Ceramide Biosynthesis Is Required for the Formation of the Oligomeric H\(^+\)-ATPase Pma1p in the Yeast Endoplasmic Reticulum*

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The yeast plasma membrane \(H^+\)-ATPase Pma1p is one of the most abundant proteins to traverse the secretory pathway. Newly synthesized Pma1p exits the endoplasmic reticulum (ER) via COPII-coated vesicles bound for the Golgi. Unlike most secreted proteins, efficient incorporation of Pma1p into COPII vesicles requires the Sec24p homolog Lst1p, suggesting a unique role for Lst1p in ER export. Vesicles formed with mixed Sec24p-Lst1p coats are larger than those with Sec24p alone. Here, we examined the relationship between Pma1p biosynthesis and the requirement for this novel coat subunit. We show that Pma1p forms a large oligomeric complex of >1 MDa in the ER, which is packaged into COPII vesicles. Furthermore, oligomerization of Pma1p is linked to membrane lipid composition; Pma1p is rendered monomeric in cells depleted of ceramide, suggesting that association with lipid rafts may influence oligomerization. Surprisingly, monomeric Pma1p present in ceramide-deficient membranes can be exported from the ER in COPII vesicles in a reaction that is stimulated by Lst1p. We suggest that Lst1p directly conveys Pma1p into a COPII vesicle and that the larger size of mixed Sec24p-Lst1p COPII vesicles is not essential to the packaging of large oligomeric complexes.

An essential protein of the yeast plasma membrane is the \(H^+\)-ATPase Pma1p. At steady state, it composes \(>25\%\) of the total protein at the plasma membrane, where it generates a proton gradient that maintains the intracellular pH and drives the import of nutrients (1). Pma1p spans the lipid bilayer with 10 transmembrane segments and belongs to the family of \(P_\text{F}_{1}\)-type ATPases, which includes the \(N^+,K^+\)-ATPases and \(Ca^{2+}\)-ATPases of the mammalian plasma membrane (2, 3).

As with other integral membrane proteins destined for the plasma membrane, Pma1p is translocated into the endoplasmic reticulum (ER) and travels through a series of transport vesicles to its final destination (4, 5). En route, Pma1p and the glycosylphosphatidylinositol-anchored protein Gas1p become associated with lipid rafts (6, 7). Interestingly, raft association of Gas1p initiates in the ER (6), unlike in mammalian cells, where glycosylphosphatidylinositol-anchored proteins enter rafts in post-ER compartments (8, 9). Exit out of the ER is mediated by COPI (coat protein complex II) vesicles, a universal mechanism in eukaryotes employing a set of cytoplasmic coat proteins (10). Budding is regulated by the small G-protein Sar1p, which recruits two complexes, Sec23p-Sec24p and Sec13p-Sec31p (10–12). Assembly of these three components on the surface of liposomes is sufficient to deform the lipid bilayer to generate small coated vesicles (11). On the ER membrane, Sar1p and Sec23p-Sec24p promote the capture of a number of cargo proteins, suggesting that Sec23p-Sec24p may function to selectively engage cargo proteins during coat assembly (13, 14).

Homologs of Sec24p have been identified in yeast and mammals (15, 16) and may act to diversify the range of cargo proteins recruited into a nascent vesicle. In yeast, the Sec24p homolog Lst1p was discovered in a screen for synthetic interactions with the sec13-1 allele (j lethal with sec-thirteen) (15). A null mutant of \(lsl1\) is viable, although sensitive to low pH resulting from a reduced flux of Pma1p out of the ER (15). Efficient incorporation of Pma1p into COPII vesicles requires a combination of both Sec24p and Lst1p complexes, unlike other cargo proteins studied to date (16). In addition to the enhancement of Pma1p packaging, Lst1p-positive vesicles are \(15\%\) larger than vesicles generated with standard COPII subunits (17).

In this study, we investigated the role of Pma1p oligomerization in Lst1p-dependent Pma1p transport. Both oligomeric and monomeric Pma1p are packaged into COPII vesicles, and both forms of Pma1p require Lst1p for efficient transport from the ER.

**EXPERIMENTAL PROCEDURES**

Reagents, Strains, and Plasmids—Reagents were purchased from Sigma unless otherwise noted. Aureobasidin A was purchased from Takara Bio Inc. (Shiga, Japan). Rich medium (yeast extract/peptone/dextrose) and minimal medium containing either 2% glucose (synthetic dextrose medium) or 2% raffinose were prepared as described (17). The yeast strains used in this study were as follows: YPH499 (MATa ade2-101oc his3::D2000 leu2-Δ1 lys2-801am trp1-63 ura3-52), RSY1578 (YPH499 pma1::HA-PMA1::LEU2), RSY1758 (YPH499 pma1::HA-PMA1::LEU2), MLY1 (MATa lebl-100 leu2 ura3 his3 pma1::HA-PMA1::LEU2) (HR2807, H. Riezman, University of Basel, Basel, Switzerland), RSY732 (MATa ura3-52 leu2-3,112 sec18-1), MLY18 (RSY372 pma1::HA-PMA1::LEU2), MLY4 (MATa ade2-1 his3-11,15 leu2-3,112 trp1-Δ1 ura3-1 pep4::TRP1 pma1::HA-PMA1::LEU2), and RSY1801 (RSY1578 slt1::HIS3). Galactose induction of hemagglutinin (HA)-tagged Pma1p carried out with pFP302 (18) in either YPH499 or RSY372.

Preparation of Proteins and Cellular Components—Microsomal membranes were prepared as described (19) unless otherwise noted. Both \(^{35}\)S-labeled and unlabeled cells were converted to semi-intact spheroplasts as described (17). Purification of Sec23p-Lst1p (17) and Sar1p, Sec23p-Sec24p, and Sec13p-Sec31p (20) is described elsewhere. Blue Native (BN)-PAGE—Membranes were solubilized with deter-
Microsomes and spheroplasts were centrifuged at 15,000 × g (Molecular Dynamics, Inc., Sunnyvale, CA). Protein extracts were separated by BN-PAGE and immunoblotted with HA-specific antibodies. B, the relative mobilities of molecular mass markers (bovine serum albumin (67 kDa), lactate dehydrogenase (140 kDa), catalase (230 kDa), ferritin (440 kDa), and thyroglobulin (670 kDa)) were used to size Pma1p complexes isolated from microsomes solubilized with n-dodecyl maltoside. The protein band of lowest molecular mass was designated as monomer (n = 1).

Electron Microscopy—The vesicle-enriched medium speed supernatant (MSS) was centrifuged at 100,000 × g for 20 min. The vesicle pellet was processed as described (12). Vesicle diameter was measured from scanned negatives (∼30,000) using the Photoshop measuring tool. A sample of 500 vesicles was measured for each condition.

RESULTS

Pma1p Forms a Large Oligomeric Complex—We used BN-PAGE to examine the oligomeric state of Pma1p in microsomal membranes. Membranes generated from a strain expressing a chromosomal HA-tagged form of Pma1p were solubilized with different detergents, and the proteins were separated by BN-PAGE (Fig. 1A). Pma1p migrated as a distinct high molecular mass complex of ∼1.8 MDa in extracts solubilized with Triton X-100, digitonin, or n-dodecyl maltoside. Pma1p solubilized with SDS migrated with an apparent molecular mass of 160 kDa, most likely corresponding to monomeric protein, which has a predicted molecular mass of ∼100 kDa.

Interestingly, two bands of intermediate size were observed in n-dodecyl maltoside extracts; the apparent sizes of these intermediates corresponded closely to those of predicted trimERIC and hexamerIC complexes of Pma1p (Fig. 1B) and may represent partial dissociation of a more abundant dodecameric species. Although the size of the 1.8-MDa species is suggestive of a homo-oligomeric complex of 12 Pma1p molecules (possibly arranged as four trimers), we cannot yet rule out the presence of additional proteins in the complex.

To determine whether the Pma1p multimer originates in the ER, we examined newly synthesized Pma1p in the sec18-1 strain, which is rapidly blocked in ER-to-Golgi transport at 37 °C. Cells were shifted to 37 °C for 5 min, and expression of epitope-tagged Pma1p was induced with galactose for 30 min. Oligomerization of the induced Pma1p was observed in both wild-type and sec18-1 extracts (Fig. 1C), whereas no protein was detected in the absence of galactose (data not shown). Although Pma1p in wild-type cells was found predominantly as...
the oligomeric species, imposition of a sec18 block resulted in approximately equal amounts of oligomeric and monomeric Pma1p. Thus, Pma1p can oligomerize in the ER, but oligomerization is somewhat impeded by a block in ER-to-Golgi transport.

**Oligomerization Is Dependent on the Ceramide Biosynthesis Pathway**—The observation that Pma1p associates with DIGs or lipid rafts (6) prompted us to investigate whether oligomerization is correlated with sphingolipid biosynthesis. To deplete cell membranes of sphingolipids, we used the lcb1-100 strain, which has a temperature-sensitive mutation in a subunit of serine palmitoyltransferase and is unable to synthesize sphingoid base, ceramide, and sphingolipids (22). Bagnat et al. (6) found that Pma1p isolated from lcb1-100 cells grown at restrictive temperature is no longer raft-associated. We examined the oligomeric state of Pma1p in wild-type and lcb1-100 cells grown either at 24 °C or after a 2-h shift to 37 °C. Unlike wild-type membranes, Pma1p isolated from lcb1-100 cells migrated almost exclusively as the monomeric form, regardless of the temperature at which the cells were grown (Fig. 2A). This is consistent with the observation that sphingolipid levels in lcb1-100 cells are considerably lower than those in wild-type cells even at 24 °C (23).

We investigated whether addition of exogenous sphingolipid base in the form of phytosphingosine (PHS) could restore the levels of sphingolipid sufficiently to allow oligomerization of Pma1p. Both wild-type and lcb1-100 cells grown at 24 °C were supplemented with various levels of PHS for 2 h and analyzed by BN-PAGE (Fig. 2B). Addition of 1–10 μM PHS to lcb1-100 cells promoted increased oligomerization of Pma1p. Interestingly, the proportion of oligomeric Pma1p in wild-type cells was also increased by addition of low levels of PHS, suggesting that oligomerization is intimately linked with sphingolipid levels in the cell.

Depletion of sphingolipid levels can also be achieved by addition of drugs such as myriocin, which inhibits serine palmitoyltransferase activity (24), and aureobasidin A (AbA), which blocks synthesis of the sphingolipid inositol phosphorylceramide from ceramide (25). To determine whether myriocin influences Pma1p oligomerization, we treated cells with up to 40 μg/ml myriocin for 2 h. Under these conditions, we were unable to observe a significant shift of the steady-state levels of oligomeric Pma1p to monomer (data not shown), suggesting either that depletion of sphingolipid was not sufficient during the 2-h treatment or that, once formed, Pma1p oligomers are resistant to a decrease in sphingolipid levels. To analyze the effects of myriocin and AbA on newly synthesized Pma1p, we used the galactose-inducible HA-tagged Pma1p plasmid described above. Wild-type cells expressing endogenous untagged Pma1p were grown in non-inducing medium at 24 °C and treated with myriocin or AbA for 15 min before induction of HA-tagged Pma1p. Unlike control cells, myriocin-treated cells showed a defect in the ability of newly synthesized Pma1p to oligomerize (Fig. 2C). However, oligomerization was not prevented in cells treated with AbA (Fig. 2C), suggesting that either ceramide or sphingoid base (but not sphingolipids per se) is required for Pma1p oligomerization. Under these conditions, AbA prevented the incorporation of 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))ceramide into inositol phosphorylceramide as determined by TLC (26).2

We assessed the effect of ceramide depletion on the stability of Pma1p by a 35S pulse-chase analysis. In wild-type cells, Pma1p remained stable throughout the chase period (Fig. 2D), consistent with its long half-life (5). In both lcb1-100 cells and myriocin-treated wild-type cells, however, Pma1p was markedly destabilized. Myriocin failed to induce degradation of Pma1p in pep4Δ cells, suggesting that monomeric Pma1p is permitted to exit the ER, but is subsequently rerouted to the vacuole.

**Lipid Raft Association in the Early Secretory Pathway**—A block in ceramide synthesis by myriocin treatment or by growth of lcb1-100 cells at nonpermissive temperature prevents the ER-to-Golgi transport of the glycosylphosphatidylinositol-anchored protein Gas1p (24, 27), which associates with DIGs before leaving the ER (6). To determine whether oligomerization of Pma1p in the ER coincides with its entry into Triton X-100-insoluble DIGs, we isolated DIGs from pulse-labeled sec18-1 cells.

At permissive temperature, the proportion of Pma1p associated with DIGs increased markedly after prolonged chase (Fig. 3A). Gas1p displayed a similar pattern, with a proportion of both the ER form (2 min) and the Golgi-modified form (15 min) in the detergent-insoluble fraction (Fig. 3A). At 37 °C, the pool of DIG-associated Gas1p increased after extended chase, whereas Pma1p was no longer observed in DIGs (Fig. 3A). This suggests that, unlike Gas1p, Pma1p does not associate with lipid rafts in the ER or, alternatively, that a block in ER export diminishes the stability of the association of Pma1p with lipid rafts.

To distinguish between these possibilities, we analyzed the detergent solubility of Pma1p in ER-derived COPII vesicles. Microsomal membranes were used in a vesicle budding reaction using purified COPII proteins (Sec13p-Sec31p) together with the Sec24p homolog Lst1p. The vesicle-enriched MSS was subjected to centrifugation at 100,000 × g to pellet vesicles, from which DIGs were isolated by flotation as described above. Vesicles generated in the presence of nucleotide showed efficient budding of Pma1p, whereas the ER resident Sec61p was not packaged into vesicles (Fig. 3B). A significant proportion of Pma1p in COPII vesicles was in a detergent-insoluble pool (fractions 5 and 6) (Fig. 3B). In contrast, the SNARE proteins Sec22p, Bet1p, and Bos1p were efficiently incorporated into COPII vesicles, but were not DIG-associated (Fig. 3B and data not shown).

To confirm that the DIG-associated Pma1p observed in the in vitro budding reaction corresponded to newly synthesized protein from the ER, we performed a budding reaction using semi-intact cells generated after a brief period of radiolabeling. Vesicle DIGs were isolated by flotation, and Pma1p was immunoprecipitated from fractions representative of detergent-insoluble and detergent-soluble material. A significant fraction (11%) of Pma1p in COPII vesicles was DIG-associated (Fig. 3C). Thus, Pma1p association with DIGs either is stabilized by entry into COPII vesicles or occurs initially in the ER, but is disrupted by the imposition of a secretion block.

**Oligomeric Pma1p Is Packaged into COPII Vesicles**—The dependence of Pma1p oligomerization on ceramide biosynthesis, together with the observation that Pma1p is associated with DIGs in COPII vesicles, prompted us to investigate the effect of ceramide depletion on the oligomeric state of Pma1p packaged into vesicles. COPII vesicles were generated from wild-type microsomes or microsomes derived from lcb1-100 cells and analyzed by BN-PAGE. In vesicles generated from wild-type microsomes, Pma1p was packaged as the large oligomeric species (Fig. 4A). Interestingly, COPII vesicles generated from lcb1-100 microsomes packaged Pma1p, but the protein remained in the monomeric form (Fig. 4A).

We investigated whether oligomerization might enhance the uptake of Pma1p into vesicles, possibly by allowing more efficient recruitment of vesicle coat components to the ER membrane. We performed budding reactions with 35S-labeled cells grown in the presence of myriocin for 2 h, a treatment that renders Pma1p monomeric (see Fig. 2C). lst1-null cells were

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used to allow an accurate titration of Pma1p budding by addition of increasing amounts of purified Lst1p and Sec24p complexes in the presence of constant levels of Sar1p and Sec13p-Sec31p. In both myriocin-treated and control membranes, Pma1p budding was stimulated by increasing concentrations of Sec24p and Lst1p complexes (Fig. 4B). No significant differences in budding efficiency were observed between wild-type and myriocin-treated membranes, regardless of the concentration of COPII components provided.

Lst1p Stimulates the Packaging of Both Oligomeric and Monomeric Pma1p—We next investigated more directly the relationship between the oligomeric state of Pma1p and the requirement for Lst1p in ER export. Shimoni et al. (17) proposed two possible mechanisms for the Lst1p-mediated stimulation of Pma1p budding. Lst1p may act as a “cargo-adapter,” binding directly to Pma1p to efficiently recruit it into a nascent vesicle. Alternatively, Lst1p may form larger vesicles with a lower membrane curvature and greater surface area, thus indirectly facilitating the packaging of oligomeric Pma1p. If so, the ability of high levels of Sec24p to overcome the requirement for Lst1p in Pma1p packaging (17) may reflect an increase in the incorporation of monomeric Pma1p into nascent vesicles. To address
this issue, we compared the oligomeric state of Pma1p in vesicles that had been generated with either a combination of Sec24p and Lst1p complexes or with high levels of the Sec24p complex alone (Fig. 4C). Analysis of vesicles by BN-PAGE revealed that the presence of Lst1p was not essential for packaging oligomeric Pma1p, which could be accommodated in Lst1p-negative vesicles given a sufficient quantity of coat proteins (Fig. 4C).

We next considered whether Pma1p rendered monomeric by ceramide depletion may bypass the requirement for Lst1p and be incorporated into the smaller vesicles generated with the standard COPII proteins. Membranes were isolated from lst1Δ cells treated with myriocin and incubated with low-to-intermediate concentrations of Sec24p and Lst1p complexes. Under these conditions, Pma1p was not significantly packaged when only the Sec24p complex was added (Fig. 4D); however, addition of equivalent amounts of both Sec24p and Lst1p complexes stimulated budding ~3-fold in both myriocin-treated and control membranes. We conclude that the oligomeric state of Pma1p does not enhance budding and that both the oligomeric complex and the monomeric protein require Lst1p for efficient incorporation into COPII vesicles.

**Packaging of Large Pma1p Complexes Does Not Determine Vesicle Size**—The requirement for Lst1p to package both oligomeric and monomeric Pma1p suggests a function as a cargo-specific adapter, rather than an indirect role in accommodating large complexes. We considered the possibility that the larger size of COPII vesicles produced with the Lst1p complex was influenced by the incorporation of large DIg-associatd Pma1p oligomers. Pma1p is a particularly abundant protein; thus, it is conceivable that its presence alone in Lst1p-positive vesicles may influence vesicle size.

Large-scale budding reactions were performed using either wild-type or lcb1-100 microsomes prepared from cells grown at 24 °C and shifted to 37 °C for 1 h. Under these conditions, Pma1p appeared monomeric in lcb1-100 membranes (see Fig. 2A). Microsomal membranes were washed to remove endogenous COPII components and then supplied with Sar1p, Sec13p-Sec31p, and either the Sec24p complex alone or a mixture of both Sec24p and Lst1p complexes, in addition to the non-hydrolyzable GTP analog GMP-PMP to preserve vesicle coats. The high speed vesicle pellet was analyzed by electron microscopy.

Vesicles with a discernible coat were generated under all conditions, and no obvious differences in coat morphology were

**FIG. 3. Association of Pma1p with lipid rafts in the ER and COPII vesicles.** A, DIG-associated proteins were isolated from sec18-1 strain MLX18 at permissive and nonpermissive temperatures. Cells were pulse-labeled for 3 min either at 24 °C or after pretreatment at 37 °C for 5 min, followed by a 2- or 15-min chase. Cells (2.5 A units) were converted to spheroplasts and gently lysed with Triton X-100, and DIGs were isolated by flotation. Representative gradient fractions of detergent-insoluble (T) and detergent-soluble (S) material were immunoprecipitated for Pma1p and Gas1p, and DIG-associated protein was quantified relative to the total (T; 1:10). ER (p) and Golgi (m) forms of Gas1p were quantitated together. B, DIGs were isolated from COPII vesicles generated from microsomal membranes (RSY1578) with Sar1p, Sec24p and Lst1p complexes, and Sec13p-Sec31p in the presence (+) or absence (−) of nucleotide. A fraction of the total reaction mixture (T; 1:100) and the MSS (+ and −; 1:40) was resolved by SDS-PAGE and immunoblotted for Pma1p, Sec22p, and Sec61p. The remaining MSS was solubilized with Triton X-100 and applied to an Optiprep gradient. Fractions (200 μl) across the entire gradient were collected, and 10 μl of each was resolved by SDS-PAGE and immunoblotted for Pma1p and Sec22p. A sizable proportion of Pma1p was found in the detergent-insoluble fraction (T). C, cells (RSY1578) were pulse-labeled for 3 min and converted to permeabilized spheroplasts. Budding reactions (cells at 5 A units) were performed as described for B. A fraction of the total reaction (T; 1:50) and MSS (+ or − nucleotide; 1:10) was used to immunoprecipitate HA-Pma1p or Sec22p, and the budding efficiency was quantified by phosphorimaging. The remaining MSS was used to isolate DIGs as described for B, and proteins were immunoprecipitated from gradient fractions 5–6 (detergent-insoluble (T) and 11–12 (detergent-soluble (S)). The proportion of DIG-associated Pma1p and Sec22p was quantified relative to the total amount present in the MSS + nucleotide.
detected (data not shown). Lst1p-positive vesicles produced from wild-type and lcb1-100 membranes were of comparable size and were 10–15% larger in diameter than Lst1p-nega-}



**DISCUSSION**

Pma1p is a particularly abundant secretory protein that uniquely requires Lst1p for packaging into COPII vesicles for ER export. We have investigated the nature of this requirement with respect to the quaternary structure of Pma1p. We found that, upon BN-PAGE, Pma1p migrated as an oligomeric complex. Our estimate that this complex represents a homodo-}



| Membranes | −Lst1p | +Lst1p |
|-----------|--------|--------|
| Wild-type | 75 ± 13 | 84 ± 14 |
| lcb1-100  | 77 ± 14 | 85 ± 16 |

<sup>*</sup> n = 500; p < 0.001.
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