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Biosynthesis of Phosphatidic Acid in Lipid Particles and Endoplasmic Reticulum of *Saccharomyces cerevisiae*

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Lipid particles of the yeast *Saccharomyces cerevisiae* harbor two enzymes that stepwise acylate glycerol-3-phosphate to phosphatidic acid, a key intermediate in lipid biosynthesis. In lipid particles of the *slc1* disruptant YMN5 (M. M. Nagiec et al., J. Biol. Chem. 268:22156–22163, 1993) acylation stops after the first step, resulting in the accumulation of lysophosphatidic acid. Two-dimensional gel electrophoresis confirmed that Slc1p is a component of lipid particles. Lipid particles of a second mutant strain, TTA1 (T. S. Tillman and R. M. Bell, J. Biol. Chem. 261:9144–9149, 1986), which harbors a point mutation in the *GAT* gene, are essentially devoid of glycerol-3-phosphate acyltransferase activity in vitro. Synthesis of phosphatidic acid is reconstituted by combining lipid particles from YMN5 and TTA1. These results indicate that two distinct enzymes are necessary for phosphatidic acid synthesis in lipid particles: the first step, acylation of glycerol-3-phosphate, is catalyzed by a putative Gat1p; the second step, acylation of lysophosphatidic acid, requires Slc1p. Surprisingly, YMN5 and TTA1 mutants grow like the corresponding wild types because the endoplasmic reticulum of both mutants has the capacity to form a reduced but significant amount of phosphatidic acid. As a consequence, an *slc1 gat1* double mutant is also viable. Lipid particles from this double mutant fail completely to acylate glycerol-3-phosphate, whereas endoplasmic reticulum membranes harbor residual enzyme activities to synthesize phosphatidic acid. Thus, yeast contains at least two independent systems of phosphatidic acid biosynthesis.

Phosphatidic acid is a key intermediate in the formation of glycerophospholipids and triacylglycerols. Two pathways of phosphatidic acid biosynthesis using either glycerol-3-phosphate or dihydroxyacetone phosphate as a substrate are being discussed (3). In the first pathway, glycerol-3-phosphate is stepwise acylated to lysophosphatidic acid and then to phosphatidic acid. In the second pathway, dihydroxyacetone phosphate is first acylated to acyl dihydroxyacetone phosphate, which is subsequently reduced in an NADPH-dependent reaction to lysophosphatidic acid (1, 16) and further converted to phosphatidic acid in a second acylation step. At present it is not known which of these two pathways is followed in vivo and whether enzymes that catalyze the acylation of glycerol-3-phosphate also accept dihydroxyacetone phosphate as a substrate and vice versa (16, 22, 23).

In mammalian cells, phosphatidic acid is synthesized from glycerol-3-phosphate by two acyltransferase reactions catalyzed sequentially by glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase. Two isoforms of glycerol-3-phosphate acyltransferases are postulated to exist in mammalian cells—one in mitochondria and the other in the endoplasmic reticulum (2). The mitochondrial glycerol-3-phosphate acyltransferase with a molecular mass of 85 kDa has been purified (28), and its cDNA has been cloned (26). The mitochondrial enzyme prefers palmitoyl coenzyme A (palmitoyl-CoA) over oleoyl-CoA as a substrate. The glycerol-3-phosphate acyltransferase of the endoplasmic reticulum has no preference for saturated or unsaturated acyl-CoAs. The activity of the second acyltransferase, 1-acylglycerol-3-phosphate acyltransferase, is ~10 times higher in the endoplasmic reticulum than in mitochondria (2, 9).

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In plant cells, glycerol-3-phosphate acyltransferase activity is present in plastids, mitochondria, and microsomes (for a review, see reference 17). Glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase have been localized to the mitochondrial outer membrane of potato tuber, whereas glycerol-3-phosphate acyltransferase of pea leaves was detected in the mitochondrial intermembrane space (7).

In the yeast *Saccharomyces cerevisiae*, the highest specific activity of the enzyme(s) acylating glycerol-3-phosphate was found in the lipid particle fraction (5, 30). Lipid particles consist of a hydrophobic core of triacylglycerols and steryl esters and are enveloped by a phospholipid monolayer. A small amount and a limited number of proteins are associated with the surface of these particles (13). Only few proteins of lipid particles have been characterized so far. One major protein of this fraction is sterol-Δ^{24}-methyltransferase (Erg6p) (13). Recently, squalene epoxidase (Erg1p) (14) and lanosterol synthase (Erg7p) (31) have been demonstrated to be lipid particle components. Thus, all proteins of lipid particles identified so far are enzymes involved in lipid biosynthesis. These proteins, however, are not exclusively localized to these particles but are also present in the endoplasmic reticulum to an extent depending on the growth phase of the cells.

Genes and proteins required for the biosynthesis of phosphatidic acid in the yeast *S. cerevisiae* are poorly characterized. Two possible acyltransferases are known. First, the product of the *SLC1* (sphingolipid compensation) gene was found to be homologous to a 1-acylglycerol-3-phosphate acyltransferase of *Escherichia coli* encoded by the *plsC* gene (19). Slc1p complements the growth defect in a *plsC*-deficient *E. coli* mutant. Second, TTA1, a mutant that was identified by a colony autoradiographic screening technique, exhibits a dramatically reduced level of glycerol-3-phosphate acyltransferase activity in vitro (27). The gene complementing the defect of the TTA1 mutant has not yet been identified.

Here we show that acylation defects in the YMN5 (harbor-

ing a deletion of *SLC1*) and TTA1 mutant become evident in the lipid particle fraction of these strains. Both mutants, however, do not show obvious growth phenotypes. Since synthesis of phosphatidic acid can be assumed to be an essential process, it is likely that as in higher eukaryotes, at least one additional system of phosphatidic acid synthesis exists in yeast. Detection of residual activity of phosphatidic acid formation in microsomes of YMN5, TTA1, and an *slc1 gat1* double mutant are in line with this hypothesis.

MATERIALS AND METHODS

Strains and culture conditions. The *S. cerevisiae* wild-type strains X2180-1A (*MAT***a** *SUC2 mal gal2 CUP1*), SJ21R (*MAT***a** *ura3-52 leu2-3,112 ade1*) and DBY747 (*MAT***a** *his3-Δ1 leu2-3 leu2-112 ura3-52 trpl-289*) and mutant strains YMN5 (*MAT***a** *ura3-52 leu2-3,112 ade1 slc1 Δ2::LEU2*) (kindly provided by M. Nagiec and R. Dickson) and TTA1 (*MATα his3-Δ1 leu2-3 leu2-112 ura3-52 trpl-289*) (kindly provided by R. Bell) were used throughout this study. An *slc1 gat1* double mutant was constructed by using standard techniques of yeast genetics (25).

Cells were grown aerobically in 2-liter Erlenmeyer flasks to the early logarithmic phase at 30°C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid), and 2% glucose (Merck). A 500-ml volume of culture medium was inoculated with 0.3 ml of a preculture grown aerobically for 48 h.

Isolation and characterization of subcellular fractions. Lipid particles (13) were obtained at high purity (enrichment factor, 700 to 800 for triacylglycerols, steryl esters, and Erg6p) from cells grown to the late logarithmic phase. Other subcellular fractions used in this study were prepared as described by Zinser et al. (30). Relative enrichment of markers and cross-contamination were comparable to the values described by Zinser and Daum (29).

Prior to protein analysis, the lipid particle fraction was delipidated. Nonpolar lipids were extracted with 2 volumes of diethyl ether. The organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were precipitated from the aqueous phase by using trichloroacetic acid (10%, final concentration). The protein pellet was solubilized in 0.1% sodium dodecyl sulfate (SDS)–0.1% NaOH. Protein was quantitated by the method of Lowry et al. (15), using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (12). Samples were dissociated at 37°C, because treatment at higher temperature resulted in hydrolysis of lipid particle proteins. Western blot analysis was carried out as described by Haid and Suissa (8), and immunoreactive proteins were detected by enzyme-linked immunosorbent assay using rabbit antiserum as the first antibody and goat anti-rabbit immunoglobulin G linked to peroxidase or phosphatase as the second antibody.

Two-dimensional gel electrophoresis. Equipment for isoelectric focusing and horizontal gel electrophoresis (Multifor II electrophoresis unit, Power Supply EPS 3500), ready-made immobilized pH gradient (IPG)- and SDS-polyacrylamide gels (Immobiline DryStrips pH 3-10 L and ExcelGel SDS 12.5%, homogeneous), and Pharmalyte pH 3-10 were purchased from Pharmacia Biotechnology, Uppsala, Sweden. Immobiline DryStrips (pH 3-10 L) were rehydrated overnight in a rehydration solution (12.0 g of urea, 130 μl of Triton X-100, 130 μl of Pharmalyte pH 3-10, and 50 mg of dithiothreitol made up to 25 ml with distilled water). Lipid particles were delipidated as described above prior to electrophoretic analysis; proteins were precipitated with acetone (50%, final concentration) and solubilized in 20 μl of lysis buffer A (540 mg of urea, 20 μl of Triton X-100, 20 μl of 2-mercaptoethanol, 20 μl of Pharmalyte pH 3-10, and 1.4 mg of phenylmethylsulfonyl fluoride dissolved in 1 ml of distilled water) and 80 μl of buffer B (540 mg of urea, 10 mg of dithiothreitol, 20 μl of Pharmalyte pH 3-10, and 5.2 μl of Triton X-100 dissolved in 1 ml of distilled water with a small amount of bromphenol blue). Proteins were applied to the rehydrated Immobiline DryStrip and electrophoretically separated for 16 h at a maximum of 5 W at 20°C. Then strips were equilibrated in a solution containing 3.6 g of urea, 3.0 ml of glycerol, 0.1 g of SDS, and 1 ml of 0.5 M Tris-Cl (pH 6.8) in a total volume of 10 ml of distilled water. The equilibration solution contained 25 to 100 mg of dithiothreitol per 10 ml depending on the amount of protein present on the strip. After treatment for 15 min, strips were incubated in 10 ml of equilibration solution containing 450 mg of iodacetamide for another 15 min. Electrophoresis in the second dimension was performed by using 12.5% ExcelGels SDS (Pharmacia) at 15°C with an upper limit of 50 mA. After electrophoretic separation, proteins were visualized by silver staining.

Enzyme analysis. Enzymatic activities of glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase were measured as described by Schlossman and Bell (23). Total yeast homogenate (200 μg of protein), lipid particles (10 to 20 μg of protein), and microsomes or mitochondria (50 μg of protein each) were used as the enzyme sources. The assay mixture contained 75 nmol of [^{14}C]glycerol-3-phosphate (0.4 μCi), 8 nmol of oleoyl-CoA, 15 μg of dithiothreitol, and 0.2 mg of bovine serum albumin in 0.2 ml of 4 mM NaF–2 mM $MgCl_2$–37.5 mM Tris-Cl (pH 7.5). After 3 and 6 min of incubation at 30°C, lipids were extracted with 3 ml of chloroform-methanol (1:2, vol/vol) in the presence of 0.7 ml of 1% perchloric acid. The organic phase was washed three times with 2 ml of 1% perchloric acid. The lipid extract was applied to high-performance thin-layer chromatography (TLC) plates (Silica Gel 60; Merck, Darmstadt, Germany), and chromatograms were developed in an ascending manner, using the solvent system chloroform-methanol-water-acetic acid (65:35:3.8:0.2, by volume). After chromatographic separation, the radioactively labeled lipids formed during the assay were detected by TLC scanning using a Tracemaster 20 Automatic TLC-Linear Analyzer (Berthold). Alternatively, lipids were visualized on high-performance TLC plates by staining with iodine vapor, bands were scraped off, and radioactivity was measured by liquid scintillation counting (Beckman 1500 Tricarb).

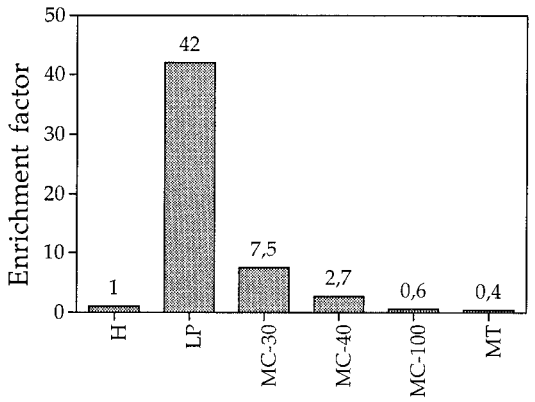

FIG. 1. Glycerol-3-phosphate acyltransferase activities in yeast subcellular fractions. The specific activity of glycerol-3-phosphate acyltransferase in the homogenate was set at 1, and the relative enrichment of specific activities in subcellular fractions was calculated. Data were obtained from at least five independent experiments with a mean deviation of ±10%. H, homogenate; LP, lipid particles; MC-30, 30,000 × *g* microsomes; MC-40, 40,000 × *g* microsomes; MC-100, 100,000 × *g* microsomes; MT, mitochondria.

Lipid analysis. Lipids of whole yeast cells were extracted by the procedure of Folch et al. (6). Individual phospholipids were separated by two-dimensional TLC on Silica Gel 60 plates (Merck), using chloroform-methanol-25% NH_3 (65:35:5, by volume) as the first, and chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume) as the second, developing solvent. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off the plate, and quantified by the method of Broekhuyse (4).

For the analysis of neutral lipids, extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (Linomat IV; CAMAG, Muttenz, Switzerland), and chromatograms were developed in an ascending manner, using the solvent system light petroleum-diethyl ether-acetic acid (70:30:2, by volume). Triacylglycerols were visualized by postchromatographic staining using a chromatogram immersion device (CAMAG). Plates were dipped for 2 s into a developing reagent consisting of 0.63 g of $MnCl_2 \cdot 4H_2O$, 60 ml of water, 60 ml of methanol, and 4 ml of concentrated sulfuric acid, briefly dried, and heated to 100°C for 30 min. Quantitation of acylglycerols was carried out by densitometric scanning at 400 nm with triolein (NuCheck, Inc., Elysian, Minn.) as a standard.

Fatty acids were analyzed by gas-liquid chromatography. Lipids extracted as described above were subjected to methanolysis using BF_3-methanol and converted to methyl esters. Fatty acyl methyl esters were separated by gas-liquid chromatography on a Hewlett-Packard Ultra 2 capillary column (5% phenyl methyl silicone) with a temperature gradient (20 min at 200°C, 10°C/min to 280°C, 15 min at 300°C). Fatty acids were identified by comparison to commercial fatty acyl methyl ester standards (NuCheck).

RESULTS

In the wild-type *S. cerevisiae* strain X2180, highest specific activity of the enzyme(s) acylating glycerol-3-phosphate was detected with lipid particles as the enzyme source (Fig. 1). In vitro, lipid particles exhibited a 42-fold enrichment of acylation activity over the homogenate. Despite this high enrichment factor, acylation of glycerol-3-phosphate must not be regarded as a biosynthetic process exclusively attributable to lipid particles, because typical lipid particle components such as triacylglycerols, steryl esters (29), or Erg6p (13) are much more highly enriched in this fraction (700- to 800-fold over the homogenate). This observation suggested that acylation of glycerol-3-phosphate may also take place in other organelles.

TABLE 1. Glycerol-3-phosphate acyltransferase activity in lipid particles and microsomes of YMN5, TTA1, and the *slc1 gat1* double mutant

Strain[a]	Lipid particles			Microsomes		
	Mean sp act (nmol/min/mg of protein) ± SD	Relative sp act[b] (%)	LPA/PA[c] (%)	Mean sp act (nmol/min/mg of protein) ± SD	Relative sp act (%)	LPA/PA (%)
SJ21R (wt)	1.15 ± 0.07	100	8:92	2.27 ± 0.33	100	4:96
YMN5 (*slc1*)	1.23 ± 0.36	107	97:3	1.05 ± 0.29	46	66:34
DBY747 (wt)	25.6 ± 3.5	100	4:96	3.84 ± 0.70	100	7:93
TTA1 (*gat1*)	0.32 ± 0.09	1.3	5:95	0.24 ± 0.04	6.3	0:100
slc1 gat1 double mutant	ND[d]	ND	ND	0.10 ± <0.01	4.1/2.4[e]	28:72

[a] SJ21R is the wild type (wt) corresponding to YMN5, and DBY737 is the wild type corresponding to TTA1.
[b] The specific activities of wild-type strains SJ21R and DBY747 were set at 100%, and the specific activity in each of the corresponding mutants was set in correlation to these values. Mean values are from three independent experiments.
[c] LPA/PA, molar ratio of lysophosphatidic acid to phosphatidic acid.
[d] ND, not detectable.
[e] The specific activity of microsomes of the *slc1 gat1* double mutant was calculated relative to both wild-type strains.

Indeed, a sevenfold enrichment of glycerol-3-phosphate acyltransferase activity was observed in the endoplasmic reticulum-derived 30,000 × *g* microsomal fraction. Taking into account that the endoplasmic reticulum contains approximately 12%, but lipid particles only 0.05 to 0.1%, of the cellular protein, 95 to 98% of the total cellular activity of glycerol-3-phosphate acyltransferase can be attributed to the endoplasmic reticulum, and only 2 to 5% can be attributed to lipid particles. Mitochondria are essentially devoid of glycerol-3-phosphate acylating activities. These data are in good agreement with previous results from our laboratory (30).

To define the protein requirements for glycerol-3-phosphate acylation in vitro more precisely, lipid particles and microsomes of the two putative yeast acyltransferase mutants, YMN5 and TTA1, were analyzed. Lipid particles isolated from YMN5, which harbors a disruption of the *SLC1* gene, exhibited an acyltransferase activity in vitro comparable to that of the corresponding wild-type strain, SJ21R (Table 1). Analysis of the reaction products formed, however, revealed that lipid particles of YMN5 could synthesize only lysophosphatidic acid, whereas those of the wild-type strain formed mainly phosphatidic acid as a product. These results indicated that in the lipid particle fraction, Slc1p acts as a 1-acylglycerol-3-phosphate acyltransferase. Since lipid particles have a relatively simple protein composition, we examined whether a protein band corresponding to Slc1p is missing in strain YMN5. By comparing two-dimensional gel electrophoresis patterns of lipid particle proteins derived from YMN5 and wild-type cells, Slc1p was identified as a lipid particle protein with an apparent molecular mass of 34 kDa and an experimental pI of approximately 8.0 (Fig. 2). These experimental data are in good agreement with protein data predicted from the DNA sequence of the *SLC1* gene (19). Two-dimensional gel electrophoresis performed with lipid particle proteins isolated from mutant TTA1 did not show a difference from the protein pattern of lipid particles from the corresponding wild type (data not shown). This, however, was not surprising because TTA1 is not a deletion mutant but harbors a point mutation (27).

Lipid particles isolated from TTA1, a *gat1* mutant strain synthesized neither lysophosphatidic acid nor phosphatidic acid in significant amounts (Table 1). These results indicate that this strain is defective either in the first step or in the first and second steps of acyl transfer required for the formation of phosphatidic acid in lipid particles. To distinguish between these two possibilities, a complementation experiment was performed. For this purpose, an acylation assay in vitro was started with lipid particles isolated from YMN5, and lipid particles isolated from TTA1 were subsequently added. Whereas lipid particles of YMN5 formed lysophosphatidic acid as the only labeled acylation product, addition of TTA1-derived lipid particles to the assay mixture compensated for this defect and led to the formation of phosphatidic acid (data not shown). Thus, the mutation in TTA1 could be pinpointed to a defect in the first step of glycerol-3-phosphate acylation.

Whereas lipid particles of TTA1 harbored essentially no glycerol-3-phosphate acylating activity, and those of YMN5 formed lysophosphatidic acid as the only acylation product,

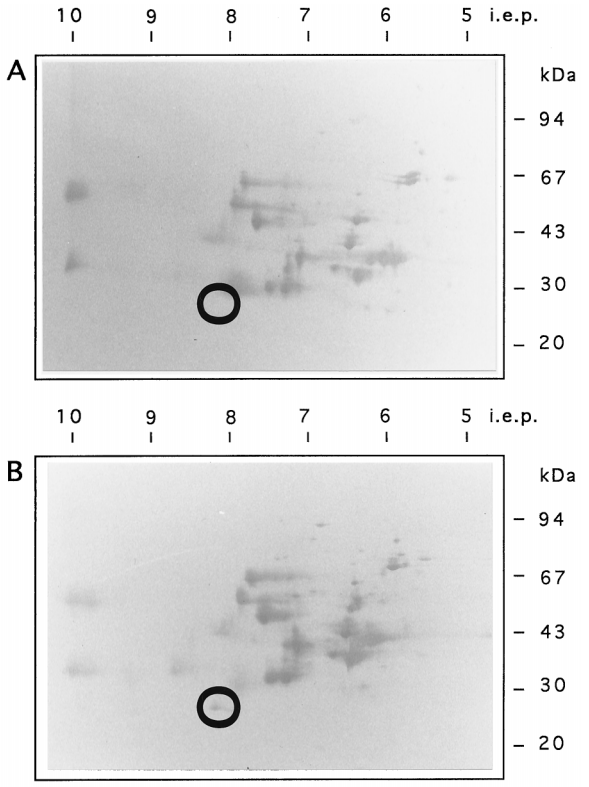

FIG. 2. Identification of Slc1p as a lipid particle component by two-dimensional gel electrophoresis. Lipid particle proteins isolated from mutant YMN5 (A) and the corresponding wild-type strain SJ21R (B) were analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. The position of Slc1p in each gel is marked by a circle. i.e.p., isoelectric point.

TABLE 2. Fatty acid composition of YMN5, TTA1, and the *slc1 gat1* double mutant

Strain	% of total fatty acids (mean ± SD)[a]				
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$
SJ21R (wt)	3.4 ± 0.6	15.1 ± 0.2	45.6 ± 1.5	3.6 ± 0.4	32.3 ± 1.8
YMN5 (*slc1*)	1.5 ± 0.4	12.2 ± 0.3	53.0 ± 1.0	2.9 ± 0.6	30.4 ± 0.3
DBY747 (wt)	2.5 ± 0.1	21.6 ± 0.3	38.8 ± 0.5	4.6 ± 0.1	32.5 ± 0.7
TTA1 (*gat1*)	1.7 ± 0.3	21.6 ± 0.4	45.0 ± 1.4	4.1 ± 0.4	27.6 ± 1.2
slc1 gat1 double mutant	1.5 ± 0.3	14.2 ± 0.3	50.0 ± 1.2	3.5 ± 0.4	30.8 ± 0.4

[a] Values are from three independent experiments.

microsomes of both mutants synthesized a significant amount of phosphatidic acid (Table 1). Similar to the situation in lipid particles, lysophosphatidic acid did not accumulate in microsomes of the TTA1 mutant. In microsomes prepared from YMN5, on the other hand, 66% of radiolabeled glycerol-3-phosphate incorporated into lipids was detected in lysophosphatidic acid, but 34% appeared in phosphatidic acid.

The decrease in the acylation activity in microsomes isolated from YMN5 and TTA1 compared to the corresponding wild types indicated that the two enzymes Slc1p and Gat1p are found not only in lipid particles but in the endoplasmic reticulum as well. Experiments using isolated lipid particles and microsomal fractions (see above) had demonstrated that Slc1p and Gat1p are distinct enzymes that cannot compensate for each other. To further test this hypothesis, glycerol-3-phosphate acyltransferase activity was measured in subcellular fractions of an *slc1 gat1* double mutant. Similar to mutants YMN5 and TTA1, which do not exhibit a specific growth phenotype, the *slc1 gat1* double mutant grows comparably to wild-type cells. Due to the lack of appropriate genetic markers of strain TTA1, we had to distinguish between an *slc1 gat1* double mutant and a *gat1* single mutant through the acylation capacity of lipid particles in a complementation experiment as described above. When lipid particles isolated from YMN5 were mixed with lipid particles from TTA1, formation of phosphatidic acid was reconstituted (see above). In contrast, the combination of lipid particles from YMN5 with those of the *slc1 gat1* double mutant did not result in phosphatidic acid synthesis (data not shown). Predictably, lipid particles of the double mutant could not synthesize any glycerol-3-phosphate acylation product. Microsomes of the *slc1 gat1* double mutant, however, still formed phosphatidic acid. These results strongly support the view that in addition to Slc1p and Gat1p, other enzyme(s) catalyzing the synthesis of phosphatidic acid must be present in the endoplasmic reticulum. These observations also explain why mutants YMN5 and TTA1 as well as the *slc1 gat1* double mutant grow like the wild type.

One could envisage, however, that a defect in the Slc1p/Gat1p acylation machinery might affect the lipid composition of the mutant strains. Since acyltransferases may have preferences for specific fatty acyl-CoAs, we first compared the fatty acid compositions of the wild-type strains to those of the two single mutants and the double-mutant strain (Table 2). In YMN5, TTA1, and the *slc1 gat1* double mutant, the amount of palmitoleic acid ($C_{16:1}$) was increased at the expense of all other fatty acids. Although these changes are not dramatic, this result may indicate that the different acylation systems have slightly different substrate specificities. The phospholipid composition of YMN5 was not significantly different from that of the corresponding wild-type strain (Table 3). In TTA1, the cellular level of phosphatidic acid was reduced and the amounts of phosphatidylcholine, phosphatidylserine, and cardiolipin were slightly increased. The phospholipid composition of the *slc1 gat1* double mutant was not significantly different from that of wild types. Thus, the Slc1p/Gat1p acylation machinery and the backup system in the endoplasmic reticulum seem to be similar with respect to product specificity.

The cellular concentration of triacylglycerols was higher in mutant strains than in wild types during logarithmic growth (Table 4). In the early stationary phase, the levels of triacylglycerols of wild types, YMN5, and TTA1 increased, although in a less pronounced way in the mutant strains. The cellular level of triacylglycerols in the *slc1 gat1* double mutant was not significantly altered from the logarithmic to the early stationary phase.

DISCUSSION

Phosphatidic acid is a quantitatively minor lipid component of eukaryotic cells, but it is an important precursor for the synthesis of CDP-diacylglycerol, which is the starting substrate for the synthesis of phosphatidylinositol, phosphatidylglycerol, and cardiolipin in mammalian cells and, in addition, phosphatidylserine in yeast (3). Moreover, phosphatidic acid converted to diacylglycerol is utilized for the synthesis of phosphatidylethanolamine and phosphatidylcholine via the Kennedy pathway or is further acylated, thus yielding triacylglycerols.

Enzymes of phosphatidic acid synthesis in mammalian and plant cells have been studied in some detail (see the introduction). In contrast, proteins and genes governing this biosynthetic pathway in *S. cerevisiae* are less well understood. The results presented in this report show that phosphatidic acid synthesis in yeast starting from glycerol-3-phosphate as a precursor occurs by two sequential steps of acylation that are catalyzed by two different enzymes. A *gat1* mutant, TTA1 (27), bears a defect in an enzyme activity that catalyzes the first step of acylation. Due to the lack of any selectable phenotype associated with mutant strain TTA1, neither the protein nor the corresponding gene has been characterized. The second acylation step is catalyzed by Slc1p, which converts lysophospha-

TABLE 3. Phospholipid composition of YMN5, TTA1, and the *slc gat1* double mutant

Strain	% of total phospholipids (mean ± SD)[a]							
	PtdCho	DMPtdEtn	PtdEtn	PtdIns	PtdSer	PA	CL	Lyso-PL
SJ21R (wt)	35.4 ± 1.5	5.1 ± 0.4	20.1 ± 1.1	18.0 ± 1.2	5.7 ± 0.2	7.8 ± 0.5	1.6 ± 0.1	2.2 ± 0.1
YMN5 (*slc1*)	37.2 ± 1.8	4.9 ± 0.5	23.7 ± 1.0	17.0 ± 0.5	4.5 ± 0.3	7.2 ± 0.3	2.2 ± 0.2	2.7 ± 0.5
DBY747 (wt)	35.3 ± 1.9	3.8 ± 0.5	20.0 ± 0.9	18.7 ± 1.2	4.3 ± 0.1	7.9 ± 0.8	1.7 ± 0.1	1.2 ± 0.1
TTA1 (*gat1*)	42.7 ± 0.5	1.7 ± <0.1	19.0 ± 0.6	21.0 ± 0.1	6.6 ± <0.1	2.7 ± 0.2	3.7 ± <0.1	2.6 ± 0.8
slc1 gat1 double mutant	39.1 ± 1.2	3.1 ± 0.3	22.1 ± 1.3	19.6 ± 0.5	5.4 ± 0.3	7.8 ± 0.4	2.1 ± 0.1	0.9 ± 0.2

[a] Values are from three independent experiments. PtdCho, phosphatidylcholine; DMPtEtn, dimethyl phosphatidylethanolamine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PA, phosphatidic acid; CL, cardiolipin; Lyso-PL, lysophospholipids.

TABLE 4. Triacylglycerols of YMN5, TTA1, and the *slc1 gat1* double mutant

Strain	Mean triacylglycerol concn (μg/mg of phospholipids) ± SD[a]	
	Logarithmic phase	Early stationary phase
SJ21R (wt)	36 ± 8	165 ± 20
YMN5 (*slc1*)	103 ± 11	148 ± 20
DBY747 (wt)	48 ± 1	99 ± 11
TTA1 (*gat1*)	103 ± 5	158 ± 7
slc1 gat1 double mutant	95 ± 4	99 ± 24

[a] Values are from three independent experiments.

tidic acid to phosphatidic acid (Fig. 3). Both Gat1p and Slc1p are enriched in lipid particles. Glycerol-3-phosphate acyltransferase appears to be rate limiting in this biosynthetic pathway in vitro, since lysophosphatidic acid never accumulates when lipid particles from wild-type cells are used as an enzyme source. This observation is in agreement with earlier reports by Bell and Coleman (2).

Do yeast cells contain only one glycerol-3-phosphate acyltransferase system, or are there redundant systems of phosphatidic acid biosynthesis in yeast as is known to be the case for higher eukaryotes? In contrast to mammalian and plant cells, yeast mitochondria do not harbor enzymes involved in the biosynthesis of phosphatidic acid. Nevertheless, two independent systems of phosphatidic acid synthesis appear to exist in yeast: one in lipid particles and one in the endoplasmic reticulum. An *slc1 gat1* double mutation leads to a complete loss of glycerol-3-phosphate acyltransferase activity in lipid particles. The double mutation also results in a dramatic decrease of acyltransferase activities in the endoplasmic reticulum, but a residual capacity (2 to 4% of the wild-type level) to form phosphatidic acid still remains in this compartment. On one hand, these results indicate that Gat1p and Slc1p are not restricted to lipid particles but are also components of the endoplasmic reticulum. The observation that microsomes of YMN5 and the *slc1 gat1* double mutant accumulate lysophosphatidic acid supports this view (Table 1). On the other hand, our data suggest that the backup system of phosphatidic acid synthesis in the endoplasmic reticulum functions independently of the Slc1p/Gat1p machinery.

Surprisingly, the reduced amount of phosphatidic acid formed in the endoplasmic reticulum of YMN5, TTA1, and the *slc1 gat1* double mutant is sufficient to maintain balanced growth of these strains. Molecular biological evidence will be required to demonstrate whether a second set of acyltransferases catalyzes synthesis of phosphatidic acid in the endoplasmic reticulum. As an alternative, the backup formation of phosphatidic acid in the endoplasmic reticulum may be catalyzed by enzymes that do not primarily acylate. Such candidate enzymes are phospholipases which in a reversed reaction of hydrolytic cleavage might form a small but sufficient amount of phosphatidic acid. It is interesting that a defect in the Gat1p/Slc1p system changes the fatty acid composition of total lipids and the phospholipid composition to only a minor extent (Tables 2 and 3). Single mutants and the *slc1 gat1* double mutant contain amounts of triacylglycerols that are comparable to or even higher than those in wild types (Table 4). This result indicates that Slc1p and Gat1p are not primarily involved in the formation of these neutral lipids.

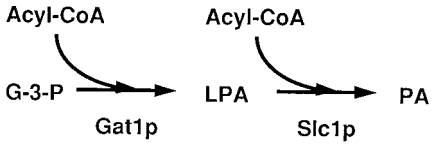

FIG. 3. Biosynthesis of phosphatidic acid from glycerol-3-phosphate. Glycerol-3-phosphate (G-3-P) is acylated to lysophosphatidic acid (LPA) and further to phosphatidic acid (PA) by catalysis of Gat1p and Slc1p. The mutant strain TTA1 is defective in enzymatically active Gat1p, and YMN5 harbors a deletion of the *SLC1* gene.

The question remains open whether an interaction of organelles occurs during synthesis of phosphatidic acid. The complementation/reconstitution experiment described in Results with lipid particles isolated from YMN5 and TTA1 indicates that lysophosphatidic acid formed by one population of lipid particles can migrate to another population of lipid particles most likely by diffusion. In vivo, exchange of lysophosphatidic acid and/or phosphatidic acid between lipid particles and the endoplasmic reticulum may be of importance. Preliminary results indicate that in vitro translocation of lysophosphatidic acid between these two fractions can occur.

Finally, the question of how newly formed phosphatidic acid is supplied from its subcellular sites of synthesis to the sites of further metabolic conversion might be addressed. Phosphatidic acid is used as a substrate for the formation of CDP-diacylglycerol, which is a key intermediate in the biosynthesis of complex phospholipids. In yeast, both microsomes (endoplasmic reticulum) and mitochondria contain CDP-diacylglycerol synthase activity (11). The mitochondrial CDP-diacylglycerol synthase, which is the product of the *CDS1* gene, has been purified (10), and recently the cDNA was cloned (24). The *CDS1* gene is essential and is therefore likely to be the only gene encoding CDP-diacylglycerol synthase. Phosphatidic acid formed in the endoplasmic reticulum can be utilized in situ for CDP-diacylglycerol biosynthesis, whereas phosphatidic acid formed in lipid particles has to be transported to the endoplasmic reticulum and/or mitochondria to supply CDP-diacylglycerol synthase with substrate. A second metabolic route for phosphatidic acid consumption is by hydrolytic cleavage yielding diacylglycerol, which is the substrate for phosphatidylcholine and phosphatidylethanolamine synthesis through the Kennedy pathway (for a review, see reference 20). Finally, diacylglycerol formed from phosphatidic acid can be converted to triacylglycerols in a third acylation step. The enzyme catalyzing hydrolysis of phosphatidic acid, phosphatidate phosphatase, has also been detected in two subcellular compartments (18, 21). Mitochondrial membranes contain a 45-kDa form of the enzyme, whereas microsomes harbor the 45- and 104-kDa forms. The activities of these isoenzymes are differentially regulated by phosphorylation. Thus, transport routes linked to the degradation of phosphatidic acid to diacylglycerol on one hand and to its conversion to CDP-diacylglycerol on the other hand are the same.

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