Phosphatidylserine Is Involved in the Ferrichrome-induced Plasma Membrane Trafficking of Arn1 in Saccharomyces cerevisiae*

Yan Guo1,1, Wei-Chun Au3, Minoo Shakoury-Elizeh1, Olga Protchenko1, Munira Basrai1, William A. Prinz4, and Caroline C. Philpott1,2

From the 1Liver Diseases Branch and 2Laboratory of Cell Biology and Biochemistry, NIDDK, and the 3Genetics Branch, NCI, National Institutes of Health, Bethesda, Maryland 20892-1800

Arn1 is an integral membrane protein that mediates the uptake of ferrichrome, an important nutritional source of iron, in Saccharomyces cerevisiae. In the absence of ferrichrome, Arn1p is sorted directly from the trans-Golgi network to the vacuolar lumen for degradation. In the presence of low levels of ferrichrome, the siderophore binds to a receptor domain on Arn1, triggering the redistribution of Arn1 to the plasma membrane. When extracellular ferrichrome levels are high, Arn1 cycles between the plasma membrane and intracellular vesicles. To further understand the mechanisms of trafficking of Arn1p, we screened 4580 viable yeast deletion mutants for mislocalization of Arn1-GFP using synthetic genetic array technology. We identified over 100 genes required for trans-Golgi network-to-vacuole trafficking of Arn1-GFP and only two genes, SER1 and SER2, required for the ferrichrome-induced plasma membrane trafficking of Arn1-GFP. SER1 and SER2 encode two enzymes of the major serine biosynthetic pathway, and the Arn1 trafficking defect in the ser1Δ strain was corrected with supplemental serine or glycine. Plasma membrane trafficking of Hxt3, a structurally related glucose transporter, was unaffected by SER1 deletion. Serine is required for the synthesis of multiple cellular components, including purines, sphingolipids, and phospholipids, but of these only phosphatidylserine corrected the Arn1 trafficking defects of the ser1Δ strain. Strains with defects in phospholipid synthesis also exhibited alterations in Arn1 trafficking, indicating that the intracellular trafficking of some transporters is dependent on the phospholipid composition of the cellular membranes.

Membrane transporters are a large class of polytopic integral membrane proteins dedicated to the transfer of small molecules across the lipid bilayers of the cell. Transporters are expressed in all organisms, and the Saccharomyces cerevisiae genome contains about 300 putative transporters (1). Membrane transporters exhibit specificity for different classes of small molecules, and the efficient regulation of these transport activities is critical to maintaining cellular metabolism and integrity. Transporters are regulated at multiple levels, and eukaryotes frequently rely on post-translational mechanisms, such as alterations in intracellular trafficking, to control transporter activity.

The activity of numerous transporters in yeast is controlled through regulated ubiquitin-mediated endocytosis and vacuolar degradation, which is typically triggered by substrate excess or changes in nutrient supply (2). In this case, transporters on the plasma membrane are covalently modified by the attachment of ubiquitin by Rsp5, the Nedd-HECT family ubiquitin ligase of yeast. Ubiquitinated transporters are recognized by epsin-like proteins Ent1 and Ent2 as well as other adaptor proteins that direct the cargo into invaginating endocytic vesicles. These endocytic vesicles undergo further covalent modification with the attachment of polyubiquitin chains linked at the lysine 63 residue of ubiquitin and reach the late endosome. Attachment of polyubiquitin chains allows for recognition by a series of heterooligomeric protein complexes, termed endosomal sorting complex required for transport (ESCRT)0, I, II, and III, which direct the ubiquitinated cargo into the luminal vesicles of the multivesicular body (MVB) (3). The MVB fuses with the vacuolar membrane, releasing the luminal vesicles into the interior of the vacuole, where they undergo proteolytic degradation.

Transporters cannot be expressed on the plasma membrane without first transiting the trans-Golgi network (TGN). There, cargo proteins are sorted into vesicles and tubular carriers that deliver cargo to the plasma membrane, endosomes, or vacuole. For some transporters, such as the general amino acid permease Gap1 (4), the uracil permease Fur4 (5), and the siderophore transporters Arn1 (6, 7) and Sit1/Arn3 (8), sorting at the TGN is regulated by the nutrient status of the cell or the presence of the transport substrate, with trafficking to the vacuole occurring under conditions where degradation of the transporter is advantageous to the cell. The sorting of

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1 Present address: Center for Molecular Physiology, Children’s National Medical Center, Suite S340, 111 Michigan Ave., NW, Washington, D. C. 20010.

2 To whom correspondence should be addressed: Bldg. 10 Rm. 9B-16, 10 Center Dr., Bethesda, MD 20892. Fax: 301-402-0491; E-mail: carolinep@intra.niddk.nih.gov.

3 The abbreviations used are: ESCRT, endosomal sorting complex required for transport; FC, ferrichrome; MVB, multivesicular body; VPS, vacuolar protein sorting; TGN, trans-Golgi network; SGA, synthetic genetic array; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DAG, diacylglycerol; DHS, dihydrosphingosine.
cargos into this vacuolar protein-sorting (VPS) pathway also requires the activity of adaptor proteins, and the list of adaptors that facilitate the sorting of cargo through the VPS pathway is rapidly expanding.

The intracellular trafficking of the siderophore transporters Arn1 and Sit1/Arn3 is particularly complex. These transporters are part of a group of four homologous transporters that facilitate the uptake of siderophore-iron chelates in budding yeast (9). These chelates are an important source of nutritional iron for yeast, and Arn1 specifically transports the fungal siderophore ferrichrome (FC). Arn1 is transcriptionally activated during iron deficiency; however, if FC is not present in the extracellular environment, newly synthesized Arn1 is directly sorted into exocytic vesicles from the TGN. One multiprotein complex, termed the exomer, is required for the trafficking of Chs3 (chitin synthase III) from the TGN to the plasma membrane at the site of the developing bud neck (13). Exomer is also required to recruit Fusi1, a protein required for cell fusion during mating, to the plasma membrane projections that develop during the mating reaction (14). Other plasma membrane proteins have not been shown to require exomer for trafficking.

In order to understand the cellular mechanisms that control transporter trafficking, we used a whole-genome approach to identify proteins involved in sorting at the TGN. Using the synthetic genetic array (SGA) approach (15), we introduced GFP-tagged Arn1 into the yeast deletion mutant strains with defects in the binding of Arn1 to the plasma membrane. At higher concentrations (micromolar) of FC, Arn1 cycles on and off the plasma membrane while actively transporting the siderophore.

Some components of the cellular machinery required to execute these trafficking patterns have been identified. The clathrin adaptor proteins Gga2, Ent3, and Ent4 are required for the sorting of Arn1 from the TGN to endosomes, although ubiquitination of Arn1 is not required at this step (10). Sorting of Arn1 from the late endosome into the MVB requires ubiquitination of Arn1 at lysine residues located in the amino terminus and also requires the ubiquitin binding activity of Gga2 (11). Cells lacking these adaptors exhibit aberrant trafficking of Arn1 to the plasma membrane in the absence of FC.

The cellular machinery required to sort Arn1 into vesicles destined for the plasma membrane in the presence of FC is largely unknown. FC binds to a high affinity site in a membrane-spanning portion of the carboxyl terminus and presumably triggers a conformational change that permits sorting to the plasma membrane (6). A cluster of phenylalanine residues in the cytosolic carboxyl terminus of Arn1 is also required for plasma membrane trafficking. Arn1-containing exocytic vesicles are dependent on the interaction of Sec1 with exocytic SNARE complexes on the plasma membrane for fusion to the plasma membrane (7, 12), but little is known about how Arn1 or many other cargo proteins are directed into exocytic vesicles from the TGN. One multiprotein complex, termed the exomer, is required for the trafficking of Chs3 (chitin synthase III) from the TGN to the plasma membrane at the site of the developing bud neck (13). Exomer is also required to recruit Fusi1, a protein required for cell fusion during mating, to the plasma membrane projections that develop during the mating reaction (14). Other plasma membrane proteins have not been shown to require exomer for trafficking.

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**MATERIALS AND METHODS**

Strains, Plasmids, Media, Antibodies, and Chemicals—Strains and plasmids used in this study are listed in Table 1. Plasmids pFA6a-GFPS65T-KanMX6 (16) and p4339 (15) were used as templates to amplify the sequences of GFP and natRMX4, respectively. The forward primer for amplifying GFP includes
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53 nucleotides homologous to the upstream of the stop codon in ARN1 followed with the sequence of primer F2 of pFA6a-GFPPS65T-KanMX6. The reverse primer for amplifying GFP was the reverse sequence of nucleotides 1101–1130 in pFA6a-GFPPS65T-KanMX6, which overlapped with the forward primer for amplifying natRMX4. The reverse primer of natRMX4 contained MX-4 followed by 55 nucleotides downstream of the stop codon in ARN1. Two products of PCR were co-transformed into Y7096 to integrate GFP-natRMX4 at the C terminus of ARN1, generating the query strain YYG001. Transformants were selected on YPD-clonNAT (100 μg/ml) plates and confirmed by PCR and fluorescence microscopy. A similar strategy was used to construct YYG006. YYG001 was mated with the deletion mutant collection and sporulated, and haploid segregants containing the yeast deletion (KAN) and Arn1-GFP (ClonNAT) were selected using the SGA approach with a Bio-Rad Versarray colony arrayer (15). To construct YYG018, YYG005, and YYG010, the ser1Δ::KanMX4 locus was amplified from the ser1Δ strain from the S. cerevisiae deletion collection (Open Biosystems) and then integrated into ARN3-GFP, ARN1-GFP, and HXT3-GFP strains (17) to generate the congenic ser1Δ strains. The arn1Δ::URA3 locus from YPH499 arn1Δ (18) was amplified and used to disrupt ARN1 in the strains YMS001 and YMS002.

Rich medium (YPD) and synthetic complete medium were prepared as described (19). Iron-poor medium containing 10 μM ferrous ammonium sulfate and 1 mM ferrrozine was prepared as described previously (20). FC was added at the indicated concentrations as the ferric chelate. The cho1Δ strains were maintained on medium containing 4 mM ethanolamine and then transferred to medium without ethanolamine for fluorescence imaging.

The following mouse monoclonal antibodies were used: anti-GFP (Roche Applied Science) and anti-PGK1 (Invitrogen). Cy5-conjugated anti-mouse IgG was used as secondary antibody (Jackson ImmunoResearch).

FC, serine, glycine, phosphatidylserine (PS), and dihydrophosphosine (DHS) were purchased from Sigma. FM4-64 was purchased from Invitrogen. ClonNAT was purchased from Werner BioAgents.

Fluorescence-based Whole Genome Screen—Yeast deletion strains containing ARN1-GFP were inoculated into 96-well plates and grown in iron-poor medium. After growth at 30 °C for 16 h, yeast cells were diluted to A600 of 0.3 for an additional 2 h of growth without or with FC (20 nM) treatment. For fluorescence microscopy, 50–100 μl cells from each well were transferred to glass bottom 96-well plates and examined using a Zeiss inverted fluorescence microscope equipped with 488- and 509-nm excitation and emission filters, a ×32 1.4 numerical aperture objective, a charge-coupled digital camera, and Axiovision imaging software. Strains in which the trafficking of Arn1p-GFP was abnormal were cultured separately, and the results were validated in independent experiments. Strains were screened by examining 6–10 high power fields, each of which contained 50–200 cells.

Microscopy of Living Cells—To visualize cells expressing GFP-tagged Arn1p, yeast cells were grown in iron-poor medium overnight at 30 °C to early log phase and then grown for an additional 2 h with or without 20 nM FC added. Images were obtained on Zeiss fluorescence microscope using a ×100 1.4 numerical aperture objective and a Hamamatsu charge-coupled digital camera controlled by IPLab software. Fluorescence images were processed using Adobe Photoshop.

Western Blotting—Western blotting was performed as described previously (21) with the following modifications. Cells were grown to 0.2–0.3 A600, and then treated with FC or without FC for 2 h. After treatment, 3 A600 of cells were used for each lysate. Cells were disrupted by glass bead lysis in 200 μl of buffer containing 1% SDS, 8 mM urea, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.01% bromophenol blue, and protease inhibitors. The lysate was incubated at 65 °C for 10 min, and cellular debris and glass beads were removed by centrifugation. Supernatants were loaded on 4–12% gradient gels. Mouse monoclonal anti-GFP (Roche Applied Science) and anti-PGK1 (Invitrogen) were used as the primary antibodies, and Cy5-conjugated anti-mouse IgG was used as the secondary antibody (Jackson ImmunoResearch).

Lipid Analysis, Ferrichrome Binding Assay, and Endocytosis Assay—For phospholipid analysis, strains YYG001 and YYG003 were grown in iron-poor medium or synthetic complete medium, and then cells were collected and washed. Total glycerol phospholipid extraction and analysis was performed as described previously (22). Individual phospholipids were quantitated using Chemstation software (Agilent). The FC binding assay was performed as described previously (10). For FM4-64 staining, lcb1-ts cells were grown at 22 °C for 9 h and then shifted to 37 °C and grown for 16 h with or without 50 μM DHS. Strains YYG001 and YYG003 were grown at 30 °C for 16 h. Cells were harvested from 1 ml of culture and resuspended in 200 μl of SD medium and then incubated with 2 μl of 8 mM FM4-64 for 10 min in the dark at 30 or 37 °C for LCB1+ cells or lcb1-ts cells, respectively, followed by centrifugation and washing with YPD medium. FM4-64-labeled cells were resuspended in 1 ml of YPD medium and chased for 20 min at 37 °C and then kept on ice and imaged immediately using a Zeiss fluorescence microscope. The FM4-64 signal was visualized by excitation at 558 nm and emission with a 734-nm filter.

RESULTS

Mislocalization of Arn1-GFP in ser1Δ and ser2Δ Strains—An Arn1-GFP fusion protein has previously been shown to be functionally similar to unmodified Arn1 (11). We inserted GFP into the carboxyl terminus of Arn1 in the SGA query strain, mated the resulting strain with the yeast deletion mutant collection and sporulated the resulting diploid strains, and analyzed the haploid strains containing Arn1-GFP and the yeast deletion mutation. Each strain was grown in iron-poor medium to induce the expression of Arn1-GFP, and then each strain was either left untreated or treated with a low concentration of Fe(III)–FC for 2 h. After growth and treatment, each strain was then individually examined by fluorescence microscopy. In wild type strains expressing Arn1-GFP without FC, fluorescent signal accumulated in the vacuolar lumen due to the protease resistance of the GFP domain from degraded Arn1. A small amount of Arn1-GFP could also be seen in
punctate structures, which represent the endosomal compartment. After treatment with FC, fluorescent signal was seen at the periphery of the cell and in the vacuole. Mutant strains that exhibited altered patterns of Arn1-GFP localization were identified and categorized according to whether mislocalization of Arn1-GFP occurred in the presence or absence of FC. We identified over 100 strains that exhibited mislocalization of Arn1-GFP to the plasma membrane in the absence of FC. We identified only two strains that failed to localize Arn1-GFP to the plasma membrane in the presence of FC, and these strains carried deletions of SER1 and SER2 (Fig. 1). In the absence of FC, fluorescent signal was primarily detected in the vacuole of the wild type, ser1Δ, and ser2Δ strains. Because Arn1 carries the GFP domain on the cytosolic face of the transporter, the GFP can only gain access to the lumen of the vacuole through the luminal vesicles of the MVB. This suggested that Arn1-GFP expressed in the ser1Δ and ser2Δ strains folded well enough to exit the endoplasmic reticulum and transit the late secretory pathway in a manner similar to the wild type strain. The addition of 20 nM FC to the medium resulted in a relocalization of Arn1-GFP to the plasma membrane in the wild type strain but no change in localization in the ser1Δ and ser2Δ strains, suggesting a plasma membrane trafficking defect. We confirmed that the trafficking defect was not due to secondary mutations produced by the SGA procedure by amplifying the ser1 deletion cassette from the ser1Δ strain of the deletion mutant collection and integrating that into the original SGA query strain (YYG001) and also into the ARNI-GFP strain from the GFP collection to produce YYG003 and YYG005. These strains carrying ARNI-GFP ser1Δ in different strain backgrounds also exhibited the FC-induced trafficking defect seen in the ARNI-GFP ser1Δ strain produced by the SGA procedure (data not shown).

SER1 and SER2 encode 3-phosphoserine aminotransferase and phosphoserine phosphatase, respectively (23). These enzymes catalyze the last two steps in the biosynthesis of serine from 3-phosphoglycerate, the major pathway of serine biosynthesis in yeast. SER1 and SER2 encode 3-phosphoserine aminotransferase and phosphoserine phosphatase, respectively. These enzymes catalyze the last two steps in the biosynthesis of serine from 3-phosphoglycerate. Serine and glycine are rapidly interconverted by serine hydroxymethyltransferases, Shm1 and Shm2. Restoration of normal Arn1p-GFP trafficking in serine- or glycine-supplemented ser1Δ cells. Cells were grown in as in Fig. 1 except that iron-poor medium was either not supplemented (top) or supplemented with 100 μg/ml l-serine (middle) or 60 μg/ml glycine (bottom) for 16 h and then treated with or without 20 nM FC for 2 h. C, increased expression of Arn1-GFP on the cell surface in the ser1Δ strain supplemented with serine. Strains YYG001 (wild type) and YYG003 (ser1Δ) were grown in iron-poor medium, without or with serine (100 μg/ml), at 30 °C to an A600 of 0.3 and then grown for an additional 2 h without or with 20 nM FC treatment. Cells were harvested, washed, and incubated with 125 nM [35S]Fe-FC on ice for 15 min. Cells were again washed, and retained 35S-Fe-FC was measured. Samples were prepared in duplicate, and the experiment was replicated twice. Error bars, S.E.; *, p < 0.05. D, increase in half-life of Arn1-GFP in the ser1Δ strain supplemented with serine. Strains Y7096 (no GFP), YYG001 (wild type), and YYG003 (ser1Δ) were grown in iron-poor medium, without or with serine and not to a loss of the activity of the enzyme with another substrate, we supplemented the iron-poor medium with serine and looked at the trafficking of Arn1-GFP in the presence and absence of FC. Serine supplementation completely restored the FC-mediated trafficking of Arn1-GFP to the plasma membrane (Fig. 2B). Serine and glycine are rapidly interconverted in S. cerevisiae by the activities of the serine hydroxymethyltransferases, Shm1 and Shm2 (24). We...
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found that glycine supplementation could also correct the FC-mediated trafficking defect of Arn1-GFP in the ser1Δ strain (Fig. 2B).

To confirm that deletion of SER1 impaired the relocation of Arn1 to the plasma membrane in the presence of FC, we assessed the amount of Arn1 expressed on the plasma membrane quantitatively by measuring the amount of radio-labeled FC that bound to Arn1 on the cell surface. Wild type cells exhibited a large increase in FC binding on the cell surface when cells were grown in the presence of 20 nM FC (Fig. C), consistent with an accumulation of Arn1 and the homologous Arn3 on the cell surface. The ser1Δ strain, however, exhibited only a small increase in FC binding when cells were grown in the presence of FC, and the FC binding was partially restored by serine supplementation. These data confirmed a significant loss of plasma membrane trafficking of Arn1 in the ser1Δ strain.

Growth in the presence of FC also produces an increase in the half-life of Arn1, due to the diversion of a portion of the protein to the cell surface, where it is stable, and away from the vacuole, where it is degraded (6). This increase in half-life was manifested as an increase in the total amount of Arn1-GFP detected by Western blot in wild type cells treated with FC (Fig. 2D). The ser1Δ strain did not exhibit an increase in the total amount of Arn1-GFP after FC treatment, although this increase in Arn1-GFP was present in the ser1Δ strain supplemented with serine. These data indicated that FC treatment did not alter the trafficking of Arn1-GFP in the ser1Δ strain.

SER1 Deletion Was Not Associated with Global Defect in Exocytosis—We questioned whether the Arn1 trafficking defect observed in the ser1Δ strain was associated with a generalized defect in plasma membrane trafficking or whether it was specific for the regulated exocytosis observed in the FC transporters. We therefore examined the localization of Hxt3-GFP in wild type and ser1Δ strains. Hxt3 is a low affinity glucose transporter of the major facilitator superfamily of transporters (25) and is structurally and evolutionarily related to the yeast siderophore transporters, which are also major facilitator superfamily members. SER1 was deleted in the Hxt3-GFP strain, and the SER+ and ser1Δ strains were examined by fluorescence microscopy. Hxt3-GFP was expressed on the plasma membrane in both strains, with some GFP signal in the vacuole (Fig. 3A), indicating that the serine deficiency in the ser1Δ strain did not affect the folding and trafficking of Hxt3-GFP. We then introduced the SER1 deletion into a strain expressing Arn3-GFP and examined its trafficking in the SER+ and ser1Δ strains. Arn3 is 47% identical to Arn1, transports both ferrioxamine and ferrichromes, and exhibits FC-mediated trafficking to the plasma membrane (9). In the absence of FC, fluorescent signal from Arn3-GFP was largely detected in intracellular vesicles in both the wild type and ser1Δ strains (Fig. 3B). The addition of FC produced the expected plasma membrane signal in the wild type background, but FC produced no plasma membrane signal in the ser1Δ strain. Expression of Arn3 on the plasma membrane can also be measured specifically by the cell surface binding of radio-labeled FC in a strain lacking ARN1. A small increase in FC binding occurred in the arn1Δ strain when FC was added to the medium (Fig. 3C), consistent with trafficking of Arn3 to the cell surface; however, no increase in FC binding was measured in the arn1Δ ser1Δ strain when FC was present in the growth medium. Thus, in strains lacking SER1, Arn3 and Arn1 exhibited similar defects in FC-mediated plasma membrane trafficking, whereas Hxt3, a more distantly related transporter that is constitutively expressed on the plasma membrane, did not.

Deletion of SER1 also did not produce a general defect in endocytosis. Internalization of the lipophilic dye FM4-64 through endocytosis occurred with similar kinetics in the wild type and ser1Δ strains (data not shown). Thus, the trafficking defect of the ser1Δ strain was not due to a defect in fluid phase endocytosis and subsequent failure to deliver FC to the TGN or endosome.

Arn1-GFP Trafficking in ser1Δ Strain Not Rescued by Purines or Sphingolipids—The mechanism by which serine deficiency might lead to transporter trafficking defects was not immediately apparent. In addition to their incorporation into aminoacyl-tRNAs, serine and glycine are required for the biosynthesis of purines, sphingolipids, and phospholipids. Amino supplementation did not rescue the trafficking defect of the ser1Δ strain (data not shown), indicating that impaired purine biosynthesis was not the cause of the trafficking defect.
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FIGURE 4. Lack of involvement of sphingolipid in the trafficking of Arn1p to the plasma membrane. A, pathway of serine incorporation into sphingolipid. B, normal trafficking of Arn1p-GFP in lcb1-ts strain. The lcb1-ts strain was transformed with pARN1-GFP, the transformants were grown at 22 °C in iron-poor medium for 9 h, and then cells were shifted to 37 °C for 16 h followed by 2 h of 20 nM FC treatment or no treatment. C, failure to rescue the trafficking of Arn1p-GFP in ser1Δ strain with DHS. The YYG003 strain (ser1Δ Arn1-GFP) was grown in iron-poor medium supplemented with DHS (50 μM) or no DHS overnight, followed by an additional 2 h of growth without or with 20 nM FC. D, confirmation of bioavailability of DHS. The lcb1-ts strain was grown at 22 °C for 9 h and then shifted to 37 °C and grown for 16 h with or without DHS (50 μM) added. After DHS treatment, lcb1-ts cells were labeled with FM4-64 and chased for 20 min at 30 °C and then kept on ice and imaged immediately.

Because lipids are known to affect the intracellular trafficking of membrane proteins, we then focused on the role of sphingolipids and phospholipids. The first step in sphingolipid synthesis is the condensation of serine with palmitoyl-CoA to form 3-keto-dihydrosphingosine, which is then converted to dihydroxydihydrosphingosine at the restrictive temperature by observing that FM4-64 failed to stain internal membranes of the lcb1-ts strain after 16 h of growth at 37 °C (Fig. 4D, left). We confirmed that DHS was successfully delivered to the cells in culture by rescuing the endocytosis defect of the lcb1-ts strain at 37 °C with 50 μM exogenous DHS (Fig. 4D, right). Having ruled out sphingolipid deficiency, we next examined the roles of phospholipids in the trafficking of Arn1.

Phosphatidylserine Requirement for Plasma Membrane Trafficking of Arn1-GFP—A large amount of cellular serine is consumed through the synthesis of phospholipids (Fig. 5A). Most of the phospholipids in yeast are derived from the reaction of serine and cytidine diphosphate diacylglycerol (CDP-DAG) to form PS. Although PS is a relatively minor phospholipid, comprising less than 10% of total cellular phospholipid, most of the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) is formed from the decarboxylation and sequential methylation, respectively, of PS (26). We tested whether the addition of PS to the medium would rescue the trafficking defect of the ser1Δ strain (Fig. 5B). Again, the addition of FC to the ser1Δ strain grown in iron-poor medium had no effect on the localization of Arn1-GFP. In contrast, the addition of PS restored plasma membrane trafficking in the presence of FC, whereas the addition of vehicle alone did not. We also observed that the addition of PS could increase the total amount of Arn1-GFP in cells treated with FC, as measured by Western blotting (Fig. 5C), and that the addition of PS to the growth medium could increase the surface binding of radiolabeled FC in cells that had also been treated with FC (Fig. 5D). These data all suggested that the trafficking defect of the ser1Δ strain could be traced to a deficiency of phospholipid.

Phospholipids in the ser1Δ Strain—Data presented in Figs. 5 and 6 suggested that the trafficking defect of the ser1Δ strain was due, at least partially, to a deficiency of PS. We directly measured levels of phospholipids in the ser1Δ strain and its congenic parent strain by growing the cells in

strain with DHS (Fig. 4C). Supplementation with 50 μM DHS had no effect on the Arn1-GFP trafficking defect of the ser1Δ strain, confirming that sphingolipid deficiency did not account for the defect.

Strains lacking sphingolipids, such as the lcb1-ts strain, exhibit a defect in the endocytosis of the lipophilic dye FM4-64 (27). We confirmed that the lcb1-ts strain was depleted of sphingolipid at the restrictive temperature by observing that FM4-64 failed to stain internal membranes of the lcb1-ts strain after 16 h of growth at 37 °C (Fig. 4D, left). We confirmed that DHS was successfully delivered to the cells in culture by rescuing the endocytosis defect of the lcb1-ts strain at 37 °C with 50 μM exogenous DHS (Fig. 4D, right). Having ruled out sphingolipid deficiency, we next examined the roles of phospholipids in the trafficking of Arn1.

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Because PS is converted to PE and PC, we questioned whether these phospholipids could also rescue the trafficking defect of the ser1Δ strain. Exogenously supplied ethanolamine and choline can be converted to PE and PC, respectively, through a salvage pathway known as the Kennedy pathway (Fig. 6A) (26). We tested whether the addition of ethanolamine or choline could restore plasma membrane trafficking to Arn1-GFP by growing the ser1Δ Arn1-GFP strain in iron-poor medium supplemented with either ethanolamine or choline and then exposing the cells to FC for 2 h (Fig. 6B). Neither of these supplements augmented the trafficking of Arn1-GFP to the plasma membrane in the presence of FC, suggesting that only PS, and not PE or PC, could rescue the trafficking defect of the ser1Δ strain.

Alteration of Phospholipid Levels in the ser1Δ Strain—Data presented in Figs. 5 and 6 suggested that the trafficking defect of the ser1Δ strain was due, at least partially, to a deficiency of PS. We directly measured levels of phospholipids in the ser1Δ strain and its congenic parent strain by growing the cells in
iron-poor medium, quantitatively extracting the lipids from crude lysates, and separating the phospholipids by HPLC. The ser1Δ strain contained only 57% of the total phospholipid that was present in the wild type strain (Fig. 7A), indicating that the serine deficiency of the ser1Δ strain was manifested in part by reduced biosynthesis of phospholipids. Measurement of the relative abundance of individual phospholipids indicated that PS levels were uniformly low in both the wild type and ser1Δ strain, only 2–3% of the total phospholipid (Fig. 7B), and probably represented a characteristic of this strain background. Relative levels of PE were lower in the ser1Δ strain than the wild type, consistent with a reduced flux of serine through the PS and PE biosynthetic steps. Relative levels of phosphatidylinositol (PI) were higher in the ser1Δ strain. CDP-DAG is consumed in the biosynthesis of both PS and PI. The increased level of PI in the ser1Δ strain was probably due to the reduced consumption of CDP-DAG for the synthesis of PS, leading to more incorporation of CDP-DAG into PI. Although we did not measure a difference in the relative proportion of PS between the wild type and ser1Δ strains, the total levels of phospholipid, including PS, were significantly lower in the ser1Δ strain, suggesting that the overall amount of PS was reduced in the ser1Δ strain.

Complex Roles of Phospholipids in the Trafficking of Arn1-GFP—We noted that several genes involved in phospholipid metabolism were identified in our screen of the deletion mutant collection; therefore, we examined genes involved in phospholipid synthesis in greater detail. Cells lacking CHO1, which encodes the sole phosphatidylserine synthase, IFigure 5. Restoration of FC-induced plasma membrane trafficking of Arn1p-GFP in ser1Δ strain supplemented with phosphatidylserine. A, pathway of serine incorporation into phospholipids. MMPE, monomethyl-PE; DMPE, dimethyl-PE. B, plasma membrane trafficking of Arn1-GFP in ser1Δ strain supplemented with PS. The YYG003 strain (ser1Δ ARN1-GFP) was grown in iron-poor medium supplemented with PS (100 μg/ml) or the same volume of DMSO overnight and then treated with FC treatment as indicated prior to fluorescence imaging. C, increase in half-life of Arn1-GFP in the ser1Δ strain supplemented with serine. Strains Y7096 (no GFP), YYG001 (wild type), and YYG003 (ser1Δ) were grown as in Fig. 2C, above, with the ser1Δ strain supplemented with PS (100 μg/ml) or the same volume of DMSO. Equivalent numbers of cells were lysed and subjected to SDS-PAGE and Western blotting. Mouse monoclonal anti-GFP and anti-PGK were used as the primary antibodies, and Cy5-conjugated anti-mouse IgG was used as the secondary antibody. D, restoration of FC-induced cell surface localization of Arn1 in the ser1Δ strain supplemented with PS. YYG001 and YYG003 strains were grown in iron-poor medium without or with PS (100 μg/ml) as in Fig. 2C. Surface binding of 55Fe-FC was measured as in Fig. 2C. Samples were prepared in duplicate, and the experiment was replicated twice. Error bars, S.E.

FIGURE 6. Failure of supplemental choline or ethanolamine to rescue the trafficking defect of Arn1-GFP in ser1Δ strain. A, Kennedy pathway of ethanolamine and choline incorporation into phospholipids. Exogenous ethanolamine and choline can be used to synthesize PE and PC, respectively. B, trafficking of Arn1p-GFP to the vacuole in choline- and ethanolamine-supplemented ser1Δ cells treated with FC. YYG003 (ser1Δ ARN1-GFP) cells were grown in iron-poor medium supplemented with either choline (4 mM) or ethanolamine (4 mM) overnight followed by an additional 2 h of FC treatment, as indicated, and then subjected to fluorescence microscopy.

FIGURE 7. Reduced levels of phospholipid in the ser1Δ strain. Strains YYG001 (wild type) and YYG003 (ser1Δ) were grown in iron-poor or synthetic complete medium, and then lipids were quantitatively extracted from an equivalent number of cells and separated by HPLC. Cells were analyzed in triplicate, and the experiment was replicated four times. Results did not differ for the two types of media, and the pooled data were analyzed. Total phospholipid (A) was calculated from the absolute amounts of the individual phospholipids shown in B. Error bars, S.E.; *, p < 0.05.
Phospholipids Control Trafficking of Arn1 in Yeast

DISCUSSION

The Role of Phosphatidylserine in the Trafficking of Nutrient Transporters—Identification of cellular factors responsible for directing cargo proteins into vesicles destined for the plasma membrane has remained elusive. Here we have shown that the phospholipid PS contributes to the trafficking of the sidrophore transporters Arn1 and Arn3 to the plasma membrane in response to the binding of FC on their receptor domains. We identified the ser1Δ and ser2Δ strains from the yeast deletion mutant collection because they failed to express Arn1-GFP on the plasma membrane when treated with FC. These gene deletions led to deficiencies of serine and glycine, which in turn were associated with reduced synthesis of the major phospholipids. Although serine is required for the synthesis of multiple cellular components, only PS rescued the trafficking defects of the ser1Δ strain.

Although PS is a minor phospholipid in yeast cells, it is concentrated in the plasma membrane and accounts for ~20% of the total phospholipid there (28). The outer leaflet of the plasma membrane is enriched for sphingolipids and ergosterol (33), whereas most of the PS is concentrated on the


gested a defect in the sorting of Arn1 from the TGN to endosomes in the absence of FC (Fig. 8B). Cho2 and Opi3 are required for the sequential methylation of PE to PC (Fig. 8A), with a cho2Δ strain exhibiting elevated levels of PE and reduced levels of PC (30) and the opi3Δ strain exhibiting elevated levels of monomethyl-PE and essentially no PC (31). Both strains exhibit variably elevated levels of PI. The cho2Δ strain accumulated Arn1-GFP on the limiting membrane of the vacuole and in bright structures next to the vacuole in both the absence and presence of FC (Fig. 8B), indicating a defect in sorting Arn1-GFP into the MVB. A similar mislocalization of Arn1-GFP is observed in strains lacking Rsp5 activity, strains expressing a mutant Gga2 that fails to bind ubiquitin, and strains expressing lysine mutants of Arn1 that are not ubiquitinated (10, 11). These data suggest that the cho2Δ strain has a defect in the ubiquitination of Arn1-GFP or in the stepwise assembly of the ESCRT complexes required for sorting into the MVB. The addition of choline to the cho2Δ strain did not rescue the trafficking defect of Arn1-GFP (data not shown), suggesting that PC deficiency was not the sole cause of this trafficking defect. The opi3Δ strain was similar to the muq1Δ strain in that Arn1-GFP was mislocalized to the plasma membrane in the absence of FC.

Not only did defects in phospholipid synthesis affect Arn1-GFP trafficking, but defects in phospholipid distribution affected trafficking as well. Drs2 is an aminophospholipid translocase that catalyzes the translocation of PS from the luminal leaflet to the cytosolic leaflet of the phospholipid bilayer in TGN vesicles, and Drs2 activity is required for clathrin-mediated trafficking from the TGN to endosomes (32). We found that the drs2Δ strain also mislocalized Arn1-GFP to the plasma membrane in the absence of FC (Fig. 8C). Thus, Arn1-GFP not only required sufficient levels of PS for FC-induced plasma membrane trafficking; it also required the asymmetric distribution of PS for the TGN-to-vacuole trafficking in the absence of FC.

A

B

C

FIGURE 8. Mislocalization of Arn1p-GFP in other phospholipid-deficient strains. A, genes involved in the biosynthesis of lipids. The degradation of sphingolipid long-chain base phosphates (LCB-P) yields ethanolamine phosphate (ETN-P), which is incorporated into PE via the CDP-ethanolamine (CDP-ETN) intermediate. Only CHO1 is an essential gene in this pathway. B, different patterns of Arn1-GFP mislocalization in strains with defects in phospholipid synthesis. Strains of the indicated genotypes were grown overnight in iron-poor medium to induce the expression of Arn1p-GFP and then treated with FC as indicated for 2 h. Cells were then subjected to fluorescence microscopy. C, mislocalization of Arn1-GFP in a strain lacking PS asymmetry. YYG025 (drs2Δ Arn1-GFP) was grown as in B, above, and subjected to fluorescence imaging.

are completely lacking PS and are dependent on the Kennedy pathway for the biosynthesis of PE and PC (Fig. 8A) (26). Although we did not identify CHO1 in our screen (CHO1 is an essential gene and not represented in the deletion collection), we did study a cho1Δ strain. We examined the trafficking of Arn1-GFP in the cho1Δ strain and found that Arn1-GFP was localized to punctate intracellular structures in both the presence and absence of FC, with no signal apparent on the plasma membrane (Fig. 8B). Muq1 catalyzes the conversion of ethanolamine phosphate to CDP-ethanolamine in the ethanolamine branch of the Kennedy pathway (Fig. 8A). Ethanolamine phosphate is produced from the degradation of sphingolipid long-chain base phosphates (28), which are involved in the trafficking of membrane proteins (29). Cells lacking MUQ1 accumulated Arn1-GFP on the plasma membrane both in the presence and in the absence of FC, which sug
inner, cytosolic leaflet of the plasma membrane (32). Thus, the distribution of PS is ideal for directing cargo proteins at the TGN into vesicles destined for the plasma membrane. This asymmetry of PS is established in the TGN by the activity of the aminophospholipid translocase Drs2. Drs2 is one of five P-type ATPases of yeast that have “flipase” activity and is specific for PS. The activities of Drs2 and other flipases are required for the formation and trafficking of vesicles in the late secretory pathway, which points to the importance of membrane lipid composition in these events.

PS differs from PE and PC in that its headgroup is negatively charged. In mammalian cells, this negative charge on the inner leaflet of the plasma membrane has been shown to contribute to the targeting of proteins with polybasic clusters to the plasma membrane, such as the tyrosine kinase Src and protein kinase C (34, 35). In yeast, PS has been shown to be important for the uptake of tryptophan, indicating that PS is required for the stability, trafficking, or activity of the Tat1 and Tat2 transporters (36). Both Arn1 and Arn3 contain a cluster of phenylalanine residues in the carboxyl terminus, and, in the case of Arn1, these residues are required for plasma membrane trafficking (6). Multiple basic residues are located within these phenylalanine clusters in both Arn1 and Arn3. One explanation for the role of PS in targeting Arn1 to the cell surface is that FC binding to Arn1 triggers a conformational change that allows the hydrophobic and basic residues of the carboxyl terminus to interact with membrane PS in a way that permits both to be sorted to the plasma membrane. Alternatively, the anionic charge on PS could facilitate the recruitment of an unidentified trafficking protein, possibly one with a polybasic cluster, that then interacts with Arn1.

The Roles of Other Phospholipids in Arn1 Trafficking—A second source of negative charges on lipid membranes are the phosphorylated PIs. Analyses of strains with mutations in the kinases and phosphatases that modify PI have revealed well-defined roles for these phospholipids in vesicle trafficking (37). Specifically, phosphorylated PIs are required for secretion, TGN-to-endosome sorting, MVB sorting, endocytosis, and autophagy. Phosphorylated PIs function by specifically binding and recruiting adaptor proteins required for vesicle formation at specific membrane sites. In our screen of the deletion mutants, we identified several strains with defects in PI biosynthesis, phosphorylation, and dephosphorylation that missorted Arn1-GFP to the plasma membrane in the absence of FC (data not shown). These findings were in accordance with the known roles of phosphorylated PIs in the VPS pathway.

Our analysis of mutants involved in the synthesis of PE and PC indicated that other perturbations in the levels of the major phospholipids and their intermediates could disrupt the trafficking of Arn1-GFP. Only a small amount of cellular PE is synthesized in the mitochondria by Psd1, whereas most of the remainder is synthesized by Psd2 in endosomal membranes (40). Deletion of Psd2, but not Psd1, results in a specific depletion of vacuolar PE and a concomitant loss of cadmium detoxification through the transporter Ycf1. Strains completely lacking in PE synthesis exhibit defects in the uptake of multiple amino acids and fail to target the Can1 arginine permease to the plasma membrane (41). Thus, there is ample evidence that PE is required for the full activity of transporter proteins in yeast. Work in Escherichia coli and in vitro systems indicates that the polar headgroup of PE is required for the stability of transmembrane domains in folding intermediates of polytopic transporter proteins (42). Our observation that the cho2Δ strain has a defect in sorting Arn1-GFP into the MVB (Fig. 8B) suggests that merely altering the levels of these lipids can affect the formation of luminal vesicles of the MVB. Remarkably, deletion of OPI3 also disrupts trafficking of Arn1-GFP through the VPS pathway but in a phenotypically different way. The appearance of Arn1-GFP on the plasma membrane and, to a lesser degree, in the lumen of the vacuole indicates a defect in TGN-to-endosome sorting similar to that seen in the muq1Δ strain. OPI3 was also identified in a screen for mutants with defects in the formation of plasma membrane microdomains containing ergosterol, Can1, and other transporters (43). These phenotypes of the opi3Δ strain are attributable to the accumulation of a different headgroup, monomethylethanolamine, in the lipid bilayer, as opposed to the trimethyllethanolamine of PC.

Taken together, our data indicate that the phospholipid composition of membranes has a critical impact on trafficking of Arn1-GFP, either through direct interactions with the transporter or indirectly through the localization and activity of other components of the trafficking machinery. Many transporters appear to have specific lipid requirements for appropriate folding, trafficking, and activity. The mechanisms by which individual lipids are directed to different membranes in the cells await investigation.

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