Phosphatidylserine Transport to the Mitochondria Is Regulated by Ubiquitination*

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Mitochondrial membrane biogenesis requires the interorganelle transport of phospholipids. Phosphatidylserine (PtdSer) synthesized in the endoplasmic reticulum and related membranes (mitochondria-associated membrane (MAM)) is transported to the mitochondria by unknown gene products and decarboxylated to form phosphatidylethanolamine at the inner membrane by PtdSer decarboxylase 1 (Psd1p). We have designed a screen for strains defective in PtdSer transport (psta1 mutants) between the endoplasmic reticulum and Psd1p that relies on isolating ethanolamine auxotrophs in suitable (psta2Δ) genetic backgrounds. Following chemical mutagenesis, we isolated an ethanolamine auxotroph that we designate pstA1-1. Using in vivo and in vitro phospholipid synthesis/transport measurements, we demonstrate that the pstA1-1 mutant is defective in PtdSer transport between the MAM and mitochondria. The gene that complements the growth defect and PtdSer transport defect of the pstA1-1 mutant is MET30, which encodes a substrate recognition subunit of the SCF (suppressor of kinetochore protein 1, cullin, F-box) ubiquitin ligase complex. Reconstitution of different permutations of MAM and mitochondria from wild type and pstA1-1 strains demonstrates that the MET30 gene product affects both organelles. These data provide compelling evidence that interorganelle PtdSer traffic is regulated by ubiquitination.

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Phospholipid Transport Regulated by a Ubiquitin Ligase

At present, no protein or gene product has been identified that mediates or regulates ER to mitochondria membrane association and/or transport of PtdSer between these organelles. Hence, the goals of our study were to 1) isolate new aminophospholipid transport mutants in the PSTA pathway, 2) characterize these transport mutants biochemically, and 3) identify the gene that complements transport-defective mutants.

In this paper, we describe the isolation of a new ethanolamine auxotrophic yeast strain, in a psdA mutant background, with the characteristics of a psdA mutation. Biochemical analysis of the phenotype and the protein product of the psdA mutation in the A. fumigatus complemented the defect in the A. fumigatus PtdSer/PSDpase and showed that the PSDpase is encoded by the MET30 gene. This defect is complemented by the A. fumigatus MET30 gene.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Media components for yeast and bacterial growth were purchased from Difco, U. S. Biological, Sigma, and Fisher. The radioisotopes, t-[3-3H]serine and t-[14C]serine, were purchased from American Biosciences. Phosphatidyl-[1-14C]serine was synthesized from t-[1-14C]serine and CDP-diacylglycerol. Phospholipid standards and CDP-diacylglycerol were purchased from Avanti Polar Lipids. Thin layer silica gel H plates were obtained from Analtech Corp.

**Yeast Strains and Media**—Yeast strains were cultured in YPLAUDE (containing per liter: 10 g of yeast extract, 20 g of peptone, 2% l-lactic acid, 20 g of adonitol, 20 mg of uracil, 3 g of ethanolamine, pH 5.5, or SL (containing per liter: 6.67 g of yeast nitrogen base without amino acids, 2% l-lactic acid, standard amino acids, pH 5.5 or SC (same as SL except 2% glucose as carbon source). Ethanolamine was added to SL medium to a final concentration of 3 mM. For the isolation of mitochondria, strains were grown in semi-synthetic lactate medium as described by Glick and Pon (25). The absorbance of liquid cultures was measured by Glick and Pon (25). The absorbance of liquid cultures was measured with a Beckman DU-640 spectrophotometer. The parent strain MSY30 (MATα, psd2Δ::KAN, met30–522L) was first outcrossed with MSY35 (MATα ura3Δ::LEU2 his3Δ::KAN met1530 psd2::KAN) and then outcrossed with MSY34 (MATα ura3Δ::LEU2 his3Δ::KAN met30–522L) and then MSY57 (MATα ura3Δ::LEU2 his3Δ::KAN met30–522L). The reaction contained 50 μCi of [3H]-sulfate and 50 μCi of [5-14C]-serine in a volume of 400 μL. The reactions were terminated by the addition of 0.5 ml of 0.25 M HSO4 to the gas-tight seal with a syringe and needle. The [14C]-recovered from the reaction that was trapped on the filter paper was quantified by liquid scintillation spectrometry.

**Phospholipid Analysis**—Yeast strains were grown in synthetic lactate plus ethanolamine medium to mid-log phase. Cultures were then washed twice with sterile water and suspended in 0.2 ml of buffer minus serine to an A600 of 0.2 in a volume of 2 ml. Radiolabeling was initiated by adding 10 μCi/μmol t-[3-3H]serine (32 Ci/mmol). Labelling proceeded at 30 °C for 4 h with vigorous shaking. Growth and metabolism were arrested by the addition of trichloroacetic acid to a final concentration of 5% (v/v), along with ~10 mg of unlabeled carrier. Cell pellets were washed twice by centrifugation with ice-cold buffer, resuspended, and extracted as previously described. Phospholipid standards were visualized by spraying the TLC plates with 0.1% 2-anilino-1-naphthalene sulfonic acid, followed by exposure to UV light. Separated lipids were spotted onto 2.5 ml of H2O, with 4.5 ml of ScintiSafe 90% scintillation fluid (Fisher). Radioactivity of the samples was measured by liquid scintillation spectrometry (Beckman catalog no. LS6500).

**In Vitro Radiolabeling and Characterization of Crude Mitochondrial Membranes**—Crude mitochondria were prepared as described (25) from 1-liter cultures grown to early log phase in semisynthetic lactate medium supplemented with 2% glucose (20 ml/g), 0.1% 2-hydroxyethane sulfonic acid, 0.1% 2-anilino-1-naphthalene sulfonic acid, and ethanolamine (3 μM) at 30 °C. Crude mitochondrial pellets were suspended in buffer C (0.6 M sorbitol, 20 mM K+ MES, pH 6.0) and assayed immediately for protein (Bio-Rad protein assay). Intactness of the outer membrane was assessed by measuring cytochrome c oxidase activity (27). Phospholipid and PSDpase enzyme activities of the crude mitochondrial preparations were assayed as described previously (5, 10). Reconstituted aminophospholipid synthesis and transport were measured using [3H]-serine incorporation into lipids as outlined by Achleitner et al. (6) with a few modifications. Briefly, crude mitochondria were diluted in buffer C (0.6 M sorbitol, 20 mM K+ HEPES, pH 7.4) to a protein concentration of 1 μg/ml. The reaction contained 50 μl of crude mitochondria, 16.7 μl of buffer C, and 113.3 μl of buffer C. Samples were equilibrated to 30 °C in a shaking water bath before initiating the reaction by adding 20 μCi (1 μCi/μl) of L-[3-3H]serine (32 Ci/mmol). After 15 min, 4 μl of 0.2 mM EDTA in buffer C was added (final concentration ~4 mM). Reactions were stopped after an additional 75 min by the addition of 1.5 ml of methanol, 1.5 ml of chloroform, and 1.2 ml of 0.2M KCl. Zero time points were obtained by adding L-[3H]serine and methanol simultaneously to the above mixture. Lipids were extracted by vortexing the samples, followed by centrifugation to separate organic and aqueous phases. The chloroform phase was washed twice, each time with 2.8 ml of practical upper phase solution (500 ml of methanol plus 450 ml of phosphate-buffered saline, pH 7.4, saturated with 50 ml of chloroform). The chloroform phase was collected and dried under a nitrogen stream. Samples were suspended in chloroform and separated by TLC on silica Gel H plates.

**In Vitro Reconstitution of Lipid Traffic between Purified MAM and Mitochondria**—Crude mitochondria were isolated from 4.5 l of culture as described above. Subsequent isolation of purified mitochondria and MAM was performed as described by Achleitner et al. (6). Briefly, crude mitochondria suspended in buffer B were layered on a density gradient composed of 20–50% sucrose constructed in 1-mL steps differing in 3.5% sucrose increments in buffer B. The gradient was centrifuged at 100,000 × g for 1 h at 4 °C using an SW 41 rotor. Mitochondria were harvested from the lower third of the gradient, diluted 3–4-fold in buffer B, and pelleted for 10 min at 12,000 × g at 4 °C. The isolated mitochondria and MAM were washed twice in buffer B and centrifuged at 4 °C. Resulting mitochondrial and MAM pellets were suspended in buffer B and buffer C, respectively, and thoroughly resuspended. Freshly isolated mitochondria and MAM were immediately assayed for protein and L-[3-3H]serine incorporation as described (27). By using a 200-μL reaction, MAM and purified mitochondria were reconstituted at final protein concentrations of 0.05 and 0.85 mg/ml, respectively. Radiolabeling was conducted in the presence of 0.2 mM CDP-diacylglycerol. Subsequent lipid analysis was performed as described above.

**Mitochondrial Phospholipid Content**—For measuring mitochondrial phospholipids, cells were first grown to log phase in synthetic lactate medium plus ethanolamine at 30 °C. The cells were washed twice with sterile water, suspended in synthetic lactate medium without ethanolamine, and incubated with shaking at 30 °C, for 14–18 h before harvesting for mitochondrial isolation. Crude mitochondria were prepared by the method of Glick and Pon (25). Lipids were extracted from 2 mg of crude mitochondrial protein, in 4 ml of methanol, 3.2 ml of 0.2 M HCl, and 1 ml of chloroform/methanol/3% aqueous HCl (13/52 by volume), and dried under a nitrogen stream with 7.6 ml of practical upper phase without phosphate solution (500 ml of methanol plus 450 ml of 0.2 M KCl, saturated with 5 ml of chloroform). The organic phase was collected and dried under a nitrogen stream. Phospholipids were separated by two-dimensional TLC on Silica 60 plates. The first solvent system contained chloroform/methanol/3% aqueous HCl (13/52 by volume), and the second solvent contained chloroform/methanol/ammonium hydroxide (13/52 by volume). Lipids were visualized by staining with iodine vapor and scraped into glass tubes. Phosphorus was quantified using the method of Rouser et al. (29).

**Mitochondrial Protein Import**—Mitochondria were prepared from

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parental and psd2Δ strains grown in semisynthetic lactate medium supplemented with 3 mM Etn using the methods of Gliek and Pen (29). Protein import was measured using the methods of Rospert and Schatz (30). The Psd1p was expressed with a carboxyl-terminal V5 epitope, from a pYES plasmid (Invitrogen), under T7 promoter regulation. The Su9-DHFR was expressed from a plasmid under SP6 promoter regulation. Both proteins were synthesized in vitro using the appropriate TNT-coupled transcription/translation system (Promega) in the presence of [35S]methionine. The radioabeled protein mixtures were incubated with isolated mitochondria in either the absence or presence of valinomycin (1 μg/ml) in an import buffer containing 0.6 M sorbitol, 50 mM HEPES-KOH, 50 mM KCl, 0.75 mg/ml methionine, 10 mM MgCl2, 1 mg/ml bovine serum albumin, 2.5 mM EDTA, and 2 mM KH2PO4, adjusted to pH 7.0. The reactions also contained 2 mM ATP and 10 mM NADH and were conducted for 3 or 10 min at 30 °C. The nonimported proteins were removed from the reactions by treatment with 100 μg/ml trypsin for 30 min at 0 °C. The action of trypsin was arrested with 200 μg/ml soybean trypsin inhibitor. Following the trypsin treatment, the mitochondria were harvested by centrifugation, precipitated in 5% trichloroacetic acid, heat-inactivated, and impregnated with 1 mM salicylate before drying. The radioabeled proteins were visualized using a Storm 860 PhosphorImager. Quantification of protein import was performed using ImageQuant 5.2 software from Amersham Biosciences.

Analysis of Met4p Ubiquitination—The Met4p gene modified to contain multiple C-terminal myc epitope tags was expressed from the chromosome under control of its endogenous promoter (31). The modified Met4p gene was expressed in strains PY725 and FY743 constructed by Dr. P. Kaiser (University of California, Irvine). In addition, we constructed the strains JCY474 (MATa ura3::KAN trp1::HIS4 MET4::his30::KAN 18myc) and JCY475 (MATa ura3::KAN trp1::HIS4 MET4::his30::KAN 18myc) from MSY30 and EAL18, respectively. The yeast strains examined were in either SC or SL medium containing 5 mM ethanolamine and supplemented with Etn as indicated. The cells were harvested by centrifugation, and 5% boiling trichloroacetic acid was added to the pellet followed by vortex mixing for 5 s. The acid-treated cells were chilled on ice and sedimented by centrifugation. The final pellets were rapidly frozen in liquid nitrogen and stored at −80 °C prior to further processing. For processing, the cell pellets were suspended in an extraction buffer consisting of 8 M urea, 50 mM Tris-HCl (pH 8.0), 0.1 mM sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, and an aliquot of Sigma anti-protease cocktail. The samples were boiled for 5 min, homogenized in a bead beater for 70 s, and centrifuged at 15,000 × g for 15 min at 20 °C. The supernatant was used as a sample. The radiolabeled proteins were visualized using a Storm 860 PhosphorImager. Quantification of protein import was performed using ImageQuant 5.2 software from Amersham Biosciences.

RESULTS

Isolation and Characterization of an Etn Auxotroph, EAL18—Yeast strains that have a psd2Δ::KAN allele depend on Psd1p for the majority of PtdEtN and PtdCho synthesis in the absence of exogenous ethanolamine or choline (see Fig. 1). We reasoned that mutants with defects in PtdSer import into and PtdEtN export from the locus of Psd1p in the inner mitochondrial membrane would require ethanolamine for growth in a psd2Δ::KAN genetic background. To generate mitochondrial phospholipid transport mutants, we mutagenized a psd2Δ::KAN strain, MSY30, and selected for ethanolamine auxotrophs on medium containing lactate as a carbon source. Lactate medium was used to avoid selection of respiration-deficient petites. Screening of the first 10,000 colonies yielded eight ethanolamine auxotrophs, of which two were psd1 mutants and one was an apparent psd1 mutant, as determined by genetic complementation and biochemical enzyme assay. One of the remaining strains, EAL18, displayed a definitive requirement for ethanolamine for growth in liquid SC medium at 30 °C, as shown in Fig. 2. In the absence of Etn, the EAL18 strain undergoes two doublings and then arrests. In the presence of ethanolamine, the mutant, EAL18, grows similarly to the parental strain, MSY30, although somewhat slower. The growth of the parental strain is essentially the same in the presence and absence of Etn. At 36 °C, EAL18 shows very little or no growth in synthetic lactate medium, regardless of ethanolamine supplementation. In addition, EAL18 is not an ethanolamine auxotroph in synthetic glucose medium at 30 °C. As shown in Fig. 3, choline supplementation does not rescue the growth defect of EAL18 in synthetic lactate medium at 30 °C, indicating a stringent requirement for Etn under these conditions. Tetrad analysis yielded a 2:2 segregation of ethanolamine auxotrophy consistent with a mutation at a single locus. Diploids that are heterozygous for the mutation are not ethanolamine auxotrophs, indicating the mutation is recessive. The above properties of the mutant are similar but not identical to psd1Δpsd2Δ strains and are consistent with defects in PtdEtN synthesis.

To test whether the ethanolamine auxotrophic mutant had a defect in the formation of aminoglycerophospholipids, cells were labeled with [3H]serine in SL medium. Incorporation of
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The EAL18 Strain Has a Defect in PtdSer Transport to the Mitochondria—We next employed an in vitro transport assay, in which a crude mitochondrial preparation containing MAM is [3H]serine labeled in the presence of 0.5 mM MnCl2. These conditions provide for an initial pulse of radiolabel incorporation into PtdSer. After 15 min, EDTA is added to a final concentration of 4 mM, allowing the chase of radiolabel into PtdSer. This finding shows that the inner mitochondrial membrane to support cell growth.

Since Psd1p synthesizes most of the cellular PtdEtn in the absence of supplemental ethanolamine, we expected that a defect in PtdSer transport to mitochondria would lead to a deficiency in cellular and mitochondrial pools of PtdEtn. Therefore, we examined the phospholipid composition of crude mitochondrial preparations by centrifugation (25). Table I shows that the mitochondrial PtdEtn pool of the outcrossed mutant strain, MSY54, is about 62% of the parental strain. A relative increase in the PtdCho and phosphatidylinositol pools of the mutant accompanies the deficiency in the PtdEtn pool. These data are consistent with the whole cell radiolabeling observations and a defect in PtdSer transport to the Psd1p.

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decrement in PtdEtn labeling of the mutant is not due to increased PtdEtn turnover to PtdCho. Fig. 6 demonstrates that the very same mitochondrial preparations of the mutant have Psdlp and Pss1p enzyme activities that are similar to the parental preparations. The minor increase in Psdlp activity may constitute some adaptation of the mutant cells to depletion of the cellular PtdEtn pool. Since the decrement in PtdEtn accumulation is not due to aberrant Pss1p or Psd1p enzyme activities, we conclude that the defect lies between these two biosynthetic steps. Taken together, these data demonstrate that the EAL18 mutant is deficient in PtdSer translocation steps of 3.3% increments from 20 to 50% sucrose. The MAM and purified mitochondria. The density gradient was constructed in 1.0-ml steps of 3.3% increments from 20–50% sucrose. The MAM and purified mitochondria were resolved by centrifugation at 100,000 × g for 1 h in an SW41 rotor. The centrifuge tubes containing the parent and psa1-1 strains are shown in the figure, and the location and percentage of the sucrose steps are shown on the right. The gradient shown is one of four prepared during this study.

A defect in PtdSer transport to the mitochondria might be expected to alter the structure of the organelle. When preparing high purity mitochondria for reconstitution studies using sucrose density gradients, we immediately noticed that mutant strains contained mitochondria of abnormal density. In Fig. 8 the results of a density gradient purification of mitochondria are shown. The mitochondria from parental cells typically sediment as a broad band between 40 and 43% sucrose. In some preparations, the band takes on the appearance of a doublet. In contrast to the parental cells, the psa1-1 strain shows a doublet of mitochondria between 43 and 47% sucrose. The major lower density band of the psa1-1 mitochondria corresponds to the higher density band of the parental mitochondria. The higher density band of the psa1-1 mitochondria is unique. The phospholipid phosphorus/protein ratio of the total purified mitochondrial pool derived from parental cells is 0.56 μmol/mg of...
protein, and that of the psa1Δ-1 cells is 0.46 μmol/mg of protein, which reflects the differences in buoyant densities. These data are entirely consistent with the psa1Δ strain having a defect in lipid traffic to the mitochondria. The data also clearly demonstrate that mitochondrial populations of at least two different densities can be found in both the parental and mutant cells.

**Mitochondria from psa1Δ-1 Cells Have a Minor Defect in Protein Import**—Since our experimental results demonstrated defects in both lipid traffic to the mitochondria and abnormal organelle density, we also examined the import of proteins into purified mitochondria prepared on Nycodenz gradients (25). For these studies, we employed an Su9-DHFR chimeric construct consisting of 69 amino acids from the N terminus of subunit 9 of the mitochondrial ATPase and mouse dihydrofolate reductase (32) that is routinely used to study the import of matrix proteins. In addition, we measured the import and processing of Psd1p. The [35S]methionine-labeled precursor proteins were produced using a coupled transcription/translation reaction (30), and the time- and membrane potential (ΔΨ)-dependent import of the proteins was measured as shown in Fig. 9. The precursor for Su9-DHFR is readily imported into the mitochondria and processed to its mature form in both parental and psa1Δ-1 cells. The rate of Su9-DHFR import into mutant mitochondria is ∼60% of that found for parental mitochondria. The precursor for Psd1p is also imported into the mitochondria and targeted to the inner membrane of both parental and mutant strains. Consistent with stepwise processing of the mitochondrial targeting sequence and the inner membrane sorting sequence, two precursor forms of Psd1p (designated pI and pII) are identifiable. The data in Fig. 9 show the appearance of the mature β subunit of Psd1p and reveal that the import into mutant mitochondria occurs at 70–80% of the rate for parental mitochondria. These studies provide evidence that the psa1Δ-1 mutant has a very mild defect in the rate of protein import into the mitochondria. When comparing the differences in protein and phospholipid import rates into the mitochondria, it is also important to understand that changes in protein import principally affect protein accumulation in the mitochondria, whereas changes in PtdSer import affect the total cellular biosynthesis of PtdEtn and PtdCho.

A Genomic Library Screen Identifies MET30 as the Gene That Complements the psa1Δ-1 Strain—The psa1Δ-1 strain, EAL18, was outcrossed twice to yield the psa1Δ-1 strain, MSY77, which was utilized to screen a multicopy vector (pSEY18) yeast genomic library for clones that could complement the ethanolamine auxotrophy of MSY77. Approximately 22,000 uracil prototrophic pSEY18 library transformants were screened for ethanolamine prototrophy. Plasmids from 45 uracil/ethanolamine prototrophs were rescued into Escherichia coli. Purified plasmids were retransformed into psa1Δ-1 strains. Of the 45 retransformed plasmids, only one conferred robust growth on selective medium, whereas other library clones yielded intermediate, poor, or no growth on selective medium. The library clone that gave the best complementation of psa1Δ-1 ethanolamine auxotrophy contained five open reading frames: PCL7, DGFI10, NEO1, SYG1, and MET30. Subsequent subcloning of the open reading frames into single copy yeast vectors, YCP50 and/or YCPlac33, demonstrated that MET30 conferred the ethanolamine prototrophy to psa1Δ-1 mutant strains. Fig. 10 shows that a single copy plasmid, YCPlac33, containing the MET30 gene, can rescue the growth defect of a psa1Δ-1 mutant in SL medium devoid of ethanolamine. The MET30 gene encodes a substrate specificity subunit of SCF ubiquitin ligase (33). Major motifs found within Met30 include an F box for interaction with the Skp1p protein subunit and WD40 domains for interaction with substrates (34, 35).

To demonstrate that MET30 can complement the PtdSer transport defect observed in our biochemical assays, we employed the in vitro transport assay with crude mitochondria using a psa1Δ-1 strain carrying MET30 on a low copy plasmid. Fig. 11 shows that MET30 on plasmid YCPlac33 can restore the accumulation of radiolabel into PtdEtn compared with the parental and psa1Δ-1 strain harboring empty vector. Taken together, the in vivo and in vitro data show that wild type MET30 can complement both the ethanolamine auxotrophy and biochemical transport-deficient phenotype of psa1Δ-1.
We examined the ubiquitination of Met4p in response to methionine supplementation in our pstA1-1 strain and a met30-6ts strain and corresponding wild type strains. A brief summary of methionine regulation of Met4p is outlined in Fig. 14A. In the presence of methionine, Met30p interacts with Met4p and the SCF complex to promote ubiquitination of Met4p (Met4p-Ubi). The Met4p-Ubi cannot be recruited to multiple MET gene promoters and essentially becomes inactive. Under some growth conditions, the Met4p-Ubi appears stable, but other growth conditions promote degradation (31, 38). In our experiments, ubiquitination of Met4p was detected using a myc epitope-tagged version of Met4p. Methionine addition produced two prominent forms of Met4p-Ubi in wild type cells, as shown in Fig. 1A. In glucose medium, the parental strain for wild type and mutant (MSY77) cells grown in semisynthetic lactate medium plus Etn at 36 °C overnight. The mitochondria were subjected to sucrose density centrifugation at 100,000 × g for 1 h in an SW41 rotor. The centrifuge tubes containing the parental and met30-2 strains are shown, and the location and percentage of the sucrose steps are shown on the right.
Met4p. In contrast, the met30-6ts strain contains significant Met4p-Ubi at 25 °C but also accumulates significant amounts of nonubiquitinated Met4p. Moreover, the met30-6ts strain displays markedly reduced Met4p-Ubi at 37 °C and accumulates very high levels of nonubiquitinated Met4p. In addition to studies in glucose medium, we analyzed Met30p function in psdT1 cells in lactate medium, since these are the conditions that produce the lethal phenotype. In lactate medium containing methionine, we demonstrated that the parental strain for psdT1 contains stable levels of Met4p-Ubi and very low levels of Met4p. In contrast, the psdT1 strain not only contains Met4p-Ubi but also accumulates relatively high levels of Met4p. Quantification of the immunoreactive Met4p reveals that the ratio of Met4p/Met4p-Ubi is 30–40-fold higher in the psdT1 strain than its corresponding parental strain. From these data, we conclude that the psdT1 strain exhibits a defect in Met4p ubiquitination. The in vivo defect in ubiquitination is more pronounced with growth of the psdT1 strain in lactate medium than glucose medium. In parallel with the above studies, we performed genetic experiments in which we constructed a psd2Δ met4Δ strain. If Met4p functions downstream of Met30p as a positive regulator of lipid traffic, then psd2Δ met4Δ mutations should reproduce the phenotype of the psdT1 strain. However, the psd2Δ met4Δ strain we constructed had no detectable phenotype. These data make it unlikely that Met4p functions as a positive regulator of lipid transport. These data also indicate that Met4p-Ubi cannot function as a positive regulator of lipid transport. These results raise the possibility that novel substrates for Met30p could be ubiquitinated and function to regulate lipid traffic.

The psdT1 Transport Defect Is Associated with Mitochondria and MAM—A previous study has demonstrated that PtdSer can be transported from yeast MAM to mitochondria in a reconstituted system containing both organelles (6). We have applied this reconstituted assay to determine whether the psdT1 transport defect is associated with the donor MAM fraction or the acceptor mitochondria. MAM and mitochondria from a psdT1 strain and the wild type parental strain were separated on sucrose density gradients. Individual reconstitution reactions were prepared consisting of wild type MAM in combination with either wild type or mutant mitochondria and vice versa. MAM and mitochondria—40% of their nascent PtdSer was followed using a [3H]serine precursor. The turnover of PtdSer and the appearance of radiolabel in PtdEtn serves as a measure of PtdSer transport from the MAM to the mitochondria. To simplify the comparison of conditions, the data are expressed as the ratio of PtdEtn/PtdSer as shown in Fig. 15. These data reveal that the transport defect observed in the intact cell and the crude mitochondrial preparations is also reproduced in the purified MAM and mitochondria reconstitution assay. Wild type cells convert ~30% of their nascent PtdSer to PtdEtn, whereas the mutant cells convert only about 10%. The wild type MAM cannot overcome the defect in a
reaction containing mutant mitochondria. In addition, wild type mitochondria cannot overcome the defect in a reaction containing mutant MAM. These results demonstrate that the transport defect of pstA1-1 cells is not exclusively associated with mitochondria or MAM but appears to reside in both organelles. From these data, we conclude that Met30p has a regulatory effect on transport constituents present in both mitochondria and MAM.

**DISCUSSION**

In this paper, we describe the isolation of a new Etn auxotroph with the characteristics expected for a mutant with a defect in PtdSer transport from the MAM to the mitochondria. This study is part of a broad genetic approach outlined in Fig. 1 that we are applying to uncover molecules involved in intermembrane lipid traffic. The new mutant, designated pstA1-1, is the first that we have discovered in the PSTA pathway, that we propose regulates aminoglycerophospholipid movement to the mitochondria.

The pstA1-1 mutant was selected for Etn auxotrophy on a lactate carbon source. This selection was biased with respect to a respiratory carbon source so as to identify strains that had an absolute requirement for fully functional mitochondria. The Etn requirement of the pstA1-1 strain cannot be replaced by choline supplementation. Despite the ability of choline to enable synthesis of PtdCho via the Kennedy pathway, this lipid cannot replace PtdEtn. This latter finding reflects both the need for critical levels of PtdEtn in the mitochondria, under respiratory conditions, and the essential role of PtdEtn in cells that have only recently been recognized (13, 14). Although the pstA1-1 strain is an Etn auxotroph, the level and rate of PtdEtn synthesis is only modestly depressed in these cells under nutrient-restrictive conditions. Greater reduction of PtdEtn levels and synthesis may simply not be tolerated under the conditions we have employed in this genetic screen. We suspect that the Etn supplementation of cells allows sufficient PtdEtn to be made by the Kennedy pathway to spare enough PtdEtn made by Psd1p to be retained and used by the mitochondria. In this regard, we highlight the observation that an independently isolated mutant, met30-2 (36), that is allelic to the pstA1-1 mutation shows a similar phenotype when it is combined with a psd22a mutation such that it is an Etn auxotroph on glucose at 36 °C.

Examination of the PtdSer traffic between organelles isolated from the pstA1-1 strain gives results that reflect the defects in lipid synthesis and composition of intact cells. In cell-free assays that monitor PtdSer synthesis in the MAM and its transport to the mitochondria, the pstA1-1 strain shows a 27% accumulation of PtdSer and greater than 63% decrement in the decarboxylation of this lipid. Although the defect in lipid traffic is not complete, it is sufficient to produce a growth phenotype in the intact cell and a highly reproducible biochemical phenotype in vitro. This lipid transport defect is also reproduced in cells harboring the met30-2 allele. The partitioning properties of (N-4-nitrobenzo-2-oxa-1,3 diazole)aminocaproyl lipids containing short acyl chains in the sn-2-position allows them to be used to rapidly load the outer mitochondrial membrane and examine lipid traffic to the inner mitochondrial membrane (28). Our findings with NBD-Ptd[1-14C]Ser demonstrate that the defect in the mutant is not between the mitochondrial membranes but occurs between the MAM and the outer mitochondrial membrane.

Consistent with the assignment of the transport lesion to events between the MAM and mitochondria are the results obtained from density gradient centrifugation that reveal that a significant fraction of the mitochondria from pstA1-1 cells has abnormally high density. The defect in PtdSer transport to the mitochondria caused the density of the organelle to increase. This increase in density indicates that the mitochondria fail to fully compensate for the PtdSer transport defect by increasing the import or synthesis of other phospholipids. The net effect is an overall change in the phospholipid/protein ratio of the mitochondria isolated from the mutant cells. This same defect is also observed in the met30-2 strain. These data further indicate that both the parental cells and mutant cells each appear to have mitochondria of two distinctly different densities. It is also noteworthy that the pstA1-1 strain not only has a unique high density mitochondrial band but also lacks the low density band associated with the parental phenotype.

Although the mechanism of lipid transfer between the MAM and mitochondria is not known, several studies have suggested that proteins on the surface of one or both membranes participate in the process (6, 21). Some evidence has also been provided to suggest that proteins with fusogenic properties may participate in the lipid transfer reaction, but none of these components has been clearly identified (39, 40). Both biochemical and morphological experiments have implicated zones of close physical association between the MAM and mitochondria as likely elements involved in the intermembrane transfer of PtdSer (4, 6, 20–24).

The gene that complements the pstA1-1 defects in growth and lipid traffic is MET30. The MET30 gene encodes a protein with an F box and five WD40 domains that dictate substrate specificity for a subset of SCF family ubiquitin ligases (33, 34). The F box is the site of interaction of Met30p with the Skp1p component of the ligase complex (35). The WD40 domains are involved in protein-protein interaction. The pstA1-1 mutation (P522L) resides in the carboxyl terminus of the molecule in a region between the fourth and fifth WD40 domain. We anticipate that this mutation will alter the recognition of one or more specific substrates and their ubiquitination. All of the current data are consistent with regulation of PtdSer transport to the mitochondria by the process of ubiquitination. However, the level at which ubiquitin ligase regulates lipid traffic is still unknown. Ubiquitin modification is now recognized to regulate cellular processes at multiple levels that include proteolysis (41), transcriptional activation (42), transcriptional inactivation (31, 38), and signaling (43, 44). Several targets for SCF-Met30p have been identified, including Met4p (31, 36), the VP16 transcriptional activation domain (42), and Swe1p (45).
Ubiquitination leads to inactivation of the transcription factor Met4p (31, 36) and activation of a heterologous VP16 transcriptional activation domain (42). The Swe1p is an inhibitory kinase that is degraded after ubiquitination (45).

Since Met4p is the best characterized substrate for Met30p-directed ubiquitin ligase, we also directly tested whether our pstA1-1 mutant was defective in Met4p modification. In experiments shown in Fig. 14, we demonstrated that Met4p ubiquitination in the pstA1-1 mutant was significantly reduced in vivo. From these data, we infer that Met30p will also be defective in recognizing and ubiquitinating other substrates as well. We also designed a specific genetic test to determine whether Met4p could act as a positive regulator of lipid traffic downstream of Met30p. In this experiment, we generated a pds2Δ met4Δ strain and tested for Etn auxotrophy. Since the strain was not an Etn auxotroph, we conclude that Met4p does not act as a positive modulator of PtdSer transport. In these results, these also eliminate a requirement for Met4p-Ubi as a positive regulator of lipid transport.

Ubiquitination is now also recognized to play an important role in membrane traffic as a signaling motif for protein sorting, endocytosis, and viral budding (46). Certainly, a provocative role for Met30p function in lipid traffic entails modification of membrane targets for the assembly of macromolecular docking complexes that facilitate apposition of donor and acceptor membranes (e.g. MAM and mitochondria) and intermembrane lipid transport. In addition to our study, another line of investigation has linked protein ubiquitination to mitochondrial maintenance and function. Using a genetic suppressor screen, Fisk and Yaffe (47) identified the ubiquitin ligase Rsp5p as an essential regulator of mitochondrial morphology and function. Thus, more than one ubiquitin ligase system appears to play a role in maintaining the structure and function of mitochondria. The Rsp5p has also been implicated in endocytosis (48), and protein sorting at the trans-Golgi network (49), and its mammalian homolog, Nedd4, participates in viral budding (50).

Our findings also indicate that the action of Met30p in regulating PtdSer transport to mitochondria is not restricted to this organelle. The reconstituted PtdSer transport system used in these studies enabled us to critically test the ultimate site of effect of Met30p. Using permutations of admixtures of wild type and pstA1-1-derived MAM and mitochondria, we mapped the lesion to both organelles. We interpret this to mean that Met30p causes distinct biochemical changes in both MAM and mitochondria, simultaneously. Hypothetically, one simple means to achieve this effect would be direct ubiquitination of critical proteins on both organelles. Clearly, more experimentation will now be necessary to determine if the effect of Met30p is direct or indirect.

In summary, we have isolated a mutant defective in PtdSer traffic between the MAM and the mitochondria. The mutation alters the PtdEtn content, the total phospholipid content, and the density of the organelle. The effects of the mutation are manifested in both the MAM and mitochondria and are reversed by the MET30 gene, which encodes a component of SCF ubiquitin ligase. These findings now identify a novel mechanism for regulating interorganellar phospholipid transport.

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