaptins and ADP ribosylation factor (ARF) proteins, and functions to select a cargo of membrane proteins, and to drive vesicle budding (Pearse and Robinson, 1990; Keen, 1990; Stamnes et al., 1993). On the other hand, nonclathrin coatomer complexes coat vesicles derived from Golgi membranes (Malhotra et al., 1989). Assembly of coatomer onto membranes is guided by ARF and functions to drive the membrane shape change that accompanies budding (Palmer et al., 1993; Orci et al., 1993). In both cases, coats disassemble prior to vesicle fusion with the target membrane (Brodsky, 1988; Orci et al., 1989). Most proteins in uncoated transport vesicles are tightly associated with the membrane. For example, uncoated synaptic vesicles contain various integral membrane proteins with distinct structural features; the most abundant are termed synaptobrevin, synaptophysin, and synaptotagmin (Südhof and Jahn, 1991). These membrane proteins are thought to promote vesicle targeting or fusion to the plasma membrane.

Insight into the components and mechanics of vesicle-mediated protein transport has been provided by genetic analysis of the yeast Saccharomyces cerevisiae. More than 25

1. Abbreviations used in this paper: ARF, ADP ribosylation factor; CPK, creatine phosphokinase; F, Ficoll; core-gpα, core-glycosylated pro-α-factor; MSS, medium speed supernatant fraction; ppα, pre-pro-α-factor; ppα, pre-pro-α-factor; s, sucrose.
proteins that play essential roles in ER to Golgi protein transport have been identified (Pryer et al., 1992) and their function tested using temperature-sensitive mutants, or by depleting cells of the protein in question. Cells that contain a mutant form of Sec12, Sec13, Sec16, or Sec23 proteins are blocked in ER to Golgi protein transport and accumulate enlarged ER membranes (Novick et al., 1980; Novick et al., 1981; Kaiser and Schekman, 1990). These proteins are thought to function in the formation of ER-derived transport vesicles. In contrast, cells that contain a mutant form of Ypt1, Sec22, Sec18, or Sec17, and cells depleted of Bos1 or Sec5 proteins are deficient in protein transport from ER to the Golgi membranes and accumulate 50–60 nm vesicles and enlarged ER membranes (Kaiser and Schekman, 1990; Shim et al., 1991; Becker et al., 1991; Hardwick and Pelham, 1992). Because these cells accumulate small vesicles, these proteins are thought to function in the targeting or fusion of ER-derived transport vesicles.

The function of proteins identified in genetic screens can be tested directly using in vitro assays that reconstitute ER to Golgi protein transport in perforated yeast spheroplasts or microsomal preparations (Baker et al., 1988; Ruohola et al., 1988). Subassays that measure vesicle budding, targeting, and fusion have allowed a direct assessment of the protein, nucleotide, and ionic requirements for each stage (Rexach and Schekman, 1991). The release of vesicles from ER membranes requires GTP and the function of Sec12p, Sarlp, Sec23/24p complex, and Sec13/31p complex (Rexach and Schekman, 1991; Oka and Nakano, 1991; d'Enfert al., 1991; Hicke et al., 1992; Barlowe et al., 1993a,b; Pryer et al., 1993; Salama et al., 1993). The stable attachment of transport vesicles to Golgi membranes requires calcium and ATP at physiological temperature (Rexach and Schekman, 1991). Specific antibodies against Sec7p and Bosp1 block vesicle targeting or fusion (Franzusoff et al., 1992; Lian and Ferro-Novick, 1993).

Here we report the purification of ER-derived transport vesicles that accumulate in vitro when perforated yeast cells of a Ypt1p-deficient strain are incubated with cytosol and an ATP regeneration system at physiological temperature. We examined the function and molecular composition of purified transport vesicles, and provide a direct biochemical demonstration that diffusible vesicles mediate selective and vectorial transport of luminal and membrane proteins from the endoplasmic reticulum to the Golgi complex.

**Materials and Methods**

The yeast strains used in this study were RSY 445 (leu3-3,112; ura3-3,52; trpl-289; prb1; pep4::URA3; gal2; his4-579; MAIo2), RSY 607 (ura3-3,52; leu2-3,112; pep4::URA3; MAIo2), RSY 453 (ypt1::LEU2, glsl-1; leu2; his3, ura3-52; MAIo2), RSY 1001 (ypt1::LEU2; glsl-1; leu2; his3; ura3-52; MAIo2; MLY 1601 ypt1::LEU2; leu2-3,112, ura3-52, pep4::URA3).

**Preparation of Perforated Yeast Spheroplasts**

Cells were grown at 24°C (ypt1ts) or 30°C (wild-type) in VP medium, 5% glucose to log phase (5-10 OD600/ml), and <600 OD600 cell units were harvested by centrifugation at 5000 rpm for 5 min at 4°C in a Sorvall GSA rotor. All subsequent steps were carried out at 4°C. Cells were resuspended in 60 ml distilled water and washed twice by sedimentation at 6000 g for 5 min and dilution in distilled water. Cell pellets were frozen at -70°C. Thawed cell pellets were washed once with B88 (20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc), resuspended in 60 ml of B88 and 20 ml portions (2000 OD600 U) aliquoted into Corex 30 ml glass tubes. Cells were sedimented as before and to each tube, 4 g of glass beads (0.5-mm diam) and 1 ml of solution A (20 mM Hepes, pH 6.8, 50 mM KOAc, 100 mM sorbitol, 5 mM MgOAc, 1 mM ATP, 0.5 mM PMSF, 1 mM DTT) were added. Cells were lysed by 6 x 30 s periods of agitation in a WVR vortexer (Scientific Industries, Inc., Bohemia, NY) at full speed, with 1-min incubations on ice in between periods. To each sample, 1.5 ml of solution B (20 mM Hepes, pH 6.8, 2 M KCl, 400 mM sorbitol, 5 mM MgOAc, 1 mM ATP, 0.5 mM PMFS, 1 mM DTT) was added and tubes were vortexed 3 x 30 s at full speed. The homogenate was clarified by centrifugation at 12,000 g for 5 min in a Sorvall SS34 rotor. The combined supernatants (9 ml) were mixed with an equal volume of 80% Nycodenz, 20 mM Hepes, pH 6.8, 150 mM KOAc, 5 mM MgOAc, and transferred into two 11.5 ml Ultracentrifuge tubes (Beckman Instruments, Palo Alto, CA) and overlaid with 25 ml of a 15% to 40% Nycodenz gradient in B88, 1 mM ATP. The sample was pumped upwards at a flow rate of 1 ml/min using a Rainin Rabbit pump (Woburn, MA), and fractions collected from the top. The eluted protein peak was pooled, niquoteted, and frozen in liquid nitrogen, and stored at -70°C. Protein concentration was usually 9 mg/ml, and was measured by the Bradford protein assay and compared to a BSA standard.

**In Vitro Transport**

Reagents used for the in vitro transport assay were obtained as described in Baker et al. (1988, 1989) unless otherwise indicated.

**Stage I: Translocation.** For each experiment a frozen aliquot of perforated yeast spheroplasts (Fig. 60 OD600 U of cell equivalents) was thawed and washed once by dilution in B88 and sedimented at 3000 g for 5 min in a refrigerated microcentrifuge (Tomy Tech USA Inc., Palo Alto, CA). The cell pellet was resuspended in 1 ml low osmotic support B88 (20 mM Hepes, pH 6.8, 50 mM KOAc, 250 mM sorbitol, 5 mM MgOAc) to maximize spheroplast lysate (>90% perforated). After 1 min at 4°C, membranes were sedimented, washed once with B88 as before, and resuspended in 0.1 ml of a yeast S-100 translation lysate that contained [35S]methionine-
prepro-α-factor (ppαf), plus 16 μl of a 10× ATP regeneration mix (Baker et al., 1988) in final volumes of 160 μl. The final concentration of components in a 25-μl translocation reaction was 75 μg of membranes (measured by the amido-black protein assay described in the Materials and Methods), 90 μg of yeast S100 lysate (measured by the Bradford protein assay), 50 μM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate (CP), and 200 μg/ml creatine phosphokinase (CPK), all dissolved in B8& The mix was incubated 15 min at 10°C to allow posttranslational translocation of ppαf, chilled to 4°C, diluted with 1 ml B88, and sedimented as before. Membranes were resuspended in 1 ml of high-salt B88 (20 mM Hepes, pH 6.8, 1 M KOAc, 250 mM sorbitol, 5 mM MgOAc) and rotated at 4°C for 7 min. Membranes were sedimented, washed once with B88 and the final membrane pellet resuspended to 160 μl (30 μg/10 μl).

Stage II. Transport Vesicle Chases. Each transport reaction (25 μl) contained 10 μl of stage I membranes, 90 μg of membrane-free cytosol, 1 mM ATP, 50 μM GDP mannose, 40 mM CP, 200 μg/ml CPK, in B88. For time courses, a 200 μl stage II mix was prepared and aliquoted in 25-μl portions into 0.5 ml microcentrifuge tubes. Reactions were incubated at 0° or 25°C for the indicated times, each tube represented one time point. Reactions were terminated by placing each tube on ice until the last time point was taken. Aliquots (15 μl) of each time point were treated with 10 μl of a trypsin solution (625 μg/ml in B88) for 10 min on ice, and then with 10 μl of a trypsin inhibitor solution (1.25 mg/ml in B88), for 10 min on ice. Each sample received 45 μl of 2× Laemmli sample buffer without reducing agent, and was heated at 95°C for 10 min. Equal aliquots of each tube were treated with either Con A-Sepharose, or protein A-Sepharose coupled with anti-cd6 antibody as described by Baker et al. (1988). Washed immunoprecipitates were heated to 95°C in 1% SDS for 5 min and dissolved in Universal ES scintillation fluid (ICN Biomedicals, Irvine, CA) for quantitation in a scintillation counter. Glycosylated forms of α-factor are the major radiolabeled proteins.

Stage III: Transport Vesicle Budding and Fusion Assay

Stage II transport reactions were terminated by placing tubes on ice (see above), and each tube was fractionated by centrifugation for 40 s at 13,000 rpm in a refrigerated microcentrifuge (Sorvall Tech USA Inc.). A 15-μl medium speed supernatant fraction (MSS) was taken from the meniscus and treated with trypsin and trypsin inhibitor, then mixed with Laemmli sample buffer without reducing agent, and aliquots were processed for stage II transport.

Vesicle Budding and Fusion Assay

Stage II transport reactions were terminated by placing tubes on ice (see above), and each tube was fractionated by centrifugation for 40 s at 13,000 rpm in a refrigerated microcentrifuge (Sorvall Tech USA Inc.). A 15-μl medium speed supernatant fraction (MSS) was taken from the meniscus and treated with trypsin and trypsin inhibitor, then mixed with Laemmli sample buffer without reducing agent, and aliquots were processed for stage II transport.

ER–Protein Sorting Assay

To measure the release or retention of luminal ER-proteins, vesicle budding assays were performed as described above, with 2% beta-mercaptoethanol (β–ME) included in the Laemmli sample buffer. To measure the release or retention of membrane embedded ER-proteins, vesicle budding assays were performed in the absence of radiolabeled [35S]pre-pro-α-factor (an S100 translation lysate without radiolabeled pre-pro-α-factor was used for Stage I). After samples were fractionated in a microcentrifuge, they were mixed with 25 μl of 2× Laemmli buffer with 2% β–ME, and heated at 65°C for 15 min. Aliquots (15 μl) were subjected to SDS-PAGE in 14% acrylamide mini-gels, and [35S]-Protein A Western blots were performed as described below.

ER–Membrane Fusion Assay

Aliquots (5 μl) of an enriched transport vesicle preparation (from pool 1) were mixed with 100 μg of wild-type membrane-free cytosol, 25 μg of wild-type yeast microsome (prepared as in Wuestehube and Schekman, 1992), and an ATP regeneration system, in a total volume of 50 μl. Chases were conducted for 1 h at 0° or 20°C in the presence of various additions (i.e., GDP-mannose, cytosol, Trition X-100). Chilled reactions were treated with trypsin and then trypsin inhibitor (as for stage II transport) to degrade glycoproteins that was not membrane enclosed. Reactions were terminated by the addition of 60 μl of 2× Laemmli sample buffer without reducing agent and samples were processed for their content of glycoprotein as described for stage II transport, or mixed with 2% β–ME and resolved in a 12.5% SDS-PAGE. Gels were fixed, dried, and exposed to phosphor plates. The percentage of glucosyl ceramide (i.e., the conversion of the 26-kD GSL-1 form of core-glycoprotein to the 29-kD GSL-2 form) was quantified using standard methods in a Molecular Dynamics PhosphorImager (Sunnyvale, CA). For a more detailed description of the ER-membrane fusion assay see Latterich and Schekman (1994).
Alami-black Protein Concentration Assay

Samples containing 0.1 to 10 μg of protein were diluted to 0.25 ml with distilled water, mixed with 0.05 ml of 1 M Tris, pH 7.5, 1% SDS, and then with 0.01 ml of 60% TCA (wt/vol). After 2 min at room temperature, the samples were spotted onto a nitrocellulose filter that was pre-soaked in 6% TCA and assembled into a Sigma dot blotter fitted to a vacuum hose. Sample wells were rinsed once with 6% TCA. Filters were removed from the dot blotter, rinsed with 6% TCA, and stained for 2-3 min in 0.1% amido black dissolved in MeOH/HOAc/H2O (45:10:45). The stained filter was rinsed with distilled water, destained in MeOH/HOAc/H2O (270:6:24), and dissolved in MeOH/HOAc/H2O (45:10:55). The stained filter was rinsed with 0.03 ml of 1 M NaOH, 0.05 mM EDTA, 50% EtOH. After 20 min, the absorbance was read at 630 nm wavelength using a spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) fitted with 0.4 ml glass cuvettes. BSA was used as a standard protein to calculate sample concentrations.

125I-Protein A Western Blots and Quantitation in PhosphorImager

Proteins in Laemmli sample buffer were heated at 65° or 95°C, resolved in 9 or 14% polyacrylamide gels, and transferred to nitrocellulose or Bio Rad PDVF membranes using a Bio Rad semi-dry blotter (75 ml of 75 mM NaCl). After 2 min at room temperature, the samples were blotted with a nitrocellulose filter that was pre-soaked in 6% TCA and assembled into a Sigma dot blotter. Filters were stained with Ponceau S, rinsed in water, cut in strips, and incubated with primary antibodies for 90 min. Blots were washed 4 x 7 min in TBS-T for 30 min, and incubated with primary antibodies for 90 min. Samples were heated to 65°C for 1 h at room temperature. Blots were washed 4 x 7 min, dried, and exposed to phosphor plates. The intensity of bands was quantified using standard software in a PhosphorImager (Molecular Dynamics).

GDPase Enzyme Assay

GDPase activity assays were performed as described in Yanagisawa et al. (1990). Samples (5–20 μl) were mixed with 100 μl of 200 mM imidazole- HCl, pH 7.4, 2 mM MgCl2, 0.1% Triton X-100, and 10 mM GDP, or GDP as control. Tubes were incubated 30 min at 30°C and reactions were terminated by adding 150 μl of 2% SDS. Inorganic phosphate was quantified by incubating the samples with 0.1 ml of 0.15% sodium deoxycholate, and then with 0.1 ml of 6% TCA. After 10 min at room temperature, the precipitates were sedimented as before, the tubes were decanted, and the pellets were washed with chilled (–20°C) acetone. Precipitates were reconstituted by sedimentation as before, the tubes were decanted, and the pellets were allowed to dry at room temperature. Pellets were finally resuspended in 1.5 Laemmli sample buffer with 2% β-β-ME. Samples were heated to 65°C for 15 min, loaded in a 10 to 15% acrylamide–SDS gradient gel, and resolved by electrophoresis at 10–15 mA. Gels were fixed in 50% ethanol, 12% acetic acid, 0.02% formaldehyde for 1 h at room temperature. Fixed gels were washed 3 x 10 min with 50% ethanol, incubated for 1 min in 0.01% sodium thiosulfate, and then washed 3 x 15 s in distilled water. Gels were precoated for 20 min in 0.1% silver nitrate, then washed 2 x 20 s in distilled water, and finally developed for 3–6 min in 3% sodium carbonate, 0.02% formaldehyde, and 0.0002% sodium thiosulfate. Developed gels were rinsed 20 s in distilled water, and color development stopped by immersion and storage in 0.01 M EDTA, pH 8.0.

Immunosolubilization of ER-derived Transport Vesicles

Aliquots (20–60 μl) of MSS fractions in 0.5 ml Eppendorf microcentrifuge tubes were incubated 1 h at room temperature with 1.25 μg of anti-Beta IgG or 0.5 μg of affinity purified anti-Sec22p antibodies (kind gift of Dieter Gall). Samples were mixed 1:1 with 6% polyacrylamide and visualized using a Philips 301 electron microscope.

Thin Section. Aliquots (250 μl) of purified vesicles from pool 3 were diluted to 1 ml with B88, and sedimented at 100,000 g for 1 h onto a “bed” of hardened low-melting agarose (1 μl of 2.5%). After sedimenting, pellets were washed with 1% gluteraldehyde dissolved in B88. Grids were washed in distilled water and spotted with 10 μl of 2% uranyl acetate. Excess stain was removed and the grids were allowed to dry at room temperature. Stained particles were visualized and photographed using a Philips 301 electron microscope.

Preparation of Antibodies against Yptl and Betl Proteins

Yptlp was overproduced in an Escherichia coli strain (Segev et al., 1988), isolated in inclusion bodies, solubilized with 8 M urea, and dialyzed against 0.1 M phosphate buffer, pH 7.4. A solution of Yptl protein (200 μg) was mixed 1:1 with Freund’s complete adjuvant, and injected into rabbits. Rabbits were boosted monthly with 50 μg of antigen dissolved in Freund’s incomplete adjuvant, and bled 2 wk after each injection. Anti-Yptl serum was used at a dilution of 1:5,000 in western blots. A synthetic peptide that contains the 12-terminal 26 amino acids of Betlp with an added cysteine at position 27 was used to prepare antibodies against Betlp. Betl peptide was conjugated to BSA in a 1:10 molar ratio using the DSP cross-linker. Conjugates were gel filtered in a column that was equilibrated in 0.1 M phosphate buffer at pH 7.4. An aliquot of the conjugate (1 mg) was mixed 1:1 with Freund’s complete adjuvant, and injected into rabbits. Rabbits were boosted monthly, twice with 500 μg of the conjugate adjuvant, and twice more with 200 μg of unconjugated peptide. Rabbits were bled 2 wk after each boost. Anti-Betlp serum was used at a dilution of 1:500 in western blots. IgG was purified using a protein A affinity matrix as described before (Harlow and Lane, 1988).
antibodies as described in the Methods for stage II transport. (B) Stage II reactions containing wild-type (Sec\textsuperscript{+}) or ypt\textsubscript{f} ts perforated cells and cytosol were incubated at 25°C for the indicated times, or at 0°C for the zero-time point control. Chilled samples were fractionated in a microcentrifuge into a medium speed pellet and MSS fractions. MSS fractions were treated with trypsin and trypsin inhibitor, and processed for their content of gpaf as in A. The release of \(^{35}\text{S}\)-gpaf from perforated cells (vesicle budding) is plotted in the left panel and the appearance of outer chain gpaf in the MSS (vesicle fusion) is plotted in the right panel. The zero time point of each set of MSS samples (less than 10% of the maximum signal) was subtracted as background from the corresponding MSS fractions to obtain the values shown.

Results

Review of In Vitro Reaction

Protein transport from the ER was reconstituted in perforated yeast spheroplasts using \(^{35}\text{S}\)-radiolabeled gpaf as a marker secretory protein (Baker et al., 1988). gpaf is post-translationally translocated into the lumen of the ER during a 15-min incubation at 10°C (stage I). Once in the ER lumen, pro-\(\alpha\)-factor (paf) is glycosylated with three N-linked core–carbohydrate chains which are then processed by a mannosidase and two glucosidases in the ER (Esmon et al., 1984; Tsai et al., 1984), and was necessary to test whether diffusible ER-derived transport vesicles are competent for fusion with ER, as well as with Golgi membranes (see below).

Perforated cell and cytosol extracts were prepared from ypt\textsubscript{f} ts and wild-type strains, and tested for the ability to support ER to Golgi protein transport in the presence of ATP at physiologic temperature. ypt\textsubscript{f} ts extracts were 10 times less efficient than wild-type in reconstituted transport at 25°C (Fig. 1 A). To determine the stage of transport that is blocked in ypt\textsubscript{f} ts reactions, we examined the efficiency of vesicle budding and vesicle fusion. Vesicle budding was measured by quantifying the release of membrane-enclosed core-gpaf from perforated cells, and vesicle fusion was measured by quantifying the subsequent appearance of Golgi-modified forms of gpaf (Rexach and Schekman, 1991). Wild-type and ypt\textsubscript{f} ts cells had equivalent vesicle budding efficiencies (Fig. 1 B, left), but vesicle fusion was 10 times less efficient in the ypt\textsubscript{f} ts (Fig. 1 B, right). Thus, ypt\textsubscript{f} ts perforated cells produce transport vesicles, but fail to consume them.

Quantitative western blot analysis was used to measure the accessibility of ER-resident proteins to transport vesicles. After 35 min of vesicle budding, more than 35% of core-gpaf was incorporated into vesicles, compared to less than 5% of Kar2p (Fig. 2, left). Kar2p is a lumenal ER-resident protein, whereas core-gpaf is a soluble secretory proprotein (Rose et al., 1989; Julius et al., 1984). Likewise, 25% of Betlp and 15% of Sec22p were incorporated into vesicles, compared to less than 2% of Sec61p (Fig. 2, right). Sec22 and Betlp proteins are embedded in the ER membrane (Newman et al., 1992; Ossig, R., unpublished results) and

Accumulation of Functional ER-derived Transport Vesicles

We previously showed that anti-Yptlp F\textsubscript{ab} antibody fragments block ER to Golgi protein transport in perforated yeast spheroplasts and prevent the attachment of ER-derived transport vesicles to Golgi membranes. The Yptlp-specific block caused the accumulation of functional transport vesicles that were physically separable from ER and Golgi membranes (Rexach and Schekman, 1991). To obtain sufficient amounts of vesicles for purification, we needed to conduct large scale in vitro incubations. Large amounts of antibody F\textsubscript{ab} fragments were required for repeated vesicle preparations. Therefore a mutation in the Yptlp protein was used as an alternate inhibitor of Yptlp function. A strain that carries a mutant form of the Yptlp protein (Schmitt et al., 1988) was examined for the ability to accumulate transport vesicles during in vitro incubations. The ypt\textsubscript{f} ts strain that we used for all experiments (referred to as ypt\textsubscript{f} ts throughout the text) carries an independent mutation in the glucosidase 1 gene (glsl-1) which renders the ER unable to trim terminal glucose residues from N-linked core–carbohydrate chains (Esmon et al., 1984). A mutation in the GLS1 gene has no effect on cell growth, protein secretion, or outer chain modification (Esmon et al., 1984; Tsai et al., 1984), and was necessary to test whether diffusible ER-derived transport vesicles are competent for fusion with ER, as well as with Golgi membranes (see below).

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are components of ER-derived transport vesicles (see below), whereas Sec61 is a resident ER membrane protein (Stirling et al., 1992; Stirling, C., unpublished results). Based on these results, we concluded that sorting of proteins in the ER must occur prior to the scission of diffusible ER-derived transport vesicles.

We examined whether ER-derived vesicles that diffuse out of perforated cells during in vitro incubations were attached or not to Golgi membranes because Golgi membranes are also released from perforated cells during in vitro incubations. Vesicle targeting is measured by quantifying the percentage of core-gp of fractions in the MSS fraction that sediments slowly (within vesicles) or rapidly (with Golgi membranes) in velocity sedimentation gradients (Rexach and Schekman, 1991). Vesicles are marked by their content of 35S-core-gp of, whereas Golgi membranes are marked by their content of GDPase activity (Yanagisawa et al., 1990), and content of outer-chain-modified gp of (Rexach and Schekman, 1991). Membranes released from wild type and mutant cells are sepa- rate by sedimentation onto a step sucrose gradient (ref.). A large mix of gp of is separated from Golgi membranes in F/s gradients. A scaled-up MSS fraction (300 μl) obtained from a wild-type (Sec') or yptl ts Stage II reaction was loaded in a F/s gradient (prepared as described in Materials and Methods) and subjected to centrifugation for 2 h at 100,000 g. 18 fractions were collected from the top. The gp of content, GDPase activity, and the density of every fraction was quantified as described in Materials and Methods. Closed circles represent core-gp of. Closed triangles represent outer-chain modified gp of. Open circles represent GDPase activity. Crosses represent the density of each fraction expressed in % sucrose (wt/wt).

**Purification of ER-derived Transport Vesicles**

Transport vesicles that accumulate in yptl ts extracts are purified using differential centrifugation and a combination of velocity and equilibrium density gradients. A flow diagram of the purification is shown in Fig. 4. A large mix of yptl ts perforated cells, cytosol, and an ATP regeneration system was incubated for 30 min at 25°C to generate ER-derived transport vesicles that contained 35S-core-gp of (see Materials and Methods). The chilled reaction mix was subjected to centrifugation at medium speed to remove perforated cells and the majority of cellular membranes. The MSS contained slowly sedimenting vesicles, and fragments of cellular membranes (see Table I below). Vesicles were concentrated by sedimentation onto a step sucrose gradient (referred to as VS T) and equilibrated as a single sharp peak at a density equivalent to 30% sucrose (wt/wt)(1.13g/ml) (Fig. 5, top). The peak of vesicles (28-32% sucrose) was pooled.

![Figure 2](image1.png)

![Figure 3](image2.png)
(pool 1), the concentration of sucrose adjusted to 15% (wt/wt) by dilution, and the sample subjected to velocity sedimentation in a F/s gradient (referred to as VS II) that resolves membranes on the basis of size. Vesicles sedimented slowly as a single peak without reaching equilibrium density (Fig. 5, middle). The peak of vesicles was pooled (pool 2), the sample density was adjusted to 35% Nycodenz (wt/vol), and vesicles were floated to equilibrium in a Nycodenz density gradient (referred to as EQ) to remove all remaining soluble proteins and particles. The vesicles equilibrated as a single peak at a density of 1.12 g/ml (Fig. 5, bottom), and were pooled (pool 3).

To assess the purity of ER-derived transport vesicles, we measured the fold enrichment of transport vesicles and the recovery of cellular markers at each purification step. A purification chart is presented in Table I. Fold enrichment was determined by calculating the ratio of protease-protected core-gpA to total micrograms of protein in each fraction. The value obtained for the total fraction was adjusted to reflect core-gpA within vesicles, because a significant portion (Fig. 6, 60%) of the core-gpA in this fraction resided within ER membranes. We calculated that ER-derived transport vesicles were enriched 900-fold from the initial mix of perforated cells and cytosol. Quantitative 125I-protein A Western blot analysis was used to determine the percent recovery of protein markers of cellular membranes and cytosol (Table II). The MSS contained slowly sedimenting vesicles, Golgi membranes, and detectable amounts of plasma membrane, vacuole, and mitochondrial markers. These contaminants were largely separated from ER-derived vesicles after isopycnic centrifugation in a sucrose gradient (Table II, VS I). Although detectable amounts of Golgi and vacuole membranes persisted throughout the purification, more than 99.5% of these membranes were removed (Table II, Pool 3).

**ER-derived Transport Vesicles Fuse with cis-Golgi, but not with ER Membranes**

The fusion competence of transport vesicles that accumulate in vitro was examined using assays that detected vesicle fusion to cis-Golgi, or to ER membranes. Because we used a glucosidase I-deficient ypt1ts strain, the transport vesicles that accumulate in vitro contain the 32-kD glucose-
Table I. Purification Table

| Fraction | Volume (ml) | Protein (μg) | Core-glycosylated 35S-pro-α-factor (cpm × 10^6) | Specific units (cpm × 10^5/μg) | Enrichment (fold) | Recovery (%) |
|----------|-------------|--------------|-----------------------------------------------|--------------------------------|------------------|--------------|
| Total    | 13          | 54,000       | 220/70                                       | 4.1/1.3                        |                  | 100          |
| MSS      | 12          | 39,000       | 66                                           | 1.7                            |                  | 96           |
| Pool 1   | 0.9         | 670          | 31                                           | 46                             | 35               | 46           |
| Pool 2   | 1.2         | 73           | 13                                           | 180                            | 140              | 25           |
| Pool 3   | 0.8         | 3.4          | 4.2                                          | 1,200                          | 950              | 11           |

The content of protein and protease-protected gPQFF (pp-gPQFF) was determined for each purification step. The protein concentration in aliquots from each pool was determined using an amido-black protein detection assay (see Materials and Methods), and the content of protease protected 35S-gPQFF was determined by treating core-gPQFF within diffusible vesicles in the Total fraction. The values given for "percent recovery" were adjusted to reflect the core-gPQFF within diffusible vesicles (right value) and was deduced from the efficiency of vesicle budding (see "Vesicle budding and fusion assay" in Materials and Methods). Fold enrichment was calculated with respect to protease-protected core-gPQFF within diffusible vesicles in the Total fraction. All values were rounded off to two significant figures after calculations were performed.

untrimmed" form of core-glycosylated pro-α-factor (referred to as gls-core-gPQFF and core-gPQFF throughout the text) instead of the 29-kD wild-type form. Altogether, nine glucose residues are trimmed from three N-linked carbohydrate chains to convert gls-core-gPQFF to the wild-type form; a terminal glucose residue is removed by glucosidase I, and two more are removed by glucosidase II after the terminal glucose has been removed (Roth et al., 1990). The gls-I and wild type forms of core-gPQFF are shown in Fig. 6 A, for reference. Fusion of ER-derived transport vesicles with wild-type ER membranes was measured by quantifying the percent glucose-trimming of radiolabeled gls-core-gPQFF by glucosidase I and II in the ER lumen (see Latterich and Schekman, 1994), whereas fusion of vesicles with cis-Golgi membranes was measured by quantifying the conversion of radiolabeled gls-core-gPQFF to "outer-chain" modified forms by α,1,6 mannosyl-transferases in the cis-Golgi lumen (see review of in vitro assay; Rexach and Schekman, 1991). A sample homotypic ER-membrane fusion experiment is presented in Fig. 6 B, for comparison. Washed gls-I ER membranes that contained radiolabeled gls-core-gPQFF were incubated with fresh wild-type ER membranes in the presence of various additions, and completed fusion reactions (except for reactions that contained Triton X-100) were treated with trypsin and then trypsin inhibitor to quantify membrane-enclosed gPQFF (Fig. 6 B, lanes 1-3). ER-membrane fusion reached 21% efficiency at physiological temperature in the presence (Fig. 6 B, lane 2) but not in the absence (Fig. 6 B, lane 1) of an ATP regeneration system, as judged by percent glucose trimming. Addition of cytosol neither stimulated nor inhibited ER-membrane fusion (not shown). When membrane barriers were removed by the presence of 1% Triton X-100 in the reaction, 92% of the gls-core-gPQFF was trimmed (Fig. 6 B, lane 3). A smear rather than a sharp band at 29 kD results from the addition of TX-100, which interferes with glucosidase activity. The smear may represent the nine intermediates in the conversion of core-gPQFF from the gls to the wild-type form, and did not result from proteolysis of gls-core-gPQFF, because a single species of deglycosylated proα-factor was formed when samples were treated with endo H (not shown).

ER-derived transport vesicles did not fuse with ER membranes. Glucose trimming was not detected when aliquots of isolated vesicles (from pool 1) were incubated at physiological temperature with an ATP-regeneration system and wild-type microsomes that contained fusion-competent ER membranes (Fig. 6 C, lanes 2 and 2). These conditions normally

Table II. Recovery of Cellular Markers

| Marker protein | Cellular location | Total | MSS (Percent recovery) | Pool 1 | Pool 2 | Pool 3 |
|----------------|-------------------|-------|------------------------|--------|--------|--------|
| core-gPQFF     | lum ERV           | 100   | 96                     | 25     | 46     | 11     |
| Sec61p         | tm ER             | 100   | 7                      | 0      | 0      | 0      |
| GDPase         | tm Golgi          | 100   | 37                     | 3.4    | 1.2    | 0.4    |
| VPM1           | tm Vac            | 100   | 29                     | 2.9    | 0.7    | 0.1    |
| PM ATPase      | tm PM             | 100   | 17                     | 0      | 0      | 0      |
| F1Bo           | pm Mito           | 100   | 28                     | 0      | 0      | 0      |
| PGK            | sol Cyt           | 100   | 88                     | 0.3    | 0      | 0      |

Aliquots of Total and MSS fractions that contained 31.25, 6.25, and 1.25 micrograms of protein, and aliquots of pools 1, 2, and 3 fractions that contained 1.25, 0.25, and 0.05 μg of protein, respectively, were subjected to SDS-PAGE, and immunoblotted with anti-Sec61p (1:5,000), anti-PGK (1:10,000), anti-PM ATPase (1:1,000), anti-VPM1 (1:500), and anti-F1Bo (1:1,000) antibodies. Immune-complexes were detected by 125I-protein A and quantified in a PhosphorImager; the values obtained were expressed as disintegrations per μl of sample analyzed, and were used to calculate the % recovery of each marker after each purification step. In the case of core-gPQFF, values were calculated as in Table I. Values obtained in GDPase assays (see Materials and Methods), were expressed as units of enzyme activity per μl of sample analyzed, and were used to calculate % recovery. Core-gPQFF is a luminal (lum) marker of ER-derived transport vesicles (ERV); Sec61p is a transmembrane (TM) protein that marks the endoplasmic reticulum (ER); guanosine diphosphatase (GDPase) marks the Golgi complex (Golgi); an integral membrane component of the vacuolar proton pump (VPM1) marks the yeast vacuole (Vac); plasma membrane ATPase (PM ATPase) marks the plasma membrane (PM); a subunit of the mitochondrial proton pump (F1Bo) is a peripheral membrane (pm) protein that associates tightly with the mitochondrial membrane (Mit); phosphoglycerolizyokinase (PGK) is a soluble (sol) protein in the cytoplasm (Cyt).
Figure 6. Fusion competence of ER-derived transport vesicles. (A) Phosphor-image displaying gis-core-gpaf and wild-type core-gpaf. Radiolabeled pppof was transloated at low temperature into microsomes prepared from glsl-1 or wild-type yeast, as indicated. Chilled microsomes were washed, treated with trypsin and trypsin inhibitor, mixed with 2× Laemmli sample buffer with 2% β-ME, heated to 95°C, and resolved in a 12.5% SDS-PAGE. The gel was fixed, dried, exposed to a phosphor plate, and visualized in a Molecular Dynamics PhosphorImager. (B) Control reactions to test the fusion-competence of ER membranes. Washed glsl-1 microsomes (75 μg) that contained radiolabeled gis-core-gpaf (prepared as in A) were mixed with microsomes (75 μg) prepared from wild-type (lane 1–3), and with an ATP regeneration system (lane 2) or 1% Triton X-100 (lane 3) in a total volume of 50 μl. Reactions were incubated for 60 min at 30°C. Chilled reactions were treated with trypsin, and then trypsin inhibitor, and processed as in A. The percent trimming (conversion of the 32-kD gis-core-gpaf to the 29-kD wild-type form) was quantified using standard software in a Molecular Dynamics PhosphorImager. (C) Fusion competence of isolated transport vesicles. Aliquots (5 μl) of an enriched transport vesicle preparation (from pool 1) were mixed with 25 μg of wild-type yeast microsomes, an ATP regeneration system, and 100 μg of cytosol (unless otherwise indicated) in the presence (lanes 1–4) or absence (lanes 1’–4’) of GDP-mannose. The reactions in lanes 2 and 2’ did not contain cytosol. Reactions were incubated for 1 h at 0°C (lanes 1 and 1’) or 20°C (lanes 2–3 and 2–3’). Chilled reactions were treated with trypsin and trypsin inhibitor, and processed as in A. The percent trimming was quantified as before, and the percent transport was quantified using precipitation with Con A and anti-outer-chain antibodies as described in the Methods for stage II transport. (D) Fusion competence of purified transport vesicles. Aliquots of purified transport vesicles (from pool 3) were mixed or not with fresh cytosol and perforated spheroplasts prepared from wild-type cells, and an ATP regeneration mix, to the same concentrations as for stage II transport. To the tubes containing no ATP, we added apyrase (0.5 U) to hydrolyze residual ATP; these tubes were supplemented with 50 μM GDP-mannose, normally added in the ATP regeneration mix. Samples were incubated for 1 h at 0° or 20°C, and reactions terminated by the addition of 2% SDS and heated to 95°C. Samples were processed for precipitation with Con A and anti-outer-chain antibodies as described in the Materials and Methods for stage II transport.

Figure 7. Morphology of ER-derived transport vesicle examined under the electron microscope. A and B show purified vesicles from pool 3 that were attached to an ionized support grid, fixed, and visualized by negative stain with 2% uranyl acetate. C and D show vesicles from the same pool 3 that were sedimented onto a hardened agarose "bed" and processed for thin section electron microscopy. Magnifications were 25,000× in A, 57,000× B, 34,000× C, and 57,000× D. Bars, 200 nm.
Figure 8. Polypeptide composition of ER-derived transport vesicles. (A) Protein from each fraction of the Nycodenz equilibrium gradient (EQ) was concentrated by precipitation with trichloroacetic acid in the presence of deoxycholate, resolved in a 10-15% acrylamide gradient gel, and stained with silver (see Materials and Methods). (B) Aliquots of pool 3 were diluted 10-fold in tubes with the indicated solutions (BBB is 20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc). A 1% Triton X-100 solution was used. Sodium carbonate was dissolved in water. After 10 min at room temperature, vesicles were sedimented at 100,000 g for 45 min. The pellets were resuspended in Laemmli sample buffer with 2% β-ME, and the proteins resolved by SDS-PAGE and visualized by silver stain. Arrows indicate the most abundant vesicle proteins, and some were assigned apparent molecular mass. ERVp is an abbreviation for ER-derived transport Vesicle protein.

Support efficient ER-membrane fusion (Fig. 6B, lane 2). Addition of cytosol did not stimulate “back-fusion” (Fig. 6C, lanes 3 and 3'). Glucosidase activity was present in membranes because 40% of the gals-core-gpof was trimmed when membrane barriers were removed by the presence of 1% Triton X-100 (Fig. 6C, lanes 4 and 4').

Transport vesicles were active for fusion with cis-Golgi membranes. Completion of protein transport was detected when aliquots of isolated vesicles (from pool 1) were mixed with cytosol, an ATP-regeneration system, GDP-mannose, and wild-type microsomes that contained fusion-competent cis-Golgi membranes (Fig. 6C). Vesicle fusion with cis-Golgi membranes was not detected at 0°C (Fig. 6C, lane 1), but reached 24% efficiency at 20°C (Fig. 6C, lane 3) in a

Table III. Fold Enrichment of Transport Proteins

| Protein   | Cellular location | Total | MSS (fold enrichment) | Pool 1 (fold enrichment) | Pool 2 | Pool 3 |
|-----------|-------------------|-------|------------------------|--------------------------|--------|--------|
| core gpoff| lum ER/ERV        | 1     | 0.49/1.3               | 24/62                    | 48/130 | 320/840 |
| Sec13p    | pm cyt            | 1     | 0.88                   | 0.62                     | 0      | 0      |
| Sec17p    | pm cyt            | 1     | 0.87                   | 0.99                     | 2.7    | 0      |
| Sec21     | pm cyt            | 1     | 1.4                    | 2.7                      | 2.5    | 0      |
| Sec22p    | tm ER/ERV         | 1     | 0.23/1.3               | 3.7/21                    | 9.4/53 | 81/450 |
| Sec23p    | pm cyt            | 1     | 4.5                    | 2.8                      | 0      | 0      |
| Arfl2p    | pm cyt/Golgi      | 1     | 1.2                    | 0                        | 0      | 0      |
| Sarlp     | pm cyt/ER         | 1     | 0.26                   | 0                        | 0      | 0      |
| Yptlp ts  | pm cyt/Golgi      | 1     | 0.95                   | 0.68                     | 0      | 0      |
| Betlp     | tm ER/ERV         | 1     | 0.62/1.3               | 4.7/10                    | 13/28  | 210/450 |
| Stylp     | pm cyt            | 1     | 0.67                   | 5.4                      | 0      | 0      |
| α-COP     | pm cyt            | 1     | 1.6                    | 12                       | 13     | 10     |
| ββγ-COP   | pm cyt            | 1     | 1.7                    | 5.0                      | 6.1    | 5.9    |
| δ-COP     | pm cyt            | 1     | 1.7                    | 8.2                      | 6.7    | 3.5    |

All aliquots of total and MSS fractions that contained 31.25, 6.25, and 1.25 μg of protein, and aliquots of pools 1, 2, and 3 fractions that contained 1.25, 0.25, and 0.05 μg of protein, respectively, were subjected to SDS-PAGE and immunoblotted with anti-Sec13p (1:1,000), anti-Sec17p (1:2,000), anti-Sec21p (1:1,000), anti-Sec22p (1:5,000), anti-Sec23p (1:1,000), anti-Arflp (1:1,000), anti-Sarlp (1:1,000), anti-Yptlp (1:2,000), anti-Betlp (1:500), anti-Stylp (1:1,000), and anti-coatomer (1:5,000) antibodies. Immune-complexes were detected by 3H-protein A and quantified in a PhosphorImager; the values obtained were expressed as disintegrations per μg of protein analyzed, and served to calculate the values shown. Fold enrichment was calculated with respect to the amount of each marker in the total fraction, which was normalized to a value of 1. The values presented for core-gpof were obtained by PhosphorImager quantitation of triply glycosylated 3H-core-gpof in nitrocellulose filters; these values include core-gpof that is not protease protected. Two values for fold enrichment are given for proteins that are highly enriched in transport vesicles: the value on the left indicates the fold enrichment with respect to the amount of marker in the total fraction, and the value on the right indicates fold enrichment calculated with respect to the amount of marker within diffusible vesicles in the total fraction.
reaction that required cytosolic protein (Fig. 6 C, compare lanes 2 and 3) and the presence of intact membranes (Fig. 6 C, compare lanes 3 and 4). The heterogeneous smear above the 32-kD g/s-core-gppase band represented outer membrane, and its formation required the presence of Golgi membranes and ATP, and was clearly distinct from the load (fractions 13–16) and fractions from all previous purification steps (not shown; see MSS in Fig. 10, lane J). The 68/55-kD protein doublet in fractions 3, 8, 9, and 10 was human keratin introduced by sample handling. The most abundant vesicle proteins were assigned an apparent molecular mass and were named "ERV" proteins, an abbreviation for ER-derived transport vesicle proteins. ERV proteins were tightly associated with the vesicle membrane and resisted extraction with 1M KOAc or 0.2 M Na2CO3 at pH 11, but not with 1% TX-100 (Fig. 8 B).

The enrichment or depletion of various proteins that function in ER to Golgi protein transport in vivo was determined for each purification step using quantitative 125I-protein A Western blot analysis (Table III). Sec13, Sec23, and Sar1 proteins were resolved early in the purification; these are peripheral membrane proteins that function to drive vesicle budding from ER membranes (Salama et al., 1993) and can form a coat around ER-derived transport vesicles (Barlowe et al., 1994). In contrast, Bet1 and Sec22 proteins were enriched 450-fold; these are membrane-embedded proteins with structural similarity to synaptobrevin (Dascher et al., 1991). Boslp was enriched to the same extent as Sec22p, judged by ECL-Western blot analysis (not shown). Sec22 and Boslp proteins function in transport vesicle docking or fusion with Golgi membranes (Kaiser and Schekman, 1990; Shim et al., 1991; Lian and Ferro-Novick, 1993). Subunits of yeast coatomer were also detected in purified vesicle fractions and were slightly enriched. We calculated that coatomer constituted only 1% of the vesicle protein by comparison to known amounts of purified coatomer (Hosobuchi et al., 1992) in 125I-Western blots (not shown). Arf proteins were absent in purified vesicle fractions; these proteins function in the recruitment of coatomer and clathrin to membranes (Palmer et al., 1993; Stamnes and Rothman, 1993). Although Yptlp is suspected of binding ER-derived transport vesicles (Segev, 1991; Lian and Ferro-Novick, 1993), the mutant form of Yptlp used in this study was not detected in vesicles, perhaps due to its inability to bind GTP (Wagner et al., 1987). Finally, neither Sec17 nor Slyl proteins were detected in purified vesicle fractions. Sec17p/αSNAP is a peripheral membrane protein that functions in transport vesicle docking or fusion (Kaiser and Schekman, 1990; Clary et al., 1990; Griff et al., 1992) and Slylp is a peripheral membrane protein that when mutated can replace Yptlp function (Dascher et al., 1991).

Because purified vesicle fractions contained detectable amounts of Betlp, Sec22p, Boslp, coatomer, and GDPase (see Tables II and III), we applied other techniques to determine whether these proteins were components of the same vesicles. Small Golgi vesicles that contained GDPase cofractionated with ER-derived transport vesicles in velocity sedimentation (Fig. 2) and equilibrium density gradients (not shown), but were separated by velocity flotation in sucrose gradients (Fig. 9). GDPase-containing vesicles floated towards equilibrium at a slower rate than ER-derived trans-

**Morphology of ER-derived Transport Vesicles**

Fig. 7 shows typical electron micrographs of transport vesicles visualized by negative stain (A and B) and thin-section microscopy (C and D). Purified vesicles in pool 3 were attached to ionized support grids, fixed, and stained with uranyl acetate; pool 3 contained a uniform population of vesicles that collapsed during dehydration, indicative of the absence of a rigid structural coat (Fig. 7, A and B). Vesicles from the same pool 3 were sedimented on a hardened agarose "bed," fixed, and processed for thin-section electron microscopy. An agarose "bed" was used because vesicle pellets were otherwise invisible. The vesicles were ~60 nm in diameter, lacked apparent protein coats surrounding the membrane, and were aggregated as a result of sedimentation (Fig. 7, C and D).

**Molecular Composition of ER-derived Transport Vesicles**

The protein composition of purified vesicle fractions was analyzed by SDS-PAGE, and visualized by silver stain. Proteins in aliquots of fractions from the last gradient (EQ) were concentrated by precipitation with trichloroacetic acid in the presence of deoxycholate, resolved in a 10 to 15% acrylamide-SDS gradient gel, and stained with silver (Fig. 8 A). The protein composition of fractions that contained transport vesicles was highly simplified (fractions 7–9), and was comprised of radiolabeled α-factor, GDPase activity, and density as described in the Materials and Methods.
Figure 10. Core-gpaf, Betlp, Sec22p, Boslp, and coatomer reside in the same vesicles. Vesicles in MSS fractions from ypl ts stage II incubations were immunosorbed onto protein A-Sepharose beads using anti-Betlp IgG (lanes 2–4, A–C) or anti-Sec22p affinity-purified antibodies (lane 5, A–C). Immunoadsorptions were conducted for 2 h in the absence of competitor peptides (lanes 2 and 5, A–C) or in the presence of the RTQLFG competitor peptide (lane 3, A–C) or the Betlp immune-peptide (lane 4, A–C). At the end of the incubations the beads were sedimented, the supernatants were saved, and beads were washed repeatedly in preparation for detergent extractions. (A) Top insert. Immunoadsorbers and supernatant fractions (unbound) were treated with trypsin and then trypsin inhibitor, and processed for Con A precipitation as described in stage II transport in the Materials and Methods. Immunoadsorption of ER-derived transport vesicles to Sepharose beads is expressed as the per cent of the ratio of protease protected core-gpaf (pp-core-gpaf) in immunoadsorbrates divided by the summation of pp-core-gpaf in the supernatant and immunoadsorbrate fractions. (A) Gel. Vesicle proteins were extracted from beads with 1% Triton X-100 in B88, resolved by SDS-PAGE, and visualized by silver stain (lanes 2–5, panel A). A diluted aliquot of a MSS fraction was loaded in lane 1 of the same gel for comparison; it is underexposed in relation to lanes 2–5. An aliquot of gradient-purified vesicles (from pool 3) was loaded in lane 6 of the same gel, for comparison; it is overexposed in relation to lanes 2–5. (B) Vesicle proteins were extracted from beads with 1% Triton X-100 in B88, resolved by SDS-PAGE, and visualized by Phosphorimaging of 125I-protein A Western blots using anti-Betlp (1:500), anti-Sec22p (1:5,000), anti-Boslp (1:2,000), and anti-coatomer (1:5,000) antibodies. Gradient-purified vesicles from pool 3 (0.3 mg) were diluted fivefold in B88, sedimented to a pellet at 100,000 g for 45 min, resuspended in sample buffer, and loaded in lane 1. gls-core-gpaf is visible because it is labeled with [35S]methionine. (C) After extracting vesicle proteins from beads with 1% Triton X-100 dissolved in B88, beads were washed twice more with the same buffer, and the remaining proteins extracted with 1× Laemmli sample buffer with 2% β-ME at 70°C for 15 min. SDS extracts were analyzed by SDS-PAGE, and visualized by Phosphorimaging of western blots using anti-Betlp (lanes 2–4) or anti-Sec22 antibodies (lane 5) at the same dilution as in B. Heavy chain IgG is visible because it binds 125I-protein A. Contiguous panels are from the same gel. Each panel was blotted separately with an antibody, or antibody mix in the case of Sec22 and Boslp, at the same dilution as in B.
of vesicles immunoisolated with anti-Betlp or anti-Sec22 antibodies was indistinguishable (Fig. 10 A, lanes 2, 3, and 5) and clearly distinct from the protein composition of MSS fractions (lane 1). Immunoisolated vesicles contained all of the ERV proteins present in gradient-purified vesicles (Fig. 10 A, lane 4), but not the RTQLFG peptide (lane 3), prevented the adsorption of vesicle proteins to beads. The presence of Betlp, Sec22p, Bosp, and coatomer in immunoisolates was tested using western blot analysis (Fig. 10, B and C) and the relative amount of each protein was compared to that in 0.3 μg of gradient-purified vesicles (Fig. 10 B, lane 1). Triton X-100 extracts of anti-Betlp immunoisolates (Fig. 10 B, lanes 2 and 3) contained Sec22p, Bosp, core-gp12f, and coatomer. Addition of RTQLFG peptide to antibodies prior to immunoisolation prevented the partial (40%) adsorption of Bosp by cross-reacting antibodies (Fig. 10 B, compare lanes 2 and 3), whereas addition of Betlp immune-peptide prior to immunoisolation fully prevented adsorption of vesicles to beads (lane 4). Antibody–antigen complexes were not extracted from beads by Triton X-100, but were subsequently extracted with 1% SDS at 65°C (Fig. 10 C). Betlp-IgG protein complexes were recovered from beads in SDS extracts (Fig. 10 C, lanes 2 and 3), except when Betlp-immune peptide was mixed with antibodies prior to immunoisolation (lane 4). The Bosp that was adsorbed onto beads by cross-reacting antibodies was also recovered in the SDS extracts and could be detected with anti-Bosp antibodies (not shown). Triton X-100 extracts of anti-Sec22p immunoisolates contained Betlp, Bosp, core-gp12f, and coatomer (Fig. 10 B, lane 5), and Sec22p-IgG complexes were recovered from beads in SDS extracts (Fig. 10 C, lane 5).

Imunoisolation with anti-Betlp antibodies was used to examine the requirements for the formation and release of vesicles. The release of ER-derived transport vesicles from ypt1 ts perforated cells required cytosol, an ATP regeneration system, physiologic temperature, and the absence of GTPyS (Fig. 11 A; Rexach and Schekman, 1991). Membranes that contain ERV proteins were released from perforated cells with the same biochemical requirements as ER-derived transport vesicles (Fig. 11 B) and were not present in the cytosol fraction used to promote vesicle budding (lane 1).

Discussion

We provide a direct biochemical demonstration that diffusible vesicles mediate the selective and vectorial transport of secretory proteins from ER to Golgi membranes. This required the purification of functional ER-derived transport vesicles, assays that reconstitute ER-membrane fusion and vesicle fusion with cis-Golgi membranes, and a molecular comparison of transport vesicle and donor membranes to establish differences in composition. A specific inhibitor of vesicle targeting was necessary to accumulate ER-derived transport vesicles that were amenable to purification, because these are only transient intermediates in reconstituted
transport (Figs. 1 and 3). Inhibition of Ypt1-function by specific antibodies (Rexach and Schekman, 1991) or by mutation (this study) was suitable, since it had no effect on the formation and release of transport vesicles from ER membranes (Fig. 1), but prevented vesicles from forming stable interactions with Golgi membranes (Fig. 3). ER-derived transport vesicles that formed in Yptlp-deficient reactions were physically separable from ER and Golgi membranes, and were free to diffuse out of perforated cells through holes in the plasma membrane. These conditions allowed the purification of vesicles which were monitored by their content of ^3S-core-gpaf.

Evidence demonstrates that ER-derived transport vesicles were purified extensively. First, vesicles that contained protease protected core-gpaf were enriched 950-fold from a mix of perforated cells and cytosol (Table I). Second, the peak of transport vesicles coincided precisely with the peak of protein in the last purification step (Fig. 8A). Third, electron microscopy shows a uniform population of 60 nm vesicles with only occasional larger membrane impurities (Fig. 7). Finally, protein markers of cytosol, ER, and Golgi membranes, as well as vacuole, mitochondria, and plasma membranes, were virtually absent in purified vesicle preparations (Table II).

Several lines of evidence establish the authenticity of the purified ER-derived transport vesicles. Transport vesicles contained only ER-modified forms of e factor and accumulated only when reconstituted ER to Golgi protein transport was blocked by a specific inhibitor (Figs. 1 and 3). Purified transport vesicles were 60 nm in size, as are the putative transport vesicles that accumulate in vivo in a subset of yeast mutant strains that are blocked in ER to Golgi protein transport (Kaiser and Schekman, 1990). The vesicles purified were not fragments of ER or Golgi membranes because they lacked protein markers of these membranes. Vesicles did not contain Sec61p or Kar2p; these proteins are dispersed throughout the ER network, mark the ER membrane and lumen, respectively, and function in protein translocation into the ER lumen (Stirling et al., 1992; Stirling, C., unpublished results; Rose et al., 1989; Preuss et al., 1991; Sanders et al., 1992). Vesicles did not contain GDPase and a-6 mannosyl-transferase activities; these enzymes reside in early compartments of the yeast Golgi complex and are part of the glycosylation machinery (Yanagisawa et al., 1990; Graham and Emr, 1991).

We demonstrate that vesicles purified from Yptlp-deficient reactions are vectorial transport intermediates on the basis of their ability to fuse with cis-Golgi membranes, but not with ER membranes. Diffusible ER-derived transport vesicles did not fuse with ER membranes under conditions that promote ER-membrane fusion (Fig. 6, B and C). Vesicle fusion with cis-Golgi membranes was detected only when purified vesicles were mixed with membranes and cytosol prepared from a wild-type strain. Fusion was efficient (20%) and required the addition of cytosolic protein, ATP, and incubation at physiologic temperature (Fig. 6, C and D). A mechanism that ensures the unidirectionality of vesicle movement is likely to be an integral component of transport vesicles. Perhaps the high concentration of Bet1, Sec22, and Boslp proteins in vesicles relative to donor membranes prevents "back-fusion" with ER membranes, while promoting docking and fusion with cis-Golgi membranes. Alternatively, the absence of Kar2p in vesicles may ensure the directionality of fusion, since Kar2p-function is required for homotypic ER-membrane fusion (Latterich and Schekman, 1994).

Sorting of membrane and lumenal proteins precedes the scission of transport vesicles from ER membranes. This follows precisely because ER-derived vesicles contained only a subset of membrane and lumenal proteins that were initially mixed in the ER membrane (Fig. 2). We find that vesicles contain Bet1, Sec22, and Boslp proteins, but not Sec61 and Kar2 proteins. The bulk flow hypothesis (Rothman, 1987) predicts that the absence of Sec61 and Kar2 proteins in vesicles is due to their active retention in the ER membrane. It will be interesting to determine if core-gpaf, Betlp, Sec22p, and Boslp are actively sorted into vesicles during budding. Quantitative electron microscopic analysis of immunostained "vesicle buds" on ER membranes should resolve this issue (Bednarek, S., L. Orci, and R. Schekman, unpublished results).

Electron microscopic analysis of thin sections that contained purified vesicles showed no apparent protein coats surrounding the vesicle membrane (Fig. 7, C and D). Nevertheless, subunits of coatmarker were consistently detected on gradient-purified (see Table III and Fig. 10 B, lane I) and immunosolated (Fig. 10 B, lanes 2, 3, and 5) transport vesicles, but altogether constituted only 1% of the total vesicle protein; at this concentration coatomer is probably scattered on the vesicle surface. It is likely that ER-derived vesicles were initially coated with a protein lattice and lost the coat proteins during purification. High molecular weight proteins detected in immunosololated transport vesicles, but not in gradient-purified vesicles, may represent components of such coats (Fig. 10 A, compare lane 6 to lanes 2, 3, and 5); these are not subunits of coatomer because equivalent amounts of coatomer were detected in gradient-purified as well as in -immunoisolated transport vesicles (Fig. 10 B, compare lane I to lanes 2-4). In other work we have shown that coatomer is not required in a reconstituted budding reaction. Instead, a distinct membrane coat formed by the Sec23/24 complex, the Sec13/31 complex, and Sarlp is found on ER-derived transport vesicles produced in the presence of nonhydrolyzable analogs of GTP (Barlowe et al., 1994). Interestingly, the gradient-purified transport vesicles described here are depleted of coat proteins (see Table III and Fig. 7), yet contain the information necessary to target and fuse with cis-Golgi membranes (Fig. 6 D); thus, it appears that coat proteins need not be maintained on vesicles to retain targeting competence.

The twelve most abundant proteins in purified ER-derived transport vesicles were tightly associated with the vesicle membrane and resisted extraction with 1 M potassium acetate or 0.2 M sodium carbonate at pH 11.5 (Fig. 8 B). These proteins were named ERV proteins. Two lines of evidence indicate that ERV proteins are components of ER-derived transport vesicles. First, the biochemical requirements for the release of membranes from perforated cells that contain ERV proteins were the same as for transport vesicles that contain ERV proteins were the same as for transport vesicles that contain radiolabeled core-gpaf (Fig. 11). Second, ERV proteins were abundant in immunosololated transport vesicles, as well as gradient-purified vesicles (Fig. 10 A; compare lanes 2, 3, and 5 to lane 6). None of the identified ERV proteins is Betl or Sec22 proteins since their absence in Triton X-100...
eluates of immuno-beads (evidenced by Western blots; Fig. 10 B, compare lane 1 to lanes 2 and 5) does not result in the absence of an ERV protein (evidenced by silver stain; Fig. 10 A, compare lane 6 to lanes 2, 3, and 5). It remains to be determined whether ERV proteins and less abundant proteins in purified vesicle fractions (Fig. 8) play a functional role in vesicular transport, or are just cargo.

Three lines of evidence confirm the presence of core-gpαf, Betlp, Sec22p, and Boslp proteins in the same transport vesicles. First, the kinetics of release from perforated cells of membranes that contained Betlp, Sec22p, and core-gpαf were almost identical, as expected for proteins that reside in the same vesicle population (Fig. 2). Second, vesicles that were purified in gradients were highly enriched in all four proteins (Table III and Fig. 10 B, lane 1). The fold enrichment of Betlp, Sec22p, and Boslp at each purification step was comparable. Nevertheless, vesicles that contain most of Sec22 and Boslp proteins were slightly denser (31% wt/wt) than the average Betlp and core-gpαf containing vesicle (30% wt/wt) in sucrose equilibrium density gradients (not shown). The small discrepancy in densities can best be explained by two possibilities; either these proteins are present in different types of ER-derived vesicles, or vesicles contain unequal amounts of all three proteins, which may influence vesicle density. To better determine whether proteins reside in the same vesicles or not, we used antibodies that recognize Betlp or Sec22p to immunoisolate ER-derived transport vesicles from MSS fractions with the premise that, if Betlp, Sec22p, Boslp, and core-gpαf reside in separate vesicles, they should not all be present in specific immunoisolates. Antibodies against Betlp, and antibodies against Sec22p, efficiently precipitated vesicles that contain all four proteins (Fig. 10, lanes 2, 3, and 5); the relative ratio of these proteins with respect to each other was similar in vesicles purified in gradients or isolated in immuno-beads (Fig. 10 B; compare lane 1 with lanes 2, 3, and 5). These results are in disagreement with those obtained by Lian et al. (1993) who reported that ER-derived transport vesicles lack detectable amounts of Betlp. This discrepancy may relate to the presence of functional Yptlp in their vesicle budding reaction. Perhaps Yptlp serves to regulate the packaging of certain vesicle membrane proteins and in its absence Betlp may be gathered into a bud. This role in protein segregation cannot be essential because vesicles purified from Yptlp-deficient reactions are competent to target and fuse with cis-Golgi membranes (Fig. 6, C and D) in a reaction that requires Yptlp-function (Rexach, M., unpublished results). In normal reactions, Yptlp protein may become associated with transport vesicles during the budding reaction, however it is clear that this timing is not obligatory and vesicles formed in the absence of Yptlp may acquire this protein in the course of targeting to the Golgi complex.

The finding that Betlp, Sec22p, Boslp, and coatomer are components of ER-derived transport vesicles, and the available genetic evidence on the various interactions between genes that encode these proteins (reviewed in Pryer et al., 1992), suggests a physical interaction between these proteins. We propose a model in which Sec22, Betl, and Bosl interact to form part of a docking complex that specifies and promotes the stable attachment of vesicles to cis-Golgi membranes. Coatomer may function to concentrate docking complexes in vesicles, or may mask active docking sites on the vesicle surface. Yptlp may monitor the subunit composition of docking complexes in ER-derived transport vesicles to promote vesicle attachment to cis-Golgi membranes.

The availability of functional ER-derived transport vesicles in highly purified form, combined with a cell-free assay that measures vesicle fusion with cis-Golgi membranes, will facilitate the further dissection of the mechanisms of vesicle targeting and membrane fusion. Because reconstituted vesicle fusion requires the addition of cytosolic factors, this provides a biochemical assay for the purification of soluble proteins that function at this stage. The generation of antibodies against surface constituents of transport vesicles should provide the tools necessary to examine the function of ERV proteins in reconstituted transport. The identification of genes that encode the ERV proteins should allow an assessment of their function in living cells.

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