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SLC1 and *SLC4* encode partially redundant acyl-Coenzyme A 1-acylglycerol-3-phosphate O-acyltransferases of budding yeast

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Phosphatidic acid (PA) is the intermediate, from which all glycerophospholipids are synthesized. In yeast it is generated from *lyso*-phosphatidic acid, which is acylated by Slc1p, a *sn*-2-specific, acyl-Coenzyme A-dependent 1-acylglycerol-3-phosphate O-acyltransferase. Deletion of *SLC1* is not lethal and does not eliminate all microsomal 1-acylglycerol-3-phosphate O-acyltransferase activity suggesting that an additional enzyme may exist. Here we show that *SLC4* (Yor175c), a gene of hitherto unknown function, encodes a second 1-acyl-*sn*-glycerol-3-phosphate acyltransferase. *SLC4* harbors a membrane bound O-acyl transferase (MBOAT) motif, and down regulation of *SLC4* strongly reduces 1-acyl-*sn*-glycerol-3-phosphate acyltransferase activity in microsomes from *slc1Δ* cells. The simultaneous deletion of *SLC1* and *SLC4* is lethal. Mass spectrometric analysis of lipids from *slc1Δ* and *slc4Δ* cells demonstrates that *in vivo* Slc1p and Slc4p generate almost the same glycerophospholipid profile. Microsomes from *slc1Δ* and *slc4Δ* cells incubated with [^{14}C]-oleoyl-Coenzyme A in the absence of *lyso*-phosphatidic acid and without CTP still incorporate the label into glycerophospholipids, indicating that Slc1p and Slc4p can also use endogenous *lyso*-glycerophospholipids as substrates. However, the lipid profiles generated by microsomes from *slc1Δ* and *slc4Δ* cells are different and this suggests that Slc1p and Slc4p have a different substrate specificity or have access to different *lyso*-glycerophospholipid substrates because of a different subcellular location. Indeed, affinity-purified Slc1p displays Mg^{2+}-dependent acyltransferase activity not only towards *lyso*-phosphatidic acid but also *lyso* forms of phosphatidylserine and phosphatidylinositol. Thus, Slc1p and Slc4p may not only be active as 1-acylglycerol-3-phosphate O-acyltransferases, but also be involved in fatty acid exchange at the *sn*-2 position of mature glycerophospholipids.

Introduction

All eukaryotes utilize glycerophospholipids (GPL) to build their membranes and stock triacylglycerols as an energy reserve when nutrition is abundant. Both of these lipid classes are made from phosphatidic acid (PA), a central metabolite, which is taken by several key enzymes into various pathways (1). As shown in Fig. 1, PA can be synthesized *de novo* through the acylation of L-glycerol-3-phosphate by Gat1p or Gat2p and subsequent acylation of the thus generated *lyso*-PA by Slc1p, a *lyso*-PA acyltransferase (LPAAT). Small variations of this general pathway can be found in various organisms: many eukaryotes including man can synthesize 1-alkenyl-2-acylglycerol-3-phosphate used for plasmalogen synthesis or 1-alkyl-2-acylglycerol-3-phosphate as alternatives to 1,2-bisacylglycerol-3-

phosphate. Also, the biosynthesis can start form dihydroxyacetonphosphate instead of glycerol-3-phosphate as depicted in Fig. 1. Furthermore, yeast can also generate PA through the breakdown of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) operated by phospholipase D (PLD) activities (2-5). PA also acts as a general regulator of lipid biosynthesis in yeast. This regulation is achieved by PA's ability to bind and inactivate the transcriptional repressor Opi1p, thereby allowing derepression of the transcription of a large variety of lipid biosynthetic enzymes when PA accumulates (6).

SLC1 was first recognized as a 1-acylglycerol-3-phosphate O-acyltransferase in a screen for second site suppressor mutations that would allow yeast to grow in the absence of sphingolipids (7). This seminal study showed that a suppressor effect was due to a point mutation in *SLC1*, that this gene encoded an LPAAT, and that the suppressing gain of function *SLC1-1* allele allowed the enzyme to utilize C26:0 fatty acids instead of the normal C18 species (7). The fact that *SLC1* was not essential and that its deletion only eliminated about 60% of microsomal LPAAT activity suggested that a second, unrelated *sn*-2-specific LPAAT may exist (8). Alternatively, the possibility was not excluded that 1-acylglycerol-3-phosphate may spontaneously transmute itself into 2-acylglycerol-3-phosphate by acyl migration and that the latter could be acylated in *sn*-1 by Gat1p/Gat2p, thus forming PA.

The two human *SLC1* homologues LPAAT-α and LPAAT-β have been demonstrated to convert *lyso*-PA to PA and humans also contain a third homologous gene, LPAAT-ζ. Similar to yeast, humans also harbor two PI4,5P_2-dependent, PC-specific PLDs, PLD1 and PLD2, which generate PA and are activated by ARF, Rho GTPases, and some PKCs (9). PA functions in multiple pathways, it acts as a cofactor in Akt/mTOR and Ras/Raf/Erk pathways, it activates protein kinases (Raf-1 and PKCε), protein phosphatases (PP1 and SHP-1), and lipid kinases such as sphingosine kinase 1 and PI4P 5-kinase, it regulates the non-receptor tyrosine kinase Fgr, plays an essential role in exocytosis and endocytosis and has numerous other signaling functions (9-12). Importantly, LPAAT-β is overexpressed in some human cancers and specific inhibitors of LPAAT-β trigger apoptosis or necrosis of tumor cells, indicating that LPAAT-β plays an important role in signaling pathways critical to tumor cell survival (10).

In plants, PA increases in response to various stresses, either through the activation of PLD or of diacylglycerolkinase. Subsequently PA acts on multitude of downstream signaling pathways to elicit the stress response (12).

Recent work on the lipid remodeling of glycosylphosphatidylinositol (GPI) anchors in yeast showed that Gup1p is required for the addition of a C26 fatty acid in the *sn*-2 position of GPI anchors (13). Gup1p harbors a membrane bound acyl transferase motif (MBOAT motif) present in a large variety of acyltransferases of bacteria as well as eukaryotes including man. Yeast also harbors 4 other MBOAT proteins, amongst which Yor175c still has no known function. Here we show that Yor175c, which we here name *SLC4,* encodes a 1-acylglycerol-3-phosphate O-acyltransferase that is partially redundant with *SLC1*.

Experimental procedures

Yeast strains, media, reagents. Strains used in this study are listed in Table IA. Cells were grown on rich medium (YPD, YPG) or defined media (SD, SG) containing 2% glucose (D) or galactose (G) as a carbon source and uracil (U), adenine (A) and amino acids (aa) at 30°C (14, 15). Media contained inositol unless indicated. Zymolyase 20T was from Seikagaku Corporation (Tokyo, Japan), lipids from Avanti Polar Lipids Inc. (Alabaster, USA): 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate, cat. number 857123P; 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate, cat. number 857128P; 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, cat. number 855675P; 1-palmitoyl-2-hydroxy-*sn*-glycero-3-

phosphoethanolamine, cat. number 856705P; 1-oleoyl-2-hydroxy-*sn*-glycero-3-[phospho-L-serine] sodium salt, cat. number 858143P. [^{14}C]-oleoyl-CoA (40-60 mCi/mmol cat. number ARC-0527), [^{3}H]-inositol (15-20 Ci/mmol, ART-116) and [^{3}H]-serine (5-25 Ci/mmol, ART-246) were from ANAWA trading SA (Wangen, Switzerland). EDTA, triethanolamine, NaN_3, sorbitol, K_2HPO_4, KH_2PO_4, NaCl, lipid-free albumin, β-mercaptoethanol, leupeptin, chymostatin, antipain and pepstatin were from Fluka Chemie GmbH (Buchs, Switzerland). DNase was from Seravac Biotech (Pty) Ltd. (Eppindust, South Africa). Deoxycholate sodium salt was from Merck (Darmstadt, Germany). Calf intestinal phosphatase and NEBuffer 3 were from New England Biolabs Inc. (Frankfurt, Germany).

Construction of plasmids and strains. The open reading frames of *SLC4* and *SLC1* were amplified by PCR from genomic DNA using primers containing rec1 and rec2 sequences. The PCR amplified *SLC4* and *SLC1* open reading frames were transfected into *slc4*Δ and slc1Δ cells, together with *Sal*I digested pGREG505 and pGREG546, respectively, to generate p$GAL1_{UAS}$-Yor175c and p$GAL1_{UAS}$-GST-SLC1 as described (16). In these transfected strains, the remaining chromosomal *SLC1* and *SLC4* genes were knocked out by transfection of *slc1::hphNT1* and *slc4::LEU2* deletion cassettes generated through PCR on plasmids pFA6a-hphNT1 (EUROSCARF) and pRS415 as templates, respectively, thus producing strains 2Δ.SLC4 and 2Δ.SLC1. For plasmid pBF27 we generated a PCR fragment with primers SLC1-F1 5'-ccaactagtctagaatacaATGAGTGTGATAGGTAGGTTCTTGTATTACT-3' and SLC1.R1 5'-ccactcgagaattcTTAATGCATCTTTTTTACAGATGAACCTTCGTTATGTGAGG-3' on genomic DNA from strain 4R3. The fragment was double digested with *Spe*I and *Xho*I, ligated into p423Met25 and cloned in the *E. coli* strain HB101 (17).

Preparation of microsomes. 100 ml of YPDUA and/or YPGUA media were inoculated with the appropriate strain to give an A_{600nm} of 0.01. After 24 h of growth at 30°C, cells were again diluted to an A_{600nm} of 0.01. Cells were grown for additional 24 h at 30°C to reach an A_{600nm} of 4 to 6. Cells (500 A_{600nm} units of cells) were washed with 50 ml ice-cold 10 mM NaN_3, resuspended in 10 ml zymolyase buffer (10 mM NaN_3, 1.4M sorbitol, 50 mM K_2HPO_4 pH 7.5, 40 mM β-mercaptoethanol, 0.4 mg/ml of zymolyase 20T) and incubated for 1 h at 25°C without shaking. Spheroplasts were collected by centrifugation at 800 g for 5 min at 4°C and washed with one volume of zymolyase buffer lacking β-mercaptoethanol and zymolyase. Spheroplasts were lysed in 10 ml lysis buffer 1 (0.2 M sorbitol, 1 mM EDTA, 10 mM triethanolamine, pH 7.2, 1 μg/ml leupeptin, 2 μg/ml chymostatine, 2 μg/ml antipain, 2 μg/ml pepstatin) containing 0.2 mg/ml DNase by vortexing 1 min, incubating on ice during 10 min and vortexing one more min. A centrifugation at 800 g for 5 min at 4°C was performed to remove intact spheroplasts. The supernatant was centrifuged at 30'000 g for 30 min at 4°C to pellet the microsomes. The supernatant was discarded, microsomes were resuspended in 3 ml of lysis buffer 1 and 0.15 ml-aliquots were snap-frozen in liquid nitrogen for storage at -80°C.

Purification of GST-Slc1p. FBY4142 cells were grown overnight in SGaa to an A_{600} of 4 and 2000 A_{600} units of cells were digested with 0.2 mg/ml Zymolyase as described above. Washed spheroplasts were solubilized at 4°C in 40 ml of lysis buffer 2 (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and protease inhibitors as in lysis buffer 1) supplemented with 1% Tween 20; lysis was promoted by vortexing and rotation on a wheel for 1 h. Insoluble material was removed by centrifugation at 4°C (Sorvall SS-34 rotor; 48'000 g x 15 min). The supernatant was added to 0.5 ml of MagneGST Glutathione Particles (Promega, Madison, WI) and left on a rotating wheel for 1 h at 4°C. The particles were then washed three times with lysis buffer 2 containing 0.1% of Tween 20 and eluted twice with 0.75 ml of the same supplemented additionally with 10 mM reduced glutathione. Glycerol was added to 25% and 10 aliquots of 0.2 ml of eluate were frozen at -20°C. The eluate contained 0.0125% of the protein loaded onto the affinity column and the purification

increased the specific activity 400 fold.

LPAAT assay (acyl-Coenzyme A: 1-acylglycerol-3-phosphate O-acyltransferase assay). Stock solutions (1 mM) of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate, 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate, *lyso*-PC, *lyso*-PE, *lyso*-PI or *lyso*-PS were prepared in lipid buffer (150 mM NaCl, 10 mM K_2HPO_4/KH_2PO_4, pH 7.5 containing 1% lipid-free albumin). *lyso*-PI was generated by treating PI with PLA_2. For the standard assay, reagents were added into a 1.5 ml plastic tube on ice to a final volume of 200 µl in the following order: 20 µl of Tris/HCl 200 mM pH 7.5, water, 10 µl *lyso*-PA (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate) or *lyso*-GPL or lipid buffer, 2μl of 2mM sodium meta-vanadate, sorbitol to a final conc. of 0.2 M, microsomes (corresponding to 20 µg of proteins unless stated otherwise) and, after a preincubation on ice for 10 min, 1 µl (0.02 µCi = 0.4 nmol) of [^{14}C]-oleoyl-CoA in 10 mM Na acetate:ethanol (1:1). Tubes were incubated at 25°C for indicated times. The concentration of [^{14}C]-oleoyl-CoA in the standard assay amounts to approximately 1.2 mol%. To stop the reaction, 520 µl of $CHCl_3$ and 260 µl of methanol were added to the 200 µl reaction mixture. After vigorous shaking and 1-min centrifugation at 12'000 x g, the lower organic phase was extracted 3 more times with upper phase from an identical mixture of $CHCl_3/CH_3OH/H_2O$ (26:13:10). The lower organic phase was evaporated, resuspended in 30 µl of $CHCl_3/CH_3OH/H_2O$ (10:10:3) and 15 µl aliquots were spotted onto a TLC plate or subjected to analytical treatments.

Microsomal assay of PI, PS and PE biosynthesis. Frozen microsomes (20 μg protein/assay), prepared as described above were incubated in 1.5 ml plastic tubes, in a final volume of 200 μl for 1 h at 25°C with either [^{3}H]-inositol (15 μCi/tube) or [^{3}H]-serine (34 μCi/tube), conditions being very similar to the LPAAT assay. The standard assay buffer contained in 20 mM Tris/HCl pH 7.5, 200 mM sorbitol, 1 mM CTP, 2 mM Mg^{2+}, 0.1 mM oleoyl-CoA and 50 μm *lyso*-PA (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate) but no vanadate. Lipids were extracted, desalted and analyzed by TLC in solvent 1 (see below).

Assaying acyltransferase activity of purified GST-Slc1p. Unless indicated otherwise, Tris-HCl buffer, *lyso*-PA or *lyso*-GPLs or only lipid buffer and vanadate were added into a 1.5 ml plastic tube on ice to a final volume of 200 µl in the same order and quantities as for microsomal assays (see above). Further additions were Tween 20 in water (0.08% final conc.), 10 μl of 100 mM $MgCl_2$, and purified protein corresponding to 5 A_{600} units of cells. After 5 min on ice, 2.5μl (0.05 µCi = 1.0 nmol) of [^{14}C]-oleoyl-CoA were added and tubes were incubated at 25°C for 120 min. Reactions were stopped as in microsomal assays.

Analytical procedures and thin layer chromatography. Lipid extracts were dried, resuspended and sonicated for 30 seconds in 100 μl NEBuffer 3 supplemented with 0.1% sodium deoxycholate. 20 U of calf intestinal phosphatase were added and the mixture was incubated at 37°C for 2 h. Phospholipase A_2 treatment was done with 5 U of PLA_2 during 2 h as described (18). GPLs were deacylated in NaOH as described (19) for 1 h at 37°C and desalted using $CHCl_3/CH_3OH/H_2O$ partitioning as described above. Ascending TLC on silica 60 gel glass plates was performed with the following solvents: solvent 1, $CHCl_3/CH_3OH$/0.25%KCl (55:45:5), and solvent 2, $CHCl_3/CH_3OH$/acetic acid/5% sodium bisulfite (67:26:4.4:2.6). Radioactivity was detected and quantified by two-dimensional radioscanning using a Berthold radioscanner or by exposing TLC plates to an image plate, which was developed by the BIORAD molecular Imager FX. Smith Waterman scores were calculated at http://pir.georgetown.edu/cgi-bin/pairwise.pl.

Mass spectroscopy analysis of lipid extracts. Exponentially growing cells were harvested when they reached an A_{600} of 2.0 and lipids were extracted as described (20), using procedure IIIB. At this stage an aliquot of a similar lipid extract from wt cells grown in [^{13}C]glucose, supplemented with [^{12}C]inositol, was added as an internal standard. Samples were dried and resuspended in $CHCl_3/CH_3OH/H_2O$

(16:16:5), heated to 65°C and stirred with a rotating pistil (30,000 rpm). Aliquots corresponding to 1 A_{600} unit of cells were immediately injected into a PVA SIL HPLC column (YMC Europe GmbH, D-46514 Scherbeck, Germany, 1 x 150 mm), which was eluted at 45 µl/min. For elution, solvents A, hexane-isopropanol (98:2); B, $CHCl_3$-isopropanol (65:35); C, CH_3OH, all containing 0.1% triethylamine (FLUKA 90335) and an equimolar amount of formic acid were changed linearly over time to give ratios A:B:C as follows: 0 min 70:30:0; 4 min 12:88:0; 10 min 9.8:74.2:16; 12 min 7.6:61.4:31; 14 through 20 min 0:0:100; 22 min 0:100:0; 26 through 33 min 70:30:0. Ions in the effluent were ionized by electrospray ionisation with an electrode potential of 3500 volt and the masses of negative ions were detected by a Bruker Esquire-LC ion trap mass spectrometer. The spectrometer automatically fragments ions and records secondary ions. This information is not used routinely but has been used to confirm the identity of signals while the method was being set up. The sum of the signals of selected internal ^{13}C standard GPLs in each lipid class (PI, PE, PS, PC, phosphatidylglycerol (PG)) was utilized to normalize the sum of signals from the corresponding lipid class in the test sample in order to make the ion counts for a given lipid class (e.g. PI) obtained in different cell lines directly comparable. (On the other hand, due to different ionization efficiency, ion counts obtained for different lipid classes (e.g. PI and PC), do not allow to estimate the relative abundance of the different lipid classes with regard to each other.

Results

Synthetic effects of *yor175cΔ*

In an attempt to find new LPAATs, we tried to delete *SLC1* in an *are1Δ are2Δ gup1Δ gup2Δ yor175cΔ* quintuple mutant lacking all 5 MBOAT proteins of yeast. While the quintuple mutant grew reasonably well, it was impossible to delete *SLC1* in this background. Further investigation showed that *gup1Δ gup2Δ slc1Δ* cells were perfectly viable but that it was impossible to delete *SLC1* in a *yor175cΔ* mutant. To test this further we crossed a haploid *slc1::KanMX4* with a haploid *yor175c::KanMX4* strain, sporulated the resulting diploid and analyzed the progeny by dissecting tetrads (supplemental Fig. S1A). In many tetrads less than 4 spores were germinating, in all tetrads producing 3 colonies, only two of them were kanamycin resistant and in all tetrads producing 2 colonies, none was kanamycin resistant (supplemental Fig. S1A). At no stage did we observe a difference in colony size between wt and slc1Δ or slc4Δ single mutants (not shown). Microscopic inspection of plates showed that spores having failed to produce colonies had germinated and produced either two or four cells but no more. These data are entirely compatible with the view that the simultaneous presence of both deletions in a haploid cell renders the cell non-viable, i.e. that the two deletions are synthetically lethal. Very bad growth of *slc1Δ yor175cΔ* double mutants, i.e. strong synthetic sickness between these two deletions has also been found in a recent global genetic interaction study (21). In the following we therefore will use *SLC4* to designate Yor175c. To generate viable double mutants we transfected *slc4Δ* and *slc1Δ* strains with centromeric plasmids containing *SLC4* and *SLC1* under the *GAL1* promoter, a promoter which initiates transcription at a high rate when galactose is present in the medium but which is repressed in the presence of glucose. In *slc4Δ* and *slc1Δ* strains containing *SLC4* and *SLC1* we deleted remaining chromosomal *SLC1* or *SLC4* genes. The resulting *slc1Δ slc4Δ* (2Δ) strains harboring a plasmid containing either *SLC1* or *SLC4* (named 2Δ.SLC1 and 2Δ.SLC4) grew well not only in galactose-, but also in glucose-containing liquid media (supplemental Fig. S1B). The 2Δ.SLC1 strain also grew well on glucose-containing agar plates (Fig. 2). Data suggest that low amounts of either Slc1p or Slc4p made on glucose media are sufficient for a relatively normal cell growth. It however was impossible to remove the GST-*SLC1*-containing plasmid by counterselection on 5-fluoroorotic acid, a compound which will kill cells unless they loose the *SLC1*-bearing plasmid (Fig. 2). This again demonstrates

that *slc1Δ* is synthetically lethal with *slc4Δ*. The data in Fig. 2 further shows that the gain of function allele Slc1-1p can functionally replace Slc1p and allows *slc1Δ slc4Δ* cells to grow at a normal rate.

Microsomes of *slc1Δ* cells efficiently transfer oleic acid to *lyso*-phosphatidic acid

About 95 - 98% of the cellular glycerol-3-phosphate acyltransferase, as measured by the generation of *lyso*-PA from C18:1-CoA and [^{14}C]-glycerol, resides in microsomes, the remainder being present in lipid bodies (8) and this activity is encoded by *GAT1* and *GAT2* (22). The incubation of microsomes with C18:1-CoA and [^{14}C]-glycerol allows for the production not only of *lyso*-PA but also of PA and such an assay thus simultaneously measures glycerol-3-phosphate acyltransferases and LPAATs (8). Using this assay we found that microsomes from 2Δ.SLC1 and 2Δ.SLC4 grown for two days on glucose produced strongly reduced amounts of PA in comparison to wt cells, but that *lyso*-PA was still made (not shown). To be able to measure *sn*-2 specific acyltranferase independently from Gat1p/Gat2p activity, we incubated microsomes with [^{14}C]-C18:1-CoA in the absence or presence of *lyso*-PA (1-palmitoyl-glycerol-3-phosphate or 1-stearoyl-glycerol-3-phosphate)(23). In the absence of *lyso*-PA, microsomes incorporated radioactivity into lipids that comigrated either with PE, PI, PC and PA standards or with free fatty acids and diacylglycerol (DAG) standards (Fig. 3A and B, lanes 1, 2, 5, 6). Interestingly, the appearance of PA, PI and DAG was strong in microsomes containing *SLC1*, whereas the synthesis of PC and PE was strongly dependent on Slc4p. The assay does not contain CTP, serine or inositol and this suggests that the labeled PI, PE and PC are not made from endogenous *lyso*-PA, which would get acylated to [^{14}C]-PA and then further processed, but that these GPLs arise through direct acylation of *lyso*-GPLs, a notion which is confirmed by another experiment shown below (Fig. 6). The addition of *lyso*-PA to the assay strongly increased the accumulation of PA and completely blocked the incorporation of label into other lipids in slc4Δ (Fig. 3A, lanes 1 - 4) and 2Δ.SLC1 (supplemental Fig. S2), whereas GPLs continued to be made in presence of *lyso*-PA in cells containing Slc4p (Fig. 3A, lane 7; Fig. 3B, lanes 3, 7; supplemental Fig. S2). Addition of *lyso*-PA blocked the incorporation of label into other lipids (PC, PI and PE), possibly by acting as competitive inhibitor of potential *lyso*-GPL substrates, since a large part of [^{14}C]-oleate (>70%) is consumed during the assay. As can be seen in Fig. 3, panels A and B, the band considered to be PA was sensitive to alkaline phosphatase, while other species identified as PE, PI and PC were not, as expected. Alkaline phosphatase shifted the label of [^{14}C]-PA to a position corresponding to DAG (Fig. 3A and 3B, lanes 4 and 8). As shown in Fig. 3C, the lipids getting labeled in microsomes of 2Δ.SLC4 were susceptible to mild alkaline hydrolysis, whereby the released label migrated at a position corresponding to [^{14}C]-oleic acid and a second, uncharacterized band of higher R_f (Fig. 3C, lanes 1, 2, 5, 6). From lipids labeled in the absence of *lyso*-PA, phospholipase A_2 (PLA_2) released the bulk of the radioactivity as [^{14}C]-oleic acid, but two relatively minor *lyso*-GPLs carrying [^{14}C]-oleic acid presumably in the *sn*-1 position were also generated (Fig. 3C, lanes 3, 4). In assays done in the presence of exogenous *lyso*-PA, almost all the label was released by PLA_2 (lanes 7, 8), and thus had been incorporated into the *sn*-2 position of *lyso*-PA.

***SLC4* expression correlates with the 1-acylglycerol-3-phosphate acyltransferase activity in *slc1Δ* microsomes**

To show that overexpression or depletion of Slc4p influenced the LPAAT activity, the kinetics of PA synthesis in microsomes from 2Δ.SLC4 grown in galactose or glucose were investigated. As shown in Fig. 4A, when cells had been on glucose, one could observe a linear increase in [^{14}C]-PA up to 32 min. A parallel increase of lipids migrating in the region of GPLs containing PI, PS and PC and of very apolar lipids migrating to the top of the TLC plate (not shown) was equally observed. When however the cells had been

grown in galactose, the LPAAT activity was so very strong that it had consumed most [^{14}C]-oleoyl-CoA already at time 0, i.e. during the time elapsing between the addition of radioactivity and addition of organic solvent to stop the reaction (about 1 min), a time during which reaction tubes were on ice. Indeed, >70% of added radioactivity was incorporated into PA at time 0. Thus, overexpression of *SLC4* on galactose media led to much a higher activity than expression on glucose media. As seen in Fig. 4B, lanes 1 - 3, decreasing the amount of microsomal proteins from 200 to 2 μg increased overall incorporation of radioactivity into lipid (by 23%), but high amounts of protein favored the incorporation into GPLs. Non-saturating concentrations of *lyso*-PA were also tested. As seen in Fig. 4B, lanes 5 - 16 and the corresponding quantification in Fig. 4C, linear incorporation of radioactivity into PA was observed during 15 min with wt microsomes and *lyso*-PA concentrations of 0.05 and 0.5 μM while again, rapid saturation was achieved at 50 μM *lyso*-PA. The incorporation of label into GPLs and DAG was not dependent on the concentration of *lyso*-PA (Fig. 4B, lanes 5 – 13, not shown). This can be rationalized by assuming that the GPLs are made by the acylation of endogenous *lyso*-GPLs. 50 μM of *lyso*-PA seemed to be inhibitory for GPL biosynthesis (Fig. 4B, lanes 14 - 16, Fig. 4C).

In the following we chose to compare activities of different strains at 1 μM *lyso*-PA corresponding to about 0.6 mol %. As shown in Fig. 5A and the corresponding quantification in Fig. 5B, the LPAAT activity in 2Δ.SLC4 microsomes was drastically reduced when cells had been grown on glucose during 48 h for depletion of Slc4 protein. It is worth noting that 2Δ.SLC4 cells grown on glucose had much less microsomal LPAAT activity than wt cells, but nevertheless grew normally (Fig. 5; supplemental Fig. S1B). This suggests that LPAAT in normal cells is not rate limiting.

On the other hand, when grown on galactose, the acyltransferase activity of 2Δ.SLC4 microsomes was as strong as the one of microsomes from wt cells grown on glucose. Significant amounts of radioactivity were also incorporated into GPLs (PI, PS, and PC) and this incorporation was significantly enhanced when *SLC4* was induced (Fig. 5B).

Phosphatidic acid generated by Slc1p and Slc4p is utilized with similar efficiency in microsomal PI, PS and PE biosynthesis.

To see if PA generated by Slc1p and Slc4p could be further metabolized in various biosynthetic pathways we modified the microsomal system such as to contain CTP and either [^{3}H]-inositol or [^{3}H]-serine. As shown in Fig. 6, [^{3}H]-inositol was incorporated efficiently into PI whereby the incorporation was totally dependent on CTP and significantly enhanced by *lyso*-PA and also somewhat better in the presence of oleoyl-CoA. This suggests that the assay allows for the incorporation of *lyso*-PA into PI along the physiological route via PA and CDP-diacylglycerol (CDP-DAG) (Fig. 1). Similarly, when [^{3}H]-serine was added instead of [^{3}H]-inositol, microsomes made PS and PE in a CTP-, oleoyl-CoA- and *lyso*-PA-dependent way (supplemental Figs. S4A, B). Again, the synthesis of PS and PE was dependent on CTP and strongly enhanced by the presence of *lyso*-PA and oleoyl-CoA (supplemental Figs. S4A, B). The absolute CTP dependency of these assays demonstrates that the PE, PI and PC species observed in Fig. 3 could not be derived from the processing of endogenous or exogenous *lyso*-PA via CDP-DAG, because these assays did not contain any CTP. We also found that the addition of CDP-ethanolamine or CDP-choline to microsomal assays containing [^{14}C]-oleoyl-CoA did not enhance the labeling of PE and PC. We therefore conclude that the PE, PI and PC species observed in Fig. 3 were generated through direct incorporation of [^{14}C]-oleate into *lyso*-PE, *lyso*-PI and *lyso*-PC. Moreover, the data shown in Fig. 6 and Figs. S4A, B argue that PA for microsomal PE, PI and PS biosynthesis via CDP-DAG can originate from either Slc1p or Slc4p, since microsomes from all different cell lines generated comparable amounts of these lipids.

***SLC4* and *SLC1* generate almost the same**

fatty acid profile on glycerophospholipids

Lipid extracts were prepared from wt, slc1Δ, slc4Δ, and 2Δ.SLC1 and 2Δ.SLC4 cells and analyzed by HPLC-ESI-MS/MS. As shown in Fig. 7A, total GPLs were slightly decreased in slc1Δ and slc4Δ mutants but overexpression of either Slc1p or Slc4p in *slc1Δ slc4Δ* cells brought total ion counts back to normal. Moreover, when all PA was made by a single LPAAT, all cell types still made each individual GPL species in quantities amounting to at least 70% of the normal amount present in wt (Fig. 7B). We also found that all the lines shown in Fig. 7 made similar amounts of triacylglycerols (supplemental Fig. S5), indicating that PA made through both, Slc1p and Slc4p, was metabolized to neutral fat.
Phosphatidylglycerol (PG), PI and PS are all derived from CDP-DAG (Fig. 1), and, as seen in Fig. 8A, the fatty acid profile of PI and PG is quite similar. However, the number of double bonds varies between PI (and PG) on the one hand and other kinds of GPLs on the other hand, since PI (and PG) mostly contain one double bond, PE and PC predominantly contain two double bonds, and PS contains about equal amounts of species with one and two double bonds in their fatty acids (Fig. 8A). Our findings, made in cells grown on galactose (YPGUA, at 30°C), are in close agreement with the observations made in a similar HPLC-ESI-MS/MS analysis of the X2180 strain, grown on glucose (YPD) at 24°C, results which are reproduced for comparison in Fig. 8B (24). In particular, in the biosynthetic pathway leading from PS to PE and to PC (depicted with thick arrows in Fig. 1) we see the same relative decrease of 34:1 and parallel increase of 32:2 species described before (24). These findings are also in agreement with the observation that the "normal" PE and PC mostly contain an unsaturated fatty acid in *sn*-1, especially when cells are grown in rich medium as was the case in here (25). The different fatty acid content between species, which of course all derive from PA, suggests a species-selective metabolism or exchange of fatty acids on GPLs. Importantly, the varying number of double bonds in different GPL species observed in wt cells (Figs. 8A, B) is equally seen in cells depending exclusively on Slc4p (slc1Δ, 2Δ.SLC4; Figs. 8C, D) or exclusively on Slc1p (slc4Δ, 2Δ.SLC1; Figs. 8E, F). Furthermore, the BY4742 as well as X2180 wt cells show a tendency to have more PI, PS and PE species with 34 C atoms than with 32 C atoms while in PC the tendency is reversed (Figs. 8A, B), and this feature again suggests some species-selective metabolism or fatty acid exchange. With regard to this phenomenon, a slight difference is observed depending on the LPAAT which is used by cells: Cells entirely depending on Slc4p for PA biosynthesis (slc1Δ, 2Δ.SLC4; Figs. 8C, D) show a relatively higher percentage of PI, PS and PE species with 32 C atoms than either wt cells (Figs. 8A, B) or cells depending exclusively on Slc1p (slc4Δ, 2Δ.SLC1; Figs. 8E, F). This minor difference suggests that Slc4p may have a better affinity for, or better access to C16:1-CoA than Slc1p. Overall, the fatty acid profiles in GPLs of all the strains are surprisingly similar.

Characterization of GST-Slc1p

We tried to purify GST-tagged versions of Slc1p and Slc4p. Purified GST-Slc4p had only very low LPAAT activity. On the other hand, purified GST-Slc1p was very active and stable on storage at – 20°C. The partially purified material contained a major band at 62 kDa, the expected molecular weight of GST-Slc1p upon SDS-PAGE and staining of gels with silver nitrate (Fig. 9A, lanes 1 - 2). This band was below the detection level in the starting microsomal extract (Fig. 9A, lanes 3, 4) and could be stained with anti-GST antibody (Fig. 9A, lanes 5, 6). The purified GST-Slc1p generated PA from [^{14}C]-oleoyl-CoA, from which [^{14}C]-oleate could be released with PLA_2 or mild base treatments (not shown). As can be seen in Fig. 9B, the activity of the purified enzyme was dependent on the addition of *lyso*-PA (lane 1 vs. 4) and was enhanced by the presence of Mg^{2+} (lane 4 vs. 6), while other divalent cations (Ca, Mn, Cu) had no effect (not shown). Short term assays allowed to calculate that Mg^{2+} enhanced the LPAAT activity of GST-Slc1p 7 to 20 fold (Fig. 9C, not shown). Yet, the acyl-Coenzyme A: 1-acylglycerol-3-phosphate O-acyltransferase activity of Slc1p present in microsomes from

slc4Δ cells was not enhanced by the addition of Mg^{2+} (not shown). GST-Slc1p was slightly more active with palmitoyl-*sn*-glycerol-3-phosphate than with 1-stearoyl-*sn*-glycerol-3-phosphate as a substrate (Fig. 9D, lanes 2, 5). Boiling for 5 min destroyed the enzymatic activity (Fig. 9B, lanes 4, 5).

GST-Slc1p can transfer acyls from CoA onto *lyso*-glycerophospholipids

As mentioned, although all GPLs of yeast are derived from PA, they contain different fatty acids (24, 25) and metabolic labeling studies have suggested that fatty acids on GPLs are turning over with half lives that are significantly shorter than the half live of GPLs as measured in $H^{32}PO_4^{2-}$ pulse chase experiments (25-27). In view of the findings of Figs. 3, 6 and S4, we were interested to see if purified GST-Slc1p was able to transfer fatty acids onto *lyso*-GPLs, i.e. if the presence of a headgroup on the phosphate of *lyso*-PA would hinder the interaction of the substrate with the enzyme. As seen in Fig. 9D, in the presence of *lyso*-PA the purified GST-Slc1p made PA and no other product, but it made PC, PE, PS and PI in the presence of the corresponding *lyso*-GPLs. The activity was relatively weak with *lyso*-PC and *lyso*-PE, but quite significant with *lyso*-PI and *lyso*-PS (Fig. 9D). In the absence of Mg^{2+} the acyltransferase activity with *lyso*-PI, *lyso*-PS and *lyso*-PA was comparable (not shown). In the presence of Mg^{2+} the activity was enhanced only 2 fold with *lyso*-PI and *lyso*-PS, but 7 to 20 fold with *lyso*-PA (Fig. 9C and D, data not shown). Mg^{2+} may have to shield the strong negative charge of *lyso*-PA and therefore be more important for the binding of this substrate than for binding of *lyso*-GPLs to the enzyme. The data suggest that Slc1p may be involved in the exchange of fatty acids on GPLs *in vivo* and offer an explanation for the labeling of GPLs with [^{14}C]-C18:1-CoA in the absence of CTP in slc4Δ cells (Fig. 3A).

Discussion

Microsomes were reported to contain an uncharacterized LPAAT, not related to *SLC1* (8, 28). Here, we show that Slc4p can account for this latter activity. The *slc1Δ* and *slc4Δ* cells grow at normal rates and mass spectrometric analysis reveals a quite normal GPL profile in both, cells depending solely on Slc1p and cells depending solely on Slc4p (Fig. 8). A previous study also indicated that slc1Δ cells have the same GPL profile as wt apart from a reduction of a minor C12:0-containing PI species (29). This data argues that PA originating from either Slc1p or Slc4p feeds into all lipid biosynthetic pathways and suffices for all the essential functions of a cell. In spite of this functional redundancy, Slc1p and Slc4p are not homologous to each other and have very different structures. Indeed, Slc1p contains a single transmembrane domain whereas 12 such domains are predicted for Slc4p.

It is likely that Slc4p itself harbors the enzymatic activity, as it contains an MBOAT motif, which has been found in many ascertained acyltransferases (30). *SLC4* also contains the proposed active site residue, which is His382. This His residue is the only strictly conserved residue throughout this large gene family and was recently shown to be required for acyltransferase activity of Gup1p, an yeast homologue of *SLC4*, involved in lipid remodeling on GPI anchored proteins (13, 30).

To date, all the known mammalian as well as bacterial LPAATs are homologous to *SLC1*. Yet, homologues of *SLC4* are found in all eukaryotes. The human genome harbors four genes predicting proteins with about 30% identity to *SLC4*, having Smith Waterman scores between 500 and 660, and for which no function is known. In comparison, the four yeast homologues of *SLC4* have scores of 105 and below. Thus, it will be interesting to investigate if the mammalian *SLC4* homologs also have LPAAT activity. The blast server at NCBI finds however no homologues of *SLC4* in bacteria.

Genetic data indicate that Slc1p and Slc4p are not functionally exchangeable in all respects. Indeed, slightly different roles for Slc1p and Slc4p are suggested by the fact that the *slc1Δ* and *slc4Δ* deletion strains

influence the effect of other gene deletions differently, i.e. that they display different synthetic interactions: When combined with either *ric1Δ* or *rgp1Δ* deletions, *slc1Δ* shows synthetic sickness, whereas *slc4Δ* shows synthetic enhancement (alleviation) (21). The *ric1Δ* and *rgp1Δ* deletions affect nucleotide exchange on Ypt6p, a G protein involved in the vesicular retrotransport from the late endosome to the Golgi.

Deletion of *SLC4* eliminated the microsomal biosynthesis of PE and PC, while deletion of *SLC1* reduced the biosynthesis of PA, PI and DAG (Figs. 3A, 3B). Due to the absolute CTP-dependence of the synthesis of these GPLs in the microsomal system (Figs. 6 and S4) we conclude that the labeling of PE, PI and PC observed in the absence of CTP (Fig. 3A, B) proceeds from direct acylation of *lyso*-GPLs. In confirmation, direct acyltransfer onto *lyso*-PI and *lyso*-PS could be demonstrated with purified Slc1p (Fig. 9D). In an earlier study, acyltransferase activity in microsomes from *slc1Δ* cells, overexpressing *SLC1* or not, was measured using [^{14}C]-oleoyl-CoA and *lyso*-PI, *lyso*-PE or *lyso*-PC as substrates (28). The incorporation of [^{14}C]-oleic acid into lipids in these *slc1Δ* microsomes was strongly dependent on the addition of exogenous *lyso*-GPLs, but was only slightly stronger (10 to 20%) when *SLC1* was overexpressed. Thus, the bulk of activity was *SLC1*-independent. Based on this and on the analysis of products, the authors concluded that it was unlikely that Slc1p itself was an acyltransferase for *lyso*-PI, *lyso*-PE or *lyso*-PC (28). The use of different assay conditions may have prevented the detection of the *lyso*-GPL acyltransferase activity of Slc1p in this former study. As the *lyso*-GPL acyltransferase activity of Slc1p seems to be able to use *lyso*-forms of PI and PS but no other *lyso*-GPLs as substrates (Fig. 9D), they provide a reasonable explanation for the relatively strong labeling of PI, but weaker labeling of PC and PE in microsomes of slc4Δ cells (containing only Slc1p)(Fig. 3A, lane 1 versus 5). The specificity of Slc1p for PI and PS may also provide part of the explanation as to why in *lcb1Δ SLC1-1* cells C26:0 fatty acids get incorporated specifically into PI and not into all other GPLs as well (7).

Exchange of fatty acids of GPLs (lipid remodeling) is a well-established phenomenon in yeast (27). In plants and yeast, the acylation of *lyso*-GPLs may be important to metabolize the *lyso*-PE and *lyso*-PC, which are generated by phospholipid-DAG-acyltransferases (PDAT) such as *scLRO1* or *AtPDAT1*. These enzymes transfer fatty acids from PE and PC onto DAG to generate triacylglycerols. Similar enzymes transfer fatty acids from GPLs onto sterols or act as phospholipase A_1 and thereby also generate *lyso*-GPLs (31-33). Several studies have reported on enzymatic activities or specific enzymes of yeast able to reacylate the *sn*-2 position of *lyso*-PC (28, 34-37). Purified Plb1p, a phospholipase B, was shown to be capable of transferring [^{14}C]-C16:0 from 1-[^{14}C]-palmitoyl-*sn*-glycerol-3-phosphorylcholine onto the *sn*-2 position of the same lipid in a CoA-independent manner (34, 35). A similar activity has been described for Taz1p. Taz1p is homologous to the human tafazzin, the loss of which causes a congenital myo- and neuropathy (Barth syndrome)(37). Plb1p and Taz1p, however, are located in the plasma membrane and the outer mitochondrial membrane, respectively, and do not need acyl-CoA. Acyl-CoA dependent *lyso*-GPL acyltransferase activities have been measured in yeast microsomes before (28, 36). Some of our data suggest that not only Slc1p but also Slc4p may contribute to this *lyso*-GPL acyltransferase activity. Indeed, the generation of labeled PE, PI and PC in presence of [^{14}C]-oleoyl-CoA was significantly enhanced in microsomes overexpressing Slc4p as compared to microsomes carrying physiological levels of Slc4p (Fig. 3A, lanes 7, 8 vs. Fig. 3B, lanes 7, 8). This suggests that Slc4p, as Slc1p, has the capacity to transfer oleate from [^{14}C]-oleoyl-CoA onto certain *lyso*-GPLs. The fact that not exactly the same *lyso*-GPLs are acylated in Slc1p- and Slc4p-containing microsomes may be due to differences in substrate specificity or subcellular localization of the two enzymes. While Slc1p has been localized to the ER and lipid bodies, two global proteome localization studies have failed to produce any data for Slc4p ((8); http://ypl.uni-graz.at/pages/home.html). Further studies are required to determine if

the acyl-CoA: 1-acyl-*sn*-glycerol-3-phosphorylcholine acyltransferase activity of *slc1Δ* microsomes is provided by Slc4p and if this function is physiologically relevant.

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Footnotes

Condensed title: *SLC4* encodes an acyltransferase

Key words not in title: LPAAT, phosphatidic acid, lipid biosynthesis, lipid remodeling, endoplasmic reticulum, *Saccharomyces cerevisiae*

Abbreviations: DAG, diacylglycerol; FOA, 5'-fluoroorotic acid; GPL, glycerophospholipid; LPAAT, *lyso*-phosphatidic acid acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PLD, phospholipase D; PS, phosphatidylserine; wt, wild type.

Tables

Table IA. Strains

Name	Genotype	Source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
slc1Δ	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 slc1::KanMX*	EUROSCARF
slc1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 slc1::KanMX*	EUROSCARF
slc4Δ	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yor175c::KanMX*	EUROSCARF
FBY4142	*his3Δ1 leu2Δ0 ura3Δ0 slc1::kanMX4* containing p*GAL1*$_{UAS}$-GST-SLC1	This study
FBY4132 and FBY4133	*ade2-1 trp1-1 his3-11,15 gup1::LEU2 gup2::URA3 slc1::hphNT1*	This study
SCY 1414	*are1::HIS3 are2::TRP gup1::LEU2 gup2::URA3*	Stephen Sturley
RSY2206	*are1::HIS3 are2::TRP gup1::LEU2 gup2::URA3 yor175c::kanMX4*	Roger Schneiter
RSY3077	*ura3Δ0 trp1::URA3 are1::kanMX4 are2::kanMX4 lro1::TRP1 dga1::Lox-HIS3-Lox* in BY4742 background	Roger Schneiter
2Δ.SLC1a (FBY4137)	*his3Δ1 leu2Δ0 ura3Δ0 slc1::kanMX4 yor175c::LEU2* containing p*GAL1*$_{UAS}$-GST-SLC1	This work
2Δ.SLC1b (FBY4138)	*his3Δ1 leu2Δ0 ura3Δ0 slc1::kanMX4 yor175c::LEU2* containing p*GAL1*$_{UAS}$-GST-SLC1	This work
2Δ.SLC1-1a (FBY4169)	*his3Δ1 leu2Δ0 ura3Δ0 slc1::kanMX4 yor175c::LEU2* containing *SLC1-1* on pBF27	This work
2Δ.SLC1-1b (FBY4170)	*his3Δ1 leu2Δ0 ura3Δ0 slc1::kanMX4 yor175c::LEU2* containing *SLC1-1* on pBF27	This work
2Δ.SLC4 (FBY4134)	*his3Δ1 leu2Δ0 ura3Δ0 slc1::hphNT1 yor175c::KanMX4* containing p*GAL1*$_{UAS}$-Yor175c	This work
4R3	*lcb1::URA3 SLC1-1 ura3-52 leu2-3,112 ade1 MEL1*	Robert Dickson

Table IB. Plasmids

Name	**Type and markers**	**Source of parent vector**
pBF27	*MET25*$_{UAS}$-SLC1-1 in p423Met25 (2μ, *HIS3*)	(17)
p*GAL1*$_{UAS}$-GST-SLC1	SLC1 under *GAL1* promoter and N-terminal GST tag in pGREG546 (*URA3*, KanMX, *ARS4*, *CEN6*)	(16)
p*GAL1*$_{UAS}$-GST-Yor175c	Yor175c under *GAL1* promoter and N-terminal GST tag in pGREG546 (*URA3*, KanMX, *ARS4*, *CEN6*)	(16)
p*GAL1*$_{UAS}$-Yor175c	Yor175c under *GAL1* promoter in pGREG505 (*LEU2*, KanMX, *ARS4*, *CEN6*)	(16)

Figure legends

Figure 1. *Biosynthesis and breakdown of phosphatidic acid in Saccharomyces cerevisiae.* Enzymes required for the generation of GPLs from diacylglycerol (DAG) and CDP-DAG are not indicated. CL, cardiolipin; DAG-PP, DAG-pyrophosphate; DHAP, dihydroxyaceton-phosphate; Gro3P, glycerol-3-phosphate; PAK, PA kinase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, PG-phosphate; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triacylglycerol.

Figure 2. *slc1Δ slc4Δ cells are not viable.* Wt (BY4742), slc1Δ, slc4Δ, and four different *slc1Δ slc4Δ* double mutant strains harboring either *GST-SLC1* on an *URA3* vector or *SLC1-1* on a *HIS3* vector were plated at ten fold dilutions on SDaa or SDaa plus 5'-fluoroorotic acid (FOA), and incubated at 30°C for 3 days.

Figure 3. *Microsomes from wt (BY4742), slc1Δ, slc4Δ, and 2Δ.SLC4 cells make phosphatidic acid from 1-acylglycerol-3-phosphate.* Microsomes were prepared from cells growing exponentially in YPGUA. A, B, Microsomes were incubated with [^{14}C]-oleoyl-CoA in the presence (+) or absence (-) of *lyso*-PA (50 μM) for 120 min. After incubation, lipids were extracted and were incubated in the presence (+) or absence (-) of calf intestinal alkaline phosphatase (AP). The combined effect of incorporation into lipids and hydrolysis of [^{14}C]-oleoyl-CoA lead to disappearance of 72 +/- 4.6 % of [^{14}C]-oleoyl-CoA during incubation in all 4 cell lines, whether or not *lyso*-PA was added. C, lipids made by microsomes from 2Δ.SLC4 cells either in the presence or absence of *lyso*-PA (2 μM) were deacylated with mild base (NaOH)(+) or treated with PLA_2 (+), or mock treated (-). Lipids were desalted and analyzed by TLC in solvent 1 followed by radioimaging. The identity of the labeled lipids is indicated and was obtained from the position of standards run in parallel, which either were radiolabeled, could be revealed by iodine vapor or were elaborated in separate metabolic labeling experiments using [^{3}H]-inositol and [^{3}H]-serine (not shown). No lipids got labeled when boiled microsomes were used (not shown). The experiment is representative of several experiments showing similar results.

Figure 4. *Assay of O-acyltransferase activity in microsomes of 2Δ.SLC4.* A, 2Δ.SLC4 were grown either on glucose (YPDUA) or galactose (YPGUA) and microsomes were prepared. A, microsomes, *lyso*-PA (50 μM final conc.) and [^{14}C]-oleoyl-CoA were placed into reaction vials on ice, vials were rapidly placed into a 25°C heating block and incubated for 0 to 64 min as indicated, whereupon the reactions were stopped by the addition of organic solvent. The 0 min vials received organic solvent after sitting 1 min on ice. The TLCs of this figure were developed with solvent 2, so that PI, PC and PS are not well resolved and migrate in the zone denoted GPL; o = origin. B, acyltransferase assays were set up with microsomes from 2Δ.SLC4 grown on YPGUA. Lanes 1 to 4, assays containing 2, 20 or 200 μg of microsomal protein and 50 μM *lyso*-PA were incubated for 30 min; a control with boiled microsomes was run in lane 4. Lanes 5 – 16, *lyso*-PA concentrations of 0.05, 0.5, 5 or 50 μM were tested and assays were incubated for 1, 4 or 15 min. C, counts present in PA and GPLs on TLC plates shown in B were determined by radioscanning.

Figure 5. *Expression levels of Slc4 protein correlate with 1-acylglycerol-3-phosphate O-acyltransferase activity in microsomes of 2Δ.SLC4.* A, microsomes were prepared from wt and 2Δ.SLC4 cells grown on YPDUA (Glc) or YPGUA (Gal). Microsomes plus limiting amounts of *lyso*-PA (1 μM final conc. = 0.6 mol%) were incubated for 0 - 64 min and lipid extracts were analyzed by TLC in solvent 2. Only the zone of the TLC plate containing PA is shown. B, TLC plates of the experiment shown in panel A as well as of an independent second experiment were scanned and counts present in PA and in mature GPLs (PC, PI, PS) were determined by radioscanning.

Figure 6. *PA generated by Slc1p and Slc4p is metabolized to PI*. The same microsomes as used in Fig. 3 were incubated with [^{3}H]-inositol in the standard assay mix containing CTP/Mg^{2+}, oleoyl-CoA and *lyso*-PA (complete) or in a mix where the ingredient indicated at the top was lacking. TLC of the extracted lipids in solvent 1 showed a single radioactive band (for illustration see supplemental Fig. S3). The counts obtained in PI when a reagent was omitted relative to the counts obtained in the complete assay are indicated below each lane (% of complete). About 5% of added [^{3}H]-inositol was incorporated into PI in all microsomes when the complete assay mixture was used.

Figure 7. *Normal amounts of all major glycerophospholipid species are made in cells lacking Slc1p or Slc4p*. BY4742 wt cells, slc1Δ, slc4Δ, 2Δ.SLC1 and 2Δ.SLC4 cells were grown in YPGUA at 30°C. Exponentially growing cells were harvested, lipid extracts were prepared, were mixed with a fixed amount of lipid extract from wt cells grown in [^{13}C]glucose, and were analyzed by HPLC-ESI-MS/MS. A, in BY4742 wt cells the total ion counts were 4.999 x 10^6 for PC, 0.302 x 10^6 for PE, 0.832 x 10^6 for PI, 0.103 x 10^6 for PG and 0.338 x 10^6 for PS. After normalization using the internal standard, the corresponding figures for all lipid species in the mutant cell lines were summed up and expressed as percentage of total ion counts in GPLs of wt, which were set as 100%. B, in the same data set, the ion counts for each individual GPL species of wt cells was set as 100% and the corrected ion counts for corresponding GPL species in mutant cells depicted as percentage of ion counts in wt sample.

Figure 8. *The fatty acid profile of major glycerophospholipid species in cells depending on only Slc1p or only Slc4p are highly similar*. From the experiment shown in Fig. 7, corrected ion counts were utilized to calculate the relative abundance of the various forms defined by the number of C atoms and double bonds present in the two fatty acids for all detectable GPL species having a given head group. In each cell line, the total of all different forms of a given GPL was taken as 100%. Data of panel B show X2180 wt cells grown at 24°C in YPD and are taken from Fig. 6 of the report of Schneiter et al., (24), but are plotted in a way to be comparable with other data in this figure.

Figure 9. *Characterization of purified GST-Slc1p*. A, the indicated amounts of protein of starting yeast lysate (lanes 3, 4, 7, 8) and partially purified GST-Slc1p (lanes 1, 2, 5, 6) were analyzed by SDS-PAGE/silver staining (lanes 1 - 4) and by Western blotting with antibody against glutathione-S-transferase (GST) (lanes 5 - 8). The most prominent band in the purified fraction is stained by anti-GST and is close to the expected molecular weight of 61'180 Da. B, acyltransferase activity of purified GST-Slc1p was assayed with native (n) or boiled (b) enzyme in the presence or absence of *lyso*-PA (50 μM) and Mg^{2+} (5 mM). In lane 7, Mg^{2+} was added after incubation. C, GST-Slc1p was assayed with short incubations ranging from 3 to 30 min. Quantitation of PA in the radiograms is shown in the graph below. D, acyltransferase activity of purified GST-Slc1p was assayed with native (n) or boiled (b) enzyme (E) in the presence of $MgCl_2$ and 50 μM *lyso*-PA, *lyso*-PC, *lyso*-PS, *lyso*-PE or *lyso*-PI. In lanes 2 and 5, 1-palmitoyl- and 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphate were used, respectively. Only 25% and 50% of the reactions were spotted for samples containing *lyso*-PA and *lyso*-PS, respectively. Radioscanning allowed to determine that the total amounts of product made from different substrates were as follows: *lyso*-PA-C16 : 19'200 cpm, *lyso*-PA-C18 : 15'500 cpm, *lyso*-PC : 460 cpm, *lyso*-PE : 400 cpm, *lyso*-PS : 3'100 cpm, *lyso*-PI : 3'300 cpm. Lipids were analyzed by TLC in solvents 2 (panel B) and 1 (panels C, D).

Supplemental figures

Figure S1. Synthetic lethality of slc1Δ with slc4Δ and growth rates in liquid media. A, *slc1::KanMX4* (*MAT*a) was crossed with *slc4::KanMX4* (*MAT*α), diploids were sporulated and tetrads were dissected on YPDUA using standard procedures. Growing colonies were replicated onto YPDUA supplemented with G418 to test for kanamycin resistance. In 22 tetrads, 6 were parental ditypes (PD, 4 colonies, all resistant to kanamycine), 14 were tetratypes (T, 3 colonies, 2 resistant to kanamycin), and 2 were non-parental ditypes (NPD, two colonies, both sensitive to kanamycin). The ratio of the various types (PD:NPD:T = 6:2:14) may indicate some centromeric linkage of the two markers. Pictures of plates taken at regular intervals and comparison with the genotypes showed that single mutant colonies have a normal growth rate on plates. B, cells were grown in SGaa or SDaa at 30°C and their concentration was determined photometrically (A_{600}).

Figure S2. *Addition of lyso-PA blocks the incorporation of* [^{14}C]-oleate *into GPLs in microsomes from 2Δ.SLC1 but not in microsomes from 2Δ.SLC4.* 2Δ.SLC4 or 2Δ.SLC1 cells were grown in YPGUA and microsomes were prepared. Microsomes (135 μg of protein), [^{14}C]-oleoyl-CoA (0.05 μCi), without (-) or with (+) *lyso*-PA (50 μM final conc.), were incubated for 120 min. Desalted lipid extracts were separated by TLC in solvent 2 and visualized by radioimaging.

Figure 3S. Complement to Figure 6. PI was the only lipid made in presence of [^{3}H]-inositol and CTP in all cell lines. For illustration the figure shows the TLC obtained for wt microsomes, of which only a small zone is shown in Fig. 6. The counts obtained in PI when a reagent was omitted relative to the counts obtained in the complete assay are indicated below each lane (% of complete).

Figure 4S. *Phosphatidic acid generated by Slc1p and Slc4p is metabolized to PS and PE.* A and B, microsomes from different strains used also in the experiment depicted in Fig. 3 were incubated with [^{3}H]-serine in the standard assay mix containing CTP/Mg^{2+}, oleoyl-CoA and *lyso*-PA (complete) or a mix wherein one of these additions had been omitted. Extracted lipids were run on TLC and the bands corresponding to PS and PE were quantitated by radioscanning. The counts obtained in PS or PE when a reagent was omitted relative to the counts obtained in the complete assay are indicated below each lane (% of complete).

Figure S5. Phosphatidic acid made by Slc1p and Slc4p is used for synthesis of triacylglycerides. Lipids were grown at 30°C until they reached the stationary phase in SDaaUA (SD), SGaaUA (SG) or YPG as indicated. Lipids were extracted and samples corresponding to 20 A_{600} units of cells were run on TLC in petroleum ether/diethyl ether/acetic acid (70:30:2) and visualized by iodine vapor. 4Δ represents RSY3077 = *are1Δ are2Δ lro1 dga1Δ* (cells which are unable to make triacylglycerides (TG)(Fig. 1) and were used as a negative control (lane 5). Depending on the genotype of microsomes, between 0.5 and 1.5% of added [^{3}H]-serine was incorporated into PS and PE when the complete assay mixture was used.

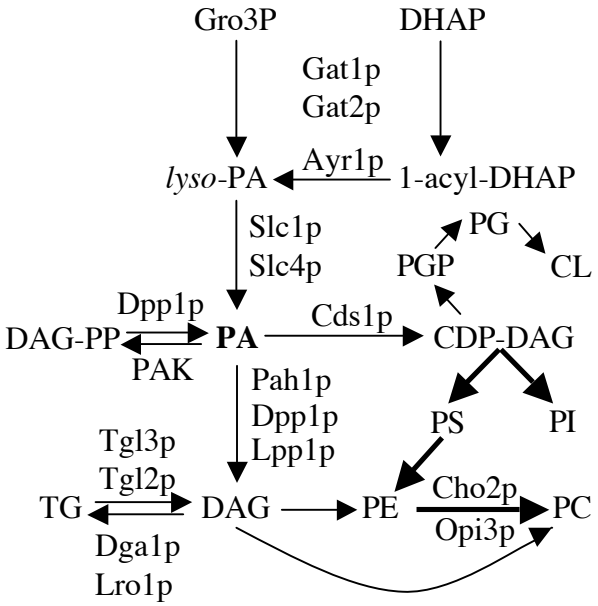


Figure 1

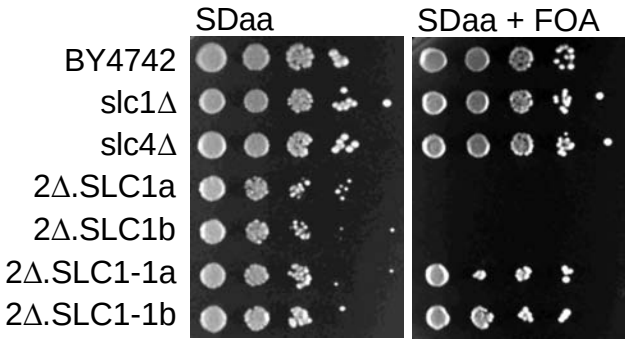


Figure 2

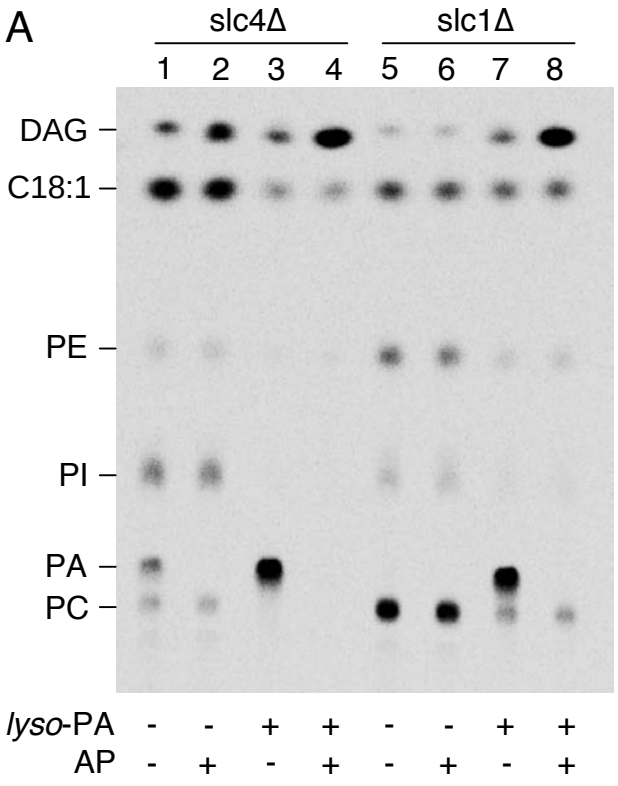


Figure 3A

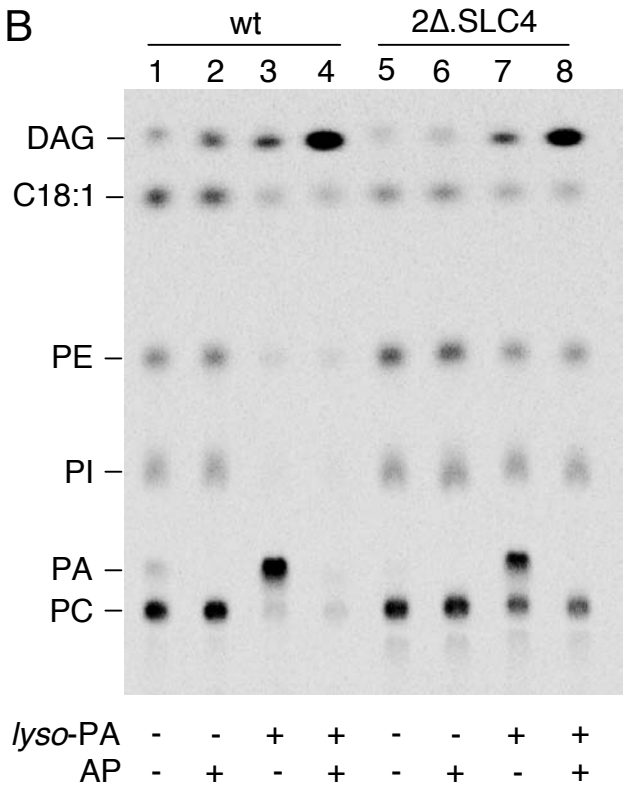


Figure 3B

C

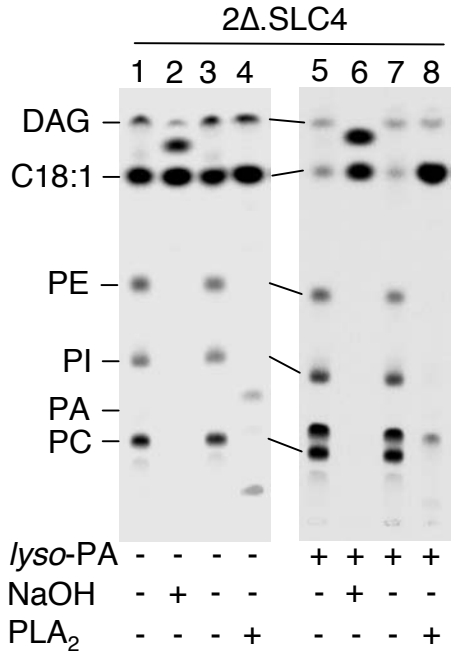


Figure 3C

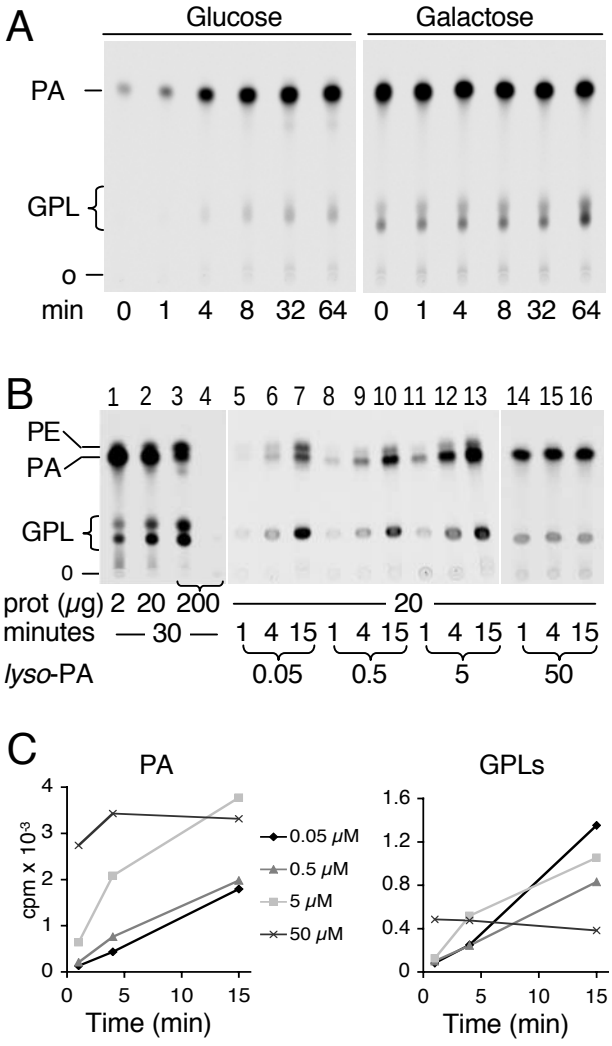


Figure 4

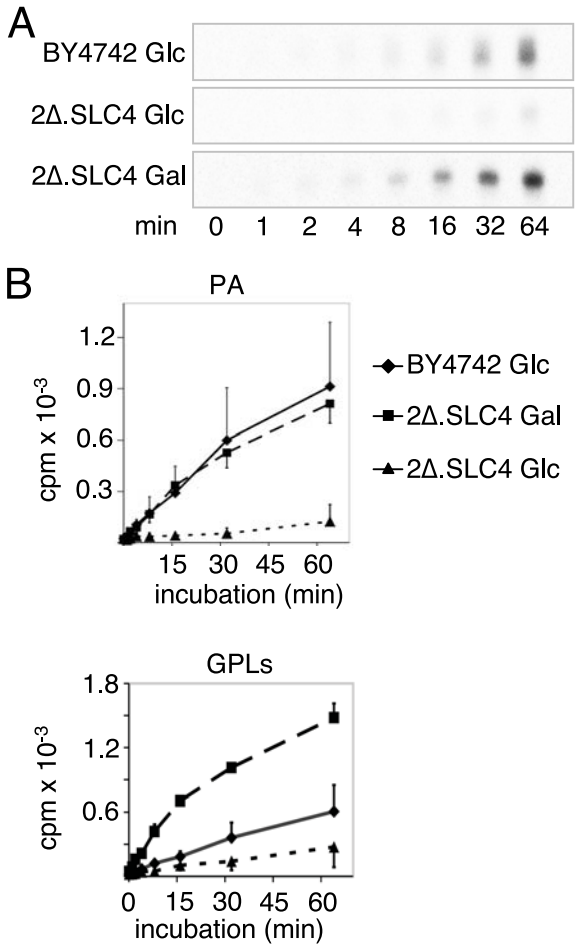


Figure 5

Figure 6

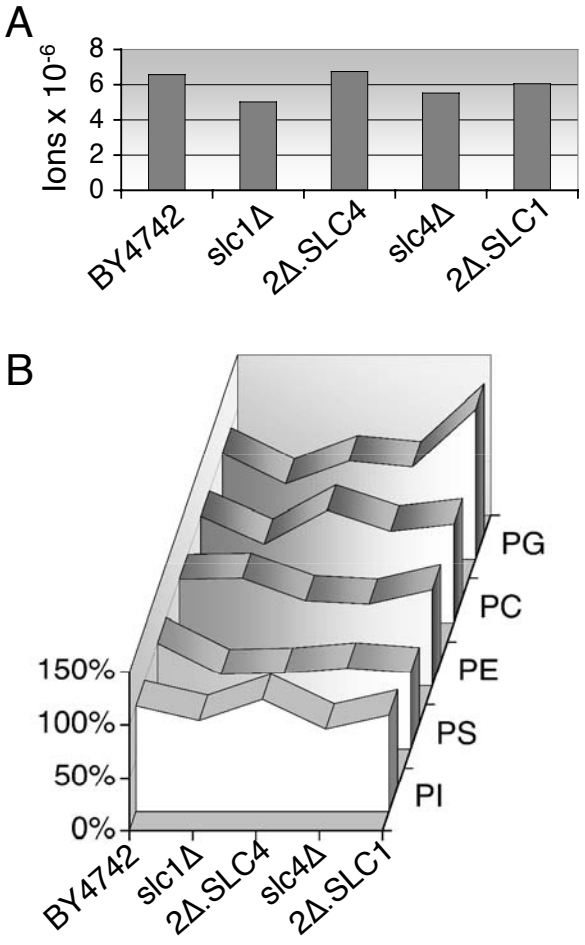


Figure 7A, B

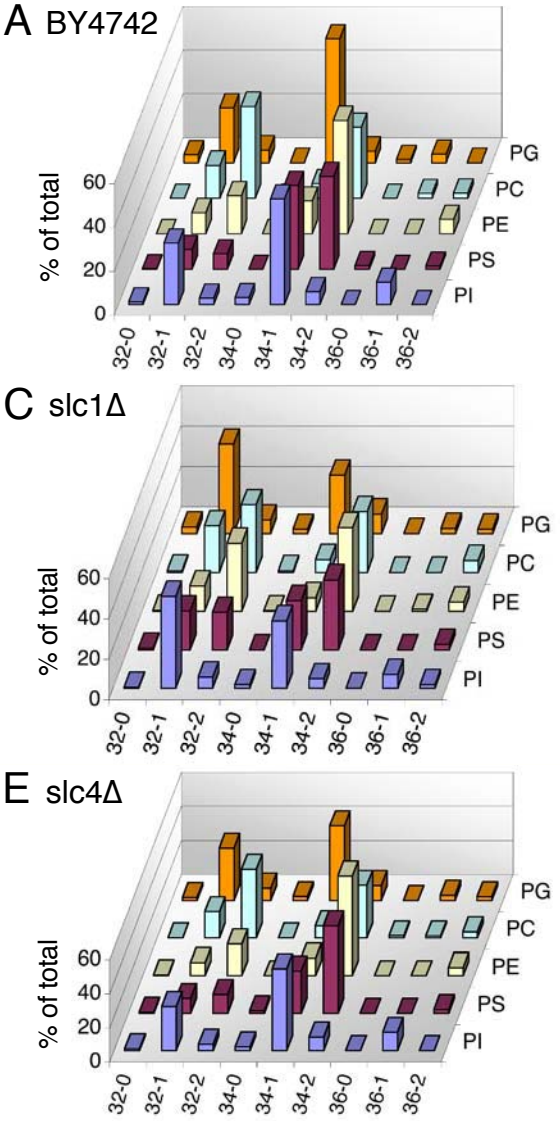


Figure 8A, C, E

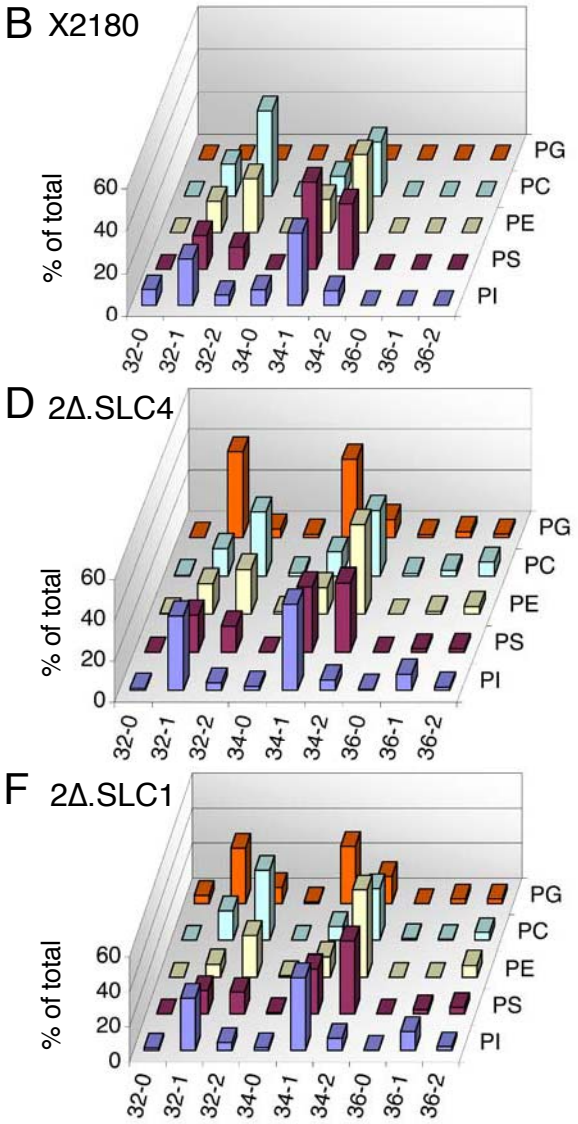
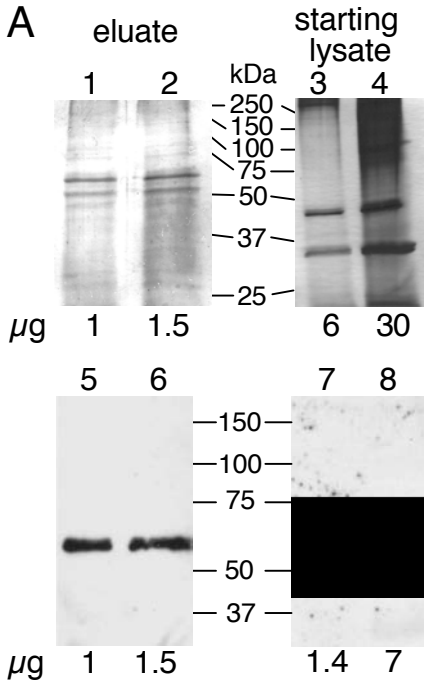


Figure 8B, D, F



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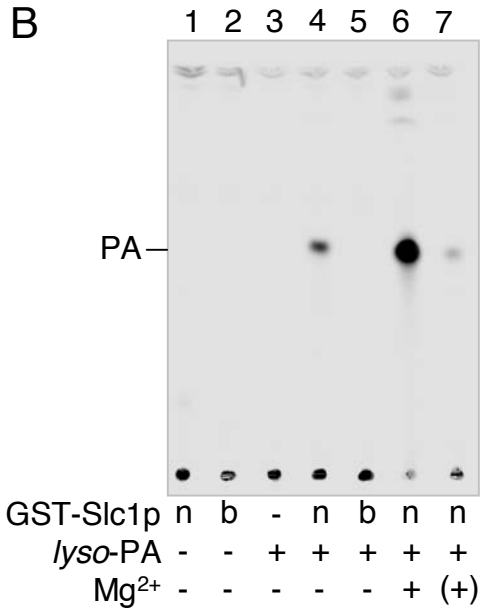

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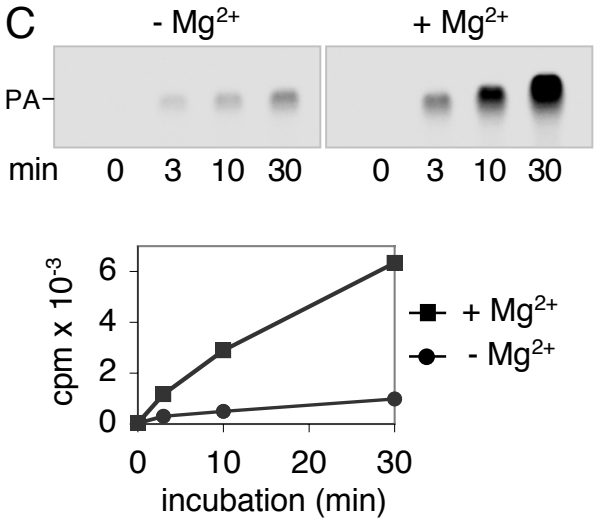

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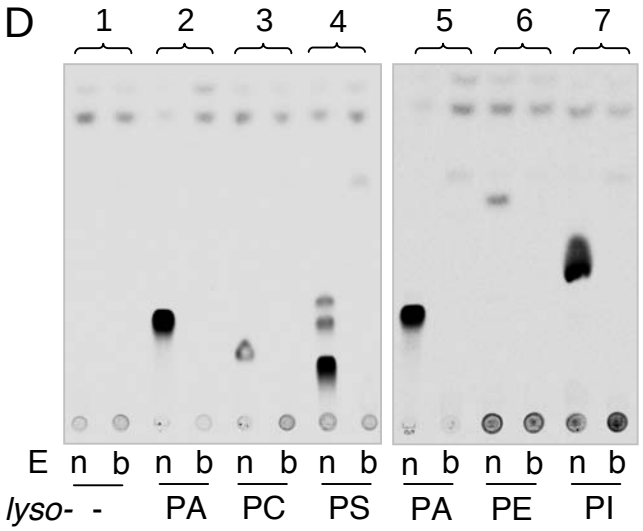

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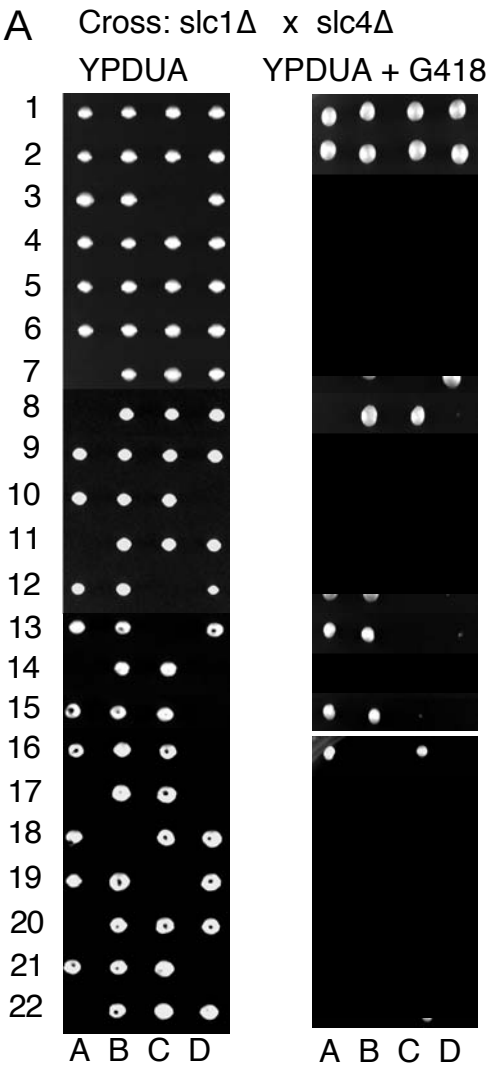
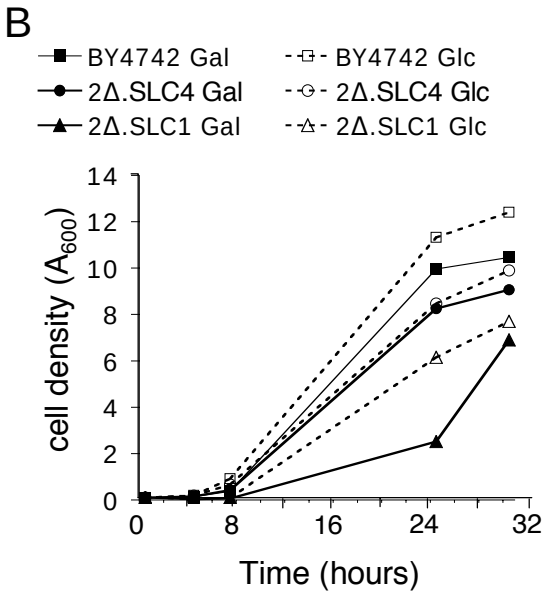
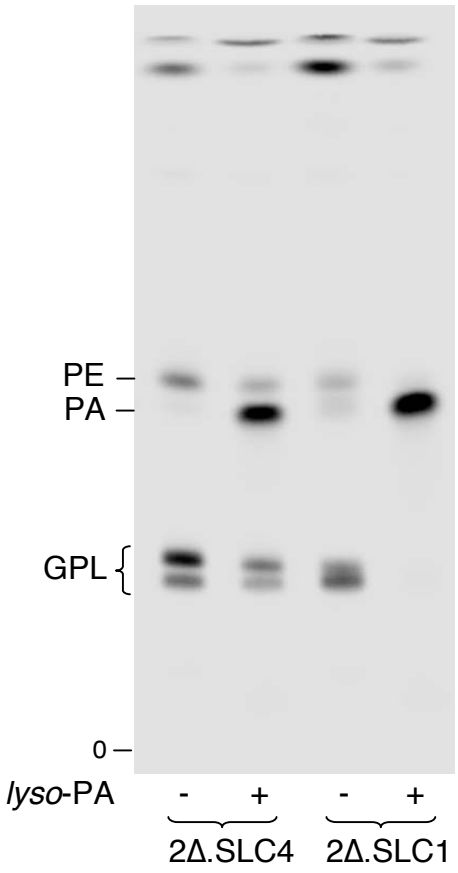


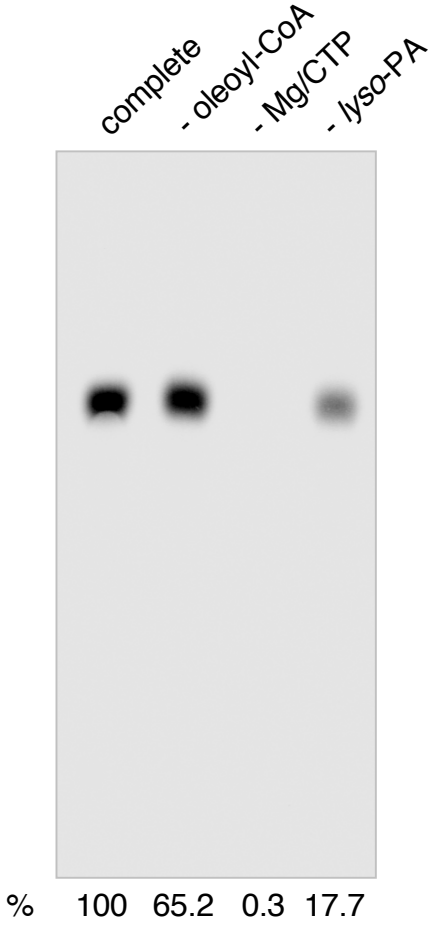
Supplemental Figure S1A



Supplemental Figure S1B



Supplemental Figure S2



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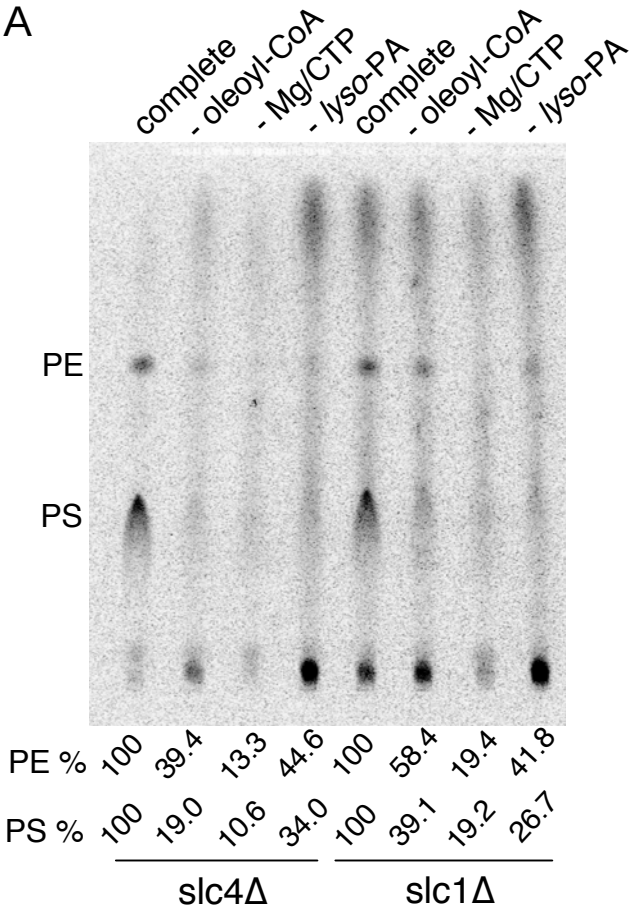
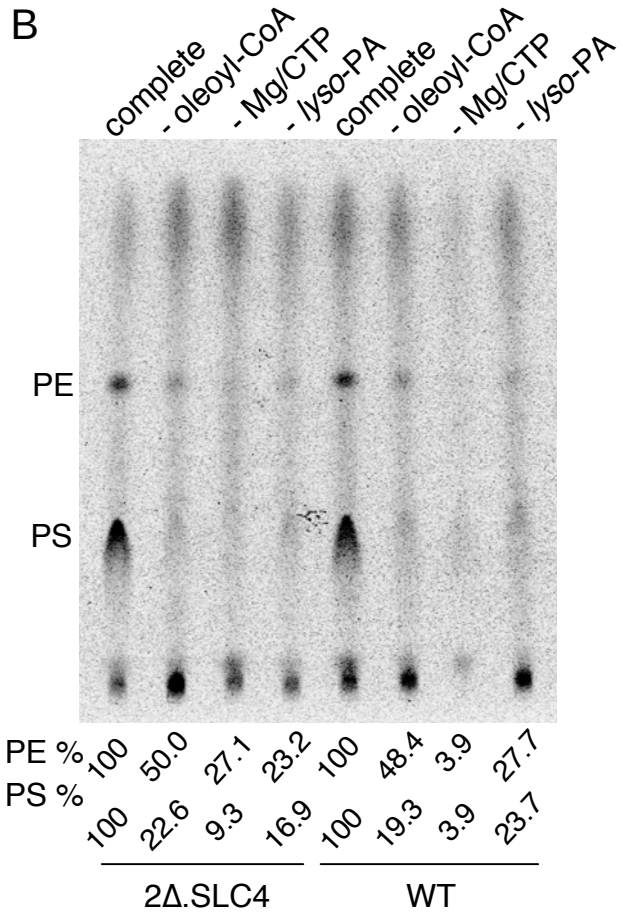


Supplemental Figure S4A



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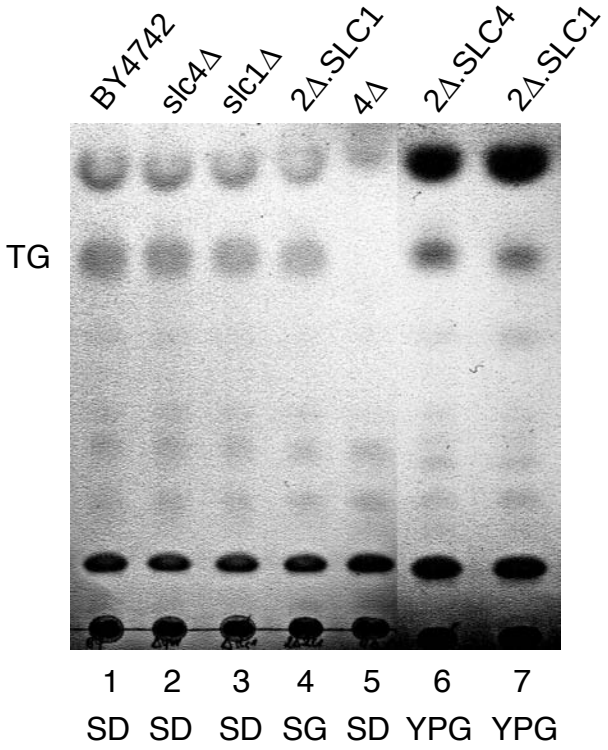

Supplemental Figure S5