Differential Partitioning of Lipids Metabolized by Separate Yeast Glycerol-3-phosphate Acyltransferases Reveals That Phospholipase D Generation of Phosphatidic Acid Mediates Sensitivity to Choline-containing Lysolipids and Drugs*

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Vanina Zaremberg and Christopher R. McMaster‡

From the Atlantic Research Centre, Departments of Pediatrics and Biochemistry and Molecular Biology, IWK Health Centre, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

In this study we demonstrate that the GAT1 and GAT2 genes encode the major glycerol-3-phosphate acyltransferase activities in Saccharomyces cerevisiae. Genetic inactivation of either GAT1 or GAT2 did not alter cell growth but inactivation of both resulted in growth cessation. Metabolic analyses of gat1 and gat2 yeast detected that the major differences were: (i) a 50% increase in the rate of triacylglycerol synthesis in gat1 yeast and a corresponding 50% decrease in gat2 yeast, and (ii) a 5-fold increase in glycerophosphocholine production through deacylation of phosphatidylcholine synthesized through the CDP-choline pathway in gat1 yeast, whereas gat2 yeast displayed a 10-fold decrease. To address why we observed alterations in phospholipid turnover specific to phosphatidylcholine produced through the CDP-choline pathway in gat1 and gat2 yeast we tested their sensitivity to various cytotoxic lysolipids and observed that gat2 cells were more sensitive to lysophosphatidylcholine, but not other lysolipids. To pursue the mechanism we analyzed their sensitivity to choline-containing lysolipids or drugs that could not be deacylated and/or reacylated. Our data showed that gat1 and gat2 yeast were resistant and sensitive to lysolecithin activating factor, platelet activating factor, and the anti-tumor lipid edelfosine, respectively, indicating that their sensitivity to these compounds was not because of differences in rates of phosphatidylcholine deacylation. As growth of gat2 cells was impaired in the presence of ethanol, a phospholipase D (Spo14p) inhibitor, we inferred that phospholipase D may play important biologic and metabolic roles in phenotypes observed in gat yeast. Genetic inactivation of the SPO14 gene resulted in increased susceptibility, whereas expression of Escherichia coli diacylglycerol kinase relieved growth inhibition, to choline-containing lysolipids and drugs. Our results are consistent with a model whereby phosphatidic acid generated from phosphatidylcholine hydrolysis by Spo14p regulates susceptibility to choline-containing lysolipid analogs and drugs.

Glycerophospholipids are found in cell membranes and are generally comprised of two fatty acid molecules attached to the first two carbons of the glycerol backbone with a phospho-head group attached to the third carbon. Glycerophospholipids provide the physical permeability barrier for cellular and organelar membranes and also serve as a reservoir for numerous second messenger signaling molecules including arachidonic acid, phosphatidic acid (PA), 1 diacylglycerol (DAG), and inositol polyphosphates (1–4). Neutral glycerolipids have only fatty acid molecules esterified to the glycerol backbone with occupancy of all three positions forming triacylglycerol (TAG) (5, 6). TAG is found in lipid droplets within the cytoplasm from which fatty acids are released to serve primarily as a source of cellular energy through fatty acid oxidation (7).

Glycerolipid synthesis is initiated by glycerol-3-phosphate (Gly-3-P) acyltransferase through the transfer of a fatty acid from fatty acyl-CoA to the sn-1 position of Gly-3-P to form lysophosphatidic acid (8–10). Lyso-phosphatidic acid can also be formed from the acylation of dihydroxyacetone phosphate and subsequent reduction of the product (11, 12). Lyso-phosphatidic acid is further fatty acylated by lysophosphatidic acid acyltransferase to form PA. PA can either be: (i) converted to DAG for subsequent incorporation into phosphatidylcholine (PC), phosphatidylethanolamine, or TAG (13–19), or (ii) metabolized to CDP-diacylglycerol for the synthesis of phosphatidylinositol in all eukaryotes (20, 21), and phosphatidylinerine in yeast (22, 23).

In mammalian cells, two isoforms of Gly-3-P acyltransferase have been identified and are localized to either the mitochondrial or microsomal subcellular compartments (24–27). The mammalian gene corresponding to the mitochondrial isoform has been isolated and was used to generate Chinese hamster ovary cells overexpressing the enzyme. Increased expression of mitochondrial Gly-3-P acyltransferase increased TAG synthesis to 470% and decreased PC synthesis to 70% of controls (27). This study concluded that mitochondrial Gly-3-P acyltransferase synthesized downstream lipids required for TAG synthesis and this pool was relatively separate from those that contribute to the synthesis of PC. A gene/eDNA coding for a mammalian microsomal Gly-3-P enzyme has yet to be isolated.

The recent identification of two Gly-3-P acyltransferases genes, GAT1 and GAT2, from the yeast Saccharomyces cerevisiae was reported and interestingly neither had a high degree of amino acid similarity to the mammalian mitochondrial Gly-

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‡ To whom correspondence should be addressed. Tel.: 902-494-7066; Fax: 902-494-1394; E-mail: cmcmaste@is.dal.ca.
3-P acyltransferase. In vitro substrate specificities were determined and the GAT1 gene product could use both Gly-3-P and dihydroxyacetone phosphate with similar efficiencies and had a broad fatty acyl-CoA specificity profile, whereas the GAT2 gene product preferred Gly-3-P over dihydroxyacetone phosphate and had a marked preference for 16-carbon fatty acyl chains (28). The precise in vivo metabolic and biological roles of the Gat1p and Gat2p enzymes are unknown. We provide evidence that yeast Gat1p and Gat2p represent the major Gly-3-P acyltransferases in yeast. The specific genetic inactivation of the GAT1 or GAT2 genes allowed for examination of the metabolic role of each Gly-3-P acyltransferase activity in the metabolism of specific glycerolipids in vivo. Differences in TAG synthesis, and PC synthesis through the CDP-choline pathway and its subsequent turnover were identified between wild type, gat1, and gat2 cells. The studies on PC turnover revealed a key role for PA produced from the hydrolysis of PC by phospholipase D in the modulation of susceptibility to choline-containing lysolipid analogues and drugs.

**EXPERIMENTAL PROCEDURES**

**Materials—**Radiolabeled [methyl-1-14C]choline was purchased from American Radiolabeled Chemicals, and [1-14C]acetate was from PerkinElmer Life Sciences. Lipids were purchased from Avanti Polar Lipids. Edelfosine was the kind gift of Medmark Pharma GmbH. Silica gel thin layer chromatography plates were purchased from Whatman.

**Plasmids and Yeast Strain Construction—**Standard molecular biology methods, yeast genetic techniques, and transformation methods were used (29, 30). Yeast complex medium supplemented to a final concentration of 2% glucose (w/v) (YPD) or galactose (w/v) (YPGal) and synthetic minimal medium using 2% glucose (w/v) (SD), 2% galactose (w/v) (SGal), 3% ethanol (SEtOH), or 3% glycerol (SGly) as carbon source supplemented as required for plasmid maintenance are described (29). Yeast strains W303-1a (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100) and W303-1A (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100) were the parental strains from which gat1 and gat2 yeast were derived. Standard yeast one-step gene disruption cassettes were constructed that replaced the entire open reading frame of GAT1 or GAT2 and were transformed into yeast to generate strains CMY201 (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat2::HIS3), CMY202 (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat2::HIS3), CMY205 (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1::HIS3), and CMY204 (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1::HIS3). A plasmid containing the GAT1 open reading frame fused to the coding region for the V5 epitope tag at its 3′ end and under control of the GAL1 promoter with URA3 as selectable marker (purchased from Invitrogen) was transformed into CMY201 strains. Ura+ transformants were mated and diploid strain bearing the plasmid was selected, sporulated, and meiotic progeny were isolated on YPGal plates to generate strain CMY228 (ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 gat1::HIS3 [pGAL1::GAT1 URA3]). Strain BY5102 (ura3 his3 leu2 met15 spo14 KanMX) was purchased from ResGen. BY5102 was mated to CMY201 and haploid progeny were generated to create strains CMY240-3A (ura3 his3 leu2 trp1 gat2::HIS3 spo14 KanMX) and CMY240-3D (ura3 his3 leu2 trp1 spo14::KanMX). Strain CMY240-3D was transformed with the gat1::TRP1 disruption cassette to generate strain CMY241 (ura3 his3 leu2 trp1 spo14::KanMX gat1::TRP1). Gene disruption events were confirmed through Northern PCR.

**The Escherichia coli DAG kinase open reading frame (dgkA) was amplified by PCR using Platinum Hi Fidelity Taq Polymerase (Invitrogen) from the DH5α E. coli strain, TA cloned into the TOPO-pcRII vector, and subcloned into pAH9 (a derivative of p416-GPD that expresses open reading frames using the constitutive glycerol-3-P dehydrogenase promoter) but adds the coding region for the 10-amino acid T7 epitope tag to the 5′ end of the subcloned open reading frame.**

**Plasmids pKK235 (spo14), pME9106 (spo14::Kan), and pME419 (spo14::Kan) were previously described (31).**

**Metabolic Labeling—**Yeast cells growing in mid-log phase were labeled with [14C]choline (10 μM, 1 × 10⁶ dpm/mmol) or [14C]acetate (30 μM, 5 × 10⁷ dpm/mmol) for the indicated time points. Subsequent to incubation with radiolabel, cells were concentrated by centrifugation, washed twice with water, and resuspended in 1 ml of CHCl₃/CH₃OH (1/1, v/v). Cells were disrupted for 1 min at 4°C using a BioSpec Multi-Med Bead Containing 0.5 g of 0.5 mm acid-washed glass beads. The beads were washed with 1.5 ml of CHCl₃/CH₃OH (2/1, v/v) and 1.5 ml of water and 0.5 ml of CHCl₃ were added to the combined supernatant to facilitate phase separation. Phospholipids in the organic phase were routinely analyzed by thin layer chromatography on Whatman Silica Gel 60A plates using the solvent system: petroleum ether/ether/acetic acid (80/20/1, v/v/v). Some plates were sprayed with ENHANCE (PerkinElmer Life Sciences) and exposed to x-ray film, whereas others had their radioactivity located and analyzed using a BioScan radiolabel imaging scanner and the corresponding bands were scraped into vials for scintillation counting. Metabolite identity was based on the mobility of known standards.

**Plate Growth Assays—**Yeast were grown to mid-log phase in minimal medium supplemented as required to maintain cell growth. Total yeast cellular membranes were prepared as described (32). Gly-3-P acyltransferase and lysophosphatidic acid acyltransferase were assayed in membrane fractions as described (33). DAG kinase activity was measured by the method of Friess et al. (34). For Western Blots, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with a 5V monoclonal antibodies coupled directly to horseradish peroxidase, or a T7 monoclonal antibody followed by incubation with a goat anti-mouse secondary antibody coupled to horseradish peroxidase. Proteins were detected using the enhanced chemiluminescence method as described by the manufacturer (Amersham Biosciences).
Lipid and Protein Mass—Lipid phosphorus was determined as described by Ames and Dubin (35), and protein mass using the Lowry protocol (36).

RESULTS

Construction of a Δgat1 Δgat2 Double Knockout Strain—

GAT1 (YKR067w) and GAT2 (YBL011w) genes share 54% similarity and 37% identity over the entire protein sequence including the presence of a putative acyltransferase motif. Other translated open reading frames in the yeast genome contain the same putative acyltransferase motif but lack similarity to Gat1p and Gat2p outside of this motif region. Inactivation of either the GAT1 or GAT2 gene in yeast did not affect cell viability. Mating of gat1::TRP1 (gat1) and gat2::HIS3 (gat2) mutants and characterization of meiotic progeny by tetrad analysis did not result in the recovery of any progeny that were simultaneously lacking both GAT1 and GAT2 genes indicating that loss of function of GAT1 and GAT2 is synthetically lethal (data not shown and Ref. 28). Synthetic lethality does not necessarily mean that Gat1p and Gat2p are the only Gly-3-P acyltransferases present in S. cerevisiae as there are several other yeast proteins that contain the acyltransferase motif.

To address the combined role of Gat1p and Gat2p to total yeast Gly-3-P acyltransferase activity we designed an inducible expression system whereby a yeast strain with genetically inactivated GAT1 and GAT2 genes was maintained by using the GAL1 promoter resulting in cessation of cell growth after approximately three to four cell divisions. The level of Gat1p after a shift to glucose was monitored by Western blot (Fig. 1) and indicated that Gat1p rapidly declined to very low levels after 3 h in glucose and was no longer detected at 12 h.

Effect of Gat1p and Gat2p Depletion on Lipid Synthesis—

Lipid analysis of the gat1::TRP1 gat2::HIS3 double knockout strain harboring the galactose-inducible GAT1 construct was assessed by labeling with [14C]acetate. Radiolabeled acetate is incorporated into de novo synthesized fatty acids for subsequent lipid acylation. Yeast were grown in galactose (which expressed Gat1p), or shifted to glucose for various time points, and were then labeled with [14C]acetate for 1 h. In cells grown in galactose, radioactivity was associated equally between phospholipids and glycerolipids with each incorporating 50% of the total radiolabel (Figs. 2 and 3). Within the phospholipid fraction 37% of the label was associated with PC, 33% with phosphatidylethanolamine, 28% with phosphatidylinositol/phosphatidylserine, and 2% with PA (Fig. 2). In the neutral lipid fraction 47% of the label was in steryl esters, 21% in TAG, 19% in DAG, and 13% in fatty acid (Fig. 3). After the shift to glucose medium radiolabeled acetate incorporation gradually shifted from the acylation of glycerolipids to sterol acylation. This indicates that fatty acids are made but cannot be incorporated into glycerolipids and are instead being stored as steryl esters. After 12 h of growth in glucose the cells took up 70% of the total radiolabeled acetate as compared with cells grown in galactose. Analysis of ratio of radiolabel indicated the label incorporated into phospholipids had decreased to 19% of the...
total with the neutral lipid fraction now containing 81% of the radiolabeled acetate. Within the phospholipid fraction 41% of the label was associated with PC, 33% with phosphatidylethanolamine, and 26% with phosphatidylserine. Thus, cells deficient in Gat1p and Gat2p showed a proportional decrease in the labeling of each of these major phospholipid classes (Fig. 3). Almost all of the label that was not incorporated into phospholipids upon inactivation of both Gat1p and Gat2p was now found in sterol esters as they now comprised 87% of the label found in the neutral lipid class, with 11% of the label in TAG, 2% in DAG, and 2% in fatty acid (Fig. 2). The large and generalized decrease in the ability to incorporate fatty acid into glycerolipids upon simultaneous inactivation of Gat1 and Gat2 is consistent with these two genes coding for the main yeast Gly-3-P acyltransferases.

To further support that Gat1p and Gat2p represent the major yeast Gly-3-P acyltransferase enzymes, the in vitro enzyme activity of Gly-3-P acyltransferase and the downstream enzyme lysophosphatidic acid acyltransferase were measured in the gat1::TRP1 gat2::HIS3 double knockout yeast harboring the galactose-inducible GAT1 construct after a shift to glucose containing medium. After 12 h in glucose the Gly-3-P acyltransferase activity was reduced to negligible levels while lysophosphatidic acid acyltransferase activity was essentially unaffected (Table I). Our above metabolic and enzymatic data, coupled with the observed synthetic lethality upon simultaneous inactivation of GAT1 and GAT2, indicate that these two genes code for the principal Gly-3-P acyltransferases in S. cerevisiae.

Partitioning of Glycerolipid Biosynthesis by GAT1 and GAT2—Although simultaneous inactivation of GAT1 and GAT2 prevented cell growth, inactivation of the GAT1 or GAT2 genes separately did not affect the rate of cell growth on rich or minimal yeast medium. The identical growth rates to wild type yeast allowed for the determination of the role of each Gly-3-P acyltransferases in the synthesis of specific glycerolipids through metabolic labeling analyses.

We initially monitored flux through the CDP-choline pathway for synthesis of PC because increased GAT2 expression was previously shown to restore growth to a yeast strain that contained a mutated choline transporter that possessed an increased \( K_m \) for choline and relied exclusively on the CDP-choline pathway for PC synthesis (37). In this study, GAT2 (referred to in the previous study as SCT1 for suppressor of choline transport) was thought to directly alter the rate of uptake of choline by the choline transporter. However, if increased GAT2 allowed for higher flux through the CDP-choline pathway by an indirect route this could also allow for restoration of cell growth to this yeast strain. We determined the rate of metabolism of radiolabeled choline into PC and its subsequent turnover into glycerophosphocholine in gat1 and gat2 yeast. As shown in Fig. 4, gat1 cells produced less radioactive phosphocholine and much higher levels of radioactive glycerophosphocholine than the wild type strain. Conversely, glycerophosphocholine labeling was almost undetectable in gat2 cells, whereas there was an increase in the radioactivity incorporated into phosphocholine. As glycerophosphocholine is an end product of PC deacylation these results indicate that flux through the CDP-choline pathway for subsequent PC deacylation is diminished in the gat2 mutant and is augmented in gat1 cells.

The DAG produced downstream of the Gly-3-P acyltransferases could also be used for the synthesis of the neutral lipid TAG. The gat1 and gat2 yeast were radiolabeled with acetate and its incorporation into de novo synthesized fatty acid for subsequent esterification into neutral glycerolipids was determined. Acetate labeling of DAG was slightly reduced in gat2 yeast and this was reflected by a 50% decrease in labeling of TAG (Fig. 5). In gat1 yeast, DAG labeling was essentially unchanged, whereas TAG labeling was increased by 50%.

The metabolism of radiolabeled ethanolamine, serine, and inositol into lipids was unchanged in gat1 and gat2 cells compared with wild type cells (data not shown). Consistent with the inositol metabolic labeling experiments was the observation that gat1 and gat2 yeast were not inositol auxotrophs.

**Table I**

| Activity relative to wild type yeast grown on glucose |
|-----------------------------------------------------|
| Glycerol-3-phosphate acyltransferase activity | Lyso phosphatidic acid acyltransferase activity |
| Wild type grown on glucose | 100⁺ | 100 |
| gat1::TRP1 gat2::HIS3 (GAL-GAT1) grown on galactose | 152 | 135 |
| gat1::TRP1 gat2::HIS3 (GAL-GAT1) grown on glucose for 12 h | 2 | 115 |

⁺ Wild type enzyme activities were 2.0 and 21.2 nmol min⁻¹mg⁻¹ for glycerol-3-phosphate and lysophosphatidic acid acyltransferases, respectively.
indicating that their ability to synthesize inositol and convert it into phosphatidylinositol was unperturbed.

Lysolipid and Edelfosine Sensitivity of gat1 and gat2 Mutants—In yeast the major PC deacylating phospholipase genes thus far identified are encoded by the PLB1–3 genes (38–40), although a fourth PC deacylating activity whose gene has yet to be identified is known to exist (42). An increase in the activity of PC/lyso-PC hydrolyzing phospholipase B (Plb2p) has been demonstrated to protect cells versus the cytostatic effects of exogenously added lyso-PC, and ablation of the PLB2 gene resulted in increased lyso-PC susceptibility (38, 39). These effects are likely through altering the rate of hydrolysis of the exogenously added lysolipid. As inactivation of GAT1 caused cells to undergo increased PC deacylation, whereas inactivation of GAT2 decreased PC deacylation we tested if there were differential sensitivities of the gat1 and gat2 strains to lyso-PC and other lysophospholipids. Of the lysolipids tested, the wild type strain was sensitive to lyso-PC, lysophosphatidylethanolamine, and lysophosphatidylserine. The gat1 and gat2 yeast were also sensitive to each of these lipids, however, gat2 cells displayed a sensitivity to lyso-PC that was significantly increased compared with wild type and gat1 cells (Table II). This difference displayed by gat2 cells was consistent with their increased PC/lyso-PC deacylation rate observed during our

Fig. 4. Phosphatidylincholine metabolism in yeast lacking Gat1p or Gat2p. Isogenic yeast with the indicated genotypes were grown to mid-log phase in minimal glucose medium containing the required supplements for cell growth. Cells were labeled with [14C]choline for the indicated time points. Lipids were extracted and the aqueous phase metabolites and lipid metabolites were separated by thin layer chromatography, located using a radiolabel imaging scanner, and scraped into scintillation vials for radioactivity determinations. There was no radiolabel associated with lysophosphatidylincholine and thus it is not included in the figure. The results are the mean of at least three separate experiments performed in duplicate. Standard errors are less than 15% of the mean for each point. Only the alterations in phosphocholine and glycerophosphocholine labeling were statistically significant (p < 0.05).
metabolic analysis. To test if PC deacylation was the means by which \textit{gat2} yeast were more sensitive to lyso-PC the sensitivity of wild type, \textit{gat1}, and \textit{gat2} strains to lyso-PC analogues containing nonhydrolyzable ether-linked fatty acids was assessed. The lyso-PC analogue lyso-platelet activating factor (lyso-PAF) is effectively taken up and reacylated to PC in yeast, however, the presence of an ether linkage at the \textit{sn}-1 position prevents lyso-PAF hydrolysis (41). If the sensitivity of \textit{gat2} yeast to lyso-PC was through a defect in lyso-PC deacylation then \textit{gat2} cells should have a similar sensitivity to lyso-PAF as wild type yeast. We observed that \textit{gat2} cells were more sensitive to lyso-PAF than wild type yeast, and surprisingly \textit{gat1} cells were more resistant (Fig. 6), indicating that their response to choline-containing lysolipids could not be explained through differences in lysolipid deacylation rates. To further test if the differences in sensitivity of \textit{gat1} and \textit{gat2} cells was because of altered lysolipid deacylation, and to test for the role of reacylation in lysolipid sensitivity, we determined their sensitivity to PAF and the anticancer drug edelfosine. Like lyso-PAF, PAF contains an ether-linked long chain fatty acid at the \textit{sn}-1 position, but also possesses an ester-linked methyl group at its \textit{sn}-2 position. Edelfosine is identical in structure to PAF except that both linkages are nonmetabolizable ether bonds. We found that \textit{gat2} cells were more susceptible than wild type yeast to both of these compounds while \textit{gat1} cells were more resistant (Fig. 6). These results indicate that the differences regarding sensitivity to choline-containing ether lipids in \textit{gat1} and \textit{gat2} cells are not because of the observed differences in the rate of deacylation or reacylation displayed when these genes are inactivated.

**Spo14p Activity Is Required to Mitigate Sensitivity to Choline-containing Lysolipids and Drugs**—A tantalizing hint as to the pathway involved in the sensitivity to choline-containing lysolipids and drugs came from our observation that \textit{gat2} cells are sensitive to growth on ethanol (Fig. 7A) but not other nonfermentable carbon sources. Ethanol serves as an alternate substrate to water in the hydrolysis of PC by the sole PC hydrolyzing phospholipase D in yeast (encoded by the \textit{SPO14} gene) resulting in the production of phosphatidylethanol instead of PA. Thus, we tested the role of phospholipase D in mediating sensitivity to choline-containing lysolipids. A yeast strain containing a genetically inactivated \textit{SPO14} gene showed increased sensitivity to PAF compared with its wild type counterpart (Fig. 7B). Similar results were observed with regard to

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**TABLE II**

| Medium composition | Wild type | \textit{gat1} | \textit{gat2} |
|--------------------|-----------|--------------|--------------|
| No lipid          | ++        | ++           | ++           |
| 40 \(\mu\text{g/ml}\) Lyso-PC | +         | +            | -            |
| 80 \(\mu\text{g/ml}\) Lyso-PC | -         | -            | -            |
| 40 \(\mu\text{g/ml}\) Lyso-phosphatidylethanolamine | +        | +            | -            |
| 80 \(\mu\text{g/ml}\) Lyso-phosphatidylethanolamine | -        | -            | -            |
| 40 \(\mu\text{g/ml}\) Lyso-phosphatidylserine | +        | +            | +            |
| 80 \(\mu\text{g/ml}\) Lyso-phosphatidylserine | -        | -            | -            |
| 40 \(\mu\text{g/ml}\) Lyso-phosphatidylinositol | ++       | ++           | ++           |
| 80 \(\mu\text{g/ml}\) Lyso-phosphatidylinositol | ++       | ++           | ++           |

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\*FIG. 5. Neutral lipid synthesis in yeast lacking \textit{Gat1p} or \textit{Gat2p}.\* Isogenic yeast with the indicated genotypes were grown to mid-log phase in minimal glucose medium containing the required supplements for cell growth. Cells were labeled with [\(^{14}\text{C}\)]acetate for 1 h. Lipids were extracted and separated by thin layer chromatography, located using a radiolabel imaging scanner, and scraped into scintillation vials for radioactivity determinations. The results are the mean of at least three separate experiments performed in duplicate. Standard errors are less then 15% of the mean for each point. Only the differences in \(^{14}\text{C}\) labeling were statistically significant (\(p < 0.05\)).
sensitivity to lyso-PAF and edelfosine (data not shown). This sensitivity was rescued by the wild type Spo14p protein, but not by a catalytically inactive point mutant (Spo14pK3H) or a mutant protein lacking 150 amino acids in its NH2 terminus (Spo14pΔN) that is enzymatically active but mislocalized (Fig. 7) (31). Together, the data implicate a requirement of a properly localized and functional Spo14p activity in attenuation of sensitivity to choline-containing lysolipid analogues and drugs.

To determine the contribution of PC turnover through Spo14p to the sensitivity of yeast to choline-containing lysolipids in the gat mutants, we genetically inactivated SPO14 in gat1 and gat2 yeast. Disruption of the phospholipase D encoding gene did not affect growth rates for any of the strains on standard complete minimal or rich medium. Similar to wild type cells, inactivation of SPO14 resulted in increased susceptibility of both gat1 and gat2 cells to choline-containing lysolipids (Fig. 7C). This data indicates that the sensitivity of yeast to edelfosine, lyso-PAF, and PAF is dependent on PC turnover through Spo14p.

Increased PA Production Improves Cell Growth in the Presence of Choline-containing Lysolipid Analogues and Drugs—The Spo14p contribution to the prevention of the susceptibility of yeast to choline-containing lysolipids could be because of these lipids being substrates of the enzyme, and/or because of a lipid product of Spo14p-mediated PC breakdown. Spo14p hydrolysis of PC produces PA that can be dephosphorylated to produce DAG. To discern between each of these possibilities the E. coli DAG kinase (an enzyme that converts DAG to PA) was ex-

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**Fig. 6. Choline-containing lysolipid sensitivity in yeast lacking Gat1p or Gat2p.** Isogenic yeast with the indicated genotypes were grown to mid-log phase in minimal or rich glucose containing medium. Identical numbers of cells were serial diluted 1:10 (starting with A600 = 1) and plated on medium containing the indicated lipids. Plates were incubated for 2–7 days at 30 °C.
pressed under control of the moderate constitutively active glycerol-3-P dehydrogenase promoter in wild type, \textit{gat1}, and \textit{gat2} cells. Ectopic expression of \textit{E. coli} DAG kinase resulted in 58 nmol min$^{-1}$/H$^1$1000 g$^{-1}$/H$^1$1000 protein of DAG kinase activity, whereas no activity was detected in control cell extracts. DAG kinase expression improved growth of all the strains in the presence of the lipids tested (while maintaining the differences in susceptibility previously detected between them) (Fig. 8). This indicates that an increase in the metabolism of DAG to PA alleviates choline-containing lysolipid cytotoxicity.

**DISCUSSION**

\textit{S. cerevisiae} Gat1p and Gat2p were recently found to possess Gly-3-P and dihydroxyacetone phosphate acyltransferase activities (28). Based on the synthetic lethal phenotype displayed by \textit{GAT1} and \textit{GAT2} genes it was speculated that they may represent the sole Gly-3-P acyltransferases in yeast (28). Because synthetic lethality is not sufficient evidence to make a firm conclusion we created an inducible system that would allow us to test this hypothesis. The data presented in this study establish that Gat1p and Gat2p correspond to the principal Gly-3-P acyltransferase activities in yeast with two new lines of evidence pointing to this conclusion. First, construction of a yeast strain with inactivated \textit{GAT1} and \textit{GAT2} genes but with a titratable promoter driving \textit{GAT1} expression revealed that in vitro Gly-3-P acyltransferase levels dropped to 2% in the wild type levels (whereas lysophosphatidic acid acyltransferase levels remained unchanged) upon inactivation of \textit{GAT1}. Second, this same yeast strain radiolabeled with acetate resulted in essentially the entire de novo fatty acid pool being incorporated into steryl esters instead of glycerolipids. The small incorporation of label into glycerolipids in combination with the remaining detectable Gly-3-P acyltransferase activity in vitro in this strain upon inactivation of \textit{GAT1} prevent us to conclude that Gat1p and Gat2p represent the entire complement of Gly-3-P acyltransferase in yeast. This residual Gly-3-P acyltransferase activity is not sufficient for life and could be because of: (i) leaky expression of Gat1p on glucose media that could not be detected by Western blot but is apparent in more sensitive assays like measurement of in vitro enzyme activity or [$^{14}$C]acetate metabolic labeling, or (ii) a third Gly-3-P acyl-

**FIG. 7.** Protective role of Spo14p in choline-containing lysolipid sensitivity in wild type, \textit{gat1}, and \textit{gat2} yeast. Isogenic wild type, \textit{gat1}, and \textit{gat2} yeast (A), BY5102 (spo14) (B), BY5102 and the derived CMY240-3A (gat2 spo14) (C), CMY240-3D (spo14) and the isogenic CMY241 (gat1 spo14) (D). Yeast were grown to mid-log phase in minimal glucose containing medium supplemented with the appropriate amino acids as required for plasmid maintenance (B–D). An identical number of cells were serial diluted 1:10 (starting with A$^600$ = 1) and plated on glucose, ethanol, or glycerol containing media (A), or medium containing the indicated concentrations of PAF (B–D). Plates were incubated for 2 (A–C) or 3 days (D) at 30°C. Similar trends were observed if lyso-PAF or edelfosine were included in the medium (data not shown).
transferase is present in yeast. Although there are no obvious Gat1p or Gat2p homologues in the yeast genome, there are several other yeast open reading frames that contain the acyltransferase motif and any of these could potentially encode for another Gly-3-P acyltransferase isoform.

Because yeast with genetically inactivated GAT1 or GAT2 genes grew at rates similar to wild type yeast we were able to directly compare the contribution of each protein toward the synthesis of specific glycerolipids. Inactivation of GAT1 increased TAG synthesis by 50% while TAG synthesis was decreased by 50% in yeast lacking GAT2. Thus, acylation through Gat2p is the major route for the downstream synthesis of TAG.

The only other lipid metabolic alteration detected in our analysis of wild type, gat1, and gat2 cells was in the synthesis/turndown of PC. Inactivation of GAT1 resulted in a 5-fold increase in glycerophosphocholine production through deacylation of PC synthesized through the CDP-choline pathway, whereas in cells containing an inactivated GAT2 there was a 10-fold decrease. Thus, PC synthesized through the CDP-choline pathway is preferentially deacylated when the PC is made from DAG acylated through Gat2p, whereas PC is not deacylated if it is made through Gat1p-acylated DAG. The alteration in the production of glycerophosphocholine in gat1 and gat2 yeast presumably occurs through alterations in PC deacylation via the action of phospholipase B or phospholipase A in conjunction with a lysophospholipase. Three phospholipase B containing genes have been identified in S. cerevisiae, Pbl1p, Pbl2p, and Pbl3p, with Pbl1p and Pbl2p catalyzing the bulk of the PC hydrolyzing activity (38–40). The Pbl proteins reside in the plasma membrane or the periplasmic space and PC turnover by these enzymes releases glycerophosphocholine into the medium, however, all of the glycerophosphocholine recovered in gat1 and gat2 yeast was intracellular. Additional PC hydrolyzing lipase(s) exist in yeast as it was observed that either heat stress or the addition of exogenous choline increased PC synthesis through the CDP-choline pathway and the PC formed was rapidly deacylated resulting in an increase in intracellular glycerophosphocholine. This CDP-choline pathway-specific hydrolysis of PC was shown to be independent of the PLB-encoded lipases (42). The alterations in CDP-choline pathway-derived PC deacylation observed in the gat1 and gat2 yeast are likely via this as yet unidentified lipase.

Eukaryotic cells, including yeast, are susceptible to cell growth arrest or death upon treatment with lysophospholipids. We observed that growth of gat2 yeast was impaired by lyso-PC (but not other lysolipids), whereas gat1 cell sensitivity was comparable with wild type yeast. As the rate of PC deacylation to lyso-PC and then glycerophosphocholine was gat1 GAT2 > wild type > GAT1 gat2 the rate of PC deacylation observed metabolically correlated with the lyso-PC susceptibility of gat2. To investigate the role of PC lyso-PC deacylation/reacylation in the sensitivity of yeast to lyso-PC we tested their sensitivity to lyso-PC analogues where nonhydrolyzable ether linkages replaced the ester bond that normally links fatty acid chains to the glycerol backbone. The gat1 and gat2 yeast were more resistant and susceptible, respectively, to lyso-PAF (which is identical to lyso-PC other than the ester bond is replaced with an unhydrolyzable ether bond) than wild type cells, implying that alterations in lysolipid deacylation may not be responsible for the differences in susceptibility of gat1 and gat2 yeast to choline-containing lysolipids. Because lyso-PAF can be effectively reacylated in yeast (41) we also tested the susceptibility of gat1 and gat2 yeast versus PAF and the anti-tumor lipid edelfosine. PAF is identical in structure to lyso-PAF but contains an ester-linked methyl group at the sn-2 position of the glycerol backbone, whereas edelfosine is identical in structure to PAF except the sn-2 methyl group is linked through a nonhydrolyzable ether bond and thus edelfosine can be neither deacylated nor reacylated. Like lyso-PAF, the gat1 and gat2 yeast were more resistant and susceptible to PAF and edelfosine, respectively, than wild type yeast. Thus, metabolism of choline-containing lysolipids and drugs through deacylation/reacylation is not the mechanism responsible for the differences in sensitivity displayed by gat1 and gat2 to these compounds.

During an exhaustive phenotypic characterization of gat1 and gat2 yeast cell growth on various media we observed that growth of gat2 cells on synthetic minimal medium containing ethanol as the sole carbon source was impaired while no differences in growth were detected on plates containing other nonfermentable carbon sources. As ethanol is a well known inhibitor of phospholipase D activity in vivo and in vitro this observation suggested a potential link between phospholipase D and the GAT genes. We demonstrated herein that wild type cells with a deleted SPO14 gene (encodes the sole yeast PC hydrolyzing phospholipase D) are more susceptible to choline-containing lysolipids and this growth impairment could only be rescued by the wild type protein and not by an inactive point mutant (Spo14pK563H) or a truncated protein (Spo14p3N) that is enzymatically active but mislocalized (31). Inactivation of SPO14 in either gat1 or gat2 yeast did not alter their growth rate on complete minimal or rich medium, but did result in an increase in the susceptibility of both gat1 and gat2 strains to the growth inhibitory effects of choline-containing lysolipids.
and drugs. Thus, an enzymatically active and properly localized Spo14p is required to alleviate cytotoxicity toward choline-containing lysolipids and drugs.

Spo14p hydrolyzes PC to PA that modulates susceptibility to choline-containing lysolipids and drugs. Expression of dgkA relieved the cytotoxicity toward choline-containing lysolipids and drugs in all three strains, but maintained the differences in the severity of the phenotype between them (i.e. gat2 > wild type > gat1). Based on the collective data reported herein we propose that hydrolysis of PC by Spo14p produces a PA pool that modulates susceptibility to choline-containing lysolipids and drugs.

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Vanina Zaremberg and Christopher R. McMaster

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