Amino Acid Permeases Require COPII Components and the ER Resident Membrane Protein Shr3p for Packaging into Transport Vesicles In Vitro

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Abstract. In \textit{S. cerevisiae} lacking SHR3, amino acid permeases specifically accumulate in membranes of the endoplasmic reticulum (ER) and fail to be transported to the plasma membrane. We examined the requirements of transport of the permeases from the ER to the Golgi in vitro. Addition of soluble COPII components (Sec23/24p, Sec13/31p, and Sarlp) to yeast membrane preparations generated vesicles containing the general amino acid permease, Gap1p, and the histidine permease, Hip1p. Shr3p was required for the packaging of Gap1p and Hip1p but was not itself incorporated into transport vesicles. In contrast, the packaging of the plasma membrane ATPase, Pma1p, and the soluble yeast pheromone precursor, glycosylated pro \(\alpha\) factor, was independent of Shr3p. In addition, we show that integral membrane and soluble cargo colocalize in transport vesicles, indicating that different types of cargo are not segregated at an early step in secretion. Our data suggest that specific ancillary proteins in the ER membrane recruit subsets of integral membrane protein cargo into COPII transport vesicles.

In \textit{S. cerevisiae} and other eukaryotes, secreted proteins move intracellularly between membrane-bounded compartments via transport vesicles (Palade, 1975; Pryer et al., 1992). Soluble and integral membrane proteins destined for subcellular compartments, the plasma membrane, or secretion are imported into the ER where folding, initial glycosyl modifications, and oligomer assembly takes place. Cargo molecules destined for transport are then targeted to the Golgi for further modification and sorting (Rothman, 1994; Schekman, 1992). The transport of membrane proteins to the plasma membrane (PM) is essential for, among other functions, the maintenance of intracellular pH and the transport of nutrients, such as amino acids into the cell. The yeast genome encodes 17 amino acid permeases of which 11 have defined substrate specificities. These proteins exhibit 30–60\% amino acid sequence homology and contain 12 putative transmembrane domains (Andre, 1995; Schmidt et al., 1994). Components of the Sec machinery are required for permease translocation into the ER (Green et al., 1992), and for export to the PM (Courchesne and Magasanik, 1983).

Shr3p, a nonessential integral ER membrane protein, is also required for the localization of amino acid permeases to the plasma membrane of \textit{S. cerevisiae} in vivo (Ljungdahl et al., 1992). Immunofluorescence localization showed that amino acid permeases were retained in the ER membrane of \(\Delta shr3\) null mutant (\(\Delta shr3\)) cells. Shr3p acts pleitropically on the entire family of amino acid permeases. As a result of diminished levels of amino acid permeases in the PM, \(\Delta shr3\) cells are resistant to high concentrations of histidine in the growth medium. The transport of soluble cargo, such as carboxypeptidase Y, \(\alpha\) factor, and invertase, and membrane proteins, such as the plasma membrane ATPase, Pma1p, and the soluble plasma membrane ATPase, Pma1p, appeared unaffected by \(\Delta shr3\) in vivo. Thus, Shr3p appears to be a specific accessory protein required for the early stage of secretion for a select group of homologous proteins, the amino acid permease family.

Vesicle-mediated transport of glycosylated \(\alpha\) factor precursor (gpaF) from the ER to the Golgi complex has been reconstituted in vitro (Baker et al., 1988). Three protein fractions comprising two hetero-oligomeric complexes (Sec23/24 and Sec13/31p) and the small ras-like GTPase Sarlp constitute the cytosolic components (COPII) required for vesicle formation (Salama et al., 1993). Sarlp-GTP and the other proteins form the COPII coat around the vesicle that disassembles upon hydrolysis of GTP by Sarlp, allowing fusion of an uncoated vesicle with Golgi membranes (Barlowe et al., 1994). Recently, these transport vesicles were also found to carry the GPI (glycosylphosphatidylinositol) anchored cell wall protein Gaslp (Doering and Schekman, 1996). In addition to cargo proteins, COPII captures the putative vSNARES, Sec22p, Betlp, and Boslp.

1. Abbreviations used in this paper: COPII, cytosolic coat components; gpaF, glycosylated pro \(\alpha\) factor; MSS, medium speed supernatant; PM, plasma membrane.
Materials and Methods

Materials and Media

Unless otherwise noted, reagents were purchased from Sigma Chemical Co. (St. Louis, MO). SUD and SPD are synthetic minimal media (6.7 g/l yeast nitrogen base without ammonium sulfate and amino acids, Difco Laboratories Inc., Detroit, MI), supplemented with 20 g/l glucose, and 1.0 g/ l urea or proline (1.0 g/l), respectively (Ljungdahl et al., 1992). Where required, SPD was supplemented with either 1 mM or 30 mM L-histidine; appropriate volumes of a filter sterilized 0.5 M L-histidine stock solution were added and the pH was adjusted to 5.5 with 10 N NaOH. The concentration of yeast nitrogen base in SUD and SPD is fourfold higher than the amount normally used in other synthetic media. MVD is a standard synthetic minimal medium (6.7 g/l yeast nitrogen base without amino acids, Difco) supplemented with 20 g/l glucose. YPD is a complex medium (10 g/l Bacto yeast extract, 20 g/l Bacto peptone, Difco) supplemented with 20 g/l glucose. Solid media were made by the addition of 15 g/l Bacto agar (Difco). 35S-labeled prepro α factor and crude RS607 yeast cytosol (~25 mg protein/ml) were prepared as described (Baker et al., 1988). COPII components were purified as described (Salama et al., 1993; Barlowe et al., 1994).

Yeast Strain and Plasmid Constructions

Yeast strains are listed in Table I and the plasmids used are listed in Table II. All yeast strains are isogenic derivatives of A A280. A spontaneous Ade scatter of AA280 was isolated and crossed to AA288, the resulting diploid was sporulated and tetrads dissected. Two spore-derived colonies with the desired genotypes resulted in PLY120 and PLY127. The Ade2 gene was deleted in PLY127 by cotransformation with BamHI linearized plP131 and the URA3 based vector YCP50. A red Ade ura4 transformant was isolated, and the loss of the YCp50 vector was selected for by growth on 5-fluoro orotic acid (5-FOA) resulting in strain PLY132 (Boeke et al., 1984). PLY170 and PLY171 are derived from meiotic seg- regants of a diploid strain obtained by mating PLY120 with PLY132. The diploid PLY116, obtained by mating PLY170 and PLY171, was transformed with a linear EcoRI-Sall fragment of DNA derived from plP288 containing shr3Δ::hisG-URA3-hisG (Alani et al., 1987). One Ura4+ transformant was sporulated and tetrads dissected. PLY214 and PLY223 are meiotic segregants of this transformant. Resistance to 30 mM histidine segregated 2:2 and was 100% linked to the URA3 marker. PLY223 was propagated on medium containing 5-FOA to attain the unmarked deletion of shr3Δ resulting in PLY229. PLY116 was transformed with Ade2 with a linear EcoRI-KpnI fragment of DNA containing hip1Δ::ADE2 derived from plasmid plP296. PLY198 is a meiotic seg- regant from one such transformant. PLY198 was subsequently trans- formed with a linear EcoRI-Sall fragment of DNA derived from plP288 containing shr3Δ::hisG-URA3-hisG to create PLY202. PLY202 was propagated on media containing 5-FOA to attain the unmarked deletion of shr3Δ resulting in PLY204. MKY951 and MKY952 were made by integrating pRS306(HA-PMA1) into the PMA1 locus of PLY214 and PLY229, respectively. The integration of Sall digested pRS306(HA-PMA1) dis- rupted gene and created a full-length version of PMA1 containing the HA epitope tag. Chromosomal integration was verified by PCR, and expression of the full-length Pma1p-HA product was monitored by immunoblotting spheroplast lysates from transformed yeast cells.

Plasmids were constructed using standard molecular biological procedures. The unmarked ade2Δ was constructed by digesting plP130 with BglII and relinking to create plP131. It should be noted that the ade2Δ construct disrupted genes flanking the Ade2 locus: 5' a protein of unknown function, and 3' the Rga1 gene was truncated (Stevenson et al., 1995). The ade2Δ allele did not noticeably affect the growth rate, mating, or sporulation of yeast strains. Importantly, the ade2Δ allele did not affect any of the shr3 mutant phenotypes. plP288 was constructed by inserting a blunt end 5-kb BglII-BamHI fragment from pSE1076 (Allen and Elledge, 1994) into HindIII-XhoI digested plP216, made blunt by treatment with Klenow fragment. plP261 was made by inserting the XbaI-Sall fragment containing shr3Δ::hisG-URA3-hisG to create PLY202. PLY202 was propagated on media containing 5-FOA to attain the unmarked deletion of shr3Δ resulting in PLY204. MKY951 and MKY952 were made by integrating pRS306(HA-PMA1) into the PMA1 locus of PLY214 and PLY229, respectively. The integration of Sall digested pRS306(HA-PMA1) disrupted gene and created a full-length version of PMA1 containing the HA epitope tag. Chromosomal integration was verified by PCR, and expression of the full-length Pma1p-HA product was monitored by immunoblotting spheroplast lysates from transformed yeast cells.

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Plasmids used to analyze Hip1p were constructed as follows. plP248 was created by inserting the 3-kb EcoRI-Sall fragment containing Hip1p from pJT19 into EcoRI-Sall digested pRS316 lacking the BamHI site in the polylinker; plP273 was constructed by inserting the larger 5.5-kb XhoI Hip1p containing fragment from pJT19 into XhoI digested pRS316 (Sikorski and Hieter, 1989). plP253 was constructed by digesting plP248 with BamHI and XhoI after which the 2.2-kb BglII fragment containing ADE2 from pASZ11 was inserted (Stotz and Linder, 1990). plP282 was made by digesting plP273 with EcoRI and XhoI. The 4-kb EcoRI-Sall fragment containing hip1Δ::ADE2 from plP253. The insert of plP282 was cloned into KpnI-EcoRI digested pBlues SK(+) (Strat- agene, La Jolla, CA) resulting in the deletion construct plP296.

The construction of plP321 containing a fully functional Hip1p-myc required multiple steps. plP302 was made by digesting plP273 with EcoRI-NotI, the ends were made blunt and religated. plP304 was con- structed by digesting plP302 with XbaI to remove the Hip1p frag- ment, following which the ends were filled in and religated. Epitope tag- ging of Hip1p was performed as described by Kolodziej and Young (1991) using plP304 as the starting vector and site directed insertion mutagenesis (Kunkel et al., 1987). plP308 contains an XbaI site introduced into the Hip1p sequence between amino acid residues 23 and 24. An oligomer en-
coding an Xbal site was annealed to single stranded pPL304 DNA prepared with helper phage M13K07 (Vieira and Messing, 1987) in the dad ung E. coli host, RZ1032 (Kunkel et al., 1987). After elongation, ligation, and transformation into a dad ung host, plasmid DNAs were screened for the presence of the new XbaI site. An XbaI flanked cloning cassette, encoding the c-myec epitope reiterated three times, was inserted into the unique XbaI site of pPL304 resulting in pPL315. To reconstruct sequences '3' of HIP1, we digested pPL315 with AvrII and the 3 kb AvrII fragment of pPL302 was inserted. This last cloning step resulted in pPL321 and reintroduced the previously deleted 1.5 kb XbaI fragment.

pRS306 (HA-PMA1) was made by introducing the 2.4 kb HindIII–BamHI fragment of pRS305 (HA-PMA1) (Ziman et al., 1996) into the multiple cloning site of pRS306 ( Sikorski and Hieter, 1989) lacking the SacI endonuclease site. The SacI site had been removed previously by SacI digestion of pRS306 followed by treatment with the Klenow enzyme and ligase. The resulting plasmid contained a 1.6 kb fragment of PMA1 encoding the epitope tagged NH2 terminus of Pma1p.

Plasmids encoding epitope-tagged proteins were transformed into yeast using the lithium acetate procedure ( Guthrie and Fink, 1991). Transformants were chosen that expressed the plasmid-encoded proteins as detected by SDS-PAGE immunoblotting of whole cell lysates. The c-myec epitope is recognized by the monoclonal antibody 9El0 (Evan et al., 1985). Preparation of extracts for immunoblotting was performed as described (Doering and Schekman, 1996).

**Microsome Preparation**

Yeast cultures of PLY204[pPL321] [pPL261], PLY198[pPL321], and PLY204[pPL321] grown at 30°C on appropriately supplemented MVD solid media were used to inoculate stock cultures of supplemented SUD broth. Stock SUD cultures grown at 24°C to 7 OD600/ml were used to inoculate YPD broth cultures grown at 24°C overnight to 10D600/ml. Cells (3 1 total, 1 OD590/ml) were harvested, washed twice in MVD, resuspended in supplemented SPD (250 ml vol with 1 × ATP mix and 0.1 mM GTP). Reactions were incubated at 25°C for 30 min. One-tenth of the sample was removed for analysis (Total), the remaining reaction mixture was centrifuged (12,000 g) at 4°C for 2 min, and 25 µl of the supernatant (MSS) was collected. Total and MSS fractions from radiolabeled membranes were analyzed by immunoprecipitation. Fractions from membranes containing transfected 35S-α factor precursor were analyzed for protease-protected gpαF as described (Rexch and Schekman, 1991).

**Semintact Yeast Spheroplasts**

Yeast cultures of PLY129[pPL269], PLY134[pPL269], MKY951, and MKY952 grown on appropriately supplemented MVD solid media at 30°C were used to inoculate stock cultures of supplemented SUD broth. Stock cultures grown to an OD600/ml of 2-6 at 24°C were used to inoculate overnight YPD broth cultures that were grown to an OD600/ml of 1-2 at 24°C. Cells were harvested, washed twice in MVD, resuspended in supplemented MVD to 5 OD600/ml, and shaken at 30°C for 15 min. The cultures were labeled with 1 µCi/60 OD600/ml 35S-Promix (1,200 Ci/mmol, Amer sham) for 3 min at 30°C. Metabolic activity was stopped by the addition of NaNO3 (20 mM final). The cells were incubated on ice for 15 min and washed with cold 20 mM NaNO3. Spheroplast preparation and lysis were performed as described (Sanz et al., 1994). Immunoprecipitation of cell wall protein (CWP) with polycyclone anti CWP, prepared by T. Doering and J. Campbell (Univ. of California, Berkeley, CA) as described (Sanz et al., 1987), and Gap1p-HA from 35S-labeled semi-intact spheroplast preparations of the isogonic pair PLY129[pPL269] and PLY134[pPL269] showed less overall protein labeling in Δshr3 cells, however the ratio of the two proteins was the same in both strains.

**Semiintact Spheroplast-based Cargo Packaging Assay**

Gently lysed spheroplasts (20-30 OD600 U/tube, ~8 µg protein/OD600U) were incubated in Stage I translocation reactions as described (Rexch et al., 1994). Unlabeled washed membranes were exposed to 35S prepro-α factor and 1 × ATP regeneration mix at 10°C for 25 min, whereas 35S metabolically labeled membranes were treated in a mock translocation reaction with only 1 × ATP regeneration mix. For urea treatment, before the budding reaction the membranes were resuspended in 2.5 M urea (100 µl), incubated for 10 min on ice, and washed twice with 1 ml B8. Membranes were resuspended in a final volume of 80 µl B8. Reaction tubes (USP, San Leandro, CA) and sedimented in the TLA100.3 for 15 min. Permease Packaging into COPII Vesicles

**Vesicle Immunoisolation**

Vesicle budding reactions using semi-intact spheroplasts performed at 4°C and 25°C were scaled up tenfold and 350 µl of the resulting MSS was collected and subjected to flotation on a 35%–30%–25%–15% Nycodenz gradient as described previously (Barlowe et al., 1994). The upper three 400-µl fractions were collected. For radiolabeled membranes, 5 µl of the total reaction, 25 µl of the MSS, and 50 µl of each 400 µl Nycodenz fraction were analyzed by immunoprecipitation. For unlabeled membranes, 5 µl of the total reaction and 335 µl of each Nycodenz fraction were precipitated in 20% TCA on ice for 5 min, and the precipitated material was collected by centrifugation (12,000 g, 20 min), washed with acetone, and sedimented again (12,000 g, 10 min). TCA precipitated material was analyzed by SDS-PAGE immunoblotting. For membranes containing transfected 35S-α factor precursor were analyzed for protease-protected gpαF. To examine the possibility of nonspecific vesicle aggregation, we mixed 200 µl of MSS containing Gap1p-HA vesicles and 200 µl of MSS containing 35S prepro-α factor vesicles on ice before Nycodenz flotation and immunolocalization. To immobilosolate vesicles, we discarded the top 100 µl of the gradient, and the following 375 µl was collected and 20 µl incubated in 5% BSA/PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4/7H2O, 1.4 mM KH2PO4 overnight at 4°C in siliconized Eppendorf tubes (USP, San Leandro, CA) with 10 µl (0.5 mg/ml) anti-HA epitope monoclonal antibody (12CA5, Boehringer Mannheim, Indianapolis, IN) or affinity-purified anti- Sec22 polyclonal antibody (1 mg/ml) (Bednarek et al., 1996), in the presence and absence of 1 mg/ml HA peptide (Boehringer Mannheim) or 0.1 mg/ml Sec22p-GST fusion protein (Bednarek et al., 1996). Immunoprecipitated material was washed 4 × 0.1% BSA/PBS, transferred to a new tube, and washed once with 50 mM NaCl 10 mM Tris-Cl, pH 7.5. For nonradiolabeled samples, immobilosolated vesicles were subjected to SDS-PAGE immunoblotting and 35S Protein A. Immunoreactive proteins were detected using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Radiolabeled Gap1p-HA in a portion of the total membrane material, the MSS, and the immobilosolated material were immunoprecipitated under denaturing conditions with the HA antibody. Immunoprecipitates were washed, subjected to SDS-PAGE, and analyzed.
**Table I. Saccharomyces cerevisiae Strains**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| AA280  | MATα ura3-52 his3Δ200 lys2Δ201 ade2 | Antebi and Fink, 1992 |
| AA288  | MATα ura3-52 leu2-3, 112 lys2Δ201 ade2 | Antebi and Fink, 1992 |
| PLY120 | MATα ura3-52 leu2-3, 112 his3 Δ200 lys2Δ201 | This work |
| PLY127 | MATα ura3-52 lys2Δ201 | This work |
| PLY129 | MATα ura3-52 leu2-3, 112 lys2Δ201 ade2 gap1Δ::LEU2 | Ljungdahl et al., 1992 |
| PLY132 | MATα ura3-52 lys2Δ201 ade2Δ2 | This work |
| PLY134 | MATα ura3-52 leu2-3, 112 lys2Δ201 ade2 gap1Δ::LEU2 shr3Δ6::URA3 | Ljungdahl et al., 1992 |
| PLY170 | MATα ura3-52 leu2-3, 112 lys2Δ201 ade2Δ2 | This work |
| PLY171 | MATα ura3-52 his3Δ200 lys2Δ201 ade2Δ2 | This work |
| PLY202 | MATα ura3-52 lys2Δ201 ade2Δ2 hip1Δ2::ADE2 shr3Δ5::hisG-URA3-hisG | This work |
| PLY204 | MATα ura3-52 lys2Δ201 ade2Δ2 hip1Δ2::ADE2 shr3Δ6 | This work |
| PLY214 | MATα ura3-52 lys2Δ201 ade2Δ2 | This work |
| PLY223 | MATα ura3-52 lys2Δ201 ade2Δ2 shr3Δ5::hisG-URA3-hisG | This work |
| PLY229 | MATα ura3-52 lys2Δ201 ade2Δ2 shr3Δ6 | This work |
| MKY951 | MATα ura3-52 lys2Δ201 ade2Δ2 pma1::HA-PMA1::URA3 | This work |
| MKY952 | MATα ura3-52 lys2Δ201 ade2Δ2 pma1::HA-PMA1::URA3 shr3Δ6 | This work |

**Table II. Plasmids**

| Name               | Description                               | Source or reference |
|--------------------|-------------------------------------------|---------------------|
| pJT19              | 9-kb fragment containing HIP1 in YEpl424  | Tanaka and Fink, 1985 |
| pPL130             | 6.2-kb BamHI fragment containing ADE2 in  | Ljungdahl et al., 1992 |
| pPL131             | ade2Δ2 in pUC19                            | This work           |
| pPL216             | shr3Δ3 in pBSII SK(+)                      | Ljungdahl et al., 1992 |
| pPL230             | SHR3::FLU2 in pRS316                       | Ljungdahl et al., 1992 |
| pPL248             | 3-kb EcoRI-SalI fragment containing HIP1 in pRS316(p BamHI) | This work           |
| pPL253             | 4-kb EcoRI-SalI fragment containing hip1Δ2::ADE2 in pRS316(p BamHI) | This work           |
| pPL261             | SHR3::FLU2 in YCP405                       | This work           |
| pPL269             | GAP1::FLU1 in YCP405                       | This work           |
| pPL273             | 5.5-kb Xhol fragment containing HIP1 in pRS316 | This work           |
| pPL282             | 6-kb KpnI-EcoRI fragment containing hip1Δ2::ADE2 in pRS316 | This work           |
| pPL288             | 5.7-kb EcoRI-EcoRI fragment containing shr3Δ5::hisG-URA3-hisG in pBSII SK(+) | This work           |
| pPL296             | 6-kb KpnI-EcoRI fragment containing hip1Δ2::ADE2 in pBSII SK(+) | This work           |
| pPL302             | 5.5-kb Xhol fragment containing HIP1 in pRS316(ΔEcoRI-NotI) | This work           |
| pPL304             | 4-kb Xhol fragment containing HIP1 in pRS316(ΔEcoRI-NotI) | This work           |
| pPL308             | 4-kb Xhol fragment containing HIP1::XI in pRS316(ΔEcoRI-NotI) | This work           |
| pPL315             | 4.1-kb Xhol fragment containing HIP1::myc3X1 in pRS316(ΔEcoRI-NotI) | This work           |
| pPL321             | 5.6-kb Xhol fragment containing HIP1::myc3X1 in pRS316(ΔEcoRI-NotI) | This work           |
| pRS305(HA-PMA1)     | HindIII-BamHI fragment containing HA-PMA1 in pRS305(ASalI) | Ziman et al., 1996 |
| pRS306(HA-PMA1)     | HindIII-BamHI fragment containing HA-PMA1 in pRS306(ASalI) | This work           |
using a Phosphorimager. To quantify colocalization of 35S-gpaF, we treated immunisolated material with protease, followed by solubilization and Con A precipitation of the protease-protected material. This quantity was compared with the amount of protease-protected, Con A--precipitable 35S-gpaF in the native immunoprecipitation supernatant.

**Immunoprecipitation and Western Blotting**

Immunoprecipitation reactions were prepared by heating the sample in B88 with an equal volume of denaturing buffer (1% SDS, 50 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM PMSE) for 10 min at 50°C. The sample was adjusted to 1 ml with IP buffer (150 mM NaCl, 1% Triton X-100, 0.05% SDS, 15 mM Tris Cl, pH 7.5, 10 µl (5 µg) HA antibody, and 50 µl 30% protein G-Sepharose in PBS, and the reactions were incubated for 2 h, rotat- ing at 25°C. Immunoprecipitates were washed as described (Baker et al., 1988). Immunoprecipitated material was heated in reducing, denaturing sample buffer at 55°C for 10 min, resolved by SDS-PAGE, and analyzed using a Phosphorimager.

For immunoblotting, proteins were heated in sample buffer at 55°C for 10 min, resolved by SDS-PAGE, and transferred to 0.2 µm nitrocellulose (Millipore, Bedford, MA) or PVDF (Millipore) at 250 mA for 1.5 h. Strips of the blot were incubated at 25°C for 1 h with 3% nonfat dry milk in TBST (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), and incubated for 2 h with affinity-purified anti-Sec61p polyclonal antibodies (1:5,000 in TBST) (Störting et al., 1992), affinity-purified anti-sec22p polyclonal antibodies (1:1,000), anti-myc epitope monoclonal antibodies (9E10, 1:3,000) (Evan et al., 1985), anti-HA antibodies (1:3,000), or puri-

**Results**

**Amino Acid Permeases Hipl and Gap1, but Not the Permease Chaperone Shr3p, Are Packaged into COPII Transport Vesicles**

The yeast phenorme precursor, glycosylated pro-α factor (gpaF), is a marker for soluble protein cargo in secretory vesicles that bud from ER membranes in vitro (Baker et al., 1988; Rexach and Schekman, 1991). In this budding assay, radiolabeled prepro-α factor is posttranslationally translo-

To investigate the packaging of amino acid permeases in vitro, we introduced single copy plasmids encoding an epitope-tagged permease into yeast strains containing a disruption of the wild-type permease gene (Tables I and II). Plasmids pPL269 and pPL321 encoding the tagged permeases Gap1p-HA and Hip1p-myc were transformed into the respective permease deletion strains PLY129 and PLY198. Epitope-tagged Gap1p-HA and Hip1p-myc are expressed and are localized to the PM where they are fully functional (Ljungdahl et al., 1992; and data not shown). Strain PLY204 which has deletions in the HIP1 and SHR3 loci was transformed with pPL261 encoding HA-tagged Shr3p (Shr3p-HA), and pPL321, encoding Hip1p-myc. Strains deleted in SHR3 and transformed with the epitope-tagged version of SHR3 behaved as wild-type strains with regard to growth on a low concentration of histidine and sensitivity to a high concentration of histi-

The strains containing epitope-tagged proteins were analyzed using a modified budding assay (see Materials and Methods) to investigate the incorporation of integral membrane proteins into transport vesicles in vitro. Microsomal membranes prepared from Δshr3 cells expressing Shr3p-HA and Hip1p-myc were incubated with either COPII proteins or whole cytosol to generate vesicles. The MSS was then subjected to high-speed centrifugation (90,000 g) to sediment the vesicles. Hip1p was detected in vesicle pellets from incubations containing nucleotide and either cytosol or the purified COPII components (Fig. 1). The cargo molecule gpaF and the putative v-SNARE Sec22p were also found in these vesicles (Fig. 1). Similar results were obtained using purified yeast nuclei, a highly enriched preparation of ER membrane (data not shown). In contrast, Shr3p and the ER resident protein Sec61p were not detected in the vesicle fractions (Fig. 1). These data indicate that Hip1p enters COPII vesicles whereas Shr3p is not transported out of the ER in vitro.

We wanted to examine the vesicle packaging of permeases quantitatively, but contamination of microsomal membranes with plasma membrane fragments prevented accurate measurement of the low level of nascent permeases in transit from the ER. For this reason, we used spheroplast membranes prepared from cells that had been pulse-radiolabeled for 3 min with [35S]methionine. This short pulse essentially labels only ER transport intermediates. Such pulses have previously been used to biochemi-

![Figure 1. Packaging of Hip1p-myc from ER membranes in vitro. Microsomal membranes isolated from PLY204[pPL261][pPL321] were used in duplicate vesicle budding reactions which contained: GTP, nucleotides alone; COPII, nucleotides and purified COPII components; Cyt, nucleotides and cytosol supplemented with Sarlp; II, COPII components without membranes; and C, Cytosol without membranes. Medium speed supernatant (MSS) fractions collected by high speed centrifugation and one-third of the total reaction mixture (Total) were resolved by SDS-PAGE, and immuno-
Figure 2. Gaplp-HA packaging into vesicles depends on each of the COPII components. PLY129[pPL269] permeabilized spheroplasts were used in duplicate vesicle budding reactions. The membranes were washed in 2.5 M urea for 10 min on ice and then washed once with B88 to decrease the amount of COPII components that remained on the membrane. Reactions contained: no nuc, COPII components; COPII, nucleotides and COPII components; Cyt, nucleotides and cytosol supplemented with Sarlp; and nucleotides and COPII reagents without Sec13/31p (13-), Sec23/24p (23-), or Sarlp (Sarl-), respectively. (A) One-fifth of the total reaction mixture (Total) and the complete MSS fractions were immunoprecipitated with anti-HA mAb. The immunoprecipitate was resolved by SDS-PAGE and analyzed with a phosphorimager. (B) Quantitation of phosphorimager signals corresponding to radiolabeled Gaplp-HA expressed as an average of the percent of Gaplp-HA in the MSS of each reaction compared with the total reaction. (C) In duplicate parallel reactions using PLY129[pPL269] permeabilized spheroplasts, the amount of trypsin resistant 35S-gpetF present in the MSS compared with the total reaction mixture was measured and the average value is shown for each of the assay conditions described above.

Shr3p Is Required for In Vitro Packaging of Permeases into Vesicles

We next examined the effect of an shr3 null mutation on the ability of the permeases to exit the ER in vitro. Hiplp packaging from SHR3 and Δshr3 membranes was investigated using microsomal membranes and an immunoblot assay. Hiplp-myc was not detected above background levels in the MSS in reactions using Δshr3 membranes with COPII or cytosol (Fig. 3 A). A quantitative difference in permease packaging from SHR3 and Δshr3 membranes was determined using 3-min pulse-labeled membranes prepared from isogenic cells expressing Gaplp-HA. Δshr3 membranes did not release immunoprecipitable Gaplp into the MSS in reactions containing either cytosol or COPII (Fig. 3 B). By quantitative phosphorimager analysis, we found that Gaplp packaging into vesicles from Δshr3 membranes was reduced at least 90% compared to SHR3 membranes (Fig. 3 C). These data demonstrate that the amino acid permeases require the ER resident membrane protein, Shr3p, to be packaged into transport vesicles in vitro.

α Factor and Pma1p Packaging into Vesicles Is Independent of Shr3p

We investigated whether the effect of the Shr3p deletion was specific to the amino acid permeases. α factor secretion and transport of the major plasma membrane proton-translocating ATPase (Pma1p) are not affected in vivo by Δshr3 (Ljungdahl et al., 1992). We looked for the presence of these cargo in vesicles produced from Δshr3 membranes in vitro. 35S-gpetF packaging was quantified from "mock-labeled" permeabilized spheroplasts that were prepared similarly to the metabolically labeled membrane preparations described above. The efficiency of gpetF precursor packaging was similar using SHR3 and Δshr3 membranes (Fig. 4 A). Although the experiment depicted in Fig. 4 A indicates a partial reduction in gpetF packaging using COPII components with Δshr3 membranes, this pattern was not seen in assays involving other isogenic SHR3/Δshr3 pairs (data not shown). Doubling the level of COPII proteins increased the efficiency of gpetF packaging from Δshr3 membranes whereas no such stimulation of Gaplp...
Figure 3. Hip1p-myc and Gap1p-HA require Shr3p for packaging into vesicles. (A) Microsomes containing Hip1p-myc were prepared from SHR3 and Δshr3 isogenic strains PLY198[pPL321] and PLY204[pPL321]. Duplicate vesicle budding reactions contained: nucl, nucleotides; COP11, nucleotides and COPII components; Cyt, nucleotides and cytosol supplemented with Sarlp. One-seventh of the total reaction mixture and the complete MSS of each reaction were analyzed by immunoblotting with anti-myc mAb and the signal visualized by ECL and autoradiography. (B) 35S-pulse labeled permeabilized spheroplasts containing Gap1p-HA were prepared from SHR3 and Δshr3 isogenic strains PLY129[pPL269] and PLY134[pPL269]. Duplicate vesicle budding reactions contained: nucl, COP11, nucleotides and COPII components; Cyt, nucleotides and cytosol supplemented with Sarlp; and 4°C, nucleotides and cytosol supplemented with Sarlp incubated at 4°C. Radiolabeled Gap1p-HA was immunoprecipitated from one-fifth of the total reaction mixture and the MSS from each reaction using anti-HA mAb. Immunoprecipitated material was resolved by SDS-PAGE and analyzed using a phosphorimager. (C) Quantitation of phosphorimager signals correspond to the percent of GapIp-HA in the MSS of each reaction compared with the total reaction. Solid (SHR3) and hatched bars (Δshr3) represent the average of three data sets.

To further characterize the population of membranes that contain the amino acid permeases, we fractionated the MSS on a Nycodenz step gradient such that small vesicles float to the top whereas larger membrane fragments remain at the bottom (Barlowe et al., 1994). Gap1p was detected in the same upper step of the gradient as gpaF and Sec22p, whereas the ER membrane marker, Sec61p, did not float (Fig. 5 A and B). These data suggest that Gap1p resides in vesicles that have the same general properties as those that contain gpaF.

Sorting of cargo molecules is thought to occur at late stages in the secretory pathway (Harsay and Bretscher, 1995; Rothman, 1994). We were interested in determining whether integral membrane and soluble cargo proteins were sorted into distinct COPII vesicles. Because the epitope tag on Gap1p is predicted to reside on the cytosolic side of the membrane, and thus be accessible to antibodies, we had the opportunity to immunoprecipitate Gap1p-HA-containing vesicles to evaluate coincident localization of gpaF. Vesicle populations were immunolabeled after flotation on a Nycodenz step gradient. Nycodenz removes most of the COPII coat (Barlowe et al., 1994) allowing better epitope recognition by the antibody. Native immunoprecipitation using a saturating amount of anti-HA antibody recovered 65% of the Gap1p-HA found in the Nycodenz-floated material; recovery was completely inhibited by the HA peptide (1 mg/ml). Anti-HA antibody coprecipitated 71% of the gpf and detectable amounts of the vSNAREs, Bet1p and Sec22p, present in the Nycodenz-floated vesicles (Fig. 5 C). Similar results were obtained using cytosol or purified COPII components to generate the vesicles. In addition, we prepared a mixture of vesicles, half carrying Gap1p-HA and not containing 35S-gpaF,
and half not carrying Gaplp-HA but containing $^{35}$S-gpaF, which were floated on a Nycodenz gradient. The presence of Gaplp-HA–carrying vesicles did not cause the coisolation of $^{35}$S-gpaF recovered with anti-HA antibody (8%, background level: 3–7%). These data indicate that only vesicles carrying the epitope tag were immunoprecipitated and thus nonspecific agglutination of vesicles did not occur during the procedure. Our results support the hypothesis that soluble and membrane cargo reside in the same ER-derived transport vesicle and suggest that protein sorting occurs at a later step in secretion.

**Discussion**

We were interested in determining the requirements for in vitro packaging of the integral membrane amino acid permease proteins into transport vesicles. By modifying methods described previously for the packaging of α factor precursor in vitro, we were able to follow newly synthesized integral membrane proteins as cargo molecules in ER-derived transport vesicles. A similar approach was used to identify Gaslp, a GPl-anchored protein, as vesicle cargo (Doering and Schekman, 1996). We found that Hiplp and Gaplp are packaged into vesicles driven by Sec proteins found in a cytosol fraction, and that the same degree of packaging of these cargo could be achieved using purified COPII components, Sec13/31p, Sec23/24p, and Sarlp (Figs. 1–3).

Pmalp, another integral plasma membrane protein, was observed as cargo in vesicles generated from ER by crude cytosol (Fig. 4, B and C). Whereas the permeases were exported as effectively by the COPII components as by whole yeast cytosol, the amount of Pmalp packaged into vesicles by the purified COPII components was only about half the amount observed using cytosol (data not shown). Thus, additional soluble factors may be required for the in vitro packaging of Pmalp into vesicles. Accumulation of plasma membrane ATPases in proliferated ER upon expression of dominant lethal pmal mutants or plant PM ATPases in yeast suggests that a limiting ER chaperone-type protein may be responsible for the transport of Pmalp to the PM (Harris et al., 1994; Villalba et al., 1992; de Kerchove d’Exaerde et al., 1995). AST1 encodes a membrane-associated cytosolic protein that suppresses the growth defect of mutant pmal cells by localizing mutant Pmalp to the PM (Chang and Fink, 1995). Immunofluorescence data show that Astlp is present in punctate cytoplasmic locations and at the cell perimeter, but is excluded from the nucleus and the vacuole. These data suggest a possible role for cytosolic Astlp in Pmalp vesicle transport.
Nycodenz flotation and immunoprecipitation of the vesicles showed that Gap1 resides in the same vesicles as a factor precursor and the putative v-SNAREs, Bet1p, and Sec22p (Fig. 5). It is not yet clear whether Pma1p resides in the same vesicles as other membrane and soluble cargo. These results show that permeases are transported out of the ER in COPII vesicles and implies that the separation of cargo with different destinations occurs at a step beyond the ER in the secretory pathway.

Shr3p is clearly required for the exit of the permeases Hip1p and Gap1p from the ER in vitro (Fig. 3). The defect in permease packaging by Δshr3 membranes is not overcome by a soluble cytosolic factor. Although immunofluorescence data demonstrated that the integral membrane protein Shr3p exists primarily in the ER (Ljungdahl et al., 1992), we considered the possibility that Shr3p entered secretory vesicles and then was recycled back to the ER. Unlike the vSNARE Sec22p (Rexach et al., 1994; Yeung et al., 1995), or the sorting accessory protein, Emp24p (Schimmoller et al., 1995), which cycle between the ER and the cis-Golgi, Shr3p is not packaged into cargo vesicles in vitro (Fig. 1). This behavior suggests that Shr3p is a true ER resident protein, interacting only transiently with the permeases before they enter cargo vesicles.

The packaging of a factor precursor or Pma1p is independent of Shr3p (Fig. 4), thus Shr3p does not appear to be a universal link between the ER membrane and the budding machinery. The result that Δshr3 membranes are not generally defective in the export of PM proteins demonstrates that Δshr3 membranes retain a normal sorting and budding apparatus.

Shr3p plays a specific, critical role in the secretion of permeases from the ER in vivo and in vitro. The presence of ER chaperones to regulate the secretion of a specific subset of proteins appears to be a wide-spread phenomenon in yeast and mammalian cells and the chaperone's function depends on the particular secreted protein. In yeast, the soluble ER chaperone BiP functions in translocation and folding of soluble proteins (Gething and Helenius, 1992). Calnexin plays a role in quality control of glycosylation before proteins are allowed to exit the ER (Hammond and Helenius, 1995). Previous studies have identified several membrane protein chaperones required for proper intracellular localization of secretory proteins, stabilizing intermediates of a growing oligomeric complex, and preventing the activity of proteins during their transit through the secretory pathway. Anp1p is an ER membrane protein involved in the correct localization of early Golgi glycosylation machinery (Chapman and Munro, 1994). Vma12p, a 25-kD integral vacuolar membrane protein, is required for vacuolar ATPase (vATPase) assembly and influences the stability of the 100-kD and 17-kD integral membrane subunits of vATPase (Hirata et al., 1993). The authors speculate that Vma12p may be present along earlier steps in the secretory route to prevent the ATPase from transporting H+ into the ER and vesicular compartments. Vma21p, another integral membrane protein responsible for vATPase assembly, is thought to cycle between the ER and the Golgi and also stabilizes the 100-kD integral membrane component of the vATPase (Hill and Stevens, 1994). In human kidney cells, the 39-kD receptor-associated protein (RAP) binds the low density lipopro-
tein receptor-related protein (LRP) in the ER to modulate the aggregation and ligand binding activity of LRP along the secretory pathway (Bu et al., 1995). In Drosophila, the interaction between the cyclophilin NinaA and the opsin Rh1 and Rh2 closely resembles the relationship between Shr3p and the permeases in yeast. In the absence of NinaA, Rh1 and Rh2, but not Rh3 and Rh4, accumulate in the ER, however, unlike Shr3p, NinaA is also found in distal compartments of the secretory pathway (Baker et al., 1994; Colley et al., 1991).

It is possible that Shr3p acts in a typical “chaperone” fashion by influencing the conformation, assembly, or activity of the associated permeases. It is unlikely that Shr3p influences translocation of the permeases, because the localization of transmembrane domains appears unperturbed in reporter-fusion constructs made with the permeases in Δshr3 cells (Gillstring, F., and P.O. Ljungdahl, unpublished data). If permeases oligomerize, Shr3p could facilitate subunit assembly in the ER or prevent premature oligomerization. Alternatively, Shr3p may act at the level of quality control by detecting a particular mature conformation achieved by the permease when it is ready to leave the ER.

A strong possibility exists that Shr3p and other transport chaperones communicate with the budding components of the Sec machinery. Shr3p could act either by contacting the permeases and the COPII components directly or by indirectly mediating permease entry into transport vesicles. A genetic approach is currently underway to investigate the interaction of Shr3p and the COPII budding components. Acting as an intermediary between the permeases and COPII, Shr3p could direct the budding components to an area of the membranes rich in permease cargo proteins, thereby participating in permease packaging without leaving the ER. Alternatively, Shr3p could mediate the interaction of the permeases with a cargo adaptor protein such as Emp24p. Shr3p could also play an active role in regulating permease secretion in response to stressful growth conditions. A cytosolic signaling molecule could modulate the interaction between Shr3p and the permeases, and thereby change the number of permeases that are directed into a budded vesicle. The analytical in vitro methods described in this paper can be used to explore many of these possibilities.

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