Lst1p and Exp1p act in parallel pathways to export the plasma membrane H^+-ATPase from the ER in *S. cerevisiae*

by

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Abstract:

Efficient transport of proteins to the correct intracellular compartment is critical for maintaining the functional integrity of the cell. Proteins destined for export from the ER are sorted from ER resident proteins and packaged into vesicles coated with the COPII protein complex. To facilitate our study of the mechanisms of protein sorting, we have selected Pma1p, the plasma membrane H+ ATPase of *S. cerevisiae*, as a model cargo protein. We found that efficient trafficking of Pma1p to the cell surface requires Lst1p, one of two yeast homologs of the COPII component Sec24p. We initially isolated *LST1* as one of a series of genes whose mutant alleles are lethal in combination with mutant alleles of the COPII gene *SEC13*. Strains deleted for *LST1* exhibit phenotypes attributable to a defect in Pma1p localization, including sensitivity to growth on acidic medium (pH 3.0) and decreased proton pumping activity. Pma1p accumulates in the ER of *lst1Δ* strains, while other cargo molecules such as invertase and CPY are transported with wildtype kinetics. Like Sec24p, Lst1p specifically binds the COPII component Sec23p. Thus, we propose that Lst1p is an alternative COPII component that selectively exports Pma1p from the ER.

We isolated *EXP1* (*ER-export of Pma1p*) as a low-copy suppressor of the lethality displayed by *lst1-1 sec13-1* double mutants. Expression of *EXP1* from a centromeric plasmid suppresses the sensitivity of *lst1Δ* strains to growth on acidic medium and restores plasma membrane localization of Pma1p. Unlike *lst1Δ* strains, *exp1Δ* strains grow normally under acidic conditions. However, *lst1Δ exp1Δ* double mutants are inviable and display severe Pma1p-trafficking defects. *EXP1* encodes a 17 KD Type III integral membrane that cofractionates with ER in wild type cells and with the Golgi in *sec21-1* mutants, indicating that Exp1p normally cycles between the Golgi and ER. Bacterially purified Exp1p fusion proteins interact with Sec23/24p in vitro. We have identified specific deletions of the cytosolic domain of *EXP1* that fail to complement the lethality of a *lst1Δexp1Δ* double mutant and to bind the Sec23/24p subcomplex. We also

demonstrated that these deletion mutants fail to cycle between the Golgi and the ER. Based on these findings, we propose that Exp1p acts to enhance the Sec24p-mediated export of Pma1p from the ER. The identification here of two parallel pathways for Pma1p export from the ER provides new insight into the molecular mechanisms driving the selective uptake of secretory cargo proteins into budding COPII vesicles.

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Chapter 1

Introduction

Overview

Eukaryotic cells are subdivided into membrane-bound compartments, or organelles, that serve distinct physiological roles within the cell. Organelles can be distinguished on the basis of lipid and protein composition, which must be preserved amidst the constant flow of intracellular membrane and protein traffic. Newly synthesized proteins must be sorted and delivered to the appropriate intracellular compartment to maintain the functional integrity of each organelle. Sorting begins at the earliest stages of protein biogenesis when hydrophobic signal sequences and transmembrane domains direct proteins for translocation into the ER lumen or incorporation into the ER membrane. Additional signals mark proteins for retention in the ER or for export to the Golgi, where yet another round of sorting separates Golgi-resident proteins from those destined for the plasma membrane, the endosome, or the vacuole. As proteins travel along the secretory pathway, they may also be subject to compartment-specific modifications such as glycosylation, phosphorlyation, or ubiquitination, that further specify their subcellular localization. The multiple sorting determinants present within each secretory protein serve as a unique mailing address that directs each protein to its final subcellular destination.

To gain a better understanding of the molecular mechanisms of protein sorting, we have focused our attention on the complex sorting events that occur early in the secretory pathway. In the ER, properly folded secretory proteins are sorted into vesicles destined for fusion with the Golgi, while steady-state levels of ER-resident proteins and misfolded secretory proteins are maintained in the ER either through direct retention or retrieval from the Golgi. Using the plasma membrane H+-ATPase Pma1p of *S. cerevisiae* as a model secretory protein, we have specifically investigated the role of the vesicle budding machinery and associated adaptor proteins in the export of membrane proteins from the ER. Herein, we report the identification of Lst1p (Appendix I) and Exp1p (Chapter 2) as novel proteins involved in the selective export of Pma1p from the ER.

COPII vesicle budding

In eukaryotic cells, secretory proteins and membrane proteins destined for the plasma membrane are incorporated into vesicles that bud from the ER and subsequently fuse with the Golgi (Kuehn et al., 1998). Over the past 20 years, genetic, biochemical, and morphological studies have led to a detailed understanding of the molecular mechanism driving ER vesicle budding. Mutants defective in protein secretion (*sec* mutants) were initially isolated by screening a mutagenized population of yeast for the internal accumulation of secretory proteins (Novick et al., 1980). Of the 23 *sec* mutants identified, 10 mutants were shown to be specifically defective in ER-to-Golgi transport (Novick et al., 1980). Subsequent analysis of these mutants by electron microscopy allowed for further categorization of the ER-to-Golgi transport mutants: *sec17, sec18,* and *sec22* were defective in vesicle consumption, as shown by the accumulation of 50nm vesicles, while mutants such as *sec12*, *sec13*, *sec16*, and *sec23* failed to accumulate vesicles (Kaiser and Schekman, 1990). Reconstitution of vesicle budding *in vitro* was used to purify and characterize the Sec proteins, confirming a role for Sec12p, Sec13p, Sec16p, and Sec23p in vesicle-mediated transport from the ER (Hicke and Schekman, 1989; Rexach and Schekman, 1991; Salama et al., 1993; Yoshihisa et al., 1993). Purification of the cytosolic Sec13p and Sec23p complexes required for vesicle formation *in vitro* led to the identification of Sec24p and Sec31p, also essential for ER-to-Golgi transport *in vivo* (Hicke et al., 1992; Salama et al., 1993). The cytosolic protein complex required for the *in vitro* formation of ER-derived vesicles is termed COPII and includes the small GTPase Sar1p and two heterodimeric subcomplexes, Sec23/24p and Sec13/31p (Barlowe et al., 1994).

The mechanism of COPII vesicle budding is conserved throughout yeast and mammalian systems. Budding is initiated when Sec12p recruits Sar1p-GDP to the ER membrane and catalyzes the exchange of GDP for GTP. Sar1-GTP remains associated with the ER membrane and attracts Sec23/24p, followed by Sec13/31p (Springer, 1999). The COPII complex polymerizes as a polygonal mesh-like network that facilitates membrane curvature and vesicle budding. The COPII coat disassembles prior to vesicle fusion with the Golgi due to Sec23p-stmulated hydrolysis of Sar1-GTP (Antonny et al., 2001; Matsuoka et al., 1998; Matsuoka et al., 2001). Sec16p interacts with Sec23/24p

and Sec31p and may act to stabilize the COPII coat and prevent premature disassembly of the coat complex (Espenshade et al., 1995; Supek et al., 2002).

Detailed structural analysis of the COPII coat components have led to the development of an atomic model of coat polymerization and vesicle budding. Rotary shadowing and high-resolution electron microscopy studies revealed that the Sec23/24p heterodimer is shaped like a bowtie, while the Sec13/31p complex forms a flexible, rod-shaped structure with globular domains at each end (Lederkremer, et al, 2001; Matsuoka, et al, 2001). As demonstrated in crosslinking studies with purified liposomes, the Sec23/24p complex lies close to the membrane, while the Sec13/31p complex occupies the outer layer of the coat (Matsuoka et al., 2001). Crystal structures of the Sec23/24p subcomplex and Sec23p bound to Sar1-GDP or Sar1-GppNHp revealed that Sec23p and Sec24p fold into similar structures, forming two halves of a bow-tie with a positively charged concave inner surface that lies parallel to the membrane bilayer (Bi et al., 2002). The Sec23/24p interface is quite small, leaving a vast surface of Sec24p available for interaction with secretory cargo. Comparison of the Sar1-GDP and Sarl-GppNHp structures suggests that GTP-activation of Sar1p induces a conformational switch that exposes an ampipathic helix for interaction with the membrane bilayer as well as several loop regions for interaction with Sec23p. The hydrolysis of Sar1p-GTP may be stimulated in part by the contact of the arginine finger of Sec23p with the nulceotide phosphate groups.

Assays monitoring the kinetics of coat assembly and disassembly *in vitro* have shown that Sec13/31p can stimulate the GAP activity of Sec23p at least ten-fold and induce coat disassembly within seconds (Antonny et al., 2001). On the basis of the intrinsic instability of the polymerized COPII, Antonny, et al. (2001) proposed a two-stage mechanism for vesicle budding. In the first stage, complexes between activated Sar1-GTP, Sec23/24p and cargo proteins would form and diffuse laterally within the ER membrane. Subsequent polymerization of the COPII coat through the bridging action of Sec13/31p would trigger rapid hydrolysis of Sar1p-GTP after incorporation of the cargo/Sec23/24p complex into the budding vesicle. Cargo proteins might then modulate the rate of Sar1-GTP hydrolysis to ensure capture into COPII vesicles (Antonny et al., 2001; Sato and Nakano, 2004).

COPI vesicle budding

Protein transport from the Golgi to the ER is mediated by a distinct cytosolic coat complex, known as COPI or coatamer. The cytosolic COPI complex is composed of 7 subunits: α (Ret1p), β (Sec26), β' (Sec27), γ (Sec21), δ (Ret2p), ε (Sec28p), and ζ (Ret3p) (Waters, et al, 1991). Genes encoding three of the COPI subunits, Sec26p, Sec27p, and Sec21p, were identified in the original screen for yeast secretion mutants, while Ret1p, Ret2p, and Ret3p were identified as mutants defective in Golgi-to-ER retrieval (Cosson et al., 1996; Duden et al., 1994; Hosobuchi et al., 1992; Kaiser and Schekman, 1990; Letourneur et al., 1994; Novick et al., 1980). COPI vesicle budding is initiated when Arf1p or Arf2p, small GTPases functionally analagous to Sar1p, is recruited to the Golgi membrane (Kreis et al., 1995). Subsequent recruitment of the cytosolic COPI complex results in coat polymerization, membrane deformation, and vesicle budding (Spang et al., 1998). Unlike the COPII coat, the COPI coat appears to bind the Golgi membrane as a previously-assembled hetero-oligomeric complex (Kreis et al., 1995).

Protein sorting at the ER

ER quality control

At the ER-to-Golgi stage of the secretory pathway, several quality control mechanisms exist to ensure that only transport-competent proteins are free to traverse the secretory pathway (Ellgaard and Helenius, 2003). Properly folded secretory proteins are sorted from misfolded proteins, incompletely assembled oligomers, and ER-resident proteins either through direct retention in the ER or signal-mediated retrieval from the Golgi. In yeast, molecular chaperones such as BiP(Kar2p) and PDI monitor and assist in the folding of nascent polypeptide chains that have been translocated into the ER (reviewed in Ellgaard and Helenius, 2003). In most cases, proteins are excluded from COPII vesicles until they have attained their native conformations, although some reports have documented the retrieval of misfolded proteins from the Golgi (Caldwell et al.,

2001; Hurtley and Helenius, 1989; Vashist and al, 2001). Proteins that fail to fold properly are targeted for ER-associated degradation or ERAD (Hampton, 2002). The molecular cues for recognition by the quality control machinery have not been well-defined, but seem to be based on structural or biophysical properties such as exposure of hydrophobic regions or formation of aggregates (Blond-Elgundi et al., 1993; Flynn et al., 1991).

Proteins involved in translocation, quality control, and ER-to-Golgi transport are localized to the ER through direct retention or at least four known mechanisms for COPI-mediated retrieval from the Golgi. Soluble ER resident proteins such as the folding chaperones BiP(Kar2p) and PDI and Type II integral membrane proteins such as Sec20p and Sed4p are retrieved from the Golgi through a signal-mediated interaction between the C-terminal HDEL motif (KDEL in mammals) and an integral membrane receptor localized to the Golgi (Hardwick et al., 1992; Lewis and Pelham, 1990; Munro and Pelham, 1987; Semenza et al., 1990; Sweet and Pelham, 1992; Townsley et al., 1994). Although HDEL is the only motif known to mediate retrieval of soluble proteins from the Golgi, recent genome-wide analysis of conserved C-terminal motifs revealed that only 11 yeast proteins contain the HDEL retrieval signal, indicating that alternative mechanisms of soluble ER-resident protein retention/retrieval must exist (Chung et al., 2003).

Membrane proteins such as Sec12p, Sed4p, Mns1p, and Sec71p contain retrieval signals within their transmembrane domains. These Type II (Sec12p, Sed4p, Mns1p) and Type III (Sec71p) integral membrane proteins require interaction with the Golgi-localized receptor Rer1p for retrieval from the Golgi (Boehm et al., 1997; Sato et al., 2001). Rer1p-independent transmembrane retrieval determinants have been identified in the SNARE protein Ufe1p, but the mechanism driving its retrieval from the ER remains unclear. Retrieval of Type I membrane proteins, such as Emp47p and members of the p24 protein family, is mediated through the direct interaction of a conserved C-terminal KKXX signal with the COPI coat (Cosson et al., 1998; Cosson and Letourneur, 1994; Fiedler et al., 1996; Letourneur et al., 1994; Schroder et al., 1995). The recycling of proteins from the Golgi also contributes to quality control in the ER by limiting the forward transport of unassembled subunits of T-cell and immunoglobulin E receptors

until assembly of heteromeric complexes masks the dilysine ER retrieval signal (Mallabiabarrena, et al, 1992; Letourner, et al, 1995).

A novel dibasic ER retention/retrieval motif has recently been identified in potassium channels and receptors, the GABAB receptor GB1, and the NMDA receptor (reviewed in Ma and Jan, 2002). In some cases, the RXR signal, like the dilysine motif, sequesters unassembled monomers in the ER until the RXR motif is masked by oligomerization (Margeta-Mitrovic, 2000; Zerangue, 1999). In KCNK potassium channels, the RXR motif appears to mediate retention in the ER through interaction with β–COP (O'Kelly et al., 2002). Interestingly, the interaction between the N-terminal RXR motif and β–COP is abolished when a 14-3-3 protein binds at a serine-phosphorylated site within the channel C-terminus, restoring cell surface expression of the otherwise ER-localized potassium channel (O'Kelly et al., 2002). A similar mechanism has been documented for the MHC Class II-associated invariant chain lip35 and the NMDA receptor (Kuwana et al., 1998; Scott, 2001). The 14-3-3 protein family is conserved in all eukaryotes and is involved in regulatory processes as diverse as apoptotic cell death and neuronal plasticity (Fu et al, 2000; Tzivion and Avruch, 2002). The involvement of these regulatory proteins in ER-to-Golgi transport raises the intriguing possibility that ER export of some cell surface proteins may be physiologically or developmentally regulated.

ER export: concentrative sorting or bulk flow?

Initially, in the absence of a defined ER-export signal researchers assumed that properly folded secretory proteins lacking ER retention motifs were exported from the ER by a default process termed bulk flow (Pfeffer and Rothman, 1987; Wieland et al., 1987). An alternative model proposes that proteins destined for anterograde transport are selectively concentrated into COPII vesicles through signal-mediated sorting (Kuehn and Schekman, 1997). Early studies measuring the rate of transport of a glycosylated acyl tri-peptide in mammalian cells provided preliminary evidence for the bulk-flow hypothesis (Wieland et al., 1987). However, later reports demonstrated that glycoprotein transport could not be accurately recapitulated using the small acyl glycopeptide (Romisch and Schekman, 1992). Subsequent analysis by immunoelectron microscopy suggested that

newly synthesized viral proteins such as VSV-G were concentrated in vesicles and at sites of ER export, but the validity of these results were later challenged on technical grounds (reviewed in Kreis, et al, 1995).

Despite these technical complications, evidence in favor of the concentrative sorting hypothesis continues to accumulate. Selective enrichment of a cargo proteins in COPII vesicles has been repeatedly observed in studies reconstituting budding reactions *in vitro* (Kuehn et al., 1998; Rowe et al., 1996; Yeung et al., 1995), and ER-exit signals have been identified in several secretory cargo proteins (Malkus et al., 2002; Otte and Barlowe, 2002; Votsmeier and Gallwitz, 2001). Furthermore, direct measurements of the selective enrichment of secretory cargo relative to bulk flow markers have recently been reported (Malkus et al., 2002). Malkus and colleagues found that the incorporation of α-factor into *in vitro*-derived COPII vesicles was almost 30-fold more efficient than packaging of the soluble neutral marker GFP-HDEL and 10-fold more efficient than packaging of a membrane-associated acyl-tripeptide (Malkus et al., 2002). Collectively, these results indicate that most secretory cargo exit the ER through signal-mediated sorting.

Bulk flow transport has been observed in dedicated secretory cells, such as pancreatic acinar cells. Quantitative immunoelectron microscopy studies showed that amylase and chymotrypsinogen were not concentrated in COPII-coated ER exit sites, but rather in tubular structures of the ER-Golgi intermediate compartment (ERGIC) (Martinez-Menarguez et al., 1999). The absence of COPI proteins in the regions of the ERGIC where amylase and chymotrypsinogen were concentrated led the authors to propose that enrichment of these abundant secretory proteins occurred through exclusion from COPI-coated retrieval vesicles. Concentration of classical secretory cargo such as SNARE proteins and the KDEL receptor could be observed at COPII coated ER exit sites, suggesting that both concentrative sorting and passive bulk flow might be operating in this cell type (Martinez-Menarguez et al., 1999). Similar studies in a variety of cell types will be needed to determine whether the bulk flow process observed in this dedicated secretory cell is common to other cell types.

Selective export of secretory cargo: the role of the COPII coat

When Sar1p locked in its activated, GTP-bound state is added to ER membranes, pre-budding complexes consisting of cargo proteins, Sar1p, and Sec23/24p can be isolated, providing a sensitive assay for cargo recognition by the COPII coat (Aridor et al., 1998; Kuehn et al., 1998). The Erv41/46p heterodimer, the general amino acid permease Gap1p, the histidine permease Hip1p, the SNARE proteins Sec22p, Sed5p, and Bet1p, the p24 family of proteins, the cargo receptors Erv14p, Erv29p, Emp47p, and the viral trimer VSV-G have all been isolated as components of pre-budding complexes (Aridor et al., 1998; Belden and Barlowe, 2001a; Kuehn et al., 1998; Miller et al., 2002; Otte and Barlowe, 2002; Powers and Barlowe, 2002). Direct interaction with Sec23/24p has also been demonstrated for the SNARE protein Bet1p, the Golgi-localized Sys1p, and ERGIC-53 (Kappeler et al., 1997; Springer and Schekman, 1998; Votsmeier and Gallwitz, 2001).

The selectivity of Sec24p was elegantly demonstrated in recent biophysical and crystallographic studies analyzing the interaction between Sec24p and SNARE peptides (Mossessova et al., 2003). Upon identifying the Sec24p-binding motifs within the SNARE proteins Sed5p and Bet1p, Mossessova and colleagues discovered that the high affinity Sec24p-binding site within Sed5p only becomes exposed and available for Sec24p interaction when Sed5p is in its fusogenic form, in complex with SNARE proteins Bos1p and Sec22p (Mossessova et al., 2003). Evidence supporting a central role for the Sec23/24p subcomplex in cargo recognition is also provided by recent biochemical and genetic analysis of cargo-selective *sec24* mutations (Miller et al., 2003). Point mutations in a highly conserved site within Sec24p, shown by x-ray crystallography to interact with peptides from Bet1p and Sys1p, produced mutant versions of Sec24p defective in the packaging of a subset of cargo molecules (Miller et al., 2003; Mossessova et al., 2003). Packaging of Emp47p, Erp1p, and the SNARE proteins Sed5p, Bos1p, Sec22p, and Bet1p into *in vitro*-derived COPII vesicles was significantly impaired in the presence of Sec24L616W, while Gap1p, Erv41/46p, and Chs3p were packaged with wild-type efficiency (Miller et al., 2003).

The existence of at least four isoforms of mammalian Sec24p and three homologs of yeast Sec24p raise the intriguing possibility that the presence of multiple Sec24p

variants enables the COPII coat to selectively export a broader range of cargo proteins (Barlowe, 2003; Higashio et al., 2000; Kurihara et al., 2000; Tang et al., 1999). Neither of the two yeast homologs of Sec24p, Lst1p and Iss1p, are essential for growth, but genetic and biochemical analysis has demonstrated a requirement for Lst1p in the efficient ER export of the essential plasma membrane H+ ATPase (Higashio et al., 2000; Kurihara et al., 2000; Roberg et al., 1999; Shimoni et al., 2000). Overexpression of *ISS1* can suppress the temperature sensitivity of *sec24-20* and *sec24-1* strains and can complement the *sec24Δ* lethality (Higashio et al., 2000; Kurihara et al., 2000). Lst1p and Iss1p can each substitute for Sec24p in *in vitro* budding assays, and Lst1p can support COPII vesicle budding from synthetic liposomes (Kurihara et al., 2000; Miller et al., 2002). However, Lst1p and Sec24p are not fully redundant, as demonstrated by the failure of Lst1p to package vesicle tethering and fusion machinery (Miller et al., 2002). Instead, Sec24p and Lst1p may have evolved to selectively export distinct, but overlapping subsets of cargo molecules. Collectively, the direct interaction of cargo with the Sec23/24p subunit of the COPII coat, the identification of cargo specific binding sites within Sec24p, and the presence of cargo selective Sec24 isoforms confirm a central role for Sec24p in cargo recognition.

The direct involvement of the COPII coat in protein sorting can be explained mechanistically by the potential catalytic relationship between cargo selection and vesicle budding. The isolation of pre-budding complexes consisting of activated Sar1p, Sec23/24p and cargo proteins on ER membranes prior to vesicle budding demonstrates that cargo recognition precedes COPII polymerization and vesicle budding (Aridor et al., 1998; Kuehn et al., 1998). When vesicle budding of a temperature sensitive VSV-G variant is monitored *in vivo*, reasearchers observed an increase in the number of ER-derived buds when cells were shifted from the restrictive to the permissive temperature, suggesting that transport-competent cargo may stimulate vesicle budding (Aridor et al., 1999). The presence of cargo is not necessary for vesicle budding *in vitro* since COPII vesicles can be formed from synthetic liposomes using only activated Sar1p, Sec23/24p, and Sec13/31p (Matsuoka et al., 1998). However, the conditions used in these assays only loosely approximate physiological conditions. Sec12p and Sec16p, although not required for COPII assembly onto synthetic liposomes, are required for vesicle budding

in vivo, and the ER membrane bilayer composition is more varied and complex than the simple phospholipid combinations used to create synthetic liposomes (Kaiser and Schekman, 1990; Zinser et al., 1993). Comparison of vesicle budding from synthetic liposomes in the presence and absence of model cargo proteins and ER resident proteins will help evaluate a potential catalytic role for cargo in the promotion of vesicle budding.

Selective export of secretory cargo: ER export motifs

The selective enrichment of secretory cargo into budding vesicles presumes the existence of ER-export signals capable of directing secretory proteins into pre-budding complexes. Two classes of ER-export motifs have been conserved in a spectrum of proteins found in yeast as well as higher eukaryotes. Di-acidic motifs, intially identified in the viral trimer VSV-G, are required for ER export of the yeast Golgi/endosome protein Sys1p and the amino acid permease Gap1p as well as the potassium channel subunit Kir2.1 (Ma and Jan, 2002; Malkus et al., 2002; Nishimura et al., 1999; Votsmeier and Gallwitz, 2001). The DxE motif is required for direct interaction of Sys1p with the Sec23/24p subcomplex, and the DID motif is required for incorporation of Gap1p into pre-budding complexes (Malkus et al., 2002; Votsmeier and Gallwitz, 2001). For VSV-G and Kir2.1, the diacidic motif forms part of a longer ER-export signal (YTDIEM and FCYENE, respectively) (Ma et al., 2001; Sevier et al., 2000).

The second class of widely conserved ER export motifs, the dihydrophobic motifs, have been identified in both subunits of the yeast heterodimer Erv41/46p, the mammalian cargo adaptor ERGIC53, and other select members of the p24 protein family, such as the yeast proteins Erp1p and the mammalian p24δ1 (Barlowe, 2003; Dominguez et al., 1998; Kappeler et al., 1997; Otte and Barlowe, 2002). Like the diacidic motifs, these dihydrophobic residues are required for recognition by the COPII coat. Interestingly, in many proteins containing diacidic or dihydrophobic ER exit motifs, such as VSV-G, Kir2.1, and Emp46p a tyrosine residue proximal to the ER exit motif also contributes to the efficiency of cargo recognition (Barlowe, 2003). Many proteins known to exit the ER contain neither of these two ER-exit motifs, suggesting that additional transport signals must also be recognized by the COPII coat.

Selective export of secretory cargo: recognition of ER-export motifs by the COPII coat

Recently, genetic, biochemical, and structural analyses revealed three spatially distinct cargo binding sites within Sec24p. Site A binds to the high affinity YNNSNPF motif of the SNARE protein Sed5p; Site B, 80 Å removed from site A, binds to the DxE motif of Sys1p and the LxxL/ME sequence of the SNARE proteins Bet1p and Sed5p, while Site C binds to the SNARE protein Sec22p (Mossessova et al., 2003). Interestingly, although Sys1p and Bet1p make similar contacts within the Site B binding pocket, significant differences were observed in the binding mode of the aspartate residue of the DxE motif of Sys1p and the 5' residues of the Bet1p ER export motif (Mossessova et al., 2003). Together, these structural data indicate that Sec24p can recognize diverse cargo molecules not only through multiple, distinct binding sites but also through multiple modes of binding at the same site.

Mutating individual residues within Sites B and C of Sec24p allowed Miller and colleagues (2003) to investigate the importance of each site in the selective uptake of a range of cargo molecules. Packaging of Bet1p and Bos1p into COPII vesicles was significantly less efficient in the presence of the Site B mutants Sec24R230A, Sec24R235A, or Sec24L616W, while packaging of α-factor remained unaffected (Miller et al., 2003). The distribution of cargo whose packaging was decreased in the presence of the Sec24L616W mutant included proteins with diacidic and dihydrophobic ER-exit motifs as well as proteins with unknown ER-export signals. Surprisingly, while the selective uptake of a Gap1-Sys1p chimera, containing the DxE motif, was significantly impaired in the presence of the Site B mutant Sec24L616W, wild-type Gap1p, containing the similar DID motif, was packaged with wild-type efficiency (Miller et al., 2003).

Site B is conserved among the Sec24p homologs, Lst1p and Iss1p, and similar mutational analysis of Lst1p confirmed a role for Site B in cargo recognition (Miller et al., 2003). Mutating residues within Site B of Lst1p abolished the packaging of Erp1p and Emp24p into COPII vesicles, while the packaging efficiency of α-factor remained unaffected. Since mutation of Site B in Sec24p similarly affected the selective uptake of Erp1p into COPII vesicles, these results indicate that some of the functional redundancy observed between Lst1p and Sec24p is achieved through a conserved binding site (Miller et al., 2003). However, despite extensive sequence conservation, Site B of Sec24p and

Lst1p are not equivalent: Lst1p fails to support the packaging of the SNARE protein Bet1p, known to bind Sec24p through conserved residues in Site B of Sec24p. Together, these findings provide critical insight into the molecular mechanism of cargo recognition and the selective capacity of Sec24p and its homologs. Additional structure/function studies will be required to address why similar ER exit motifs are not equivalently recognized by Sec24p and how subtle differences between the Sec24p homologs account for the preferential recognition of distinct subsets of cargo molecules.

Selective export of secretory cargo: the contribution of multiple sequence and structural determinants

A simple model for signal-mediated sorting predicts that ER-export signals, like the HDEL and KKXX retrieval motifs, would be composed of a conserved, linear sequence of amino acids that were both necessary and sufficient for ER-export. However, many of the ER-exit motifs described above fail to meet one or both of these criteria, suggesting that multiple, complex determinants, including secondary, tertiary, and quartenary structural signals, may contribute to the concentrative sorting of secretory proteins in the ER.

For some proteins, such as Erv41/46p, Gap1p, and VSV-G, residues surrounding the identified ER-exit motif also contribute to the efficiency of export. Both subunits of the Erv41/46p heterodimer contain ER-export motifs, and the presence of both signals is required for efficient incorporation of the heterodimer into COPII vesicles (Otte and Barlowe, 2002). Interchanging the C-terminal ER exit motifs between subunits of the heterodimer resulted in a complex that could no longer be exported from the ER (Otte and Barlowe, 2002). Similarly, the C-terminal DID motif of Gap1p cannot substitute for the C-terminal DXE motif of Sys1p in promoting the incorporation of Sys1p into COPII vesicles (Miller et al., 2003). Sequence context also contributes to the efficiency of VSV-G export from the ER. While mutation of the DxE motif of VSV-G results in a significant reduction in the rate of ER export, additional mutation of neighboring residues reduced the rate of export even further (Nishimura et al., 1999; Sevier et al., 2000). Appending this larger ER-export motif (YTDIE) to a neutral transport reporter promoted export of the resulting chimeric protein from the ER at near wild-type rates (Sevier et al.,

2000). Together, these results indicate that sequence context can significantly modulate the efficiency of cargo recognition by the ER export machinery. Interestingly, replacing the 13 C-terminal amino acids of VSV-G, including the extended ER export motif, with 13 alanine residues resulted in a protein that could be exported from the ER at near wild-type rates (Sevier et al., 2000). A linear series of alanine residues is predicted to assume an α-helical conformation , leading Sevier and colleagues (2000) to propose that secondary structure also contributes to the efficiency of VSV-G export from the ER.

Oligomerization and structural rearrangements may also alter the context in which an ER export motif is presented, thereby increasing the affinity of cargo molecules for the COPII coat (Barlowe, 2003). The high affinity ER export motif of Sed5p only becomes accessible to the COPII coat when Sed5p undergoes a conformational change due to oligomerization with Bet1p and Bos1p (Mossessova et al., 2003). Similarly, the potassium channel subunit Kir3.1 is retained in the ER unless co-expressed with subunits Kir3.2A or Kir3.4A, which contain functional ER-exit motifs (reviewed in Ma and Jan, 2002). Reports have also implicated oligomerization in the efficient ER export of ERGIC53 and its yeast homolog Emp47p (Sato and Nakano, 2003). The coordinate effects of multiple ER export determinants within the same secretory protein may enhance the fidelity of protein sorting at the ER.

Selective export of secretory cargo: the role of accessory proteins

While some cargo proteins interact directly with the COPII coat, soluble secretory proteins and some integral membrane cargo require accessory proteins for efficient uptake into COPII vesicles. These accessory proteins can be divided into three classes: (1) ER-exit chaperones, proteins that mediate cargo-COPII interaction without being incorporated into the budding vesicle (2) cargo receptors that recruit lumenal proteins into budding vesicles and (3) cargo adaptors that recruit integral membrane cargo into budding vesicles.

The most well-characterized ER-exit chaperone, Shr3p, is required for the export of amino acid permeases from the yeast ER. Shr3p is absent from pre-budding complexes and COPII vesicles but is required for the packaging of amino acid permeases into budding vesicles, although the amino acid permease Gap1p can be properly folded

and topologically inserted into the membrane in the absence of Shr3p (Gilstring et al., 1999; Kuehn et al., 1998; Kuehn et al., 1996). Shr3p interacts directly with the Sec23/24p subcomplex and is required for the incorporation of Gap1p into pre-budding complexes, suggesting that Shr3p may facilitate Gap1p packaging into COPII vesicles by mediating an interaction between Gap1p and Sec23/24p (Gilstring et al., 1999; Kuehn et al., 1998).

The selective enrichment of lumenal cargo proteins into COPII vesicles requires the action of integral membrane receptors that bind the COPII coat and cargo. ERGIC53, a Type I integral membrane protein, interacts directly with the Sec23/24p subcomplex and may function as a cargo receptor for a subset of mammalian secretory glycoproteins such as cathepsin Z and coagulation factors V and VIII (Appenzeller et al., 1999; Cunningham et al., 2003; Zhang et al., 2003). Emp24p, also a Type I membrane protein, may act similarly in the export of the yeast GPI-linked protein Gas1p. Emp24p can be isolated in pre-budding complexes, and studies have demonstrated that Emp24 can be cross-linked to Gas1p in *in vitro*-derived COPII vesicles (Kuehn et al., 1998; Muniz et al., 2000). In yeast, soluble proteins such as CPY, proteinase A, and α-factor are selectively concentrated into COPII vesicles by the cargo receptor Erv29p. In the absence of Erv29p, these soluble secretory cargo are inefficiently exported from the ER, and *in vitro*, Erv29p is required for the packaging of α-factor into COPII vesicles (Belden and Barlowe, 2001b).

Some integral membrane proteins, although topologically oriented for direct interaction with the COPII coat, also require accessory proteins for selective export from the ER. In the absence of Erv14p, Axl12p accumulates in the ER and cannot be incorporated into ER-derived vesicles *in vitro*, while lumenal cargo proteins CPY and Gas1p are exported at wild-type rates (Powers and Barlowe, 1998; Powers and Barlowe, 2002). Erv14p can be isolated in pre-budding complexes with Sec23/24p, and an interaction between Erv14p and Axl2p can be observed with Erv14p deletion mutants incapable of binding Sec23/24p (Powers and Barlowe, 2002). Together, these findings indicate that Erv14p selectively recruits Axl2p into budding vesicles. Interestingly, overexpression of *AXL2* in *erv14Δ* strains failed to complement all of the pleiotropic phenotypes displayed in *erv14Δ* strains, suggesting that Erv14p may be involved in the

transport of additional cargo proteins (Powers and Barlowe, 1998). Erv14p is homologous to the product of the *Drosophila melanogaster* gene *cornichon*, which has been proposed to contribute to the ER export of Gurken, a signalling molecule required for polarity establishment during *Drosophila* oogenesis (Powers and Barlowe, 1998; Powers and Barlowe, 2002; Queenan et al., 1999).

The role of ER-export defects in human pathophysiology

A growing number of human diseases have been attributed to defects in protein sorting at the ER (reviewed in Aridor and Hannan, 2000). Distinct from the large class of diseases caused by misfolded secretory proteins that are retained in the ER and degraded by the quality control machinery, these ER-trafficking diseases are due to defects in the ER-export machinery (Aridor and Hannan, 2000). Mutations linked to a rare heritable form of hemophilia have been mapped to the human homolog of ERGIC53 and its binding partner (Cunningham et al., 2003; Nichols et al., 1998; Zhang et al., 2003). In the absence of this heterodimeric cargo receptor, cells fail to secrete adequate levels of blood coagulation factors V and VIII (Cunningham et al., 2003; Nichols et al., 1998). Similarly, a human homolog of a yeast TRAPP complex subunit, involved in tethering and fusion of vesicles to the Golgi, has been implicated in the skeletal disease spondylo-epiphyseal dysplasia tardais, which is caused by defective collagen transport (Gedeon et al., 1999). The identification of additional cargo receptors and other non-essential components of the ER export machinery may expand our understanding of the genetic foundations of a variety of human secretory diseases.

The plasma membrane H+-ATPase: a model cargo protein

To facilitate our understanding of the cargo sorting in the ER, we have chosen the plasma membrane H+ ATP-ase (Pma1p) of *S. cereviesiae* as a model cargo protein. Pma1p accounts for more than 25% of the total protein at the plasma membrane, and recent studies using large-scale tandem affinity purification analysis estimate that 1.26 x 10^6 copies of Pma1p are present in a single logarithmically growing yeast cell (Serrano,

1991; Ghaemmaghami, et al, 2003). At the plasma membrane, Pma1p couples ATP-hydrolysis to proton transport, establishing the proton gradient used to drive the uptake of cellular nutrients by proton symporters (Serrano et al., 1986). The catalytic extrusion of protons from the cytosol also enables Pma1p to de-acidify the cytosol (Serrano et al., 1986). Pma1p is essential for cell viability, and reports suggest that at least 30% of wild-type Pma1p activity is required for cell growth (Ambesi et al., 1997). Reduced levels of Pma1p activity at the cell surface result in pleiotropic phenotypes such as resistance to the antibiotic hygromycin B, sensitivity to growth on low-pH medium, and aberant multi-budded cellular morphology (Cid et al., 1987; Perlin et al., 1988).

Pma1p is a member a large super family of P-type ATPases that include the Na+, K+ ATPases and Ca++ ATPases of mammalian cells (Lutsenko and Kaplan, 1995). These ATP-dependent ion transporters are functionally related by the formation of an aspartyl-phosphate intermediate as a part of their reaction mechanism (Pederson and Carafoli, 1987). Structural analyses have confirmed that *PMA1* and its homolog in the related yeast *Neuorspora crassa* encode 100kD polypeptides anchored in the membrane through ten hydrophobic helices (Scarborough, 2000). The four N-terminal transmembrane domains are separated from the six C-terminal transmembrane domains by a large cytoplasmic domain containing the ATP binding site and the catalytic aspartate (Ambesi et al., 2000).

At the cell surface, Pma1p exists as a >1 MD homo-oligomer associated with membrane domains enriched in sphingolipds and ergosterol known as membrane rafts (Bagnat et al., 2001; Lee et al., 2002). Inhibition of sphingolipid biosynthesis disrupts raft association and oligomerization of Pma1p, both of which are required for proper delivery of Pma1p to the cell surface (Bagnat et al., 2001; Bagnat et al., 2000; Lee et al., 2002). Although oligomerization of Pma1p appears to be dependent on raft association, monomeric and oligomeric, raft-associated Pma1p exit the ER with equal efficiency (Lee et al., 2002). Proper folding of monomeric Pma1p is controlled by the ER quality control machinery, including the PDI homolog Eps1p (Wang and Chang, 1999). Efficient export of Pma1p from the ER requires the Sec24p homolog Lst1p (see Appendix II). In the absence of Lst1p, cells become sensitive to growth on low pH medium and accumulate Pma1p in the ER (Roberg et al., 1999). Lst1p is required along with Sec24p for efficient

packaging of Pma1p into COPII vesicles (Shimoni, et al 2000; Miller, et al 2002). The presence of multiple quality control and selective export determinants within Pma1p provide an excellent opportunity to investigate the mechanisms cells have evolved to preserve the fidelity of protein sorting in the ER.

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Chapter 2

A membrane protein adaptor that enhances Sec24p-mediated export of Pma1p from the endoplasmic reticulum

Preface

This chapter represents primarily my own work. Kevin Roberg initially isolated *EXP1* as a low-copy suppressor of the *lst1-1 sec13-1* lethality, constructed the *EXP1* deletion, and performed preliminary genetic analysis on *exp1Δ* strains.

This chapter is in preparation for submission as: Michelle Crotwell Kirtley, Kevin J. Roberg, and Chris A. Kaiser. A membrane protein adaptor that enhances Sec24p-mediated export of Pma1p from the endoplasmic reticulum.

Abstract

We previously reported that the plasma membrane H+-ATP-ase (Pma1p) of *S. cerevisiae* requires the Sec24p homolog Lst1p for its efficient export from the ER. Here we report identification of a novel gene, named *EXP1* (*ER export of Pma1p*) that under conditions of overexpression can restore full localization of Pma1p to the plasma membrane to *lst1Δ* strains. Strains carrying a deletion of *EXP1* grow normally, but *lst1Δ exp1Δ* double mutants are inviable and display severe Pma1p-trafficking defects. These genetic interactions indicate that Exp1p acts in parallel to Lst1p in a pathway for export of Pma1p from the ER.

EXP1 encodes a 17 kD Type III integral membrane protein which fractionates with ER in wild type cells and with the Golgi in *sec21-1* mutants, indicating that Exp1p normally cycles between the Golgi and ER. Recombinant Exp1p binds to the Sec23/24p COPII subcomplex. We have identified specific deletions of the cytosolic domain of *EXP1* that fail to complement the lethality of a *lst1Δexp1Δ* double mutant and to bind Sec23/24p. We propose that these deletions define a region of Exp1p that binds to Sec23/24p and enhances the recruitment of this coat complex to pre-budding structures that contain Pma1p. Thus Exp1p appears to be a cargo adaptor required for incorporation of Pma1p into Sec23/24p containing vesicles, but not for Sec23/Lst1p containing vesicles.

Introduction

In eukaryotic cells, proteins destined for the plasma membrane are captured into vesicles that bud from the ER and subsequently fuse with the Golgi (Kuehn et al., 1998). Vesicle budding is mediated by the COPII protein complex, composed of two heterodimeric subcomplexes, Sec23/24p and Sec13/31p, a cytosolic GTPase Sar1p, and a peripheral membrane protein Sec16p (Barlowe et al., 1994). *In vitro* budding assays and analysis of secretion mutants in *S. cerevisiae* have revealed the following mechanism for COPII vesicle budding common to yeast and mammalian cells (Barlowe, 2002): The process is initiated when the ER membrane protein Sec12p generates Sar1p•GTP by catalyzing exchange of the guanine nucleotide bound Sar1p. Sar1p•GTP binds to the ER membrane and recruits first the Sec23/24p protein complex followed by the Sec13/31p protein complex. Polymerization of COPII proteins onto the membrane has been shown to be sufficient to drive formation of COPII coated membrane vesicles (Matsuoka et al., 1998; Matsuoka et al., 2001). Subsequent Sec23p-stimulated hydrolysis of Sar1•GTP results in coat disassembly prior to vesicle fusion with the Golgi (Antonny et al., 2001). Sec16p interacts with Sec23/24p and Sec31p and may act to stabilize the COPII coat and prevent premature disassembly of the coat complex (Espenshade et al., 1995; Supek et al., 2002).

Secretory cargo proteins are concentrated into COPII vesicles at the earliest stages of vesicle formation (Belden and Barlowe, 2001; Kuehn et al., 1998; Matsuoka et al., 1998). The Golgi/endosome protein Sys1p and the SNARE proteins Sed5p, Sec22p, and Bet1p are recruited to COPII vesicle buds through specific binding of their cytosolic domains to membrane-associated Sec23/24p subcomplexes (Miller et al., 2002; Miller et al., 2003; Mossessova et al., 2003; Peng et al., 1999; Springer and Schekman, 1998; Votsmeier and Gallwitz, 2001). Recent mutational studies of *SEC24* combined with structural analysis of Sec23/24p bound to peptides derived from cargo proteins show that Sec24p can bind to multiple ER-export motifs through at least three spatially distinct sites (Miller et al., 2003; Mossessova et al., 2003).

Although integral membrane proteins may interact directly with the COPII proteins, recent evidence suggests that some integral membrane proteins also require the

action of accessory proteins (also called cargo adaptors) in order to be incorporated into the budding vesicle. One type of cargo adaptor is an ER-resident protein that promotes the export of a specific protein or related set of proteins from the ER without being packaged into COPII vesicles itself. This type of cargo adaptor is often called an ER-exit chaperone, the best characterized example being the *S. cerevisiae* ER membrane protein Shr3p, which is required for the export of amino acid permeases from the ER (Gilstring et al., 1999; Gilstring et al., 1996; Kuehn et al., 1998; Kuehn et al., 1996; Ljungdahl et al., 1992). Shr3p is not essential for proper membrane insertion or folding of Gap1p and interacts directly with the COPII coat, suggesting that Shr3p may facilitate Gap1p packaging into COPII vesicles by mediating an interaction between Gap1p and Sec23/24p (Gilstring et al., 1999). A second type of cargo adaptor actively recruits integral membrane proteins into COPII vesicles. For example, Erv14p, a membrane protein that is packaged into COPII vesicles, is required for the export of the polytopic bud-site selection protein Axl2p from the ER. (Powers and Barlowe, 1998; Powers and Barlowe, 2002). Erv14p interacts specifically with Axl2p and the COPII coat and escorts Axl2p into the budding vesicle (Powers and Barlowe, 2002).

To facilitate our study of the mechanisms of protein sorting, we have selected Pma1p, the plasma membrane H+ ATPase of *S. cerevisiae*, as a model cargo protein. Pma1p is an essential integral membrane protein that uses energy derived from ATP hydrolysis to pump protons across the plasma membrane, a process that establishes the proton gradient necessary for the uptake of nutrients from the extracellular medium (Serrano et al., 1986). Pma1p also regulates the pH of the cytosol (Serrano et al., 1986). Cells partially defective for Pma1p grow poorly on acidic medium, presumably because of protons generated by metabolism are not efficiently expelled from the cytosol. Previously, we reported that efficient trafficking of Pma1p from the ER requires the Sec24p homolog Lst1p (Roberg et al., 1999). Strains with a deletion of *LST1* are viable, but display a phenotype attributable to a partial defect in Pma1p localization to the plasma membrane, including sensitivity to low pH and decreased proton pumping activity. *In vitro* analysis confirmed that both Sec24p and Lst1p are required to efficiently package Pma1p into COPII vesicles (Shimoni et al., 2000). During the initial isolation of the *LST1* gene, we identified a gene that is not linked to *LST1*, but can

efficiently suppress some of the growth defects of a *lst1-1* mutant (Roberg et al., 1999). Here we show that this novel suppressor gene, named *EXP1* (*ER-export of Pma1p*), participates in the selective export of Pma1p from the ER and may act to enhance Pma1p entry into COPII vesicles through specific interaction with the Sec23/24p subcomplex.

Materials and Methods

Media, Strains, and Plasmids

S. cerevisiae strains used in this study are listed in Table I. Cells were grown in either rich medium (YPD) or supplemented minimal medium (SMM), as described in Kaiser, et al, 1994. For growth under acidic conditions, YPD or SMM media was adjusted to pH 3.0 with HCl. Plasmids used in this study are described in Table I. pKR10 carries a 3.4 kb KpnI – SpeI fragment of Chromosome IV containing the *EXP1* gene (*YDL121c*) in the centromere plasmid pRS316 (*URA3*). pMC5 contains the same KpnI – SpeI fragment on the multi-copy vector pRS426 (*URA3*). pMC18, which contains *EXP1* expressed from the *GAL10* promoter was constructed by ligating a BamHI – SacII PCR fragment, amplified from pKR10, into pCD43 (*URA3*).

The *exp1::URA3* disruption was created as follows. A KpnI – SacI fragment from p21-3, the original *EXP1*-containing clone described in Roberg et *al*, 1999, was ligated into the integrating vector pRS306 (*URA3*) to create pKR7. A 5 kb BglII – BamHI fragment of pSE1076 containing a URA3 disruption cassette was inserted into pKR7. The resulting plasmid was digested with XhoI and MscI to delete most of the *EXP1* ORF and was religated to form pKR11. An EcoRI – BamHI fragment containing the disrupted *EXP1* allele was transformed into a diploid (KRY8 x KRY9) and sporulated. URA+ spores were tested for the integration of the *EXP1::URA3* disruption by Southern blotting with a probe homologous to the EcoRI – BamHI fragment used to generate the mutant allele.

Immunoblotting

Proteins were solubilized in Sample Buffer (2% SDS, 10 % glycerol, 80 mM Tris HCL (pH 6.8, 0.1 mg/ml bromophenol blue, 100 mM DTT) and resolved by SDS-PAGE according to standard protocols (Harlow and Lane, 1988). For transfer of Gas1p to nitrocellulose membranes, methanol was omitted from the transfer buffer. Rabbit anti-Pma1p (a gift of A. Chang, Albert Einstein College of Medicine) was used at a dilution of 1:500; an alternate Pma1p antibody (gift of S. Losko and R. Kölling, Heinrich-Heine-Universitat, Düsseldorf, Germany) was used at 1:2500 dilution. Rabbit anti-Gas1p (a gift

of H. Riezman, University of Basel) was used at a dilution of 1:10,000, and mouse monoclonal anti-Dpm1p (Molecular Probes) was used at a dilution of 1:250. Rabbit anti-Sec23p (a gift of L. Hicke, Northwestern University) and anti-Sec24p (gift of T. Yoshihisa, Nagoya University, Japan) were used at 1:500. HA-Sec31p was detected using mouse monoclonal 12CA5 anti-HA at 1:1,000. Rabbit anti-PDI (a gift from T. Stevens, University of Oregon, Eugene, Oregon) was used at a dilution of 1:1000. Rabbit antiserum to Exp1p was raised against GST-Exp1p produced in *E. coli.* Anti-Exp1p was used at a dilution of 1:250.

Microscopy

Digital photomicrographs were taken with Hamamatsu Digital camera attached to a Nikon Eclipse E800 microscope and visualized with OpenLab software (Improvision). Cell morphology was examined using DIC microscopy of cells treated with 3.7% formaldehdyde. Aberrant, multi-budded morphology was quantitated by counting the percentage of cells with three or more buds. 600 cells were examined for each strain; percentages shown represent the average of two separate experiments.

Localization of Pma1p by immunofluorescence was performed as described in Roberg, et, al, 1999. Anti-Pma1p was affinity-purified as described in Roberg, et al, 1999 and used at a 1:5 dilution. Alexa-conjugated anti-rabbit IgG (Molecular Probes) was used at a dilution of 1:200. Mounting medium was supplemented with 4',6-diamidino-2-phenylindole (DAPI).

Cell fractionation

Subcellular distribution of Exp1p was examined as follows. Wild-type cells (CKY 8) were grown to exponential phase in YPD, converted to spheroplasts, and then gently lysed by agitation with glass beads in lysis buffer (50 mM Tris-HCl [pH7.5], 1mM EDTA, 0.2 M sorbitol) containing a protease inhibitor cocktail (1mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 2 μg/ml aprotinin). The cell extract was sequentially centrifuged at 500 x *g* for 10 minutes 13,000 x *g* for 10 minutes, and 100,000 x *g* for 30 minutes. Proteins solubilized in sample buffer were separated with SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antiserum to Exp1p, PGK, or Dpm1p.

Release of Exp1p from the particulate/membrane bound fraction was performed as described in Kaiser, et al 2002 with the following modifications. CKY833 cells growing logarithmically in YPD were harvested and converted to spheroplasts. Spheroplasts were lysed on ice by douncing in lysis buffer supplemented with 1mM PMSF, 0.5μg/ml leupeptin, 0.7 μg/ml pepstatin, and 2μg/ml aprotinin. After a clearing spin (5 minutes at 500 x *g*), cell extracts were treated with either 0.1M Na_2CO_3, pH 11.5; 1 M NaCl; 2.5 M urea; 1% Triton; or buffer alone and incubated on ice for 1 hour. Treated extracts were centrifuged at 100,000 x *g* for 75 minutes to separate membrane bound and soluble proteins. Samples were solubilized in sample buffer and analyzed by immunoblotting with antiserum to Exp1p or Dpm1p.

Sucrose density gradient fractionation was performed as described in Kaiser, et al, 2002. The presence of Pma1p, Gas11p, Exp1p, and Dpm1p in each fraction was detected by SDS-PAGE followed by immunoblotting. The relative amounts of each protein in cell fractions were determined using an Image Station 440CF (Kodak Digital Sciences) and 1D Image Analysis Software (Kodak Digital Sciences). The presence of Golgi GDPase activity was detected by standard methods as described in (Kaiser et al., 2002).

Protease accessibility of Exp1p

Wild type (CKY8) cells were converted to spheroplasts with lyticase in 1.2 M sorbitol, 10 mM Tris-HCl [pH 7.4] at 30°C after treatment for 15 minutes with 0.5% β-mercaptoethanol. Washed spheroplasts were lysed in lysis buffer (250mM sorbitol, 150 mM potassium acetate, 20mM HEPES [pH 6.8], 1 mM magnesium acetate) by Dounce homogenization on ice, and cell debris was removed by centrifugation at 500 x g for 5 minutes. Microsomal membranes were collected by centrifugation of cleared cell extracts for 15 minutes at 13, 000 x g and treated with 0.75 mg/ml Trypsin in either the presence or absence of 1% Triton X-100. Proteolysis was terminated with 25 mM Pefabloc (Roche) after 5, 15, and 30 minutes. Proteins were separated with SDS-PAGE and analyzed by immunoblotting with antiserum to Exp1p or Pdi1p.

Site-Directed Mutagenesis

Deletion mutations in *EXP1* were created by PCR amplification of the flanking regions on the 5' and 3' sides of deletion in separate reactions. For generation of the

deletion mutation in pMC3 (*GST-EXP1)*, an EcoRI site was introduced into the 3' end of the 5' fragment and the 5' end of the 3' fragment. The two PCR fragments were ligated into pGEX5-3. A similar strategy was used to generate the same deletion mutation in the yeast vector pRS316 (*EXP1 CEN)* and pRS426 (*EXP1 2μ*) with the following modifications. The two PCR fragments were ligated together using a BamHI site, and the resulting deletion was introduced into pKR10 or pMC5 either by subcloning an EcoRI/MscI fragment from the deletion construct into pKR10 or pMC5, or by homologous recombination with linearized pKR10 or pMC5. Presence of the deletion was confirmed by sequence analysis. Point mutations were generated using the Stratagene *Quik-Change* PCR mutagenesis kit.

Binding of Exp1p to Sec23/24p

EXP1 was fused to the glutathione-S-transferase gene under the control of the *lac* promoter in the *E. coli* expression vector pGEX2-T (Pharmacia). Purification was performed as described in Smith, et al, 1993 with the following modifications. Wild type and mutant Exp1p fusion proteins were purified from CKB175 (*ompT- lon::ΔTn10,* a gift of T. Opperman, M.I.T.) grown at 24°C to minimize proteolysis. Fusion proteins were induced with 1 mM IPTG for 5-6 hours at 24°C then suspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.5% Triton X-100, 1 mM PMSF), and frozen at –80°C. Bacterial cells in lysis buffer were incubated with 5 mg/ml lysozyme and then lysed by sonication. Cell debris was collected by centrifugation at 10,000 x g for 15 minutes, and the supernatant was added to glutathione-sepharose beads (Pharmacia). After incubation at 4°C for one hour, beads were washed six times in GST-wash buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% Triton X-100, 0.02% NaN_3) and suspended in GST-wash buffer. The quantity of protein bound to glutathione-sepharose beads was estimated with the Bradford protein assay (BioRad) and the purity of the protein preparation was assessed by polyacrylamide gel electrophoresis followed by Coomassie staining.

For protein-binding assays, yeast cytosolic extracts were prepared as described in (Gimeno et al., 1996). Exponentially growing cells from *pep4Δ* or *SEC31-HA* strains were harvested and lysed in immunoprecipitation buffer (20 mM HEPES [pH 6.8], 80

mM potassium acetate, 0.2 M NaCal, 0.02% Triton X-100, 5 mM DTT, 1 mM EDTA) containing 1mM PMSF, 0.5μg/ml leupeptin, 0.7 μg/ml pepstatin, and 2μg/ml aprotinin. Cell debris and intracellular membranes were removed by centrifugation at 13, 000 x *g* and 100, 000 x *g*. Total protein concentration was determined by Bradford assay. Purified Exp1p fusion proteins bound to sepharose beads were added to the cell extracts and incubated for 1 hour at 4°C. The beads were washed four times in immunoprecipitation buffer (200 mM NaCl, 80 mM potassium acetate, 20 mM HEPES [pH 7.5], 5 mM magnesium acetate, 0.02% Triton X-100, 5 mM dithiothreitol, 1 mM EDTA) and suspended in 30 μl of Sample Buffer for analysis by SDS-PAGE. COPII proteins were detected by immunoblotting with 12CA5 antibody or antiserum to Sec23p or Sec24p.

Phenotypic analysis of lst1Δ exp1Δ strains

lst1Δ and *exp1Δ* strains expressing *EXP1* from the *GAL10* promoter were mated, and diploids were sporulated and dissected on galactose-containing medium. For growth analysis and microscopy, P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains grown in galactose medium were transferred to raffinose containing medium for 8 hours prior to the addition of glucose.

Sequence comparison and alignment

Sequence comparisons to related yeast species were provided by the *Saccharomyces Genome Database* based on the work of Kellis, et al, 2003, and Cliften, et al, 2003. Preliminary sequence data for the *Dictyostelium discoideum* genome was obtained from the Welcome Trust Sanger Institute www.sanger.ac.uk, Baylor College of Medicine http://dictyogenome.bcm.tmc.edu/, The University of Cologne http://www.uni-koeln.de and the Department of Genome Analysis in Jena of the Institute of Molecular Biotechnology http://genome.imb-jena.de. Most of this data was generated at the aforementioned institutes with a small part of it produced at the Institut Pasteur http://www.pasteur.fr.

Results

Isolation of EXP1

LST1 (lethal with *sec thirteen*) was identified in a genetic screen for mutations that were lethal in combination with the COPII mutation *sec13-1* (Roberg et al., 1999). We isolated the *LST1* gene by screening for clones that could restore viability to *sec13-1 lst1-1* strains. We also identified a second complementing locus that appeared to be unrelated to *LST1*. The genomic region from clone p21-31, a representative of this second complementing locus, was inserted into an integrating vector (pRS306), linearized, and integrated at its chromosomal locus into CKY 564, which contains wild-type copies of *SEC13* and *LST1*. The resulting integrant was crossed to a *sec13-1 lst1-1* strain, and the diploids were sporulated and dissected. Tetrad analysis showed that the locus containing the inserted plasmid was not linked to *LST1* and, therefore, encoded an extragenic suppressor of the lethality displayed by *sec13-1 lst1-1* strains (data not shown). We sequenced the p21-31 clone and isolated the suppressing locus as YDL121c, which we have named *EXP1* (*ER-export of Pma1p*).

Overexpression of EXP1 restores plasma membrane localization of Pma1p in a lst1Δ mutant

In the absence of *LST1*, a decreased delivery of Pma1p to the cell surface causes yeast cells to grow poorly on acidic medium (pH$\leq$4.0), particularly at high temperatures (Roberg et al., 1999). Expression of *EXP1* from a centromeric plasmid restored the ability of *lst1Δ* strains to grow at pH $\leq$ 4.0 at either 30°C or 37°C (Fig. 1A). To test whether overexpression of *EXP1* can also suppress the Pma1p-trafficking defect of *lst1Δ* strains, localization of Pma1p was examined by immunofluorescence microscopy. In *lst1Δ* cells, Pma1p staining is observed in the ER at the nuclear periphery as well as at the cell surface (Roberg et al., 1999). As shown in Figure 1B, overexpression of *EXP1*, either from a centromeric plasmid or a 2μ plasmid, completely restored cell surface localization of Pma1p in a *lst1Δ* genetic background.

As an independent test for Pma1p localization, extracts from *lst1Δ* strains ectopically expressing *EXP1* were fractionated on a linear sucrose density gradient. In wild type cells all of the Pma1p is located in plasma membrane fractions, whereas in *lst1Δ* cells, about half of the total Pma1p fractionates with the ER marker Dpm1p, indicative of a delay in export from the ER. When *lst1Δ* strains expressed additional copies of *EXP1*, all the detectable Pma1p cofractionated with the cell surface marker Gas1p (Fig. 1C). On the basis of these localization experiments, we conclude that *EXP1* overexpression suppresses the low-pH sensitivity of *lst1Δ* strains by bypassing the requirement for Lst1p in the export of Pma1p from the ER.

lst1Δ exp1Δ double mutants exhibit a severe defect in Pma1p trafficking

Unlike *lst1Δ* strains, *exp1Δ* strains grew normally on acidic medium (pH < 4.0) and efficiently exported Pma1p to the cell surface as demonstrated in immunofluorescence and cell fractionation experiments (data not shown). However, *lst1Δ exp1Δ* double mutants were inviable at all temperatures (Fig. 2A). To determine the severity of the Pma1p-trafficking defect in the *lst1Δ exp1Δ* double mutant strain, we conditionally expressed *EXP1* under the control of the *GAL10* promoter in *lst1Δ exp1Δ* cells and examined the localization of Pma1p in cells depleted of Exp1p. Within six hours after addition of glucose, which represses expression of P_{GAL10}-*EXP1*, Exp1p could no longer be detected in P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains as determined by immunoblotting with anti-Exp1p (data not shown). After growth in glucose for 14 hours, a P_{GAL10}-*EXP1 lst1Δ exp1Δ* strain exhibited a significant reduction in growth rate indicating that the cells were no longer growing exponentially (Fig. 2B). The time lag between full depletion of Exp1p from P_{GAL10}-*EXP1 lst1Δ exp1Δ* cells and the cessation of exponential growth corresponded to about 8 hours which is the estimated half-life of Pma1p (Bagnat et al., 2001). These observations suggest that upon depletion of Exp1p, newly synthesized Pma1p cannot be delivered to the cell surface, and cells cease dividing as pre-existing Pma1p at the cell surface is endocytosed and degraded.

A striking phenotype of cells defective in Pma1p activity at the cell surface is the presence of cells exhibiting an aberrant, multi-budded morphology (Cid et al., 1987).. Examination of P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains by phase contrast microscopy after

incubation in glucose minimal medium for 14 hours revealed that more than 30% of these cells displayed a multi-budded morphology compared to only 3% of *lst1Δ* cells and 1% of wild-type cells grown under the same conditions (Table I). Immunofluorescence microscopy was used to examine the location of Pma1p in *lst1Δ exp1Δ* mutants. After 14 hours of growth in glucose, Pma1p staining could not be detected in the nascent buds of P_{GAL10}-*EXP1 lst1Δ exp1Δ* cells, and in mother cells Pma1p was only located in ER at the nuclear periphery, not the plasma membrane (Fig. 2C). (Because the buds of the multi-budded cells have completed cytokinesis, the dramatic multi-budded morphology of these strains was not preserved after digestion of the cell wall in preparation for microscopy(Roberg et al., 1999).) Finally, to directly demonstrate that Pma1p is found exclusively in the ER in *lst1Δexp1Δ* cells, we attempted to use subcellular fractionation to localize a pulse-labeled pool of Pma1p synthesized after P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains were depleted of Exp1p. This approach was not feasible because cells depleted of Exp1p exhibited a severe defect in protein synthesis and did not incorporate radiolabeled amino acids into protein. Together, these results indicate that *lst1Δexp1Δ* double mutants are completely defective for Pma1p trafficking out of the ER. The residual slow growth of P_{GAL10}-*EXP1 lst1Δ exp1Δ* cells depleted of Exp1p can be explained by the presence of Pma1p that had been delivered to the plasma membrane prior to depletion of Exp1p.

Exp1p is a type III integral membrane protein

EXP1 encodes a 149 amino acid protein with a sequence of hydrophobic amino acids at the N-terminus, predicted to serve either as a transmembrane domain or a signal sequence, and a highly charged C-terminus (Kyte and Doolittle, 1982) (Fig. 3A). As an experimental test of the intracellular distribution of Exp1p, lysates from wild-type cells were converted to spheroplasts and subjected to a series of centrifugation steps designed to separate soluble proteins from those that are membrane bound. At both the 13,000 x *g* and 100,000 x *g* centrifugation steps, Exp1p was located in the pellet, along with the membrane bound marker Dpm1p, indicating that Exp1p is tightly associated with cellular membranes (Fig. 3B).

To confirm that Exp1p is an integral membrane protein, we subjected spheroplasted cell lysates to chemical treatment prior to centrifugation at 100,000 x *g*.

Incubation of cell lysates with high salt (0.5M NaCl), high pH (0.2 M Na_2CO_3 [pH 11.5]), or 2 M urea, treatments known to perturb the interaction of peripheral membrane proteins with cell membranes, had no effect on the association of Exp1p with the 100,000 x *g* insoluble fraction. Only treatment with 1% Triton was sufficient to release Exp1p into the soluble fraction (Fig. 3C).

The TopPred algorithm for membrane topology prediction, which chiefly relies on the "positive-inside rule" for charged residues flanking the transmembrane domain, indicated that Exp1p is a type III membrane protein whose C-terminus localized to the cytosol (Claros and von Heijne, 1994; von Heijne, 1992). The orientation of Exp1p was experimentally determined by testing the accessibility of Exp1p in microsomes to digestion with Trypsin. Exp1p in intact microsomal membranes was efficiently degraded by Trypsin, suggesting that the C-terminus of Exp1p is accessible to protease and, therefore, is oriented to the cytosolic face of the membrane (Fig. 4). As a control for the integrity of the microsomes under these conditions, we also examined the protease accessibility of the lumenal ER chaperone PDI and found that PDI remained stable and not accessible to protease unless the membranes had first been solubilized by detergent (Fig. 4). Together, these results demonstrate that Exp1p is a type III integral membrane protein, anchored to the membrane through its N-terminus with the C-terminus facing the cytosol.

Localization of Exp1p

To determine the subcellular localization of Exp1p, we subjected cell extracts to fractionation on sucrose density gradients. In the presence of magnesium, ribosomes remain associated with ER membranes, causing the ER membranes to sediment with the dense fractions near the bottom of the gradient. In the presence of EDTA, the ribosomes are released from the ER, causing the ER membranes to equilibrate at a relatively low density (Sanderson and Meyer, 1991). More than 75% of Exp1p co-fractionated with the ER marker Dpm1p in the presence and in the absence of EDTA, indicating that most of Exp1p is localized to the ER (Fig. 4). These results are consistent with previous proteomic studies in which epitope-tagged Exp1p was localized to the ER by immunofluorescence microscopy (Huh et al., 2003; Kumar et al., 2002; Rout et al.,

2000). Exp1p also partially co-fractionated with Golgi marker proteins, suggesting that at steady state a small portion of Exp1p may localize to the Golgi.

Colocalization of a small proportion of Exp1p with Golgi marker proteins suggested that Slo1p might be actively cycling between the Golgi and the ER. As an experimental test of the recycling activity of Exp1p, we compared the subcellular localization of Exp1p in wild-type strains and in strains expressing a mutant version of the COPI component Sec21p. In cells expressing *sec21-1* at the semi-permissive temperature (30°C), retrograde transport from the Golgi to the ER is blocked while anterograde transport from the ER to the Golgi remains unaffected (Gaynor and Emr, 1997). Under these conditions, proteins that are normally recycled back from the Golgi to the ER accumulate in the Golgi, in later secretory compartments such as the vacuole, or are secreted from the cell. Cell extracts from wild type and *sec21-1* strains grown at a semi-permissive temperature (30°C) were fractionated on a linear sucrose gradient under conditions that separate Golgi and vacuolar membranes from the ER. In *sec21-1* cells, approximately 40% of Exp1p co-fractionated with the Golgi marker GDPase, whereas in wild type cells, most Exp1p co-fractionated with ER markers (Fig. 6). Thus, steady-state localization of Exp1p in the ER requires Exp1p to be recycled from the Golgi in a COPI-dependent manner.

Exp1p mutagenesis

Exp1p is homologous to predicted proteins in several related yeast species and in the slime mold *Dictyostelium discoideum* (Cliften et al., 2003; Kellis et al., 2003). We deleted the regions of Exp1p most highly conserved among the Exp1p orthologs, creating a series of five short deletions together spanning most of the cytosolic domain of Exp1p (Fig. 7A). The functionality of each deletion mutation was tested by its ability to support growth of a P_{GAL10}-*EXP1 lst1Δ exp1Δ* strain on glucose medium. Deletion alleles *exp1-Δ 86-102*, *exp1-Δ121-149* and *exp1-Δ107-119* were unable to suppress the *lst1Δexp1Δ* lethality despite stable expression of their protein products, while *exp1-Δ49-58* and *exp1-Δ61-68* suppressed *lst1Δexp1Δ* lethality as well as wild-type *EXP1* (Fig. 7B; data not shown). We also examined the effect of each of the mutant *EXP1* alleles on the prevalence of the multi-budded rosette phenotype in P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains

depleted of Exp1p. Approximately 20-25% of cells expressing the deletion alleles *exp1-Δ 86-102*, *exp1-Δ121-149*, and *exp1-Δ107-119* exhibited the multi-budded morphology compared to only 2-5% of cells expressing *exp1-Δ49-58* , *exp1-Δ61-68* , or wild-type *EXP1*, indicating that the C-terminal domain of Exp1p is required for its function in the export of Pma1p from the ER (Table II).

We next sought to determine whether the deletion mutations interfered with the intracellular trafficking of Exp1p by comparing the intracellular distribution of each of the deletion mutants in wild type and *sec21-1* strains. Exp1p-Δ49-58 and Exp1p-Δ61-68 displayed a significant shift in distribution in the presence of the *sec21-1*allele and were indistinguishable from wild type Exp1p (Fig. 8). In contrast, Exp1p-Δ107-119 and Exp1p-Δ121-149 fractionated with ER marker proteins even when Golgi-to-ER traffic was blocked with the *sec21-1* allele, suggesting that these deletion mutants were unable to exit the ER. Interestingly, Exp1p-Δ86-102 fractionated with the Golgi marker protein GDPase in both *sec21-1* and wild type strains, indicating that this deletion mutation may be partially defective for retrieval from the Golgi to ER. Together, these observations establish a strong correlation between role of Exp1p in Pma1p trafficking and the ability of Exp1p to exit the ER.

Exp1p binds to Sec23/24p in vitro

Genetic, biochemical, and structural studies have established a central role for the Sec24p and its homologs in cargo recognition and selection into budding vesicles. To assay interactions between Exp1p and components of the COPII coat, a version of Exp1p was constructed in which the glutathione S-transferase protein (GST) replaced the transmembrane domain of Exp1p. We expressed GST-Exp1p in *E. coli* and purified the hybrid protein using glutathione-sepharose beads. Glutathione beads with bound GST-Exp1p were then incubated with cytosolic extracts from yeast, and proteins that remained bound to the GST-Exp1p beads after stringent washing were analyzed by SDS-PAGE and detected by immunoblotting with antibodies to various components of the secretory pathway (Fig. 9A). Sec23p and Sec24p bound to beads carrying GST-Exp1p but not to beads carrying GST alone. This interaction appeared to be specific for the Sec23/24p

subcomplex since the GST-Exp1p beads failed to associate with the COPII subunit Sec31p.

If Exp1p exits the ER through direct interaction with Sec24p, then the deletion mutants that fail to exit the ER should be unable to interact with Sec23/24p in the *in vitro* binding assay. To test this prediction, we replaced the transmembrane domain of each deletion mutant with GST, purified the mutant protein from *E. coli* using glutathione-sepharose beads, and added the purified beads to wild-type yeast cell extracts. GST-Exp1p-Δ107-119 and GST-Exp1p-Δ121-149 failed to interact significantly with Sec23/24p in this assay, while GST-Exp1p-Δ49-58, GST-Exp1p-Δ61-68, and GST-Exp1p-Δ86-102 bound as much Sec23/24p as GST-Exp1p (Fig. 9B). These tests of the mutants for binding to Sec23/24p verified the significance of this interaction for function *in vivo*; the two deletion alleles that did not complement a *lst1*Δ *exp1*Δ double mutant also exhibited a defect in binding to the Sec23/24p subcomplex.

Since Exp1p does not require Lst1p for its function, we hypothesized that Exp1p may interact selectively with the Sec23/24p subcomplex but not with Sec23/Lst1p. Contrary to this expectation, Lst1p-HA interacted with GST-Exp1p as efficiently as Sec24p, and similar amounts of Lst1p-HA were precipitated by each of the Exp1p deletion mutants (data not shown). We also tested for interaction between the COPI subunit Sec26p and GST-Exp1p. While a significant interaction with Sec26p-HA was observed in this assay, we were unable to establish the specificity of the interaction since all deletion mutants tested interacted with Sec26p-HA equally well (data not shown).

To identify the residues of Exp1p specifically required for interaction with the Sec23/24p subcomplex, we performed alanine-scanning mutagenesis of residues 107-119, a subdomain rich in acidic residues containing the DID motif known to be essential for the interaction of Gap1p with Sec24p (Malkus et al., 2002). Each point mutant was tested for its ability to complement the *lst1Δ exp1Δ* lethality and interact with Sec23/24p in the *in vitro* binding assay. Surprisingly, none of the point mutations were defective; each of the thirteen point mutants fully suppressed the *lst1Δ exp1Δ* lethality and bound to Sec23/24p as efficiently as wild-type Exp1p (data not shown).

Genetic interactions between exp1Δ and secretion mutants

To further investigate the relationship between Exp1p and the COPII coat proteins, we combined *exp1Δ* and thermosensitive alleles of known secretory genes. Mutations in COPII component genes are synthetically lethal with other COPII mutant alleles, but not with mutant alleles of genes involved in vesicle tethering or fusion to the Golgi, or COPI mediated retrieval. We found that *exp1Δ* exacerbates the temperature sensitivity of the COPII alleles *sec13-1*, *sec23-1*, and *sec24-2*, but not *sec12-4*, *sec16-1*, *sec31-1*, or the COPI allele *sec21-1* (Table III). Powers and Barlowe (2002) observed a similar pattern of genetic interaction between the cargo adaptor allele *erv14Δ* and COPII mutant alleles. We also tested the effect of *exp1Δ* on the sensitivity of COPII mutant strains to growth on low pH media at the permissive temperature. Interestingly, *sec23-1*and *sec24-2* strains grew poorly on acidic medium (pH 2.8-3.0) at the permissive temperature, and the presence of the *exp1Δ* allele further inhibited growth of these COPII mutant strains (Table III). Similarly, *sec13-1*, which grew normally on acidic medium, grew poorly on acidic medium when combined with *exp1Δ*. An *exp1Δ* mutation did not cause increased sensitivity to acidic medium of the COPII alleles *sec12-4*, *sec16-1*, *sec31-1*, or the COPI allele *sec21-1*. The selective genetic interaction between the *exp1Δ* allele and mutant alleles of the early acting COPII components suggests that Exp1p may act in the earliest stages of vesicle budding.

Discussion

We previously reported the identification of Lst1p as a Sec24p homolog involved in the selective export of Pma1p from the ER (Roberg et al., 1999). During the cloning of *LST1*, we isolated an unlinked suppressor of *lst1-1,* which we have named *EXP1* (*ER export of Pma1p*). Expression of *EXP1*, even from a centromeric plasmid, completely suppressed the Pma1p trafficking defect of *lst1Δ* strains, whereas deletion of *EXP1* in a *lst1Δ* genetic background strain was lethal. As diagrammed in Figure 10, the simplest interpretation of these genetic interactions is that Exp1p and Lst1p function in distinct parallel pathways to export Pma1p from the ER.

A role for Exp1p in the export of Pma1p from the ER is further supported by examination of double mutants, which show that the Pma1p trafficking defect is more severe in *lst1Δ exp1Δ* double mutants than in *lst1Δ* single mutants. When compared to *lst1Δ* strains, P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains that were depleted of Exp1p exhibited a ten-fold increase in the occurrence of the multibudded morphology characteristic of strains defective in Pma1p activity (Table I). Moreover, P_{GAL10}-*EXP1 lst1Δ exp1Δ* cell cultures that had been depleted of Exp1p displayed a significant decrease in growth rate, closely resembling the behavior of P_{GAL}-*PMA1 pma1Δ* strains depleted of Pma1p (Fig. 2B) (Cid et al., 1987). Finally, localization of Pma1p in P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains depleted for Exp1p revealed an accumulation of Pma1p in the ER and an absence of Pma1p in new cell buds (Fig. 2C). The severity of the Pma1p-trafficking defect exhibited by *lst1Δ exp1Δ* strains can be explained if the combined absence of *LST1* and *EXP1* causes a complete or nearly complete block in Pma1p-trafficking out of the ER. Under these conditions, mother cells that contain a stable pool of Pma1p in their plasma membranes continue to divide, while the daughter cells are inviable due to a failure to deliver newly-synthesized Pma1p to the emerging bud.

How does Exp1p facilitate the export of Pma1p from the ER? Evaluation of the *EXP1* gene product has led to the following findings, which provide evidence that Exp1p acts as a cargo adaptor to promote the selective export of Pma1p from the ER: (i) Exp1p is a type III integral membrane protein located in the ER membrane, (ii) Exp1p is transported from the ER to the Golgi and recycles to the ER in COPI-dependent vesicles,

and (iii) Exp1p binds to COPII subunit Sec24p and a region of Exp1p required for its function and trafficking out of the ER is also necessary for Sec24p binding. Thus, we propose that Exp1p facilitates Pma1p export from the ER by enhancing Sec24p-mediated capture of Pma1p into COPII vesicles, probably through a direct interaction between the cytosolic portion of Exp1p and the Sec24p subunit of the COPII vesicle. Exp1p appears to accompany Pma1p into the budding vesicle in a manner similar to the cargo adpator Erv14p rather than the ER-exit chaperone Shr3p, which promotes interaction between cargo and the COPII coat while remaining in the ER (Gilstring et al., 1999; Gilstring et al., 1996; Powers and Barlowe, 1998; Powers and Barlowe, 2002).

Like Erv14p, Exp1p is a small, integral membrane protein with a significant portion of the protein localized to the cytoplasm, available for interaction with the COPII coat (Powers and Barlowe, 2002). Erv14p was initially identified as a component of purified COPII vesicles; since Exp1p is a 17 kD protein that is exported from the ER, Exp1p may correspond to either Erv16p or Erv17p, unidentified proteins purified from COPII vesicles along with Erv14p (Belden and Barlowe, 1996; Powers and Barlowe, 1998). Interestingly, both *erv14Δ* and *exp1Δ* exacerbate the thermosensitivity of *sec13-1*, *sec23-1*, and *sec24-2* but not *sec16-1, sec12-4,* or *sec31-1* (Table II) (Powers and Barlowe, 2002). Similarly, combining *exp1Δ* with the *sec13-1, sec23-1*, and *sec24-2* alleles enhances the sensitivity of these strains to growth on acidic medium, indicative of a Pma1p trafficking defect (Table II). Such genetic interactions specific for alleles of COPII coat components that act earliest in vesicle budding may prove to be a hallmark of cargo adaptor mutants.

Cargo adaptors such as Erv14p are thought to actively recruit cargo molecules into COPII vesicles by specifically binding cargo proteins and the COPII coat (Powers and Barlowe, 2002). We have attempted through coimmunoprecipitation and crosslinking experiments to test for the analogous physical interaction between Exp1p and Pma1p, but we have not been able to detect a robust interaction between these two proteins. Pma1p is 1000 times more abundant that Exp1p, and the interaction between a cargo adaptor and its cargo is predicted to be weak and transient (Ghaemmaghami, 2003; Powers and Barlowe, 2002). Thus, we may have been unable to detect an interaction between Exp1p and Pma1p because the fraction of Pma1p bound to Exp1p may be small

relative to the total amount of Pma1p in the cell or because of inefficient crosslinking of the two proteins. An alternative possibility is that Exp1p does not bind directly to Pma1p but induces a conformational change in Sec24p that increases the affinity of Sec24p for Pma1p.

On the basis of recent structural and genetic analyses, Miller, et al. (2003) have proposed that differences between Sec24p and Lst1p in their affinity for cargo proteins may partially account for the preferential packaging of distinct subsets of cargo proteins by the Sec24p homologs. One of the three cargo-binding sites recently identified within Sec24p (Site B) is highly conserved in Lst1p, and mutations of the corresponding residues in Lst1p interfere with the packaging of some, but not all, of the proteins known to interact with Sec24p at Site B. Although the residues involved in the possible Pma1p – Lst1p or Pma1p – Sec24p interactions have not yet been identified, Miller et al. (2003) have hypothesized that Pma1p may interact with Lst1p and Sec24p at a common, conserved site, but that Pma1p has a greater affinity for the binding site on Lst1p than for the corresponding site on Sec24p. The work we have presented here suggests that the binding of Exp1p to Sec24p may enhance the Sec24p-mediated export of Pma1p from the ER by modulating the affinity of Pma1p for a coat complex containing Sec23/24p.

Previously we showed that overexpression of *SEC24* can suppress the Pma1p-trafficking defect of *lst1Δ* strains (Roberg et al., 1999), but in similar tests we have found that overexpression of *SEC24* will not suppress lethality of *lst1Δ exp1Δ* (data not shown). These *in vivo* experiments imply that Sec24p-mediated incorporation of Pma1p into COPII vesicles in the absence of Lst1p has an absolute requirement for Exp1p. In parallel *in vitro* assays, Shimoni et al (2000) found that optimum packaging of Pma1p into COPII vesicles occurred when both Sec23/Lst1p and Sec23/24p were present. In the absence of Lst1p, a 20-fold higher concentration of Sec24p was required to package Pma1p into COPII vesicles as efficiently as when both Lst1p and Sec24p were included in the assay (Shimoni et al., 2000). All such budding reactions have been performed using ER membranes that should have contained Exp1p; repeating these experiments using membranes derived from an *exp1Δ* strain to would provide a direct test for the requirements for Exp1p in Pma1p packaging *in vitro*.

In an attempt to identify more precisely the residues of Exp1p that contact Sec24p, we performed alanine scanning mutagenesis across the region of Exp1p that had been found by deletion analysis to be required for Sec24p binding. However, none of the 13 Exp1p alanine substitution mutants that we tested either interfered with the ability of Exp1p to function *in vivo* (as determined by complementation of a *lst1Δ exp1Δ* double mutant) or to bind to Sec24p *in vitro*. These alanine substitutions included mutations in a Asp-Ile-Asp motif (residues 114-116) that is similar to a cytosolic diacidic motif shown to be necessary for the export of Gap1p (Malkus et al., 2002). Our failure to find point mutations defective for Exp1p interaction with Sec24p suggests redundancy in this binding interaction. The highly charged cytosolic domain of Exp1p contains a number of potential diacidic motifs, and alternative sites on Exp1p may be able to substitute for one another in binding to Sec24p. Alternatively, two or more discrete sequences of Exp1p may bind to distinct sites within Sec24p, any one of which may be sufficient for a productive binding interaction. A precedent for this type of complex binding interaction is the binding of two separate sequences within the cytosolic tail of Sed5p to two spatially distinct binding sites on Sec24p (Miller et al., 2003; Mossessova et al., 2003).

Recent evidence indicates that multiple sequence motifs and structural determinants may contribute to the specificity of cargo recognition by the COPII coat. Otte and Barlowe demonstrated that the ER export signals contained within the C-termini of Erv41/46p required specific contextual information for proper recognition, since the respective ER exit motifs could not be interchanged even between the subunits of the heterodimer (Otte and Barlowe, 2002). Similarly, Sevier and colleagues (2000) showed that replacing residues 17-29 of the VSV-G cytoplasmic tail with 13 alanine residues restored wild-type export of VSV-G, suggesting that secondary structure may be a key determinant in the recognition of the VSV-G tail by the ER export machinery (Sevier et al., 2000).

Further evidence for the importance of higher order structural or contextual information in cargo recognition is provided by studies comparing the interaction between cargo molecules and Sec24p. Erv41p and Erp1p both require the dihydrophobic motif , Ile-Leu, for efficient incorporation into COPII vesicles, but the packaging efficiency of Erp1p is drastically reduced in the presence of the Sec24R616W mutant,

while Erv41p is packaged as efficiently as with wild-type Sec24p (Miller et al., 2003; Otte and Barlowe, 2002). Similar results were obtained when comparing the diacidic motifs Asp-Xaa-Glu of Sys1p and Asp-Ile-Asp of Gap1p (Malkus et al., 2002; Votsmeier and Gallwitz, 2001). Sys1p is not efficiently packaged into COPII vesicles in the presence of the Sec24R616W mutant, while the packaging of Gap1p remains unaffected (Miller et al., 2003). Conformational state can also alter the efficiency with which cytosolic motifs are recognized. The strongest of two COPII binding motifs found in the t-SNARE Sed5p becomes available for interaction with Sec24p only when Sed5p undergoes a conformational change due to interaction with Bos1p and Sec22p (Mossessova et al., 2003). Together these results suggest that multiple, complex determinants contribute to the fidelity of protein sorting in the ER and may explain why we have not yet been able to identify individual residues required for Exp1p export from the ER. Additional crystallographic analysis of Exp1p along with a variety of cargo molecules in complex with Sec23/24p will be necessary to elucidate the precise nature of these structural, contextual signals.

Like *LST1*, *EXP1* is non-essential, and the two proteins appear to act in redundant, parallel pathways to export Pma1p from the ER. Since Pma1p is one of the most abundant secretory proteins, and since its function is essential for fundamental physiological processes such as nutrient uptake and de-acidification of the cytosol, redundant pathways for Pma1p export from the ER may have evolved to ensure adequate delivery of Pma1p to the cell surface (McCusker et al., 1987; Serrano et al., 1986; Vallejo and Serrano, 1989). Alternatively, Sec23/Lst1p and Sec23/Sec24p/Exp1p may each be involved in transporting distinct but overlapping sets of cargo molecules including Pma1p. Since Exp1p is not necessary for the Lst1p-mediated export of Pma1p, we were surprised to detect an interaction between Lst1p and Exp1p (data not shown). However, because each of the Exp1p deletion mutants we constructed bound to Lst1p-HA in our *in vitro* assay, the detected Lst1p—Exp1p interaction may not be specific. An alternative possibility is that Exp1p interacts with Lst1p to modulate its affinity for cargo proteins other than Pma1p.

Why do yeast cells require two proteins, Exp1p and Sec24p, to perform a function that Sec24p could, theoretically, perform on its own? One possibility is that Exp1p is

part of a family of adaptor proteins, and various combinations of the Sec24p homologs with these adaptor proteins broaden the range of cargo proteins selectively exported by the COPII coat. Such a combinatorial model for cargo selection has already been suggested as an explanation for the inclusion of different Sec24p homologs, each of which preferentially packages a distinct subset of cargo proteins, into the COPII coat (Barlowe, 2003). The presence of Exp1p-like adaptor proteins may further increase the selective capacity of the COPII coat, allowing each Sec24p homolog to interact with a broader range of cargo molecules.

Pma1p is a member of a large super-family of ion pumps with close homologs in most fungal and plant species, and more distant homologs in metazoans, including humans (Lutsenko, 1995; Pederson, 1987). In higher eukaryotes, the expression and localization of P-type ATPases are often developmentally or physiologically regulated. Recently, O'Kelly and colleagues demonstrated that 14-3-3 proteins, implicated in the regulation of cellular processes as diverse as apoptotic cell death and neuronal plasticity, can specifically bind to the C-terminal tail of potassium channel subunits, mediating the regulated export of the channel subunits from the ER (O'Kelly et al., 2002). Interestingly, the *Dictyostelium discoideum* homolog of Pma1p, PAT2 is expressed only under acidic conditions (Coukell et al., 1997). The *Dictyostelium* genome is also predicted to encode a very close homolog of Exp1p (>75% identity), raising the possibility that this Exp1p homolog may be involved in the regulated expression and transport of PAT2.

The identification of Exp1p expands our understanding of the complex mechanisms used by the cell to ensure efficient delivery of Pma1p to its proper cellular location and elaborates on the multiple layers of partial redundancy that work in parallel to preserve the fidelity of cargo selection. Screening for mutations synthetically lethal with *lst1Δ*, *exp1Δ*, or *iss1Δ* may uncover alternative cargo adaptors and yield further insight into the range of cargo selectivity among the various members of the cargo recognition pathway.

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Figure 1. Overexpression of *EXP1* suppresses the Pma1p-related phenotypes of *lst1Δ* strains. (A) *lst1Δ* strains with the indicated plasmids were grown for three days on SMM, pH 3.0 or SMM, pH 4.5. (B) *lst1Δ* strains containing the indicated plasmids were fixed and labeled for immunofluorescence with affinity-purified Pma1p antibody. Alexa®488-conjugated goat anti-rabbit secondary antibody. Nuclear DNA was stained with DAPI, and cell bodies were visualized DIC microscopy. (C) *lst1Δ* strains containing the indicated plasmids were grown in SMM at 30°C, and cell extracts were fractionated on 20-60% sucrose density gradients containing 10 mM EDTA. Fractions were collected from the top of the gradient. Relative levels of Pma1p, Gas1p, and Dpm1p in each fraction were quantitated by immunoblotting and densitometry. GDPase activity in each fraction was assayed enzymatically.

Figure 1

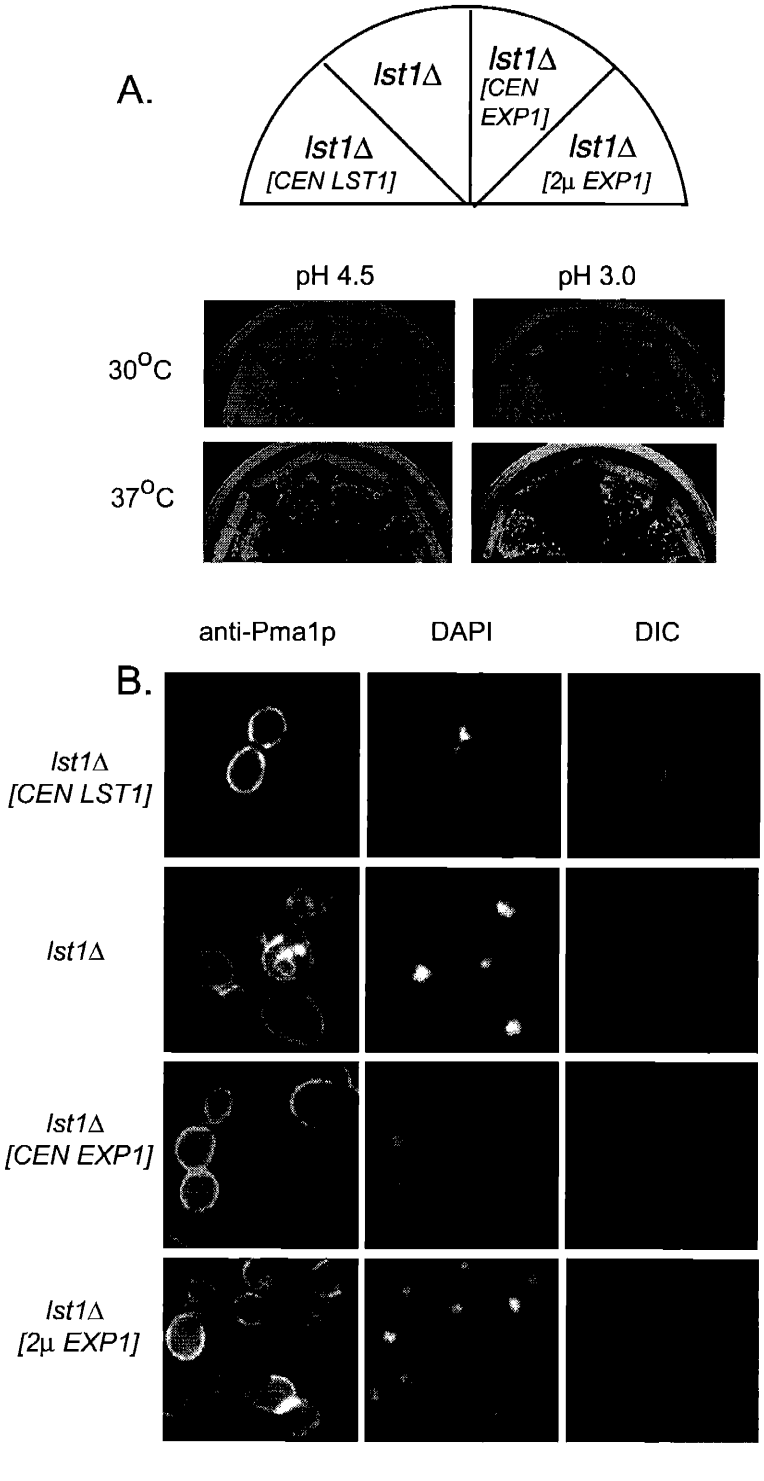
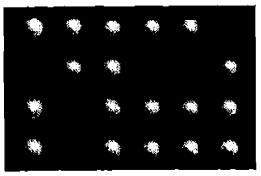

A.
lst1Δ
lst1Δ [CEN EXP1]
lst1Δ [CEN LST1]
lst1Δ [2μ EXP1]
pH 4.5
pH 3.0
30°C
37°C
B.
anti-Pma1p
DAPI
DIC
lst1Δ [CEN LST1]
lst1Δ
lst1Δ [CEN EXP1]
lst1Δ [2μ EXP1]
C.
Pma1p
lst1Δ [CEN LST1]
lst1Δ
lst1Δ [CEN EXP1]
lst1Δ [2μ EXP1]
% of total
marker proteins
Gas1p
Dpm1p
GDPase
fraction number

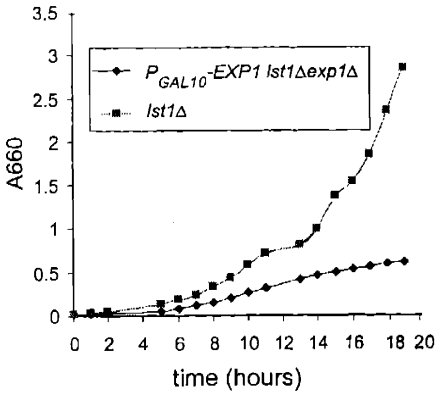
Figure 2. *lst1Δ exp1Δ* strains are inviable and accumulate Pma1p in the ER. (A) A *lst1::LEU2 leu2-3,112 ura3-52* strain was crossed to a *exp1::URA3 leu2-3,112 ura3-52* strain. Tetrads were dissected and spores analyzed for the presence of the *URA3* or *LEU2* makers. According to the segregation pattern of these markers, each of the inviable spores shown should contain both *lst1::LEU2* and *exp1::URA3* disruptions. (B) After growth to exponential phase in SMM with 2% raffinose, cells were transferred to SMM containing 2% raffinose and 2% glucose (t = 0). The optical density at 660 nm of each culture was measured at regular time intervals. (C) After growth to exponential phase in SMM (2% raffinose), cells were transferred to SMM media containing 2% raffinose and 2% glucose (t = 0). After 14 hours of growth in glucose, cells were fixed with formaldehyde and visualized with differential interference phase contrast microscopy. Representative examples of cells displaying the multi-budded rosette phenotype are shown from three separate fields. (D) *lst1Δ*, *exp1Δ*, and P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains were grown and fixed as described above. Cells were stained with affinity-purified Pma1p antibody followed by Alexa® 488-conjugated anti-rabbit secondary antibody. Nuclear DNA was visualized with DAPI staining, and cell bodies were visualized by DIC microscopy.

Figure 2

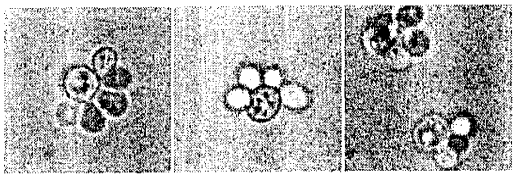
A. *lst1Δ x exp1Δ*



B. Growth in 2% glucose



C.



P_{GAL10}-EXP1 lst1Δ exp1Δ
after 14 hours in glucose

D.

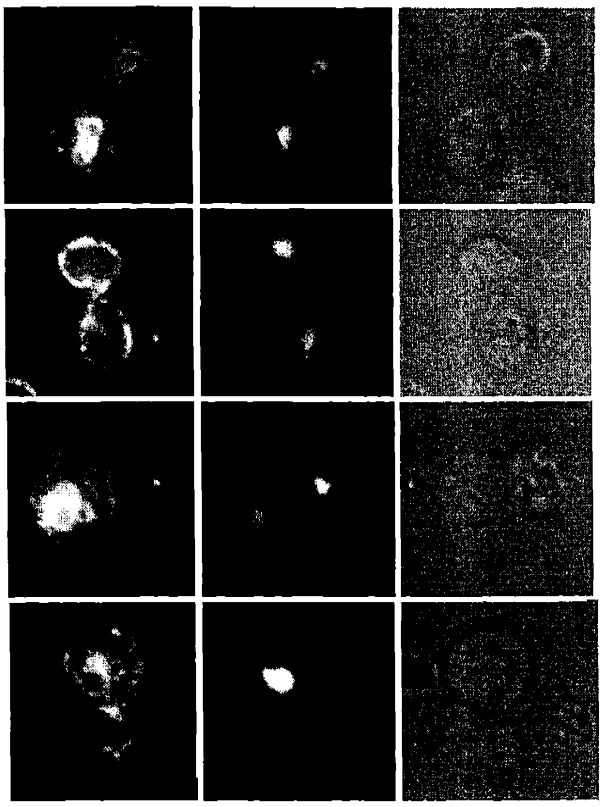


Figure 3. Exp1p is an integral membrane protein. (A) Predicted amino acid sequence of Exp1p (translation of *YDL121c*). (B) Wild type cells (CKY 8) were grown to exponential phase in YPD. Cleared cell extracts were sequentially centrifuged at 500 x *g* for 10 minutes, 13,000 x *g* for 10 minutes, and 100,000 x *g* for 30 minutes. An aliquot of the cleared cell extract was removed prior to centrifugation (TOTAL). Proteins in the soluble and particulate fractions after each spin were analyzed by immunoblotting with antisera raised against Exp1p, PGK, or Dpm1p. (C) Wild type cell extracts were treated for 1 hour at 4°C with 1% Triton X-100, 0.1M sodium carbonate (pH 11.5), 2.5 M urea, 0.5 M NaCl, or buffer alone. Treated samples were then separated into soluble (S) or particulate (P) fractions by centrifugation at 100,000 x *g* for one hour. Samples were solubilized in sample buffer and analyzed by immunoblotting with anti-Exp1p or anti-Dpm1p.

Figure 3

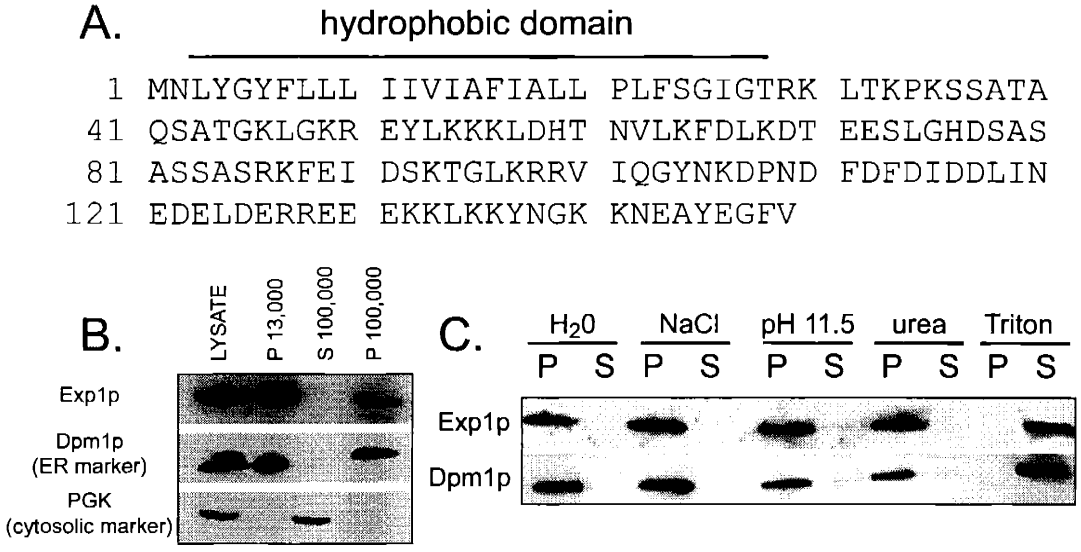

A.
hydrophobic domain
1 MNLYGYFLLL IIVIAFIALL PLFSGIGTRK LTKPKSSATA
41 QSATGKLGKR EYLKKKLDHT NVLKFDLKDT EESLGHDSAS
81 ASSASRKFEI DSKTGLKRRV IQGYNKDPND FDFDIDDLIN
121 EDELDERREE EKKLKKYNGK KNEAYEGFV
B.
LYSATE
P 13,000
S 100,000
P 100,000
Exp1p
Dpm1p
(ER marker)
PGK
(cytosolic marker)
C.
H20
NaCl
pH 11.5
urea
Triton
P S P S P S P S P S
Exp1p
Dpm1p

Figure 4. Protease accessibility of Exp1p. Microsomes generated from wild type cell extracts were digested with 0.75 mg/ml Trypsin in the presence or absence of 1% Triton X-100. Proteins were analyzed by immunoblotting with antiserum to Exp1p or Pdi1p, an ER lumenal protein.

Figure 4

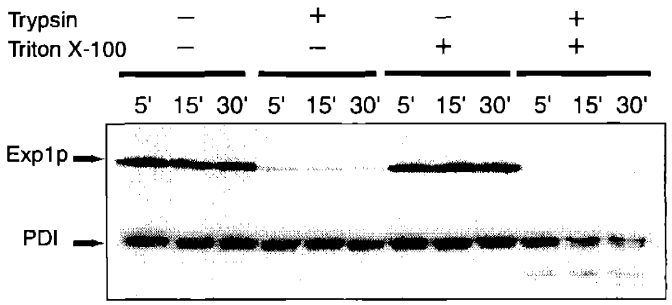


Figure 5. Exp1p is located in the ER. Wild type cell extracts were fractionated on 20-60% sucrose density gradients containing either 10 mM EDTA or 2 mM $MgCl_2$. Fractions were collected from the top of the gradient. Relative levels of Pma1p, Exp1p, and Dpm1p in each fraction were quantitated by immunoblotting and densitometry. GDPase activity in each fraction was assayed enzymatically.

Figure 5

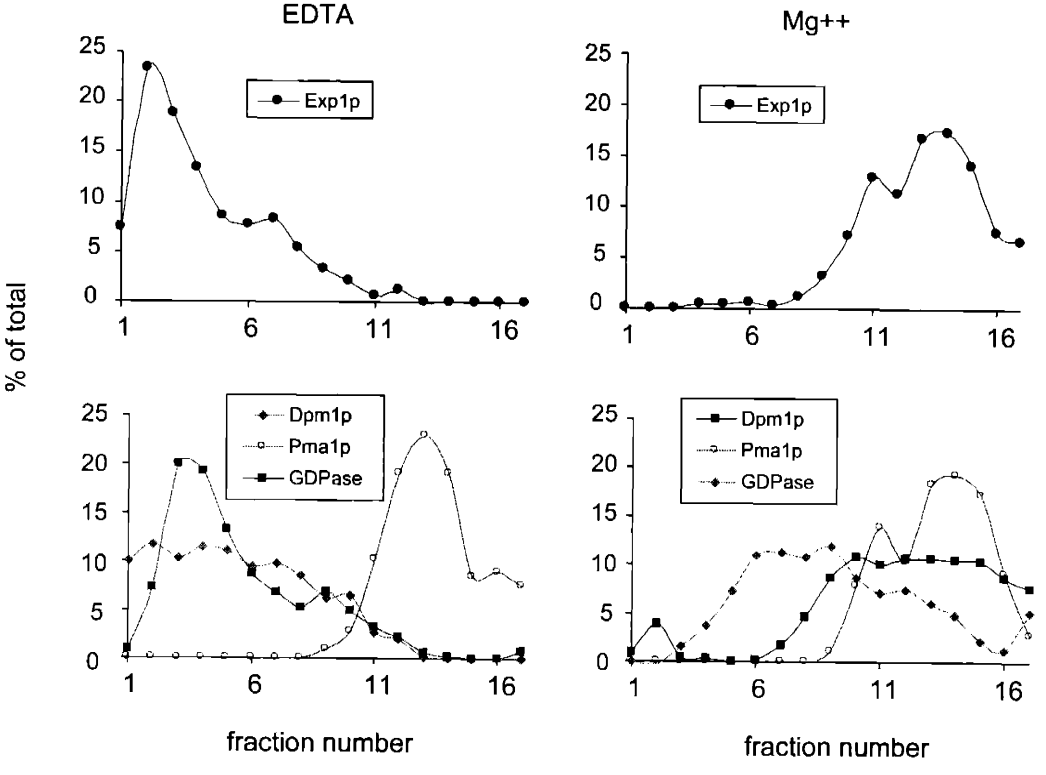

EDTA
Mg++
Exp1p
Exp1p
Dpm1p
Pma1p
GDPase
Dpm1p
Pma1p
GDPase
% of total
fraction number
fraction number
0
5
10
15
20
25
1
6
11
16

Figure 6. Exp1p cycles from the Golgi to ER. *pep4Δ* or *pep4Δ sec21-1* strains were grown in YPD at the semipermissive temperature of 30°C, and cell extracts were fractionated on 20-60% linear sucrose density gradients containing 2mM $MgCl_2$. Relative levels of Pma1p, Exp1p, and Dpm1p in each fraction were quantitated by immunoblotting and densitometry. GDPase activity in each fraction was assayed enzymatically. Note the shift in intracellular distribution of Exp1p in the presence of the *sec21-1* allele.

Figure 6

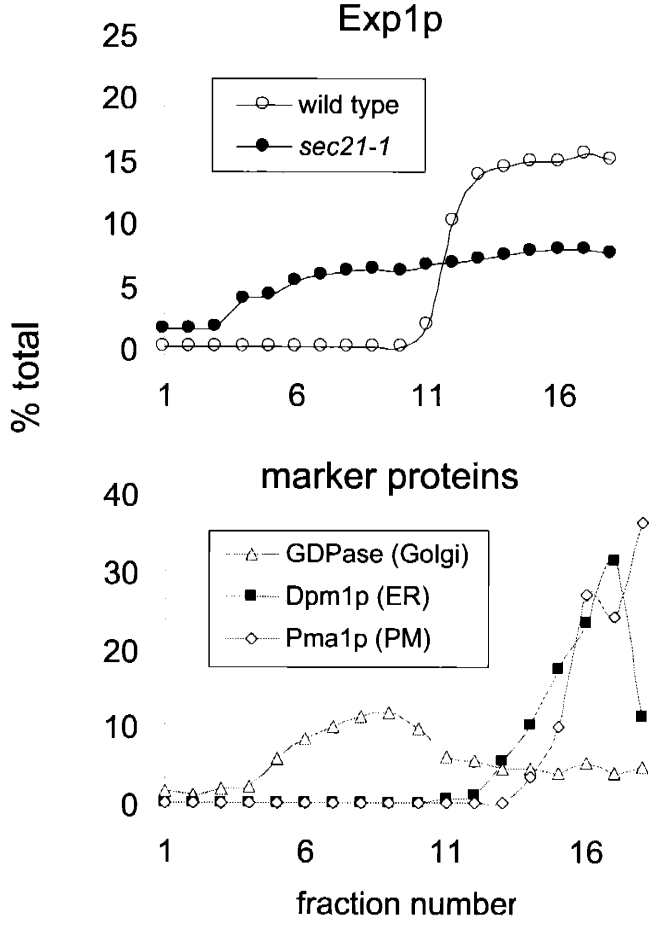
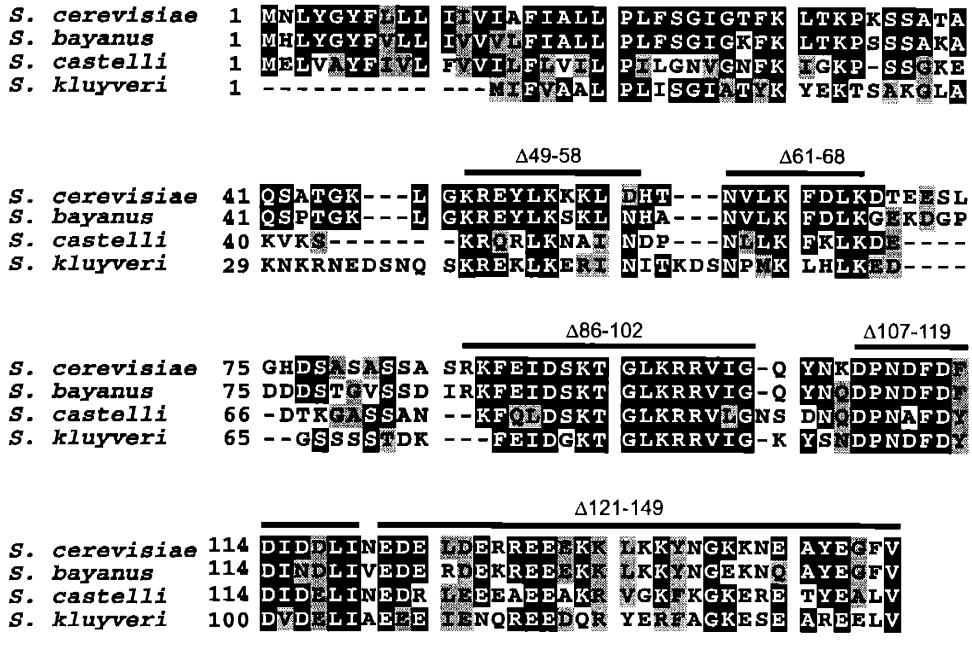

Exp1p
25
20
15
10
5
0
wild type
sec21-1
% total
1
6
11
16
marker proteins
40
30
20
10
0
GDPase (Golgi)
Dpm1p (ER)
Pma1p (PM)
1
6
11
16
fraction number

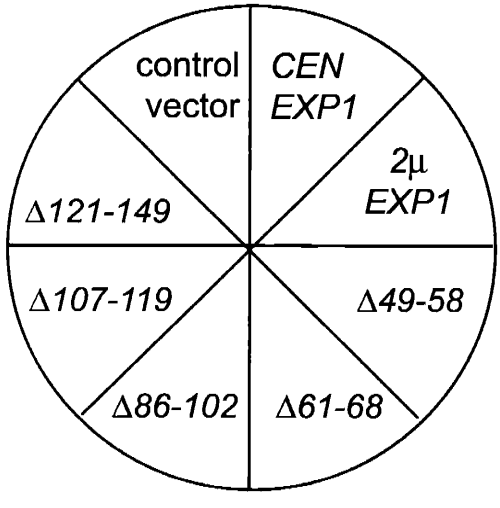
Figure 7. Exp1p mutagenesis. **A.** ClustalW alignment of *EXP1* and orthologs from related yeasts and the slime mold *Dictyostelium discoideum*. A series of five deletions, spanning the conserved cytosolic domain of Exp1p were generated by PCR. **B.** The resulting deletions were tested for their ability to complement lethality of *exp1Δ lst1Δ* double mutant strains. The indicated mutant alleles were expressed ectopically on 2μ plasmids in P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains and assayed for growth on glucose media at 30°C.

Figure 7

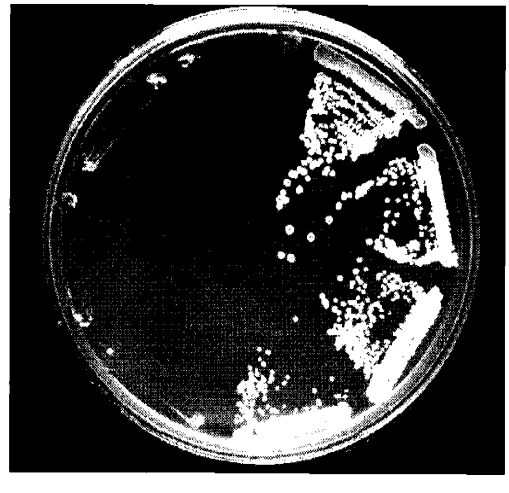
A.



B.



P_{GAL10}-*EXP1 lst1Δ exp1Δ*



2% glucose

Figure 8. Localization of Exp1p deletion mutants. *exp1Δ pep4Δ* and *exp1Δ pep4Δ sec21-1* strains expressing the indicated deletion alleles were grown in SMM at 30°C (a semi-permissive temperature for *sec21-1* strains). Cell lysates were fractionated on 20-60% sucrose density gradients containing 2mM $MgCl_2$ to maximize the separation between ER and Golgi-containing fractions. Fractions were collected from the top of the gradient, and the relative levels of Exp1p, Dpm1p, and Pma1p were quantitated by immunoblotting and densitometry. Dpm1p and Pma1p localization are represented by the bars labeled ER and plasma membrane, respectively. GDPase activity was assayed enzymatically and is represented by the bar labeled Golgi.

Figure 8

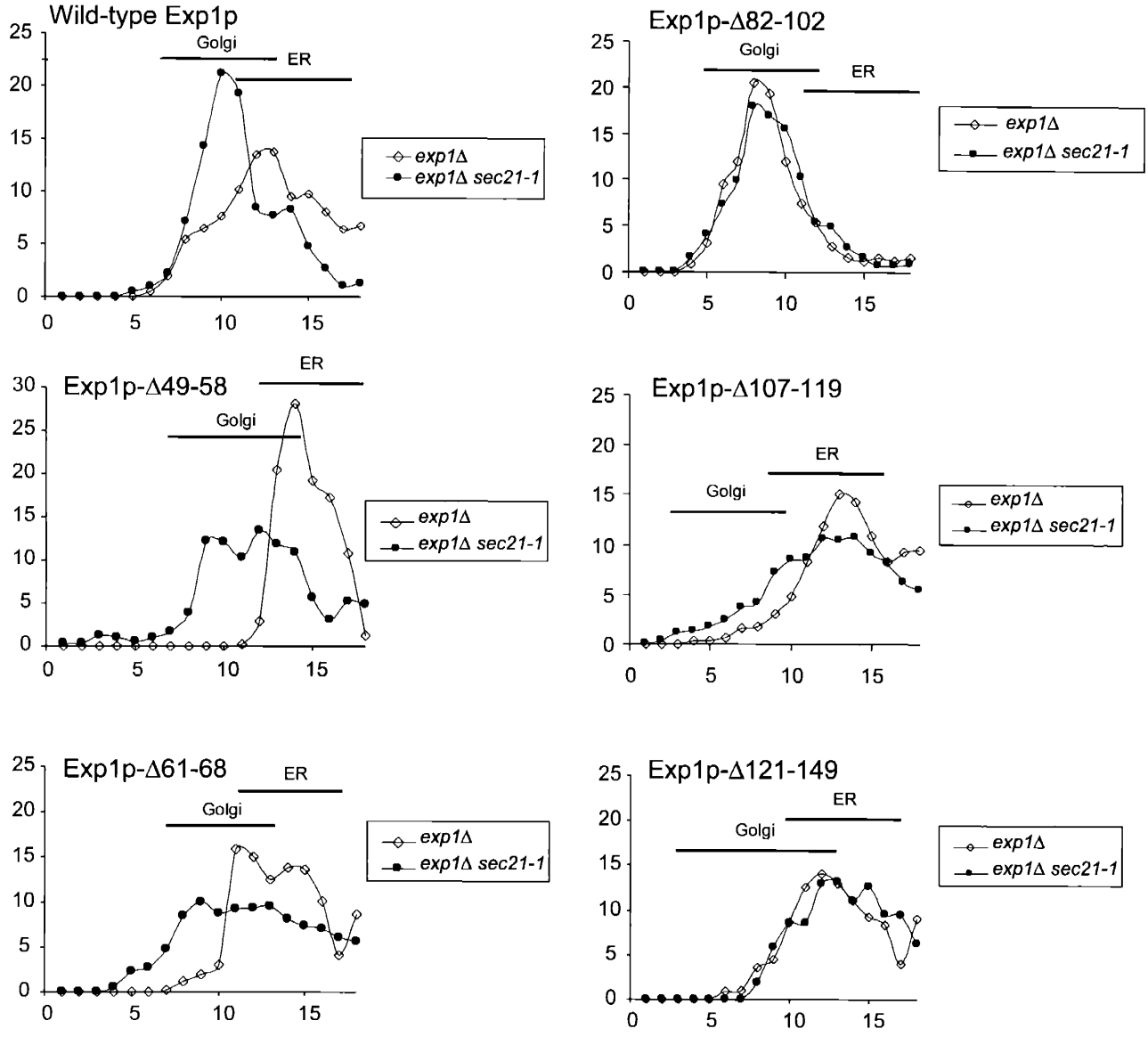

Wild-type Exp1p
Golgi
ER
exp1Δ
exp1Δ sec21-1
Exp1p-Δ82-102
Golgi
ER
exp1Δ
exp1Δ sec21-1
Exp1p-Δ49-58
ER
Golgi
exp1Δ
exp1Δ sec21-1
Exp1p-Δ107-119
ER
Golgi
exp1Δ
exp1Δ sec21-1
Exp1p-Δ61-68
ER
Golgi
exp1Δ
exp1Δ sec21-1
Exp1p-Δ121-149
ER
Golgi
exp1Δ
exp1Δ sec21-1

Figure 9. Exp1p binds to Sec23/24p. (A) Sec23/24p interacts with in-vitro purified GST-Exp1p. Glutathione-sepharose beads bound to bacterially-purified GST-Exp1p or GST alone were added to wild-type yeast extracts or extracts from *SEC31-HA* strains. Proteins bound to the glutathione-sepharaose beads after stringent washing were detected by SDS-PAGE followed by immunoblotting with anti-Sec23p, anti-Sec24p, or anti-HA (12CA5). An aliquot of the total cleared lysate (Total) representing 1% of the total number of cell equivalents used in each binding assay was removed prior to the addition of the protein-bound Glutathione-sepharose beads. (B) Interaction of Sec23/24p with Exp1p deletion mutants. Each deletion allele was fused to GST, and the resulting fusion protein was purified from *E. coli* with glutathione-sepharose beads. Equivalent amounts of protein-bound beads were added to wild-type yeast cell extracts and tested for binding to Sec23/24p as described in (A).

Figure 9

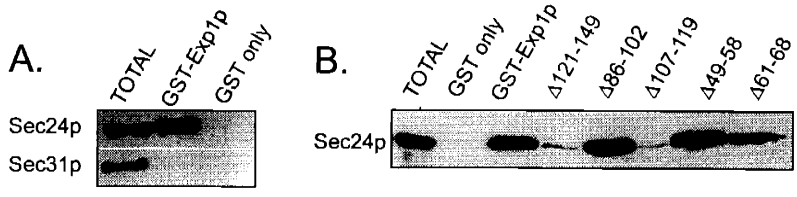
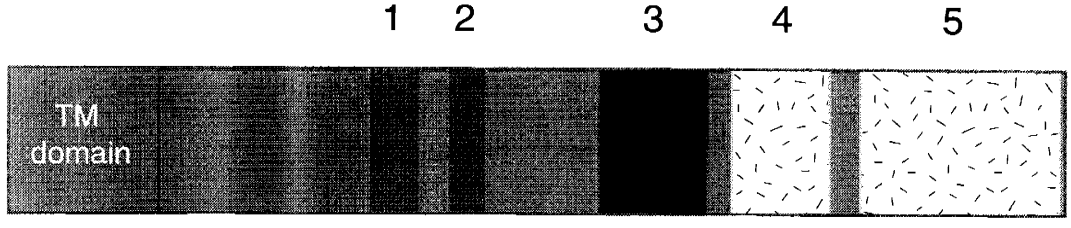

A.
TOTAL
GST-Exp1p
GST only
Sec24p
Sec31p
B.
TOTAL
GST only
GST-Exp1p
Δ121-149
Δ86-102
Δ107-119
Δ49-58
Δ61-68
Sec24p

Figure 10. A summary of Exp1p mutagenesis. Shaded boxes 1-5 represent deletion mutations Δ49-58, Δ61-68, Δ86-102, Δ107-119, and Δ121-149, respectively.

Figure 10



	1	2	3	4	5
required for *in vivo* function	-	-	+	+	+
required for exit from the ER	-	-	-	+	+
required for retrieval from the Golgi	-	-	+	-	-
required for Sec24p binding	-	-	-	+	+

Figure 11. A model diagramming the two distinct, parallel pathways for the export of Pma1p from the ER. The Sec24p-mediated pathway is dependent on the adaptor Exp1p, which is required for efficient trafficking of Pma1p out of the ER in the absence of Lst1p. The Lst1p pathway is not dependent on Exp1p or Sec24p. Exp1p may bind Pma1p directly or may facilitate the ER export of Pma1p indirectly by modulating the affinity of Pma1p for the Sec23/24p complex.

Figure 11

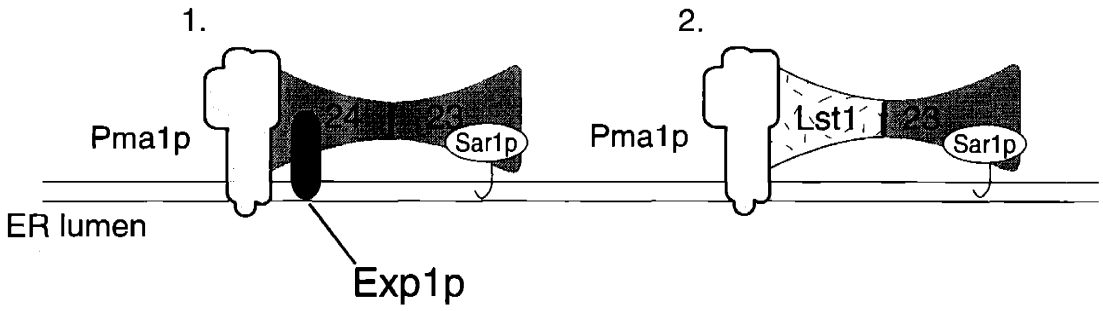


Table I *lst1Δ* strains depleted of Exp1p develop multi-budded morphology.

Strain	carbon source	Percentage of cells with multi budded morphology
wild type	raffinose	1.3
lst1Δ	raffinose	2.8
P_{GAL10}-*EXP1 lst1Δ exp1Δ*	raffinose	14.7
P_{GAL10}-*EXP1 lst1Δ exp1Δ*	glucose	34.3

After growth to exponential phase in SMM with 2% raffinose, cells were transferred to SMM medium containing 2% raffinose +/- 2% glucose. After 14 hours, cells were fixed in 1% formaldehyde and transferred to microscope slides. The proportion of cells with three or more buds was analyzed using phase contrast microscopy. 600 cells were counted for each strain. Numbers reported above are the average of two separate experiments.

Table II Occurrence of the multi-budded morphology in *lst1Δ* strains depleted of Exp1p in the presence of *EXP1* deletion alleles.

Strain	Percentage of cells with multi budded morphology
P_{GAL10}-*EXP1 lst1Δ exp1Δ [pRS316]*	43.2
P_{GAL10}-*EXP1 lst1Δ exp1Δ [EXP1 CEN]*	2.8
P_{GAL10}-*EXP1 lst1Δ exp1Δ [EXP1 2μ]*	1.8
P_{GAL10}-*EXP1 lst1Δ exp1Δ [exp1-Δ49-58]*	4.6
P_{GAL10}-*EXP1 lst1Δ exp1Δ [exp1-Δ61-68]*	3.0
P_{GAL10}-*EXP1 lst1Δ exp1Δ [exp1-Δ86-102]*	20.0
P_{GAL10}-*EXP1 lst1Δ exp1Δ [exp1-Δ107-119]*	23.3
P_{GAL10}-*EXP1 lst1Δ exp1Δ [exp1-Δ121-149]*	26.0

After growth to exponential phase in SMM with 2% raffinose, cells were transferred to SMM medium containing 2% raffinose + 2% glucose. After 14 hours, cells were fixed in 1% formaldehyde and transferred to microscope slides. The proportion of cells with three or more buds was analyzed using phase contrast microscopy. 600 cells were counted for each strain.

Table III Genetic interaction between *exp1Δ* and *sec* mutant alleles.

growth at pH 3.0[a] of *sec* mutant		growth at pH 3.0[a] of *sec exp1Δ* double mutants		difference in restrictive temperature[b]
sec12-4	++	*sec12-4 exp1Δ*	++	0
sec13-1	++	*sec13-1 exp1Δ*	+/-	-3
sec31-1	++	*sec31-1 exp1Δ*	+/-	0
sec23-1	+/-	*sec23-1 exp1Δ*	-	-3
sec24-2	+/-	*sec24-2 exp1Δ*	-	-3
sec18-1	++	*sec18-1 exp1Δ*	++	0
sec21-1	++	*sec21-1 exp1Δ*	++	0

[a] The indicated strains were grown on YPD adjusted to pH 3.0 for three days at 24°C.

[b] The COPII mutant strains and the COPII *exp1Δ* double mutant strains were grown on YPD at 24°C, 27°C, 30°C, 33°C, and 37°C.

Table IV Strains used is this study.

Strain	Genotype	source
CKY8	*MATα ura3-52 leu2-3,112*	
CKY290	*MATα ura3-52 leu2-3,112 his3Δ200 Gal+*	
CKY536	*MATa ura3-52 leu2-3,112 lst1::LEU2*	
CKY761	*MATa ura3-52 pep4::kanMX6*	
CKY802	*MATa ura3-52 leu2-3,112 his3Δ200 Gal+ lst1::LEU2*	
CKY803	*MATα ura3-52 leu2-3,112 his3Δ200 Gal+ lst1::LEU2*	
CKY804	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 exp1::kanMX6*	Euroscarf
CKY807	*MATa ura3-52 leu2-3,112 his3Δ200 Gal+ exp1::URA3*	
CKY808	*MATα ura3-52 leu2-3,112 his3Δ200 Gal+ exp1::URA3*	
CKY810	*MATa ura3-52 leu2-3,112 his3Δ200 Gal+ lst1::LEU2 ($_{GAL10}$-EXP1 (HIS3))*	
CKY811	*MATa ura3-52 leu2-3,112 his3Δ200 Gal+ lst1::LEU2 ($_{GAL10}$-EXP1 (HIS3))*	
CKY812	*MATa ura3-52 leu2-3,112 his3Δ200 Gal+ lst1::LEU2 exp1::URA3 pMC18*	
CKY814	*MATa ura3-52 leu2-3,112 exp1::URA3 his4-619 sec13-1*	
CKY816	*MATa ura3-52 leu2-3,112 exp1::URA3 sec23-1*	
CKY818	*MATa ura3-52 exp1::URA3 sec12-1*	
CKY820	*MATα ura3-52 sec24-2 exp1::URA3*	
CKY821	*MATα ura3-52 sec21-1 exp1::URA3*	
CKY823	*MATa ura3-52 sec19-1 exp1::URA3*	
CKY825	*MATa ura3-52 leu2-3,112 sec16-1 exp1::URA3*	
CKY827	*MATα ura3-52 leu2-3,112 sec18-1 exp1::URA3*	
CKY829	*MATa ura3-52 sec22-3 exp1::URA3*	
CKY831	*MATa ura3-52 sec17-1 exp1::URA3*	
CKY862	*MATα ura3-52 pep4::kanMX6 sec21-1*	
CKY896	*MATα ura3-52 exp1::kanMX6 pep4::kanMX6 sec21-1*	
CKY897	*MATa ura3-52 exp1::kanMX6 pep4::kanMX6*	
CKY900	*MATa ura3-52 leu2-3,112 his3Δ200 Gal+ lst1::LEU2 exp1::kanMX6 pMC18*	

All strains were generated in the course of this study unless otherwise indicated.

Chapter 3

Prospectus

We have focused our research on the molecular mechanisms driving the selective export of Pma1p from the ER. Continuing studies initiated in the lab by Kevin Roberg, we have described two parallel pathways for Pma1p export from the ER: one dependent on Sec24p and the novel cargo adaptor Exp1p (Chapter 2) and one dependent on the Sec24p homolog Lst1p (Appendix I). Efficient incorporation of Pma1p into COPII vesicles requires Lst1p [NOTE:(Appendix I)] (Shimoni et al., 2000). However, in the absence of Lst1p, the added presence of Exp1p can enhance the Sec24p-mediated export of Pma1p from the ER. Chromosomal deletion of *EXP1* in a *lst1Δ* genetic background is lethal, and the Pma1p-trafficking defects displayed by P_{GAL10}-*EXP1 lst1Δ exp1Δ* strains depleted of Exp1p are more severe than those displayed by *lst1Δ* alone. Exp1p cycles between the ER and Golgi and specifically binds Sec23/24p. Mutant versions of Exp1p that fail to complement the *lst1Δ exp1Δ* lethality also fail to bind Sec23/24p, establishing a strong correlation between the Pma1p-trafficking function of Exp1p and its interaction with the COPII coat. On the basis of these findings, we propose that Exp1p and Lst1p act in distinct, parallel pathways act to selectively export Pma1p from the ER.

Analysis of *in vitro* budding assays using ER-derived microsomes and synthetic liposomes have provided extensive detail into the molecular mechanisms underlying the selective capacity of the Sec24p homologs. Lst1p and Sec24p preferentially package discrete, but overlapping subsets of cargo molecules, suggesting that the presence of both homologs within the COPII coat expand the range of cargo selectively exported from the ER. The identification here of Exp1p provides new insight into the multiple layers of partial redundancy cells have evolved to preserve the fidelity of cargo sorting at the ER. To gain a better understanding of the role of Exp1p and Lst1p in the selective export of Pma1p and other cargo proteins, the following questions should be addressed: (1) Is Exp1p required for the Sec24p-mediated export of Pma1p in vitro? (2) Does Exp1p enter COPII vesicles in vitro and can it be isolated in prebudding complexes with Sec24p or Lst1p? (3) How does Exp1p improve the efficiency of the Sec24p-mediated export of Pma1p from the ER? What residues within Sec24p and Exp1p mediate their interaction? (4) How is Pma1p selectively exported by Lst1p and Sec24p? Are the Pma1p binding sites within each homolog conserved? (5) Does Exp1p enhance the export of other cargo

proteins? (6) What is the range of cargo selectively exported by Lst1p? (7) Is Exp1p part of a larger family of adaptor proteins?

In vitro analysis of the role of Exp1p in the Sec24p-mediated export of Pma1p from the ER

To confirm that Exp1p promotes the export of Pma1p through direct interaction with Sec24p, the requirements for Exp1p in the Sec24p-mediated packaging of Pma1p into budding vesicles should be examined in vitro. Shimoni and colleagues (2000) and Miller and colleagues (2002) have demonstrated that Pma1p is most efficiently incorporated into COPII vesicles when both Lst1p and Sec24p are present, while in the absence of Lst1p, Sec24p could only package wild-type levels of Pma1p when added in 20-fold excess. Our model predicts that overproduction of Exp1p may decrease the amount of Sec24p needed to efficiently package Pma1p into COPII vesicles, while the absence of Exp1p may render Sec24p incapable of packaging Pma1p in the absence of Lst1p. These predictions can be tested in the *in vitro* budding assay using donor membranes from *exp1Δ* and Exp1p-overproducing strains.

At the earliest stages of vesicle budding, pre-budding complexes consisting of activated Sar1p, Sec23/24p and cargo proteins can be isolated. If Exp1p enhances the recognition of Pma1p by Sec24p, then pre-budding complexes consisting of activated Sar1p, Sec23/24p, Pma1p, and Exp1p may form on the ER membrane. The role of Exp1p in the Sec24p-mediated recognition of Pma1p could be directly examined by analyzing the formation of Pma1p-containing pre-budding complexes in the presence and absence of Exp1p. The presence of Exp1p in COPII vesicles could be confirmed by testing whether Exp1p could be co-immunoprecipitated with Sec24p from vesicles generated in vitro. The effect of Exp1p on the Lst1p-mediated export of Pma1p should also be examined in the *in vitro* budding assay.

Towards a molecular understanding of the selective export of Pma1p from the ER

The specific signals driving the uptake of Pma1p into COPII vesicles have not been identified, and we were unable to find a small, linear export motif within Exp1p. The molecular signals mediating direct interactions between Pma1p and Lst1p or Sec24p and between Exp1p and Sec24p could be investigated using the elegant structure/function

approach recently described by Mossessova, et al. (2003) and Miller, et al. (2003). By screening for *lst1* alleles synthetically lethal with *exp1Δ*, specific regions of Lst1p required for its Pma1p-export activity may be identified. *lst1* mutants generally impaired in vesicle trafficking could be distinguished from *lst1* mutants with specific Pma1p-export defects by examining the export of other cargo proteins known to be recognized by Lst1p, such as Gas1p, Erp1p, Emp24p, or Erv41p (Miller et al., 2002; Miller et al., 2003).

Extensive study into the structure and molecular mechanism of Pma1p has generated a wealth of data that could direct a targeted mutagenesis approach for the identification of the Pma1p ER-export motif (Morsomme et al., 2000). Electron crystallographic analysis has provided detailed information about cytoplasmically-exposed residues along the surface of Pma1p that may be available for interaction with the COPII coat (Scarborough, 2000). Furthermore, researchers have identified several partial loss-of-function Pma1p mutants that may be impaired in their ability to exit the ER (see Morsomme et al., 2000 for a categorization of mutant *pma1* alleles). Integrating the structural and mutagenic information available about Pma1p will allow researchers to identify small subdomains of Pma1p that may interact with the COPII coat. If Pma1p interacts with Sec24p or Lst1p through a short, linear motif, then the corresponding binding site on Sec24p or Lst1p could be identified by co-crystallizing a Pma1p peptide with Sec24p or Lst1p. Similar studies could be performed with Exp1p to identify the ER-export motif of Exp1p and the corresponding binding site on Sec24p. Co-crystallization of a Pma1p peptide with Sec24p in the presence or absence of the Exp1p interacting peptide may yield information about conformational changes in the Pma1p-binding site of Sec24p that may be induced by Exp1p.

Exploration of the range of cargo recognized by Exp1p and Lst1p

A simple, brute-force proteomic approach could be used to asses the range of cargo selectively exported by Exp1p and Lst1p. The protein composition of vesicles generated in a large scale budding assay in the absence of Lst1p or Exp1p could be compared to the protein composition of wild-type vesicles using high-throughput mass spectrometry. Candidate cargo proteins could then be epitope tagged and expressed in

lst1Δ or *exp1Δ* strains to analyze *in vivo* trafficking defects by immunofluorescence microscopy. A similar approach comparing proteins packaged in the presence of Sec24p and Sec24p cargo-binding site mutants may reveal which cargo proteins interact at the known Sec24p binding sites.

Identification of additional cargo adaptor proteins

Exp1p may be part of a family of adaptor proteins that modulate the affinity of the Sec24p homologs for cargo proteins. Since these adaptor proteins may act in partially redundant, parallel pathways, screening the yeast genome for deletions of non-essential, ER/Golgi-localized genes that are lethal in combination with *sloΔ* or *lst1Δ* may uncover cargo adaptors whose activity overlaps with Exp1p or Lst1p. Genetic interaction with thermosensitive *sec24* alleles or *sec24* binding site alleles may be used as a secondary criteria for candidate cargo adaptors. Phenotypic analysis of the candidate cargo adaptor mutants may provide information about the specific cargo proteins whose export is compromised by the cargo adaptor mutations.

Conclusion

The co-crystallization of Sec24p in complex with peptides from cargo proteins, and the isolation of *sec24* mutants defective in selective export of specific cargo molecules have confirmed a central role for the Sec24p subunit of the COPII coat in cargo recognition (Miller et al., 2003; Mossessova et al., 2003). The identification here of a Sec24p homolog, Lst1p, and a Sec24p-interacting cargo adaptor, Exp1p, expands our understanding of how the COPII coat can selectively export such a broad range of secretory cargo proteins using multiple, diverse ER export motifs. Further study of the molecular mechanisms underlying the selective capacity of Lst1p and Sec24p/Exp1p will provide insight into the specific requirements for Pma1p export from the ER and will serve as useful tools for elucidating general mechanisms for cargo-recognition in the ER.

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Appendix I

LST1 is a *SEC24* homolog used for selective export of the plasma membrane ATPase from the ER

Preface

Appendix I represents primarily the work of Kevin Roberg. I contributed Figures 3 and 5.

Appendix I has been published in its entirety in the Journal of Cell Biology as:
Roberg, K.J., M. Crotwell, P. Espenshade, R. Gimeno, and C.A. Kaiser. 1999. *LST1* is a *SEC24* homologue used for selective export of the plasma membrane ATPase from the endoplasmic reticulum. *J Cell Biol.* 145:659-72.

Abstract

In *S. cerevisiae*, vesicles that carry proteins from the endoplasmic reticulum (ER) to the Golgi are encapsulated by COPII coat proteins. We identified mutations in ten genes, designated *LST*, that were lethal in combination with the COPII mutation *sec13-1*. *LST1* showed synthetic lethal interactions with the complete set of COPII genes, indicating that *LST1* encodes a new COPII function. *LST1* codes for a protein similar in sequence to the COPII subunit Sec24p. Like Sec24p, Lst1p is a peripheral ER-membrane protein that binds to the COPII subunit Sec23p. Chromosomal deletion of *LST1* is not lethal, but inhibits transport of the plasma membrane proton-ATPase (Pma1p) to the cell surface, causing poor growth on media of low pH. Localization by both immunofluorescence microscopy and cell fractionation showed that the export of Pma1p from the ER is impaired in *lst1Δ* mutants. Transport of other proteins from the ER was not affected by *lst1Δ*, nor was Pma1p transport found to be particularly sensitive to other COPII defects. Together these findings suggest that a specialized form of the COPII coat subunit, with Lst1p in place of Sec24p, is used for the efficient packaging of Pma1p into vesicles derived from the ER.

Introduction

The plasma membrane H^+-ATPase (Pma1p) is an essential integral membrane protein that couples ATP hydrolysis to the translocation of protons across the plasma membrane (Serrano et al., 1986). The proton gradient generated by Pma1p then drives the uptake of nutrients such as amino acids from the extracellular medium (Vallejo and Serrano, 1989). A second physiological function of Pma1p is to maintain the cytosol at a neutral pH, and in medium of low pH the growth rate is limited by the amount of cellular Pma1p (McCusker et al., 1987; Portillo and Serrano, 1989). Pma1p transport to the cell surface depends upon the secretory pathway defined by the *sec* genes, (Brada and Schekman, 1988; Chang and Slayman, 1991), and Pma1p is one of the most abundant cargo molecules of the secretory pathway, constituting 25 to 50% of the total plasma membrane protein (Serrano, 1991). Because of its abundance and physiological importance one might expect that yeast cells would have specialized mechanisms to ensure efficient transport of Pma1p through the secretory pathway. Such a function has been suggested for two proteins, Ast1p and Ast2p, in the transport of Pma1p from the Golgi to the plasma membrane (Chang and Fink, 1995). For early steps in the secretory pathway, proteins that are specifically required for the transport of Pma1p have not yet been identified.

Proteins destined for the plasma membrane are transported from the ER to the Golgi by vesicles coated with a set of proteins known as COPII (Barlowe et al., 1994). These COPII coats are thought to both cause the deformation of the membrane into a vesicle and to recruit cargo molecules into vesicle buds (reviewed by Schekman and Orci, 1996). The stepwise recruitment and assembly of the COPII coat onto the membrane is thought to occur as follows. Action of the ER-resident membrane protein Sec12p, a guanine nucleotide exchange factor for Sar1p, causes Sar1p to bind to the ER membrane (Barlowe and Schekman, 1993). Membrane associated Sar1p, in turn, recruits the soluble Sec23p/Sec24p and Sec13p/Sec31p complexes (Matsuoka et al., 1998). Sec16p resides on the ER membrane and binds to both the Sec23p/Sec24p and Sec13p/Sec31p complexes, likely organizing their assembly onto the membrane (Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). To examine the role of different COPII coat subunits in recruitment of cargo molecules to vesicles, partially assembled COPII

complexes have been tested for their ability to associate with cargo proteins. Association of a membrane bound complex of Sar1p and Sec23p/Sec24p with integral membrane proteins indicates that cargo proteins may laterally partition into the vesicle membrane by virtue of their affinity for the Sec23p/Sec24p protein complex (Kuehn et al., 1998, Aridor et al., 1998).

An early indication that the COPII coat subunits would physically interact came from specific genetic interactions between mutations in COPII genes. When temperature sensitive mutations in COPII genes are combined the resulting double mutants are almost always much more restrictive for growth than the component single mutations and are usually inviable at 24°C. These synthetic-lethal interactions are restricted to genes involved in COPII vesicle formation and do not occur when mutations in genes required for vesicle formation are combined with genes required for vesicle fusion (Kaiser and Schekman, 1990). The specificity of this type of genetic interaction suggested that synthetic lethality with known COPII mutations would be a useful criteria to identify new mutations involved in the assembly of the COPII coat.

We screened for mutations that were lethal with the COPII mutation *sec13-1*, and identified ten *LST* genes (Lethal with *sec-thirteen*). As we describe elsewhere, most of the *LST* genes are related to an unanticipated role for *SEC13* in the regulated delivery of specific amino acid permeases to the cell surface (Roberg et al., 1997a; Roberg et al., 1997b). Accordingly, these *LST* genes display synthetic lethal interactions with *SEC13*, but not with the other COPII genes. On the other hand, mutations in *LST1* were lethal with the full set of mutations defective in COPII vesicle budding but not with mutations defective in vesicle fusion, indicating that *LST1* does participate in vesicle budding at the ER. Here we show that *LST1* encodes a homolog of the COPII subunit, Sec24p, and that Lst1p is a peripheral membrane protein localized to the ER that can form complexes with Sec23p. The *LST1* gene is not essential, but by examination of the phenotypes of *lst1Δ* mutants we show that *LST1* is required for the efficient export of Pma1p from the ER to

the Golgi. These results suggest a specialized form of the vesicle coat that is responsible for recruitment of Pma1p into COPII coated vesicles.

Materials and Methods

Media, Strains, and Plasmids

The *S. cerevisiae* strains used in this study are listed in Table I. Rich medium (YPD) and supplemented minimal medium (SMM) were prepared according to Kaiser et al. (1994). To evaluate growth at low pH, YPD was adjusted to pH 3.8 with HCl (this medium remained at pH 3.8 throughout the growth of a yeast culture). For some experiments SMM was buffered to pH 6.5 using 50 mM MOPS, 50 mM MES. Genetic manipulations were performed according to standard protocols (Kaiser et al., 1994). DNA manipulations were carried out as described in Sambrook et al. (1989). pAF70 carries the *SEC24* gene in the centromere vector pCT3 (*URA3*) (Gimeno et al., 1996). pKR34 and pKR41 carry the 3.8-kb, KpnI/SalI fragment containing the *SEC24* gene from pAF70 in the 2μ vectors pRS426 (*URA3*) and pRS425 (*LEU2*), respectively. pKR17 carries the *LST1* gene on a 3.5-kb fragment in the centromere vector pRS316 (*URA3*). A subclone of the *LST1* gene from pKR17 into the 2μ vector pRS426 gave yeast transformants at very low efficiency because of the toxicity of *LST1* sequences when present at high copy. To study the toxic effects of *LST1*, pKR35 was constructed which contains the entire *LST1* coding sequence expressed from *pGAL1* on pCD43 (*URA3*). pKR35 will prevent growth under conditions of full induction on galactose medium, establishing that overexpression of Lst1p is toxic to yeast cells. Under conditions of partial induction of *pGAL1-LST1* in cells grown on raffinose, pKR35 will complement *lst1Δ::LEU2* for growth on acidic medium. This shows that the *LST1* ORF carried on pKR35 still posses *LST1* function.

Epitope-tagged *LST1* was constructed as follows. First, the NotI site in the polylinker of pKR17 was deleted with a 350-bp SmaI/NaeI fragment (pKR17Δ), then a 12-bp linker carrying a NotI site (#1127; New England BioLabs, Beverly, MA) was inserted at the Eco47 III site (at codon 13 of *LST1*) of pKR17Δ to make pKR17N.

pKR17HA carries the 100-bp NotI fragment from pGTEPI (Tyers et al., 1993), which encodes three tandem copies of the hemagglutinin (HA1) epitope, inserted into the NotI site of pKR17N. Restriction analysis using sites flanking the point of insertion revealed two 100-bp inserts (six HA epitopes) were present in pKR17HA. pKR17HA was transformed into CKY536 to make CKY535 (*MATa lst1Δ::LEU2 leu2-3,112 ura3-52* [pKR17HA]).

Synthetic Lethal Screen

The following plasmids and strains were constructed for use in the *sec13-1* synthetic-lethal screen. The plasmid pKR1 carries *SEC13* on a 1.8-kb SalI/BamHI fragment excised from pCK1313 (Pryer et al., 1993), inserted into pRS316 (Sikorski and Heiter, 1989). pKR4 carries the same 1.8-kb SalI/BamHI fragment and a 3.8-kb NheI-BamHI fragment containing *ADE3* from pDK255 (Koshland et al., 1985), both inserted into the vector pRS315 (Sikorski and Heiter, 1989). CUY563 and CKY45 were crossed to produce a *MAT***a** *ade2 ade3 leu2 ura3 sec13-1* segregant, which was transformed with pKR4 to give CKY423. The mating type of CKY423 was switched by ectopic expression of the *HO* gene (Herskowitz and Jensen, 1991) to give CKY424.

Cultures of CKY423 and CKY424 were mutagenized by irradiation with a germicidal UV lamp at a dose that gave 10% cell survival (Lawrence et al., 1991) and mutagenized cells were plated on YPD at a density of 150 colonies per plate. After five days of growth at 24°C, colonies with a solid red color and no white sectors were selected for further analysis. The dependence of the nonsectoring phenotype on the *sec13-1* mutation was tested by transforming candidate mutants with either pKR1 or pRS316. Strains that sectored after transformation with pKR1, but not after transformation with pRS316, were scored as *sec13-1* dependent.

Complementation tests were performed by mating mutants isolated from CKY423 with those isolated from CKY424. Zygotes isolated by micromanipulation were scored for their ability to form sectored colonies on YPD plates. The genes defined by these complementation groups were designated *LST* for lethal with *sec-thirteen.* All *lst* mutant strains were backcrossed to a parental strain twice.

The *lst sec13-1* double mutants were converted to *lst* single mutants by integration of a wild-type copy of *SEC13* at the *sec13-1* locus using the integrating plasmid p1312 (*SEC13 URA3*) (Pryer et al., 1993). The integrants were grown on YPD and cells from white sectors (indicating loss of pKR4) were isolated. The integration of a wild-type copy of *SEC13* was confirmed by the ability of the cells from white sectors to grow at 36°C, a temperature that is restrictive for the *sec13-1* mutation. Owing to the poor growth of *lst9* strains, we were not able to construct a *lst9* single mutant by this method.

To test for synthetic-lethal interactions between *lst* mutations and mutations in *sec* genes, *lst* mutants CKY435 (*lst1-1*), CKY436 (*lst2-1*), CKY437 (*lst3-1*), CKY438 (*lst4-1*), CKY439 (*lst5-1*), CKY440 (*lst6-1*), CKY441 (*lst7-1*), and CKY442 (*lst8-1*) were crossed to the *sec* mutants CKY45 (*sec13-1*), CKY50 (*sec16-2*), CKY78 (*sec23-1*), and CKY450 (*sec31-2*). Inviability of a given *lst sec* double mutant was inferred from crosses where lethality segregated as a two-gene trait (most tetrads giving a segregation pattern of 1:3 for lethality), an outcome that was easily detectable since crosses to wild-type typically gave greater than 95% spore viability. The segregation pattern of the *sec* mutation in the surviving sister spores was used as an additional test to establish that the inviable spores always carried the *sec* mutation and were therefore not the result of random spore death.

Construction of lst1Δ mutants

Replacement of the chromosomal *LST1* gene with an allele disrupted with the *LEU2* gene was constructed as follows. pKR18 carries the 5' half of *LST1* on a 2.0 kb Xho1-HindIII

fragment inserted into pRS316. A 2.0-kb HindIII/BamHI fragment containing the *LEU2* gene from plasmid pJJ252 (Jones and Parakash, 1990) and a 250-bp BclI/SacI fragment from the 3'-noncoding region of *LST1* were inserted into pKR18 to construct pKR28. The N-terminal coding region of *LST1* (except for codons 1-13) was removed by deleting a 1.7-kb Eco47 III/MscI fragment from pKR28 to generate pKR28Δ. The *lst1Δ::LEU2* construct, liberated from pKR28Δ by digestion with XhoI, was transformed into the wild-type diploid strain CKY348 (*MATa/α leu2-3,112/leu2-3,112 ura3-52/ura3-52*). On sporulation and dissection this diploid gave four viable spore clones and haploid segregants carrying *lst1Δ::LEU2* were confirmed by Southern blotting. One such segregant was further backcrossed to our S288C genetic background to give strains CKY536 and CKY542.

Proton efflux from intact yeast cells

Pma1p activity was assayed by proton efflux from intact cells into the external medium. Cells were grown to exponential phase in YPD at 37°C and were washed and then stored in deionized water at 4°C overnight. Cell number was measured by light scattering and a total of 25 A_{600} units (about 5 x 10^8 cells) were suspended in 5 ml of 100 mM KCl, 10 mM glycine (pH 4.0). The pH of the cell suspension was measured using a combination electrode at 25°C with constant stirring. Once the pH had stabilized after about 10 minutes, glucose was added to a final concentration of 40 mM and the ensuing drop in pH was recorded at 30 second intervals over 15 minutes. In comparison of wild-type (CKY443) and *lst1Δ* (CKY536) strains, both suspensions had identical cell concentration as measured by light scattering and showed the same response to calibration pulses with HCl.

Immunofluorescence microscopy

The intracellular location of Pma1p in wild-type (CKY443) and *lst1Δ* (CKY536) cells was examined by indirect immunofluorescence microscopy using techniques described

previously (Pringle et al., 1991; Espenshade et al., 1995). Strains were grown exponentially in SMM medium (pH 7.2) at 30°C. Cells were fixed in 3.7% formaldehyde and then converted to spheroplasts by digestion with lyticase. Both primary and secondary antibody incubations were for one hour at 25°C. Affinity purified anti-Pma1p antibody was prepared as follows. A crude preparation of yeast membranes was resolved by preparative SDS-PAGE and after transfer of proteins to a nitrocellulose membrane by electrophoresis the strip of membrane that contained Pma1p was excised. Rabbit antiserum to Pma1p was applied to the nitrocellulose strip, and after the strip was washed with 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5%Tween 20, the bound antibody was eluted with 100 mM glycine (pH 2.8), 500 mM NaCl, 0.5%Tween 20. Affinity purified Pma1p was used at a 1:100 dilution and FITC-conjugated anti-rabbit IgG was used at 1:200 dilution. Mounting medium was supplemented with DAPI. Micrographs were taken with a Nikon Eclipse TE300 microscope with a Hamamatsu Orca C4742-95 CCD camera.

For the localization of Lst1p-HA, CKY535 was grown on SMM to exponential phase and prepared as described above. For visualization of Lst1p-HA the 12CA5 antibody (BAbCO, Richmond, CA) was used at a 1:5000 dilution and FITC-conjugated goat anti-mouse IgG was used at a 1:50 dilution. Rabbit anti-Kar2p polyclonal serum (a gift of M. Rose, Princeton University, New Jersey) was used at a 1:1000 dilution and rhodamine-conjugated goat anti-rabbit IgG was used at a 1:200 dilution. Samples were viewed and imaged using a Nikon Optiphot 2 microscope and a Photometric ImagePoint CCD camera. Images were recorded using IP-Lab software (Molecular Dynamics, Sunnyale, California).

Cell Fractionation

Cell organelles were fractionated on equilibrium density gradients as previously described (Roberg et al., 1997a). Cultures were grown exponentially at 24°C and then

shifted to 37°C for three hours. 1.6 x 10^9 cells were collected by centrifugation and suspended in 0.5 ml STE10 (10% [w/w] sucrose, 10 mM Tris-HCl (pH 7.6), 10 mM EDTA) with a protease inhibitor cocktail (1 mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin) and lysed by vortexing with glass beads. An additional 1ml of STE10 was added and the lysate was cleared of unbroken cells and large cell debris by centrifugation at 300 *g* for two minutes. The cleared extract (300 μl) was layered on top of a 5 ml, 20-60% linear sucrose gradient in TE (10 mM Tris-HCl [pH 7.6], 10 mM EDTA) prepared for an SW50.1 rotor (Beckman Instruments, Palo Alto, CA). Samples were centrifuged 100,000 *g* for 18 hours at 4°C and fractions of 300 μl were collected from the top of the gradient. Protein was precipitated from each fraction by the addition of 100 μl of 0.15% deoxycholate and 100 μl of 72% trichloroacetic acid. Protein pellets collected by centrifugation at 13,000 *g*, washed with cold acetone, and then solubilized in ESB (60 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% SDS, 10% glycerol, 0.02% bromophenol blue). Pma1p, Gas1p, and Sec61p were resolved by SDS-PAGE and were detected by immunoblotting. The relative amount of each protein in cell fractions was determined by densitometry using an Ultroscan 2202 (LKB Instruments, Inc., Gaithersburg, MD). The Golgi GDPase activity was assayed in gradient fractions prior to protein precipitation using standard methods (Abeijon et al., 1989).

The subcellular distribution of Lst1p-HA was examined using techniques described in detail previously (Espenshade et al., 1995). CKY535 carrying pKR17HA, which expresses Lst1p-HA, was grown to exponential phase in SMM without uracil. 2x10^9 cells were harvested and converted to spheroplasts and then gently lysed by glass beads in 500 μl of cell lysis buffer (20 mM MES [pH 6.5], 100 mM NaCl, 5 mM $MgCl_2$) including the protease inhibitor cocktail. The cell extract was sequentially centrifuged at 500 *g* for 20 minutes, 10,000 *g* for 20 minutes, and 150,000 *g* for 60 minutes, to give one soluble and three particulate fractions.

Release of Lst1p-HA from the particulate fraction was examined by treating cell extracts with 500 mM NaCl, 100 mM sodium carbonate (pH 11.5), 2.5 M urea, or 1% Triton X-100. After 1 hour of incubation at 4°C, samples were centrifuged at 50,000 *g* for 30 minutes to separate soluble and particulate fractions. Fractions from both experiments were solubilized in sample buffer and analyzed by immunoblotting.

Immunoblotting

Samples of 10 to 30 µl in ESB were resolved by SDS-PAGE and immunoblotting was conducted according to standard protocols (Harlow and Lane, 1988). For transfer of Lst1p to nitrocellulose membranes, 0.1% SDS was included in the transfer buffer. The following antibodies were used: mouse monoclonal 12CA5 anti-HA at 1:1,000 dilution; rabbit anti-Pma1p (a gift of A. Chang) at 1:500 dilution; rabbit anti-Gas1p (a gift of H. Riezman, University of Basel, Switzerland) at 1:10,000 dilution; rabbit anti-Sec61p (a gift of R. Schekman, University of California, Berkeley, California) at 1:3,000 dilution; rabbit anti-Gdh2p (a gift of B. Magasanik, Massachusetts Institute of Technology, Cambridge, MA) at 1:1,000 dilution; HRP coupled sheep anti-mouse Ig and HRP coupled sheep anti-rabbit Ig (both Amersham Corp, Arlington Heights, IL) at 1:10,000 dilution. Blots were developed using chemiluminescence detection system (Amersham).

Pulse-chase kinetics of invertase maturation

The strains used for radiolabeling all carried the plasmid pNV31, which carries the *SUC2* gene under the constitutive *TPI1* promoter (a gift of M. Lewis, M.R.C. Laboratories of Molecular Biology, Cambridge, U.K.). Wild-type (CKY540) and *lst1Δ* (CKY542) strains were grown in SMM without methionine (buffered with 50 mM MES and 50 mM MOPS to pH 6.5) at 24°C to exponential phase and then shifted to 37°C three hours prior to labeling. A *sec12-4* strain (CKY541) was similarly grown to exponential phase at 24°C, but was shifted to 37°C five minutes prior to the addition of label. Radiolabeling

and immunoprecipitation of invertase was performed as previously described (Gimeno et al., 1995; Elrod-Erickson and Kaiser, 1996).

Two-Hybrid Interactions

The yeast two hybrid assay was used to test potential protein-protein interactions as previously described (Gyuris et al., 1993; Bartel and Fields, 1995). Interactions were tested between either Lst1p or Sec24p fused to the LexA DNA-binding domain and Sec23p fused to an acidic transcriptional-activation domain. The following plasmids were used: pPE81 carries *SEC23* fused to the acidic activation domain of pJG4-5 (Espenshade et al., 1995). pRH286 carries *SEC24* (codons 34-926) fused to the *lexA* DNA-binding domain in pEG202 (Gimeno et al., 1996). pKR37 carries *LST1* fused to the *lexA* DNA-binding domain in pGilda (a derivative of pEG202 with *pGAL1* provided by D. Shaywitz).

Combinations of control and fusion-protein plasmids, along with the reporter plasmid pSH18-34, were transformed into the strain EGY40 (Golemis and Brent, 1992). Strains were grown exponentially in SMM with 2% raffinose as the carbon source. Galactose was added to a concentration of 2% and incubation was continued for 10 hours to induce fusion proteins expressed from *pGAL1*. Assays for β-galactosidase activity were performed on cells lysed by disruption with glass beads (Rose and Botstein, 1983). Activity was normalized to total protein determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Binding of Lst1p to Sec23p

A gene fusion expressing Lst1p fused to glutathione-*S*-transferase was constructed by inserting the 3.0 kb BamHI/XhoI fragment of pKR17HA into pRD56 (a gift of R. Deshaies, California Institute of Technology, Pasadena, California) to construct pRH254, which gives GST-HA-Lst1p (aa 14-927 of Lst1p) fusion expressed from *pGAL1*. pPE123 is the *SEC23* gene expressed from *pGAL1* in pRS315 (Gimeno et al., 1996).

Binding interactions were tested from extracts of CKY473 transformed with pRH254 (GST-HA-Lst1p) and either pCD43 (vector) or pPE123 (Sec23p).

Cells were grown to exponential phase in SMM with 2% raffinose, galactose was added to 2%, and incubation was continued for two hours at 30°C to induce *pGAL1* expression. 5 x10^8 cells were converted to spheroplasts as previously described (Espenshade et al., 1995) and then gently lysed using glass beads in IP buffer (20 mM HEPES-KOH [pH 6.8], 80 mM KOAc, 5 mM magnesium acetate, 0.02% Triton X-100) containing the protease inhibitor cocktail. The extract was diluted to 1 ml with IP buffer, and membranes were collected by centrifugation at 500 *g* for 20 minutes. This pellet was extracted with 1 ml of IP buffer and 600 mM NaCl for 10 minutes at 0°C to release membrane-bound protein complexes. After clarification by centrifugation at 90,000 *g* for 10 minutes the extract was diluted three-fold with IP buffer, and a 1 ml aliquot was removed and incubated at room temperature for one hour with glutathione Sepharose 4B beads (Pharmacia, Piscataway, NJ). The beads were washed twice with 200 mM NaCl, 20 mM HEPES-KOH [pH 6.8], 80 mM KOAc, 5 mM magnesium acetate, 0.02% Triton X-100 and once in IP buffer without Triton X-100. Proteins were released from glutathione Sepharose 4B beads by solubilization in ESB. Samples of total lysate were prepared by adding 2X ESB to an equal amount of the diluted extract from the salt washed membranes. Samples were analyzed by immunoblots probed with anti-Sec23p antibody.

For analysis of the membrane association of GST-Lst1p and Sec23p, cells expressing GST-Lst1p, or Sec23p, or both GST-Lst1p and Sec23p from *pGAL1* were grown in 2% raffinose and then induced by the addition of 2% galactose as described above. Two hours after induction, 2 x 10^7 cells were collected by centrifugation and resuspended in 20 µl of cell lysis buffer (20 mM MES [pH 6.5], 100 mM NaCl, 5 mM $MgCl_2$) with protease inhibitor cocktail. Cells were lysed by vigorous agitation with glass beads and an additional 500 µl of lysis buffer was added. The lysate was cleared of

unlysed cells and large cell debris by centrifugation at 300 *g* for 3 minutes. 50 µl of the supernatant was reserved for a total extract sample and the remainder was centrifuged to pellet ER membranes at 10,000 *g* for 30 minutes at 4°C in a microcentrifuge. An equal number of cell equivalents of total extract, membrane-pellet, and supernatant fractions were solubilized in ESB and analyzed by immunoblotting. The cytosolic protein Gdh2p was found only in the soluble fractions, demonstrating cell lysis was complete (data not shown).

Results

Mutations Synthetically Lethal with sec13-1

To find new genes required for the budding of COPII vesicles we screened for mutations that displayed synthetic lethality with the COPII mutation *sec13-1* using a plasmid sectoring assay (Roberg et al., 1997b). Strain CKY423 has the chromosomal mutations *ade2 ade3 sec13-1* and harbors the plasmid pKR4 which carries wild-type copies of *SEC13* and *ADE3*. This strain accumulates a red pigment because of the *ade2* mutation, but the spontaneous loss of pKR4 during the growth of a colony gives white sectors of *ade2 ade3* segregants. In this strain, a mutation that is lethal with *sec13-1* will produce a nonsectoring colony. Mutagenesis of CKY423 and the isogenic strain of opposite mating type, CKY424, yielded 139 nonsectoring mutants (Fig. 1). These strains were then tested for restored ability to sector after transformation with pKR1, which carries wild-type *SEC13*, but lacks the *ADE3* gene. By this test, 57 of the mutants had synthetic-lethal mutations that could be rescued by wild-type *SEC13*. In backcrosses, 52 mutants gave a segregation pattern indicating that the trait was due to a single nuclear mutation (Fig. 1).

Matings between mutants identified 11 complementation groups using colony-sectoring of the diploid as the criterion for allelic complementation. These complementation groups were designated *LST* for lethal with *sec-thirteen* (Table II). One of the complementation groups was shown to comprise recessive lethal mutations in the *SEC13* gene itself (Roberg et al., 1997b). Tests for rescue of the nonsectoring phenotype by plasmids carrying known *SEC* genes showed that *LST10* was *SEC16* (Roberg et al., 1997b).

Synthetic interactions of the lst mutations

To perform further genetic tests on the *lst* mutations, the *lst sec13-1* double mutants were converted to *lst* single mutants by integration of a wild-type copy of *SEC13* at the *sec13-*

1 locus (see Methods). Representative *lst* single mutants were then crossed to *sec16, sec23*, and *sec31* mutants. For mutations in *LST2, LST3*, *LST4*, *LST5*, *LST7*, and *LST8*, only crosses to *sec13-1* gave a segregation pattern indicative of a synthetic lethal interaction (Table III). We have subsequently shown that these *LST* genes relate to a function of *SEC13* in the sorting of amino acid permeases in the late secretory pathway, and the analysis of these genes is described elsewhere (Roberg et al., 1997a; Roberg et al., 1997b). Mutations in *LST1* were inviable when combined with *sec16*, *sec23*, and *sec31* mutations, and mutations in *LST6* were inviable with *sec16* and *sec31* (Table III). Importantly, mutations in *LST1* and *LST6* did not show synthetic lethality in parallel crosses to mutations in *SEC17* or *SEC18*, genes required for fusion of COPII vesicles. Given that synthetic lethal interactions usually occur between mutations in genes involved in the same step of the secretory pathway, the tests for genetic interactions indicated that *LST1*, and probably also *LST6*, participate in vesicle budding from the ER.

Lst1p is homologous to Sec24p

The *LST1* gene was isolated by its ability to restore sectoring to CKY426 (*MATa lst1 sec13-1 ade2 ade3 leu2 ura3* [pKR4]), a strain that forms solid red, nonsectoring colonies because of the presence of the *lst1-1* mutation. CKY426 was transformed with yeast genomic libraries and 34 colonies that regained the ability to form white sectors were identified among 97,000 Ura^+ transformants. We expected this screen to yield plasmids carrying either *SEC13* or *LST1*, and about half of the complementing plasmids were shown to carry *SEC13* by restriction site mapping and by the ability to complement the temperature sensitivity of *sec13-1*. The restriction maps of the remaining rescuing plasmids showed that they represented two unrelated chromosomal regions. The clones p21-31 and p77-2 were selected as representatives of each region. The genomic sequence from p77-2 (a clone in the pλYES vector; Elledge et al., 1991) was inserted as an XhoI

fragment into the integrating vector pRS306 to produce pKR20. For chromosomal integration, pKR20 was linearized by digestion with HpaI and transformed into CUY564 (*MATα ade2 ade3 leu2 ura3*). The resulting strain was crossed to the *lst1-1* mutant CKY426 (*MATa lst1-1 sec13-1 ade2 ade3 leu2 ura3* [pKR4]). After sporulation, and dissection the integrated pKR20 was found to be completely linked to the *LST1* locus: sectoring segregated 2:2 and all sectored colonies were Ura^+ whereas all nonsectored colonies were Ura^-. Thus, p77-2 carries the *LST1* gene. In parallel, the genomic sequence from p21-31 (a clone in the pCT3 vector; Thompson et al., 1993) was inserted as a EcoRI/HindIII fragment into pRS306 to produce pKR7. pKR7 was integrated at its chromosomal locus after linearization with MscI and was then checked for linkage to *lst1-1*. Tetrad analysis showed that pKR7 was not linked to *LST1* and we concluded that pKR7 carries an unlinked suppressor gene.

The 3.5-kb insert of p77-2 was inserted into the XhoI site of the centromeric vector pRS316 to construct pKR17. The base sequence of this insert was determined and found to contain a single open reading frame encoding a protein of 929 amino acids. This sequence corresponds to the open reading frame YHR098c located on Chromosome VIII (Saccharomyces Genome Database, Cherry et al., 1997). The predicted amino acid sequence of *LST1* shows significant similarity to *SEC24* (YIL109C). The two proteins share 23% sequence identity that extends over most of their length (Fig. 2), suggesting that Lst1p may have a function similar to that of Sec24p as a subunit of the COPII vesicle coat.

Phenotypes of lst1Δ

One copy of the *LST1* gene in the wild-type diploid strain CKY348 was disrupted to generate a *lst1Δ::LEU2 /LST1* heterozygote. Sporulation and dissection of this diploid gave >95% spore viability on YPD medium and the *LEU2* marker segregated 2:2,

showing that *LST1* is not essential for growth. A *lst1Δ::LEU2* mutant spore clone was crossed to *sec* mutants to test for synthetic lethality. In these crosses both the temperature sensitivity of the *sec* mutation and the *lst1Δ* allele marked by *LEU2* could be followed independently. In crosses of *lst1Δ* to *sec12*, *sec13*, *sec16*, *sec23*, *sec24,* or *sec31* mutants, inviability segregated as a two-gene trait (segregation patterns for dead:viable spore clones were 2:2, 1:3, and 0:4). Tests of the genotype of the surviving sister spore clones showed that the inviable spores in these crosses were always *lst1Δ sec* double mutants. Crosses between *lst1Δ* and *sec17* or *sec18* produced viable double mutants. These findings confirmed and extended our earlier tests for synthetic lethality with *lst1-1* and demonstrated that *lst1Δ* was synthetically lethal with all the known genes required for COPII vesicle formation, but not with genes required for vesicle fusion.

We evaluated the growth of *lst1Δ::LEU2* mutants under a variety of conditions. On rich medium (YPD), the *lst1Δ::LEU2* strain grew as well as an isogenic wild-type strain at temperatures ranging from 14°C to 37°C. However, on synthetic medium with added amino acids (SMM) the *lst1Δ ::LEU2* strain grew poorly at temperatures above 30°C. Since our rich and synthetic media differed markedly in pH (SMM is pH 3.8 whereas YPD is pH 6.5), we suspected that *lst1Δ* mutants may be particularly sensitive to an acidic environment and we tested the effect of pH on the growth of *lst1Δ* mutants. Although *lst1Δ* mutants grew as well as wild-type on YPD at all temperatures, when YPD was brought to pH 3.8, *lst1Δ* mutants grew much more slowly than wild-type at 37°C (Fig. 3 *A*). Conversely, on SMM buffered to pH 6.5, the *lst1Δ* strain grew as well as wild-type even at 37°C (data not shown). These results demonstrated that at high temperature, growth of the *lst1Δ* mutant was sensitive to acidic conditions.

Having identified conditions where *LST1* was needed for growth, we investigated whether overexpression of *SEC24* could supply the function lost in *lst1Δ*. Some restoration of function was indicated by the ability of a *lst1Δ* mutant grow on acidic medium when provided with extra copies of *SEC24* provided on either centromeric or 2μ

plasmids (Fig. 3 *B*). These findings imply some functional overlap between *LST1* and *SEC24*. In parallel tests for suppression, we found that the genes *SEC12*, *SEC13*, *SEC31*, or *SEC23* when expressed from 2μ plasmids could not restore the ability of a *lst1Δ* mutant to grow on acidic medium. As discussed below, we found that the *lst1Δ* mutation caused a selective defect in the trafficking of Pma1p from the ER and we also examined the ability of overexpressed *SEC24* to suppress this phenotype caused by the *lst1Δ* mutation. By immunofluorescence microscopy, the proper localization of Pma1p to the cell surface was restored in a *lst1Δ* strain that also carried *SEC24* on a 2μ plasmid (Fig. 5).

In an attempt to test the effect of overexpression of *LST1*, we found that *LST1* on a 2μ plasmid severely impaired growth of wild-type yeast cells. To examine the response of cells to different doses of Lst1p, we designed a way to express different levels of Lst1p according to the amount of galactose in the growth medium. A wild-type strain (CKY473) carrying a plasmid that expressed *LST1* from *pGAL1* (pKR35) was spread on a SMM plate with 2% raffinose, a carbon source that allows yeast growth without repression of the *GAL1* promoter. When these cells are exposed to a gradient of galactose concentrations, from 3 mg of galactose in a filter disk on top of the lawn, growth was inhibited in a halo 1.5 cm beyond the edge of the filter (Fig. 3 *C*). A strain that did not contain pKR35 grew uniformly up to the edge of the filter showing that the galactose itself was not inhibitory. Given the similarity of Lst1p to Sec24p, we asked whether the overexpression of *SEC24* could compensate for overexpression of *LST1*. Cells carrying both the *pGAL1-LST1* plasmid (pKR35) and the *SEC24* gene on a 2μ plasmid (pKR41) were tested in an identical halo assay and were found to be resistant to the effect of galactose (Fig. 3 *C*). Suppression by SEC24 appeared to be specific, since parallel tests of 2μ plasmids carrying *SEC12*, *SEC13*, *SEC31*, or *SEC23* failed to show suppression. (It is worth noting that *SEC23* expressed from a 2μ plasmid significantly slows the growth of our yeast strains. So any suppression afforded by overexpression of

SEC23 might be counteracted by this inherent toxicity of *SEC23*.) A simple conclusion that can be drawn from these overexpression studies is that too great of a stoichiometric excess of Lst1p over Sec24p is lethal. This observation can be explained if Lst1p and Sec24p compete with one another in the assembly of vesicle coat complexes and that excess Lst1p causes sequestration of vesicle components into complexes that fail to satisfy some essential function of COPII.

lst1Δ diminishes the activity of the plasma membrane proton-ATPase

The sensitivity of *lst1Δ* mutants to low pH suggested the involvement of Pma1p, which has been shown to be the limiting cell component for growth on acidic medium (McCusker et al., 1987; Portillo and Serrano, 1989). The dependence of Pma1p activity on *LST1* was supported by the observation that *lst1Δ* mutants exhibited an unusual morphology characteristic of *pma1* mutants. When *lst1Δ* mutants were grown at low pH (SMM or YPD brought to pH 3.8) and at 37°C, about 10% of the cells formed multi-budded rosettes; in some cases, as many as 15 daughters radiated from a single large mother cell (Fig. 4 *A*). The unseparated daughter cells contained nuclei that could be stained with DAPI and could be separated from their mothers by micromanipulation, indicating they had completed cytokinesis. Cells depleted of Pma1p produce similar multi-budded cells with attached daughters that had completed cytokinesis. In this case, multi-budded rosettes are thought to form because a mother cell formed with sufficient Pma1p in the plasma membrane will continue to bud, whereas daughter cells formed after Pma1p transport is compromised will have insufficient Pma1p to form buds themselves (Cid et al., 1987). The morphology of *lst1Δ* cells grown at relatively high pH (YPD or SMM buffered to pH 6.5) at 37°C appeared normal, with few cells having more than one attached daughter.

As a more direct test of the effect of *lst1Δ* on the activity of Pma1p, we measured the capacity of mutant cells to pump protons into the external medium. Wild-type and *lst1Δ* strains were cultured in YPD at 37°C, conditions under which both strains grow equally well. After starvation by prolonged incubation in water, the cells were placed in a weakly buffered medium and proton efflux on addition of glucose was measured as a drop in extracellular pH. For both wild-type and *lst1Δ* strains, addition of glucose produced a sharp decline in pH (after a 30 second lag) which began to level off after about 5 minutes (Fig. 4 *B*). Although the response of wild-type and *lst1Δ* cells were qualitatively similar, proton efflux from *lst1Δ* cells was compromised: in the first 5 minutes after addition of glucose the rate of change in pH produced by the *lst1Δ* mutant was 65% of that of wild-type. These findings indicate that the *lst1Δ* mutant grown at 37°C, has about half of the Pma1p activity as wild-type cells.

LST1 is required for efficient transport of Pma1p from the ER to the Golgi

To determine whether the reduced Pma1p activity in *lst1Δ* mutants was due to a defect in the transport of Pma1p to the cell surface, we compared the localization of Pma1p in wild-type and *lst1Δ* mutant cells by immunofluorescence microscopy. Cells grown at 30°C in YPD medium to avoid possible secondary effects due to the pH sensitivity of *lst1Δ* mutants. In *lst1Δ* cells, Pma1p was primarily at the nuclear periphery and at the cellular rim, indicating that a large proportion of Pma1p remains in the ER (Fig. 5). This pattern of localization differed markedly from the surface localization of Pma1p in wild-type cells incubated at 30°C (Fig. 5), or in *lst1Δ* cells incubated at 24°C (data not shown).

We also examined the subcellular distribution of Pma1p in *lst1Δ* cells by cell fractionation. Lysates from cells grown at 37°C for three hours were fractionated on sucrose density gradients under conditions where the ER and plasma membrane are well separated on the basis of their buoyant density. Pma1p from wild-type cells was located

in dense fractions of the gradient, in a peak that was coincident with that of Gas1p, a GPI-linked plasma membrane protein (Nuoffer et al., 1991). In contrast, less than 35% of the total Pma1p from *lst1Δ* cells coincided with the plasma membrane marked by Gas1p protein, and the majority of Pma1p was located in fractions containing the ER (Fig. 6). Interestingly, the ER from *lst1Δ* mutants (marked by Sec61p) reproducibly resolved into two peaks of different density, suggesting that accumulation of Pma1p segregates ER membranes into subdomains of relatively high and low density. Given that most of the Pma1p was located in the ER peak of higher density, it is possible that the density of the ER had been increased because of the accumulation of Pma1p. A similar increase in density of a portion of the ER is caused when folding mutants of *PMA1* are retained within the ER (Harris et al., 1994).

The fact that transport of Pma1p, but not of Gas1p, was affected by deletion of *LST1* suggested that *LST1* may be specifically required for the export of Pma1p from the ER. The absence of a general protein secretion defect in *lst1Δ* mutants was implied by the normal growth of *lst1Δ* mutants at 37°C in medium of pH 6.5 (the doubling time of both *lst1Δ* and wild-type was 1.75 hours in YPD), indicating a normal rate of expansion of the plasma membrane. As a specific test for the rate of ER to Golgi transport, pulse-chase experiments were performed to follow the rate of maturation of invertase from its core glycosylated ER form to the Golgi and secreted form. No delay in invertase transport was observed in *lst1Δ* mutants that had been grown at 37°C for three hours, conditions that caused the accumulation of Pma1p (Fig. 7). Similarly, no defect in the maturation of carboxypeptidase Y (CPY) from the ER form to the Golgi and vacuolar forms of the enzyme could be detected (data not shown).

We also considered the possibility that transport of Pma1p may be particularly sensitive to any subtle defect in vesicle formation. We addressed this possibility by examining the localization of Pma1p in *sec24-1* and *sec31-2* mutant cells at the semi-permissive temperature of 28°C. Although the growth rate of both mutants was

compromised at this temperature (doubling time on rich medium of 2.9 hours for *sec24*, and 2.4 hours for *sec31* as compared to 1.7 hours for wild-type), no accumulation of Pma1p was detected in the perinuclear region of either mutant by immunoflouresence microscopy (data not shown). Thus partial defects in COPII functions did not lead to the extensive accumulation of Pma1p in the ER that was observed for *lst1Δ* mutants. Taken together, comparisons between the *lst1Δ* mutation and COPII gene mutations indicate that the *lst1Δ* mutation is unusual in its ability to inhibit Pma1p exit from the ER without interfering with the transport of other cargo proteins.

Localization of Lst1p

To examine the intracellular distribution of Lst1p, an epitope tagged derivative was constructed by inserting six copies of the ten amino acid hemagglutinin epitope (HA) near the N-terminus of Lst1p. The HA-tagged *LST1* was functional as demonstrated by its ability to complement *lst1-1* in a sectoring assay and to restore the ability of a *lst1Δ* mutant to grow on acidic medium at 37°C (not shown). In cells expressing Lst1p-HA that were fixed for immunofluorescence microscopy, staining was found primarily at the nuclear periphery (Fig. 8). No signal was seen in cells expressing untagged Lst1p, verifying that the origin of the staining pattern was due to Lst1p-HA. Although Lst1p-HA staining largely coincided with the ER marker Kar2p, there were subtle differences in their patterns of localization: Kar2p appeared in a relatively uniform distributed around the nuclear periphery whereas Lst1p-HA staining had a more punctate appearance indicating that Lst1p might be concentrated in particular regions of the ER. In addition, weak punctate staining was observed throughout the cell body, some of which may correspond to ER membranes near the cell periphery.

The intracellular distribution of Lst1p was also examined by subcellular fractionation. Cells expressing Lst1p-HA were converted to spheroplasts and then gently

lysed. This cell lysate was subjected to differential centrifugation and most of Lst1p-HA was found to pellet at either 500 *g* or 10,000 *g* (Fig. 9 *A*). All of the soluble marker protein Gdh2p (Miller and Magasanik, 1990), was found in the 150,000 *g* supernatant fraction, indicating complete cell lysis (data not shown). The association of the Lst1p protein with the sedimenting fraction was analyzed by chemical treatment of cell lysates before centrifugation at 50,000 *g*. Incubation of cell extracts in 1% Triton X-100, 2.5 M urea, 100 mM sodium carbonate (pH 11.5), or 500 mM NaCl, resulted in the release of a portion of the Lst1p-HA into the soluble fraction (Fig. 9 *B*). The partial dissociation of Lst1p-HA from the sedimenting fraction by these agents suggested that Lst1p is a peripheral membrane protein that adheres tightly to the membrane.

Lst1p binds Sec23p

Sec24p was first identified as a protein that formed a 400-kD complex with Sec23p (Hicke et al., 1992). Because of the similarity of Lst1p to Sec24p we investigated whether Lst1p could also bind to Sec23p. To assay potential interactions by the yeast two-hybrid assay, *LST1* was fused to the *lexA* DNA-binding domain (pKR37), and *SEC23* was fused to an acidic activation domain (pPE81). Interaction between the two fusion proteins was tested by assaying for activation of a *lacZ* reporter gene. Induction of β-galactosidase was observed when the *LST1* and *SEC23* fusions were co-expressed, but not when either was expressed alone (Table IV). The level of induction caused by interaction of *LST1* and *SEC23*, was similar to that seen for interaction of *SEC24* and *SEC23* (Gimeno et al., 1996).

To confirm the interaction between Lst1p and Sec23p, association of these proteins was examined in yeast cell extracts. The coding sequence of *LST1* (codons 14-927) was fused to GST and expressed in yeast from the *pGAL1* promoter. *SEC23* was also expressed from *pGAL1*. Since both proteins are largely associated with intracellular

membranes (see Fig. 10 *B*), membranes prepared from cells overexpressing both Sec23p and GST-Lst1p were first extracted with 600 mM NaCl to release protein complexes from the membrane, then the salt extracts were clarified by centrifugation at 90,000 *g* and diluted to give a final concentration of 200 mM NaCl. GST-Lst1p was isolated from the extracts by affinity to glutathione Sepharose beads. Sec23p was found in association with GST-Lst1p, but not in control extracts prepared from cells expressing Sec23p and GST alone (Fig. 10 A). Together these experiments show that Lst1p, like Sec24p, can form a complex with Sec23p.

Sec23p and Sec24p have been shown to assemble onto the ER membrane as a complex (Matsuoka et al., 1998). While working out conditions to optimize recovery of Sec23p bound to GST-Lst1p, we discovered that assembly of an Lst1p-Sec23p complex appears to enhance the association of both proteins with the ER membrane. When both GST-Lst1p and Sec23p were overexpressed in the same cell, more than 60% of the Sec23p, and 70% of the GST-Lst1p were found in a fraction that pelleted at 10,000 *g* (Fig. 10 *B*). This pellet contains most of the ER as marked by the ER membrane protein Sec61p (data not shown). When material that pelleted at 10,000 *g* was suspended in 60% sucrose and applied to the bottom of a sucrose density gradient, more than 90% of the GST-Lst1p and Sec23p cofractionated with the ER resident membrane protein, Sec61p, at a density corresponding to 45% sucrose, showing that GST-Lst1p and Sec23p were associated with membranes (data not shown). In contrast to the case when Sec23p and GST-Lst1p were expressed together, less than 10% of the Sec23p pelleted at 10,000 *g* in lysates from a strain overexpressing Sec23p alone. Similarly, less than 20% of the GST-Lst1p pelleted at 10,000 *g* in lysates from a strain expressing GST-Lst1p alone (Fig. 10 *B*). Thus, when either Sec23p or GST-Lst1p were overexpressed alone, most of the overexpressed protein was soluble, but when both proteins were expressed together, most of the proteins were associated with the ER membranes. These data support the

observation that Lst1p can form a complex with Sec23p, and that the Lst1p-Sec23p complex has affinity for ER membranes.

Discussion

By screening for mutants that exhibited synthetic lethal genetic interactions with the COPII mutation *sec13-1*, we identified the *LST1* gene. Subsequent genetic tests showed that *lst1Δ* is lethal when combined with mutations in genes required for COPII vesicle budding from the ER (*SEC12*, *SEC13*, *SEC16*, *SEC23*, *SEC24*, and *SEC31*), but *lst1Δ* is not lethal when combined with mutations in genes that are required for vesicle fusion with the Golgi (*SEC17* and *SEC18*). This pattern of genetic interactions indicated that *LST1* participates in the process of vesicle budding from the ER, an expectation that was born out by the examination of the *LST1* gene and its product. The following observations, reported here, indicate a role for Lst1p as part of a COPII-like vesicle coat: (i) *LST1* encodes a 90 kD protein that is homologous to the COPII-coat subunit Sec24p. The two proteins share 23% amino acid identity over their entire lengths. (ii) Lst1p is a peripheral ER membrane protein as shown by immunofluorescence microscopy and cell fractionation. (iii) Lst1p, like Sec24p, can bind to Sec23p as shown by tests for two-hybrid interaction and affinity purification of a complex of GST-Lst1p and Sec23p. (iv) Assembly of the Sec23p-Lst1p complex appears to enhance the membrane association of both Lst1p and Sec23p: when both proteins are overexpressed together most of both proteins associate with membranes, whereas either protein overexpressed alone is mostly cytosolic. (v) Although strains with chromosomal deletion of *LST1* are viable and appear normal for secretion of marker proteins, these mutants show a pronounced accumulation of the plasma membrane protein Pma1p in the ER, indicating a selective defect in ER to Golgi traffic. Based on these findings we propose that Lst1p takes the place of Sec24p in a specialized COPII coat complex that is used for the recruitment of Pma1p into vesicles.

Strains carrying *lst1Δ* have the phenotypic hallmarks of a deficiency in Pma1p activity, including sensitivity to growth in an acidic environment, the formation of multi-budded cells, and a decreased rate of proton efflux from intact cells. All three traits are

expressed only at temperatures of 30°C and above, indicating that *LST1* is only required for Pma1p activity at high temperature. Localization of Pma1p in *lst1Δ* cells by immunofluorescence and sucrose density cell fractionation demonstrate that the transport of Pma1p from the ER is compromised in *lst1Δ* at 37°C.

Export of Pma1p from the ER can not be completely dependent on Lst1p, since Pma1p transport appears normal in *lst1Δ* mutants at 24°C. Even at 37°C, the block in Pma1p transport may not be complete since about 35% of the total Pma1p fractionates with the plasma membrane – although some of the Pma1p detected in the plasma membrane in this experiment was probably synthesized before the shift to restrictive temperature. It therefore seems likely that Lst1p and Sec24p share the burden of transporting Pma1p from the ER. At 24°C it appears that Sec24p (or some other protein) can compensate for the absence of Lst1p, but at temperatures of 30°C or higher compensation is no longer possible unless extra copies of Sec24p are provided by expression from a multi-copy plasmid.

The transport defect caused by deletion of *LST1* appears to be specific for Pma1p. Under conditions where a defect in Pma1p transport was observed in *lst1Δ* mutants transport of Gas1p, carboxypeptidase Y, and invertase was unaffected. Using growth as a more general assay for trafficking defects, we found that *lst1Δ* mutants grew at an identical rate to wild-type at 37°C when we compensated for the defect in Pma1p transport by using media at pH 6.5. This indicates rate of expansion of the plasma membrane, including the transport of all essential plasma membrane proteins, is not significantly affected by the absence of *LST1*.

We also considered the possibility that there may be differences among cargo molecules in their response to general defects in the protein transport machinery. Of particular concern, was the possibility that Pma1p transport might be particularly sensitive to slowed ER to Golgi transport such that a defect in transport that was too subtle to have an effect on our standard marker proteins might have a significant effect on

the rate of transport of Pma1p. If this were the case, then partial defects in other COPII components should also interfere with Pma1p transport. We therefore examined *sec24* and *sec31* mutants, but could find no evidence for a defect in Pma1p transport even at semi-permissive temperatures where the rate of growth was inhibited. Although Pma1p was the only essential protein for which we could detect a transport defect in *lst1* mutants, a defect in the transport of any nonessential protein could have been overlooked by our analysis.

Factors required for the transport of specific membrane proteins have been documented in a number of other cases. The *SHR3* gene encodes an ER resident protein that is required for the transport of amino acid permeases out of the ER, but is not required for the transport of a variety of other proteins (Ljungdahl et al., 1992; Kuehn et al., 1996). A set of ER proteins, Vma12p, Vma21p, and Vma22p, are required for transport from the ER of the integral membrane subunit of the vacuolar ATPase (Hill and Stevens, 1994, 1995; Jackson and Stevens, 1997). Similarly, mutational studies have shown that the small ER membrane protein Erv14p is specifically required for transport of the plasma membrane protein Axl2p out of the ER (Powers and Barlowe, 1998). Finally, Ast1p has been suggested to be a factor specifically needed for the transport of Pma1p from the Golgi to the plasma membrane (Chang and Fink, 1995). In all of these cases the question remains whether Shr3p, the Vma proteins, Erv14p, or Ast1p act directly in vesicular transport of their respective cargo molecules, or whether they are primarily involved in protein folding and influence protein sorting indirectly through quality control mechanisms. Because Lst1p appears to be a component of a vesicle coat, Lst1p seems more likely to have a direct role in the sorting of Pma1p rather than in its folding.

Expression of a variety of dominant *PMA1* mutations can cause accumulation of both mutant and wild-type Pma1p in proliferated ER (Harris et al., 1994; Portillo, 1997). Similarly, the transport of wild-type Pma1p from the ER is blocked when *PMA2* (an

isoform of *PMA1*) or plant plasma membrane H^+-ATPases are overexpressed in yeast (Supply et al., 1994; Villalba et al., 1992; de Kerchove d'Exaerde, et al., 1995). One proposal was that a special factor may be required for the transport of Pma1p from the ER in a manner analogous to the requirement for Shr3p in the transport of amino acid permeases (Supply et al., 1994). The specific role of Lst1p in the transport of Pma1p suggests that it may be the factor depleted by the expression of dominant forms of Pma1p. In the future, it may be possible to test this idea by evaluating the ability of Lst1p overexpression to reverse the effects of dominant *PMA1* mutations.

The mechanism by which Lst1p acts in the transport of Pma1p may be inferred from recent studies examining the recruitment of cargo molecules into COPII vesicles. Using ER-derived microsomes and purified COPII components, Kuehn et al. have shown that the Sec23p/Sec24p complex, along with Sar1p, associate with amino acid permeases and other integral membrane protein that are destined for the plasma membrane (Kuehn et al., 1998). In parallel experiments using mammalian microsomes, mammalian Sec23p/Sec24p and Sar1p, were found to bind to microsomal membranes and form a complex that contains the cargo protein VSV-G (Aridor et al., 1998). The conclusion from both experimental systems is that the Sec23p/Sec24p complex contains specific binding sites for the capture of membrane cargo proteins within the plane of the ER membrane. Based on the data presented here, Lst1p appears to be an isoform of Sec24p that is adapted for selection of Pma1p. This provides the first evidence that Sec24 family members carry information specifying the type of cargo molecules that are accepted by ER-derived vesicles.

We have looked for association of Lst1p with ER derived vesicles, but under the conditions of an *in vitro* budding reaction a large quantity of Lst1p-HA is released from the membrane in soluble form. Soluble Lst1p-HA gives a high background in vesicle fractions preventing us from reliably determining whether there is a specific association of Lst1p with vesicles. In future experiments, it may be possible to isolate vesicles

coated with Lst1p by performing an *in vitro* budding reaction using purified cytosolic components including a purified complex of Lst1p and Sec23p. It may also be possible to determine whether vesicles that are formed using a Sec23p/Lst1p complex more efficiently incorporate Pma1p than vesicles formed using the Sec23p/Sec24p complex. Finally, it will be of interest to determine if there is direct binding of Lst1p to Pma1p.

The identification of a Sec24p homolog that also acts in transport from the ER raises the possibility that the coats of ER-derived vesicles may be heterogeneous. It is possible that Sec23p/Lst1p complexes act to form a class of vesicle that is distinct from those formed by Sec23p/Sec24p complexes. Alternatively, it is possible that the two complexes assemble together forming vesicles with coats of mixed composition. The identification of additional homologs of Sec23p and Sec24p suggest the existence of coats with even greater combinatorial complexity. We have identified a third Sec24p family member which we call Iss1p, as a protein that binds to Sec16p. Iss1p (YNL049c) also binds Sec23p and appears to be associated with the ER membrane (Gimeno, 1996). In addition, the *Saccharomyces* genome contains an uncharacterized ORF (YHR035w) that is 21% identical to Sec23p (Saccharomyces Genome Database, Cherry et al., 1997). If each of the Sec23p and Sec24p homologs carry different determinants for cargo selection, and if mixed coats can form, the possible combinations of Sec23p and Sec24p homologs should allow the formation of a wide variety of COPII-like vesicles with different capacities to carry different cargo molecules.

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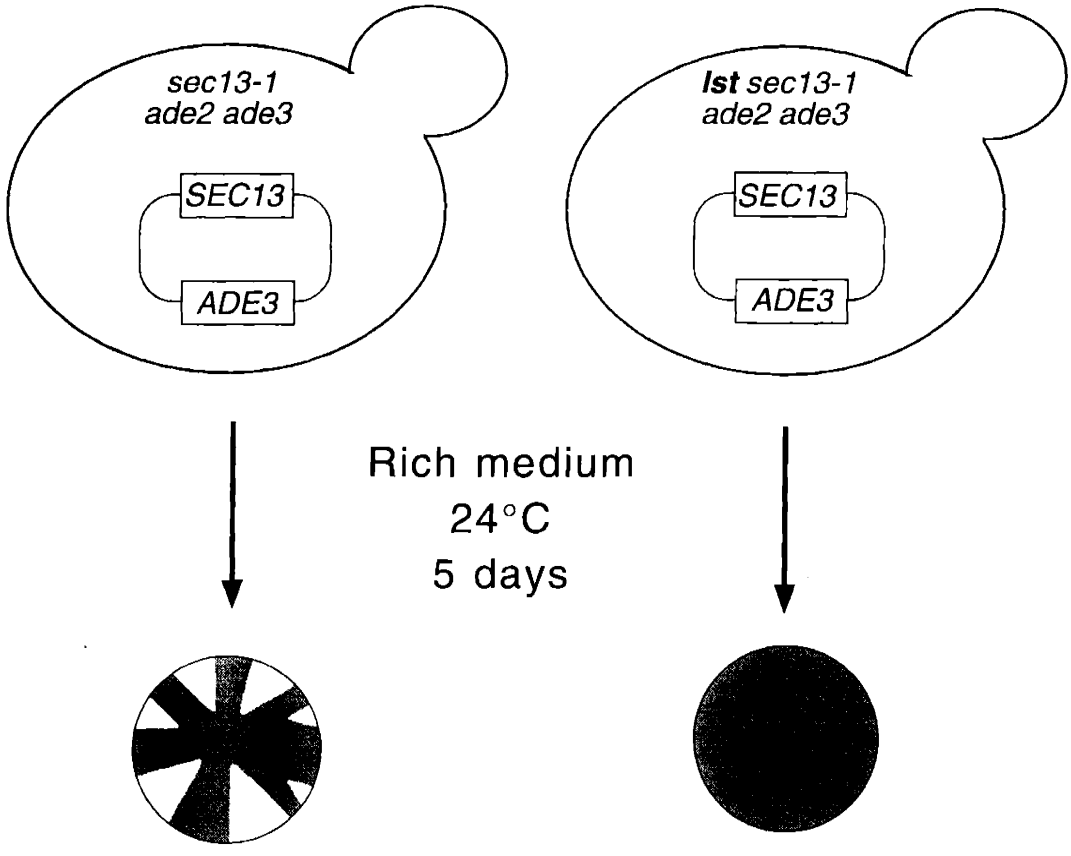
Figure 1. Colony-sectoring screen for mutations that are lethal with *sec13-1*. CKY423 (*ade2 ade3 leu2 ura3 sec13-1* [pKR4: *SEC13*, *ADE3*]) can lose the plasmid pKR4 when grown at 24°C on YPD, to give *ade2 ade3* segregants that form white sectors within a red colony. Mutagenized cells that have acquired a *lst* mutation can not grow without the pKR4 plasmid and form nonsectoring, solid red colonies. Of 132 nonsectoring colonies, the sectoring in 57 was restored by transformation with a second *SEC13* bearing plasmid (pKR1).



78,000	Colonies screened
139	Non-sectoring colonies
57	*SEC13* dependent

Figure 2. Comparison of *LST1* and *SEC24* sequences. Identities are indicated by solid lines and similarities are indicated by dotted lines. The overall amino acid identity is 23%.

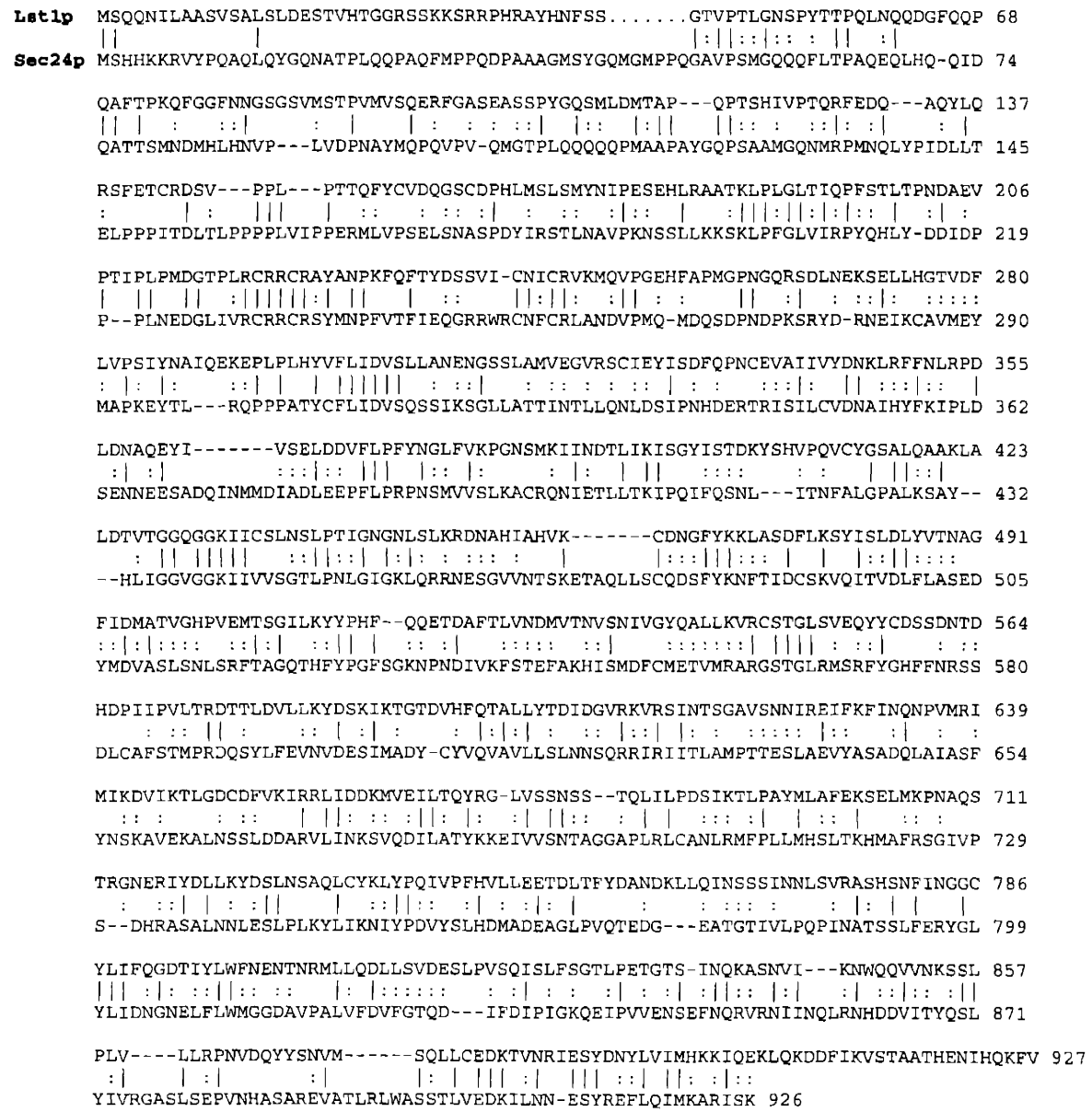

Lst1p
Sec24p

Figure 3. Functional relationships between *LST1* and *SEC24*. (*A*) Sensitivity of *lst1Δ* mutants to acidic medium. Equal numbers of wild-type (CKY443) or *lst1Δ ::LEU2* (CKY534) cells were spotted onto YPD medium (pH 6.5), or acidic YPD medium (brought to pH 3.8 by the addition of HCl). Plates were photographed after incubation at 37°C for two days. (*B*) A *lst1Δ::LEU2* strain (CKY552) was transformed with: vector only (pRS316), *LST1* on a centromeric plasmid (pKR17), *SEC24* on a centromeric plasmid (pAF70), or *SEC24* on a 2μ plasmid (pKR34) and streaked onto YPD medium (pH 3.8) and colonies were photographed after growth at 37°C for two days. (*C*) A wild-type strain (CKY473) was transformed either with a plasmid carrying *pGAL1 - LST1*(pKR35) and vector control (pRS425), or with pKR35 and *SEC24* on a 2μ plasmid (pKR41). Transformants were plated at a density of 800 cells/cm^2 on SMM plates containing 2% raffinose then 3 mg galactose solution was placed on a sterile 1 cm filter on top of the lawn. The plates were photographed after growth at 30°C for two days.

lst1Δ
pRS316

lst1Δ
[CEN LST1]

lst1Δ
[2μ SEC24]

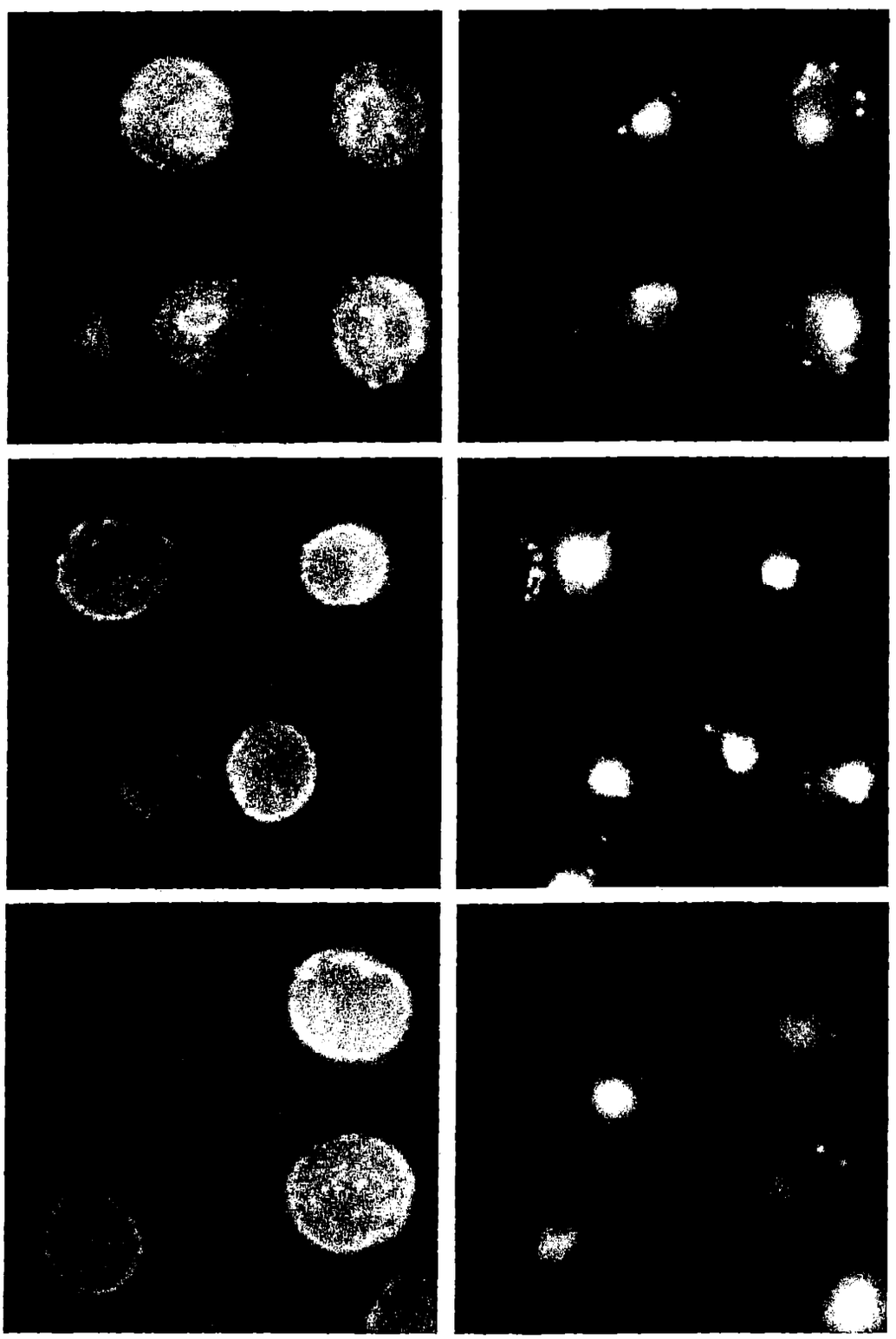
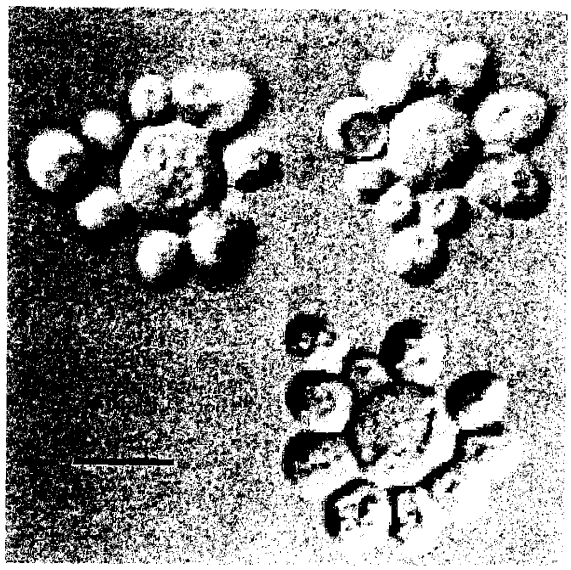


Figure 4. Plasma membrane proton ATPase defects caused by *lst1Δ*. (*A*) *lst1Δ* cells (CKY534) were photographed using differential interference contrast microscopy after growth at 37°C on YPD (pH 3.8). A montage of multi-budded cells is shown. Cells of this type comprise about 10% of a *lst1Δ* culture, but are never seen for wild-type grown under the same conditions. The bar represents 10 μm. (*B*) Reduced capacity for proton pumping by *lst1Δ* cells. Wild-type (CKY443) and *lst1Δ* (CKY536) were grown to exponential phase in YPD medium (pH 6.8) at 37°C. Cells were incubated in water overnight and then suspended in 10 mM glycine buffer at pH 4.0. Proton efflux from the cells after addition of glucose was recorded as a decrease in the pH of the external medium. Based on the average rate of change in pH over the first 5 minutes after glucose addition, *lst1Δ* cells exhibited 65% the rate of proton efflux as wild-type.

A



B

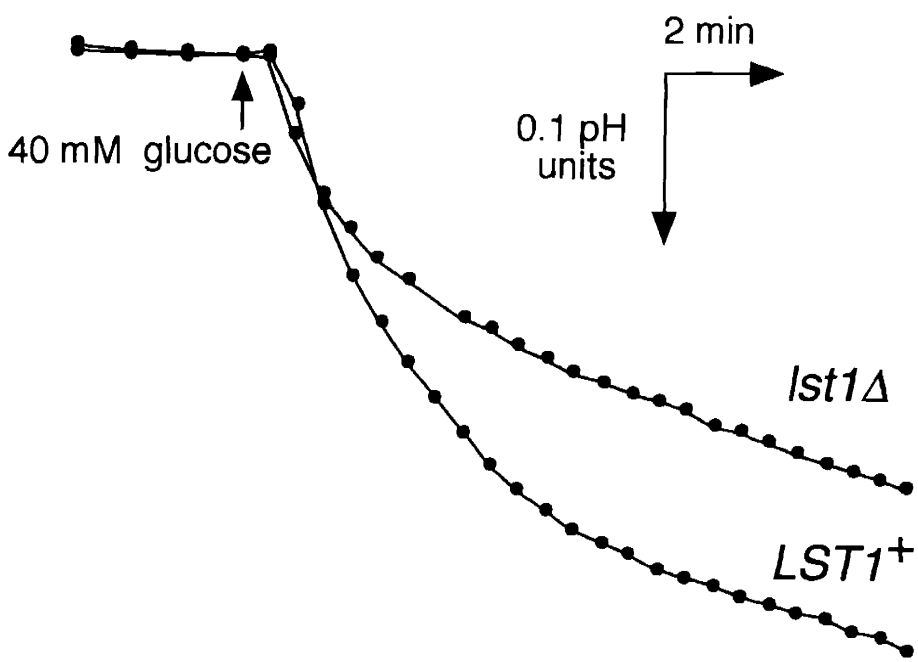


Figure 5. Pma1p accumulates the ER in *lst1Δ* cells and this accumulation is suppressed by overexpression of *SEC24*. Cells grown in SMM at 30°C were fixed with formaldehyde and then stained for immunofluorescence microscopy with affinity purified anti-Pma1p antibody and FITC-conjugated secondary antibody. The same fields of cells stained with DAPI to label the nuclear DNA are also shown. The top panels are a montage of *lst1Δ* cells (CKY536 carrying the empty vector pRS316); the middle panels are genotypically wild type cells (CKY536 carrying the *LST1* plasmid pKR17); the bottom panels are *lst1Δ* cells suppressed by SEC24 (CKY536 carrying the 2μ SEC24 plasmid pKR34). The bar represents 5 μm.

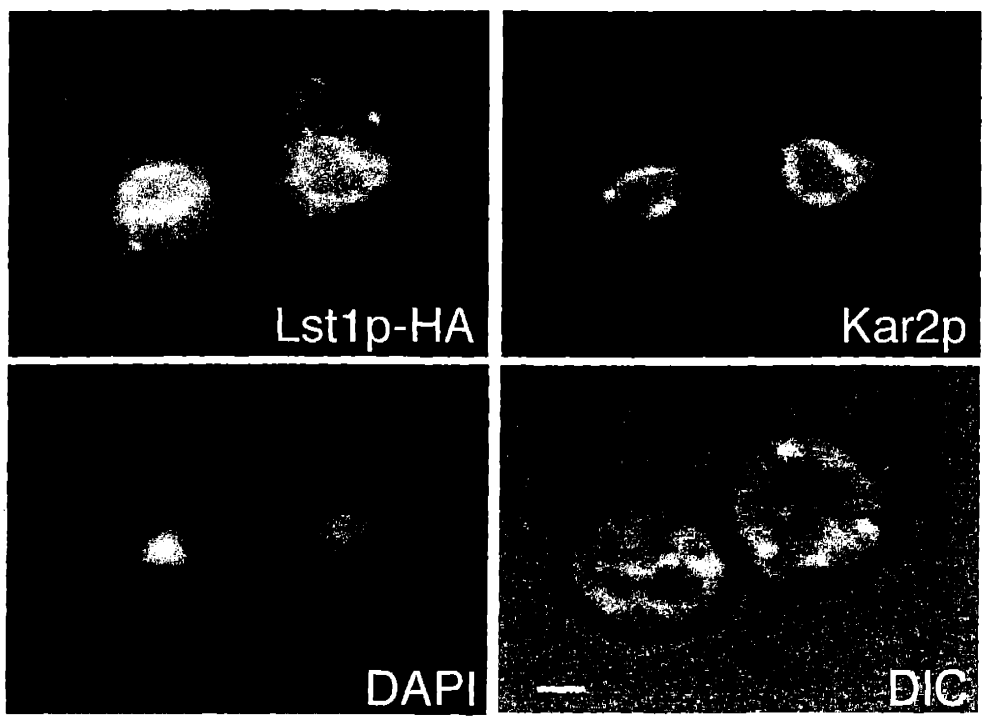

Lst1p-HA
Kar2p
DAPI
DIC

Figure 6. Cell fraction to localize Pma1p in *lst1Δ* cells. Wild-type (CKY443) and *lst1Δ* (CKY536) cells were grown in YPD at 24°C and then were shifted to 37°C for three hours. Cell lysates were fractionated on density gradients of 20-60% sucrose. Relative levels of Pma1p, Gas1p (plasma membrane marker), and Sec61p (ER marker) in each fraction were quantitated by immunoblotting and densitometry. GDPase (Golgi marker) was determined by enzymatic assay.

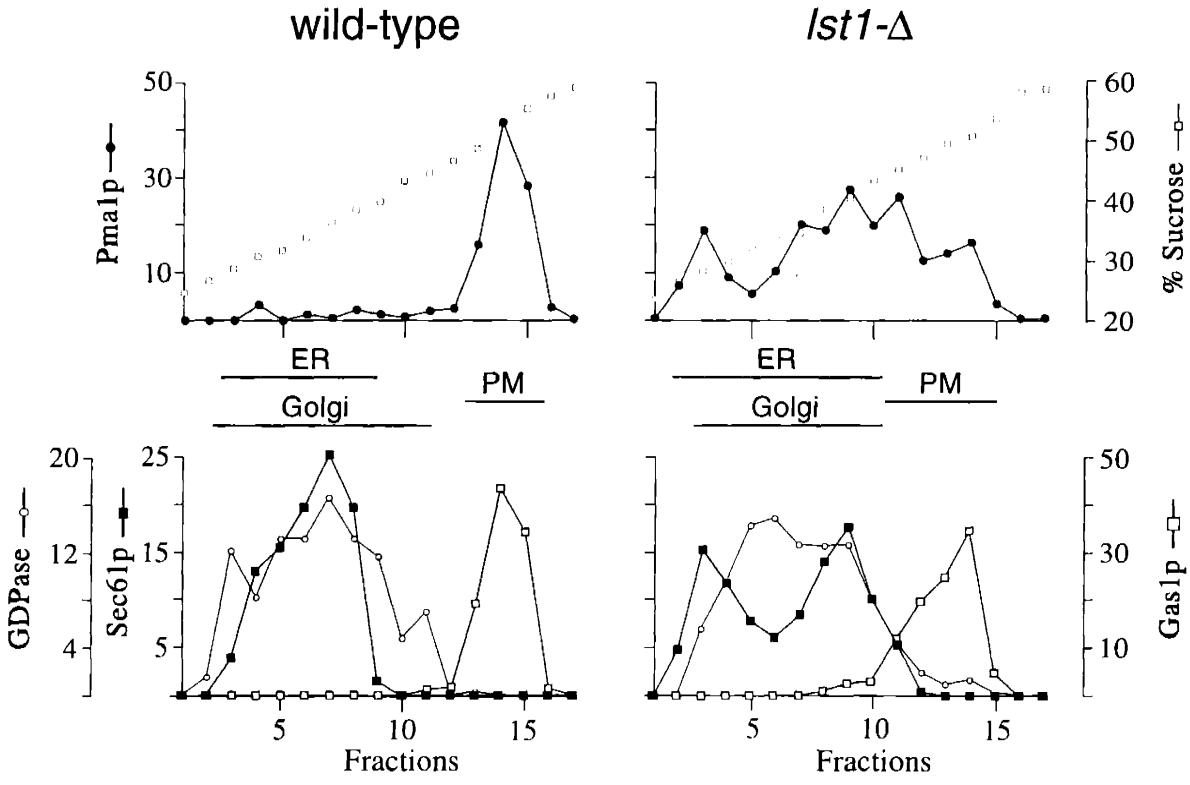

wild-type
lst1-Δ
Pma1p
% Sucrose
ER
Golgi
PM
GDPase
Sec61p
Gas1p
Fractions
Fractions

Figure 7. Transport of invertase is not affected by *lst1-Δ*. Wild-type (CKY540), *lst1Δ* (CKY542) and *sec12-4* (CKY541) strains expressing invertase from the constitutive *pTPI1-SUC2* fusion, were grown to exponential phase at 24°C in SC medium (pH 6.5) without methionine. Wild-type and *lst1Δ* strains were shifted to 37°C and grown for 3 hours, and the *sec12-4* (CKY541) strain was shifted to 37°C five minutes before labeling. Cells were pulse labeled with [^{35}S] methionine and cysteine for 5 minutes and then chased by the addition of an excess of unlabeled methionine and cysteine. Invertase was immunoprecipitated from labeled extracts and resolved by SDS-PAGE. The positions of the core-glycosylated ER form and the mature Golgi and secreted forms of invertase are indicated.

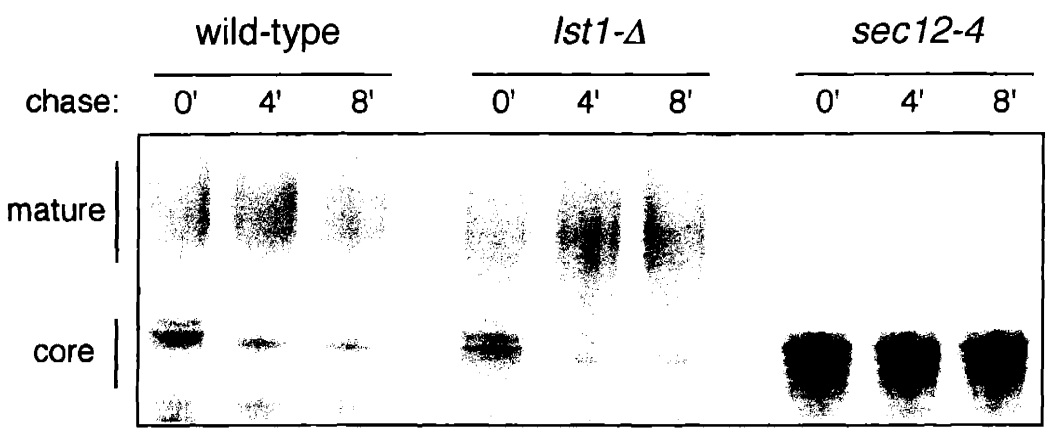

wild-type
lst1-Δ
sec12-4
chase:
0'
4'
8'
0'
4'
8'
0'
4'
8'
mature
core

Figure 8. Immunolocalization of Lst1p-HA. CKY535 (*MATa lst1Δ::LEU2 leu2-3,112 ura3-52* [pKR17HA]), expressing Lst1p-HA from a centromeric plasmid, was fixed and labeled with mouse anti-HA and FITC-conjugated anti-mouse antibodies and with rabbit anti-Kar2p and rhodamine-conjugated anti-rabbit antibodies. Nuclear DNA was visualized by DAPI staining. Cells bodies were visualized by differential interference contrast microscopy (DIC). The bar represents 1 μm.

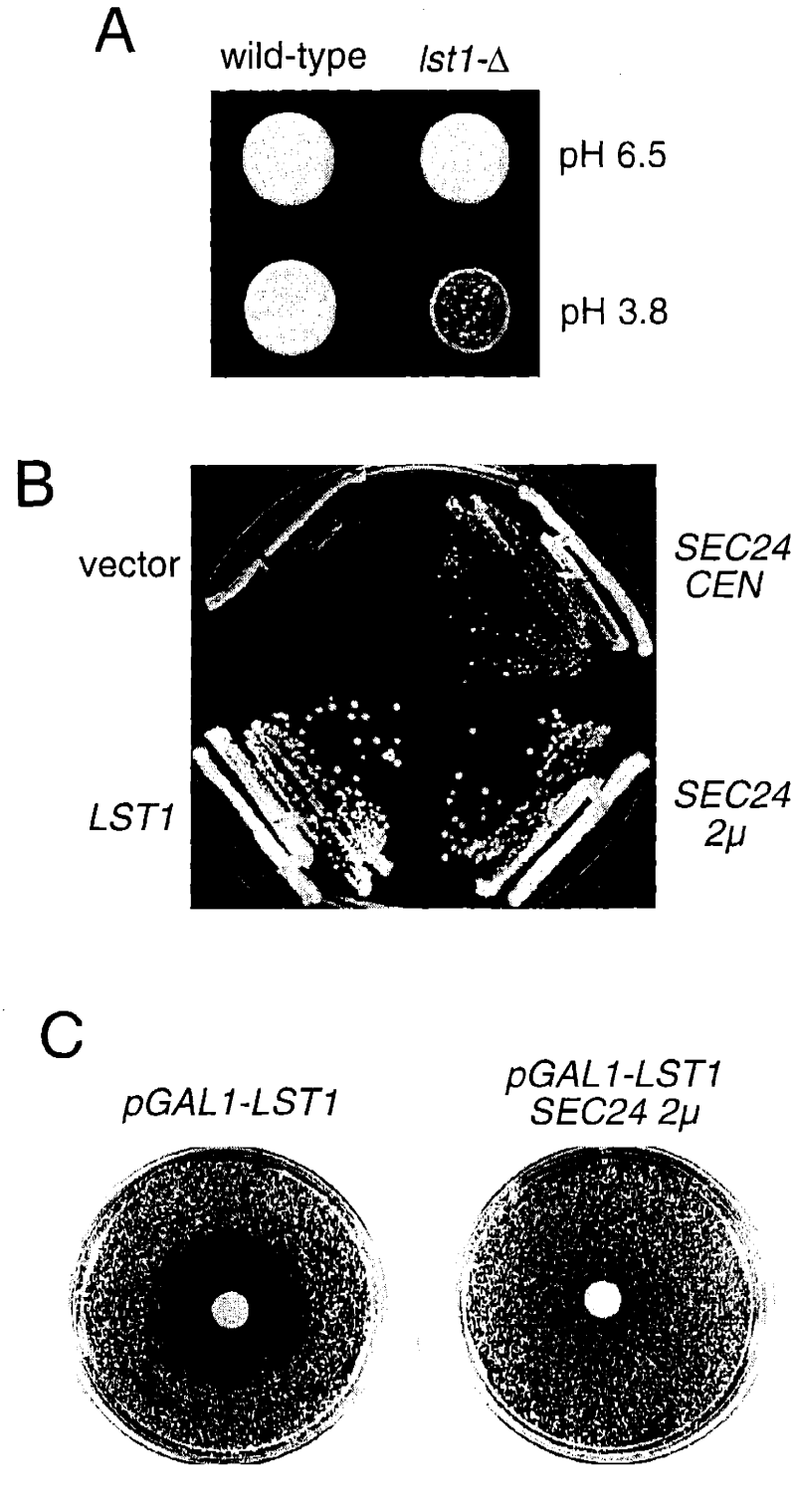

A
wild-type
lst1-Δ
pH 6.5
pH 3.8
B
vector
LST1
SEC24
CEN
SEC24
2μ
C
pGAL1-LST1
pGAL1-LST1
SEC24 2μ

Figure 9. The intracellular distribution of Lst1p. (*A*) Cells expressing Lst1p-HA from a centromeric plasmid (CKY535) were gently lysed and subjected to sequential centrifugation steps giving 500 *g*, 10,000 *g* and 150,000 *g* pellets fractions (P) and a 150,000 *g* supernatant fraction (S). Each sample contains extract from the same number of cells. (*B*) Cell lysates were treated for one hour at 4°C with either 2.5 M urea, 500 mM NaCl, 100 mM sodium carbonate (pH 11.5), or 1% Triton X-100. Pellet (P) and supernatant (S) fractions were then separated by centrifugation at 50,000 *g*. Lst1p-HA was detected by SDS-PAGE and immunoblotting with anti-HA antibody.

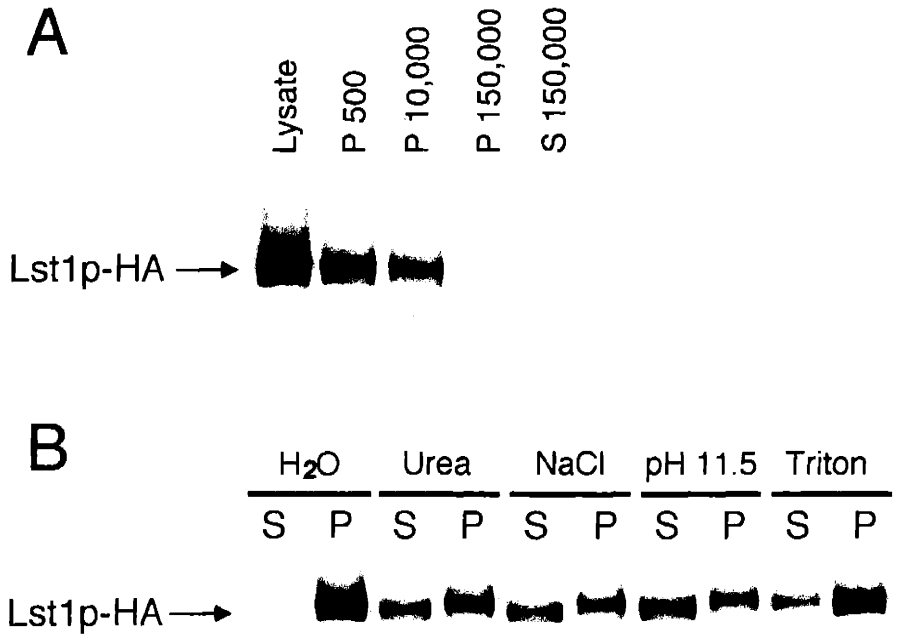
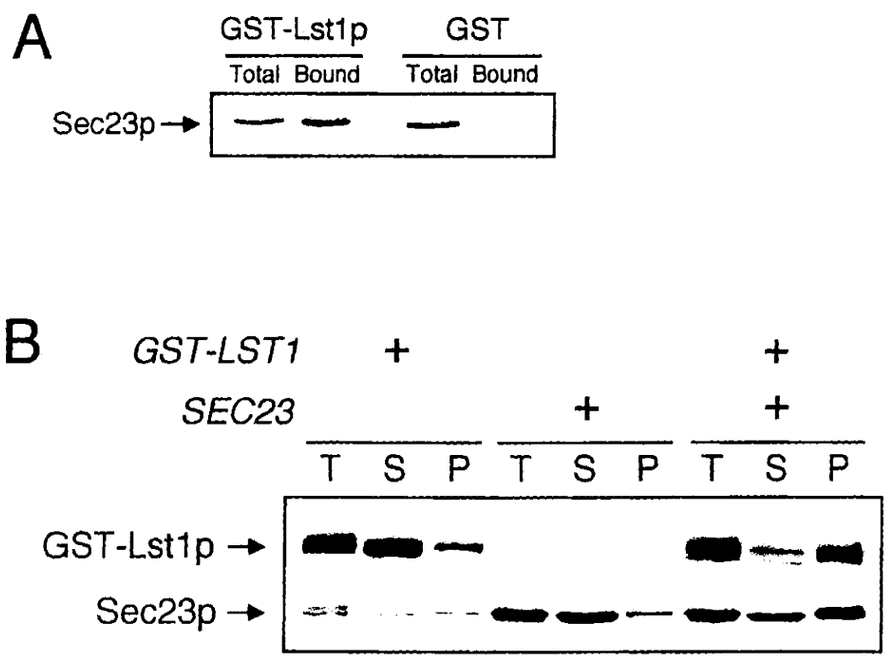

A
Lysate
P 500
P 10,000
P 150,000
S 150,000
Lst1p-HA
B
H2O
Urea
NaCl
pH 11.5
Triton
S P S P S P S P S P
Lst1p-HA

Figure 10. Lst1p-Sec23p complex is membrane associated. (*A*) Affinity isolation of Lst1p-Sec23p complexes. GST-Lst1p-HA, or GST alone, were coexpressed with Sec23p and isolated by affinity to glutathione-Sepharose beads. Proteins bound to glutathione beads were loaded in lanes 2 and 4. One-sixth of the total lysate was loaded in lanes 1 and 3. (*B*) GST-*LST1*, or *SEC23*, or both were expressed from the *GAL1* promoter. Cell lysates were cleared of cell debris by centrifugation at 300 *g* for 2 minutes. Pellet (P) and supernatant (S) fractions from cleared cell lysates were separated by centrifugation at 10,000 *g* for 30 minutes. An aliquot of the total cleared lysate (T) was removed prior to centrifugation. An equal number of cell equivalents was loaded for each sample. The GST-Lst1p-HA fusion was detected using anti-HA antibodies. For both A and B the Sec23p protein was detected using anti-Sec23p antibodies.


A
GST-Lst1p
GST
Total Bound
Total Bound
Sec23p
B
GST-LST1
SEC23
T S P T S P T S P
GST-Lst1p
Sec23p

Appendix II

Further analysis of Exp1p

Overview

This appendix represents the summary of additional experiments conducted in the course of our analysis of the *EXP1* gene product. The data described here are preliminary, and, therefore, not shown, but may be of interest to those pursuing the study of Exp1p and Lst1p.

Genetic and phenotypic analysis of EXP1

Strains deleted for *EXP1* were tested for a variety of Pma1p-related phenotypes, including sensitivity to hygromycin B and growth on low-pH medium, but were indistinguishable from wild-type strains under all conditions tested, presumably because of the efficient export of Pma1p from the ER of *lst1Δ* cells. Strains deleted for *EXP1* were also tested for defect in Gas1p maturation, but preliminary results indicated that Gas1p maturation was unaffected by the *EXP1* deletion. A sensitive assay for defects in the secretory pathway is intracellular retention of an 80 kD precursor of the cell wall protein ICWP (Elrod-Erickson, unpublished observations). While *lst1Δ* strains accumulate the ICWP precursor, *exp1Δ* strains do not. Interestingly, preliminary results indicated that overexpression of *EXP1* does not rescue the ICWP retention phenotype of *lst1Δ* strains, suggesting that the retention of ICWP is not an indirect effect of the accumulation of Pma1p in the ER.

Dominant-negative Pma1p mutants accumulate wild-type and mutant Pma1p in the ER. If Exp1p acts to accelerate the export of Pma1p, then overproduction of Exp1p may abrogate the lethality caused by expression of dominant negative *PMA1* alleles. To test this prediction, we ectopically expressed the dominant negative *PMA1D378N* under the control of an galactose-inducible promoter and assayed growth of Exp1p overproducing strains in the presence of galactose. *EXP1* overexpression had no effect on the dominant negative phenotype of P_{GAL1}*PMA1D378N* strains grown in 2% galactose. However, we found that overproduction of Exp1p in P_{GAL1}*PMA1D378N* strains decreased the diameter of the zone of growth inhibition caused by galactose in a plate assay. These preliminary results suggest that Exp1p can enhance the export either

of wild-type Pma1p before it binds to the dominant negative form of Pma1p or of mixed oligomers before they are recognized by the quality control machinery.

Overproduction of wild-type Pma1p in a *lst1Δ* genetic background is toxic to cells, presumably because the accumulation of Pma1p in the ER titrates essential components of the folding and secretion machinery or because of the negative effects of proton pumping at the ER membrane. This toxicity can be suppressed by expressing additional copies of *EXP1*, either from a centromeric or 2μ plasmid (data not shown), consistent with the proposed role for Exp1p in the export of Pma1p from the ER.

Role of Exp1p in Pma1p oligomerization

Pma1p exits the ER as a lipid-raft associated oligomer, although neither oligomerization or raft association is necessary for its export from the ER (Bagnat et al., 2000; Lee et al., 2002). To investigate the relationship between Pma1p oligomerization and Exp1p or Lst1p-mediated export from the ER, we assayed the oligomeric state of Pma1p in the presence and absence of Lst1p and Exp1p. We first used 10-30% linear sucrose gradients to separate oligomeric and monomeric Pma1p. Preliminary results indicated that Pma1p monomer accumulated in *lst1Δ* strains. Overexpression of *EXP1* only slightly reduced the amount of monomeric Pma1p in *lst1Δ* strains. Raft association of Pma1p has been assayed experimentally on the basis of detergent solubility since raft-associated Pma1p is insoluble in 1% Triton (Bagnat et al., 2000). In *lst1Δ* strains, as in wild-type strains, Pma1p remained associated with the Triton-insoluble particulate fraction. The overexpression of *EXP1* had no effect on the solubility of Pma1p in 1% Triton. On the basis on these preliminary results, we concluded that Exp1p does not alter the oligomerization or raft association of Pma1p.

References

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