Identification of a novel GPCAT activity and a new pathway for phosphatidylcholine biosynthesis in *S. cerevisiae*

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Abstract **Turnover of phospholipids in the yeast *Saccharomyces cerevisiae* generates intracellular glycerophosphocholine (GPC). Here we show that GPC can be reacylated in an acyl-CoA-dependent reaction by yeast microsomal membranes. The lysophosphatidylcholine that is formed in this reaction is efficiently further acylated to phosphatidylcholine (PC) by yeast microsomes, thus providing a new pathway for PC biosynthesis that can either recycle endogenously generated GPC or utilize externally provided GPC. Genetic and biochemical evidence suggests that this new enzymatic activity, which we call GPC acyltransferase (GPCAT), is not mediated by any of the previously known acyltransferases in yeast. The GPCAT activity has an apparent V_{max} of 8.7 nmol/min/mg protein and an apparent K_m of 2.5 mM. It has a neutral pH optimum, similar to yeast glycerol-3-phosphate acyltransferase, but differs from the latter in being more heat stable. The GPCAT activity is sensitive to *N*-ethylmaleimide, phenanthroline, and Zn^{2+} ions. In vivo experiments showed that PC is efficiently labeled when yeast cells are fed with [^{3}H]choline-GPC, and that this reaction occurs also in *pct1* knockout strains, where de novo synthesis of PC by the CDP-choline pathway is blocked. This suggests that GPCAT can provide an alternative pathway for PC biosynthesis in vivo.**—Stålberg, K., A. C. Neal, H. Ronne, and U. Ståhl. **Identification of a novel GPCAT activity and a new pathway for phosphatidylcholine biosynthesis in *S. cerevisiae*. *J. Lipid Res.* 2008.** 49: **1794–1806.**

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Cell membranes contain a carefully balanced mixture of different phospholipid classes, each with a certain acyl composition. This composition is important for the physical properties of the membrane, such as its fluidity, which in turn may alter with variations in the temperature and other environmental conditions (1–3). Cells therefore need to be able to adapt to such changes by altering the composition of their membranes. This is achieved by remodeling, in which existing phospholipids are deacylated and reacylated in a process known as the Land's cycle (4). The intermediate lysophospholipid is rapidly converted back to its corresponding phospholipid. Recently, an enzyme known as lysophospholipid acyltransferase (LPLAT) has been identified in yeast and *Arabidopsis*, as well as mammals, which can acylate all major lysophospholids 5–12.

Glycerophosphodiesters are produced by complete deacylation of phospholipids. One important glycerophosphodiester is glycerophosphocholine (GPC), which accumulates in yeast cells in response to elevated temperature (13) or osmotic stress (14). Complete deacylation of phosphatidylcholine (PC) to GPC is catalyzed by Nte1, a yeast esterase which is activated during high temperature stress (15, 16). Nte1 seems to act primarily on PC derived from the CDP-choline pathway (**Fig. 1**), because a knockout of the *PCT1* gene blocks the formation of GPC (13). GPC can also be excreted from the yeast cell, a process that is dependent on the three phospholipase B enzymes Plb1, Plb2, and Plb3 (17–19). Extracellular GPC can be taken up by the Git1 transporter and then used as a phosphate source (Fig. 1). This requires both Git1 and the Gde1 glyc-

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Abbreviations: DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; GPC, glycerophosphocholine; GPCAT, glycerophosphocholine acyltransferase; GPE, glycerophosphoethanolamine; GPG, glycerophosphoglycerol; GPI, glycerophosphoinositol; GPS, glycerophosphoserine; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPLAT, lysophospholipid acyltransferase; NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; WT, wild type.

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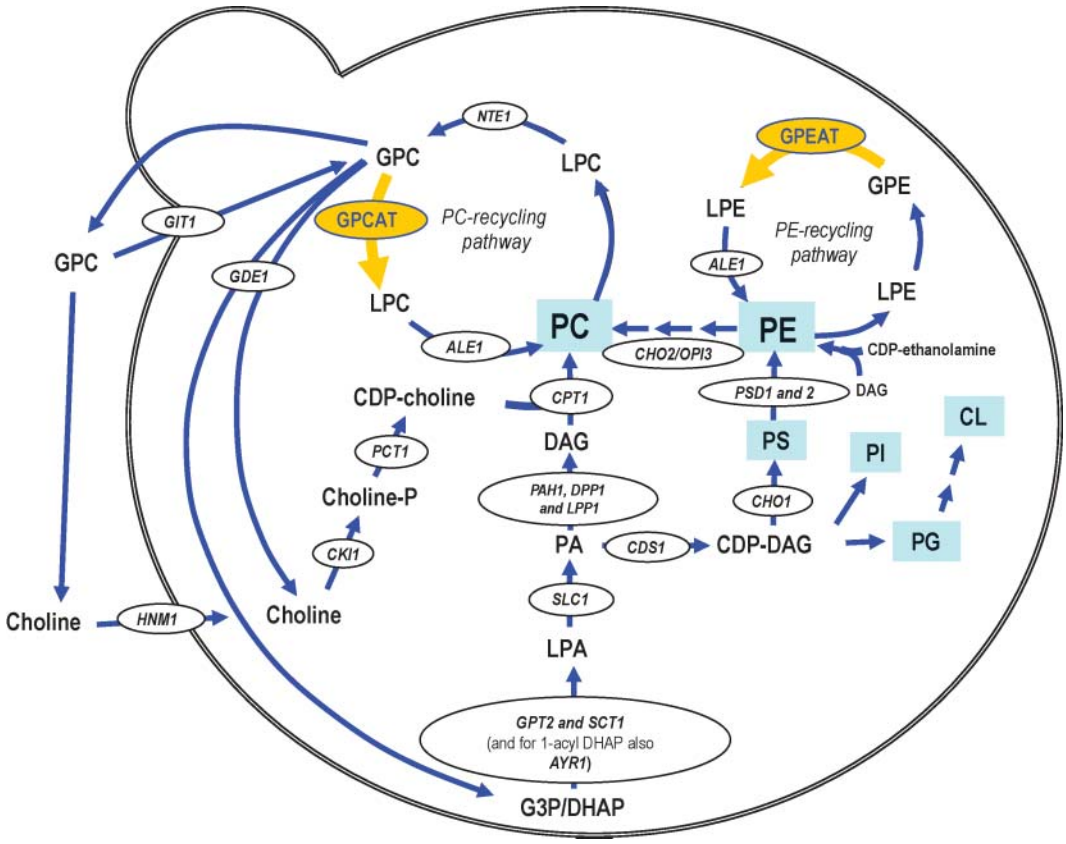

Fig. 1. Schematic outline of the major pathways for phospholipid biosynthesis in yeast, including the phosphatidylcholine/phosphatidylethanolamine (PC/PE) reacylation pathways and the glycerophosphocholine acyltransferase (GPCAT) and glycerophosphoethanolamine acyltransferase (GPEAT) activities described in the present paper. CL, cardiolipin; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

erophosphodiesterase, which cleaves GPC into glycerol-3-phosphate (G3P) and choline (20, 21). Intracellular reutilization of GPC as a choline source is also partially dependent on Gde1 (16). GPC has furthermore been proposed to function as an osmoregulator both in plants (22) and in human renal cells (23). Interestingly, GPC accumulates intracellularly in some brain disorders, such as Alzheimer's disease (24) and schizophrenia (25), which suggests that an increase in phospholipid turnover may occur under these conditions.

Early studies suggested that G3P is the main acyl acceptor for de novo lipid biosynthesis in eukaryotic cells (26). More recently, two yeast genes encoding a G3P acyltransferase (GPAT) were isolated and cloned, *GPT2* and *SCT1*, and both gene products were shown to be able to use both G3P and dihydroxyacetone phosphate (DHAP) as acyl group acceptors (27). The possibility that GPC could act as an acyl group acceptor was tested in early in vivo labeling experiments in rats, but these results were negative, and it was therefore assumed that eukaryotic cells lack the ability to acylate GPC (28). Instead, phospholipid remodeling was proposed to involve cleavage of GPC into G3P and choline, followed by de novo synthesis of PC from these sources (16, 21).

In the present paper we show, however, that GPC can be acylated by yeast microsomal membranes in an acyl-CoA dependent reaction to form lysophosphatidylcholine (LPC), which is then further efficiently acylated to form PC. We call this novel enzymatic activity GPC acyltransferase (GPCAT). Accordingly, a direct recycling pathway for PC exists in eukaryotes, which involves deacylation of PC followed by reacylation of the resulting GPC. The implications of this novel finding for phospholipid remodeling and membrane biology are discussed.

MATERIALS AND METHODS

Yeast strains, plasmids, and chemicals

All yeast (*Saccharomyces cerevisiae*) strains used (**Table 1**) were congenic to the BY4741 and BY4742 background from the Euroscarf collection (29). For generation of overexpression constructs, genomic DNA from wild-type (WT) yeast was used as a template to amplify the coding regions of the *GPT2* and *SCT1* genes, using a 2:1 mixture of *Taq* and *Pfu* DNA polymerases. The primers used for the amplification of *GPT2* were 5′-GGA TCC ATG TCT GCT CCC GCT GCC-3′ and 5′-GGA TCC TCA TTC TTT CTT TTC GTG TTC T-3′ and for *SCT1*, 5′-GGA TCC ATG CCT GCA CCA AAA CTC AC-3′ and 5′-GGA TCC CTA CGC ATC TCC

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
WT (BY4742)	MAT α his3D1 leu2D0 lys2D0 ura3D0	Euroscarf
gpt2	MAT α his3D1 leu2D0 lys2D0 ura3D0 YKR067w::kanMX4	Euroscarf
sct1	MAT α his3D1 leu2D0 lys2D0 ura3D0 YBL011w::kanMX4	Euroscarf
pct1	MAT α his3D1 leu2D0 lys2D0 ura3D0 YGR202c::kanMX4	Euroscarf
hnm1	MAT α his3D1 leu2D0 lys2D0 ura3D0 YGL077c::kanMX4	Euroscarf
gde1	MAT α his3D1 leu2D0 met15D0 ura3D0 YPL110c::kanMX4	Euroscarf
git1	MAT α his3D1 leu2D0 met15D0 ura3D0 YCR098c::kanMX4	Euroscarf
pct1 gde1	MAT α his3D1 leu2D0 lys2D0 ura3D0 YGR202c::kanMX4 YPL110c::kanMX4	This study
gpt2/pGAL1-GPT2	Strain *gpt2* transformed with the plasmid pGAL1-GPT2	This study
gpt2/pGAL1-SCT1	Strain *gpt2* transformed with the plasmid pGAL1-SCT1	This study
gpt2/pYES2	Strain *gpt2* transformed with the plasmid pYES2	This study

WT, wild type.

TTC TTT CC-3′. *Bam*HI sites were introduced at the ends of the resulting PCR products. The amplified DNA fragments were gel purified, incubated with *Taq* polymerase, and cloned into the pCR2.1 TOPO vector (Invitrogen, Abingdon, UK), generating the two plasmids pUS95 and pUS99. The two constructs were then excised from the cloning vector by *Bam*HI digestion and cloned into the *Bam*HI site behind the strong inducible *GAL1* promoter in the multi-copy plasmid pYES2 (Invitrogen), thus generating the plasmids pGAL1-GPT2 and pGAL1-SCT1. A *gpt2* deletion strain was transformed with the two overexpression plasmids and, as a control, with the empty vector, pYES2.

Unlabeled lipids were obtained from Sigma-Aldrich (Stockholm, Sweden) or from Larodan Fine Chemicals (Malmö, Sweden). 1,2-Dipalmitoyl-phosphatidyl[*N*-methyl-^{14}C]choline (62 mCi/mmol), 1,2-dioleoyl-phosphatidyl[2-^{14}C]ethanolamine (62 mCi/mmol), 1,2-dipalmitoyl-phosphatidyl[*N*-methyl-^{3}H]choline (85 mCi/mmol), L-[U-^{14}C]G3P (100 mCi/mmol), [1-^{14}C]palmitoyl-CoA, and [1-^{14}C]oleoyl-CoA were obtained from Amersham Radiochemicals (Piscataway, NJ).

Growth of yeast cells

WT and knockout yeast strains were inoculated from overnight precultures and grown by rotary shaking at 30°C in liquid YPD (1% yeast extract, 2% peptone, and 2% glucose) to stationary phase (OD_{600} 6-9) or as indicated in figure legends. The *GPT2* and *SCT1* overexpression strains were precultured overnight in synthetic media lacking uracil, supplemented with 2% glucose. Cells were pelleted, washed once, and then grown for 5 h in synthetic media lacking uracil, supplemented with 2% galactose.

Preparation of cell extracts

Yeast cells were harvested by centrifugation for 10 min at 1,000 *g*. The cell pellet was washed once with 0.15 M NaCl and resuspended in an equal volume of ice-cold breaking buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA). The cell slurry was mixed with 1 ml of 0.5 mm zirconium beads (BioSpec Products, Inc., Bartlesville, OK) in a 2.5 ml microcentrifuge tube and heavily shaken in a Mini Beadbeater (BioSpec Products, Inc.) three times for 1 min with 3 min cooling on ice in between. The broken cells were centrifuged at 1,000 *g* for 5 min at 4°C, and the supernatants, referred to as yeast extracts, were frozen in aliquots and stored at −70°C. Microsomal fractions were obtained by centrifugation of yeast extracts at 100,000 *g* for 1 h. The microsomal (crude membrane) pellet was resuspended in breaking buffer using a glass homogenizer, and frozen in aliquots and stored at −70°C. The protein concentration in the extracts was determined using the Bradford method (30) with BSA as standard.

Generation of glycerophosphodiesters

GPC, glycerophosphoethanolamine (GPE), glycerophosphoserine (GPS), glycerophosphoinositol (GPI), glycerophosphoglycerol (GPG), [^{3}H]choline-GPC, [^{14}C]choline-GPC, and [^{14}C]ethanolamine-GPE were synthesized by mild chemical hydrolysis of their corresponding phospholipids (31). Phospholipids dried under N_2 were dissolved in 0.1 M NaOH in methanol (dry) solution and incubated at 37°C for 2 h. The solution was then neutralized by the addition of 1 M acetic acid, and the generated glycerophosphodiesters were separated from fatty acid methylesters by butanol extraction. For this step, 3.75 vol water and 5 vol 1-butanol were added to the samples, followed by thorough end-over-end shaking and centrifugation at 14,000 *g* for 1 min. The bottom (water) phase was saved, and the butanol phase was reextracted with water, after which the two water phases were pooled. The combined water phase was evaporated on a warm sand bath under N_2 gas in order to reduce the volume and remove traces of 1-butanol. In the case of [^{14}C]choline-GPC synthesis, scintillation counting of the water and 1-butanol phases showed that the hydrolysis was almost complete, and TLC analysis of the water phase revealed no traces of [^{14}C]LPC or [^{14}C]PC.

GPAT and GPCAT assays

Acylation of GPC and G3P was assayed using a slightly modified version of the GPAT assay of Tillman and Bell (32). Unless otherwise stated, the assays contained either yeast extract (50 μg) or microsomes (20 μg), 25 mM Tris-HCL, pH 7.6, 2 mg/ml BSA (essentially fatty acid free), 1 mM DTT, 8 mM NaF, 2 mM $MgCl_2$, 2 mM of either [^{3}H]choline-GPC (6,000 dpm/nmol) or [^{14}C]G3P (2,000 dpm/nmol), and 0.1 mM 16:0-CoA, all in a total volume of 100 μl. The reactions were incubated at 30°C, for 10 min in the GPCAT assay and for 5 min in the GPAT assay. In assays where cold glycerophosphodiesters were added as acyl acceptors, we used 0.1 mM of [^{14}C]16:0- or [^{14}C]18:1-CoA (5,000 dpm/nmol) as acyl donors. The assays were stopped by the addition of 400 μl of methanol-chloroform-glacial acetic acid (50:50:1) (33). For phase separation, 80 μl H_2O was added and the tubes were thoroughly shaken and centrifuged for 1 min at 13,000 *g*. The bottom (chloroform) phase (about 160 μl) was removed and combined with a second chloroform phase obtained by reextraction of the upper methanol-water phase with chloroform. The combined chloroform phases were either dried under a stream of N_2 gas, dissolved in chloroform and spotted and analyzed by TLC, or else washed with a fresh water phase and then transferred to a scintillation vial. For TLC, we used precoated silica gel 60 plates (Merck Chemicals, Darmstadt, Germany) which were developed in chloroform-methanol-acetic acid-water (85:15:10:3.5). The developed TLC plates were either analyzed in a phosphorimager [BIO-RAD (Hercules, CA) Molecular Imager FX, using the Quantity One 1-D Analysis Software, version 4.4] or else briefly exposed to iodine vapor in order to stain lipids. Stained lipids were identified by authentic standards. For quantification by scintillation counting, the stained lipid spots were scraped off the silica gel plate after having been sprayed gently with water in order to facilitate removal of the silica gel. In experiments with ^{3}H-labeled substrates, the chloroform was evaporated under a stream of N_2 gas prior to scintillation counting.

The counting efficiency for the ^{3}H-labeled compounds was about 30%. For calculating GPCAT and GPAT activity in assays with labeled acyl-CoA substrates, the radioactivity recovered in LPC and lysophosphatidic acid (LPA), respectively, was added to half the radioactivity recovered in PC and phosphatidic acid (PA), respectively. The reason for the latter correction is the fact that PC and PA will be doubly labeled, and the second acyl group is not added by GPCAT or GPAT but rather by the LPLAT and lysophosphatidic acid acyltransferase activities present in the yeast microsomes.

In vivo labeling of yeast cells with [^{3}H]choline-GPC

Yeast strains were precultured on YPD plates overnight, washed once in 0.15 M NaCl, and then used to inoculate either liquid complete synthetic media supplemented with 2% glucose and 33 μM [^{3}H]choline-GPC (30,000 dpm/nmol) or synthetic phosphate-free media supplemented with 2% glucose, 75 μM inositol, 200 μM KH_2PO_4, and 10 μM [^{3}H]choline-GPC (30,000 dpm/nmol). The cultures were started at an OD_{600} of 0.1 and grown as indicated in the legend to Fig. 7. The cells were harvested by centrifugation and washed three times in 0.15 M NaCl. To the cell pellet was added 1 ml of 0.5 mm zirconium beads (BioSpec Products, Inc.) and 1 ml of methanol-chloroform (2:1; v/v). The cells were broken by heavily shaking in a Mini Beadbeater, as described above. The extract was removed, and the beads were washed once with 0.4 ml of methanol-chloroform (2:1; v/v). To the combined methanol-chloroform extract, water and chloroform were then added to achieve phase separation between chloroform and methanol-water, as described previously (33). The bottom (chloroform) phase was removed and combined with a second chloroform phase obtained by reextraction of the methanol-water phase. Aliquots of the combined chloroform phases and the methanol-water phases were added to scintillation vials together with 5 ml of scintillation fluid, and radioactivity was quantified by scintillation counting. Radioactive compounds in the chloroform phases were also separated using TLC, after which regions comigrating with lipid standards were scraped off for scintillation counting.

RESULTS

Yeast extracts possess an acyl-CoA-dependent GPCAT activity

The starting point for our work was the finding that the two yeast GPATs, Gpt2 and Sct1, though partially redundant in function (27), appear to have opposite effects on GPC accumulation. Thus, *gpt2* cells accumulate more GPC than WT cells, whereas *sct1* cells hardly accumulate any GPC at all (34). It has been proposed that this might reflect an increased flux through the CDP-choline pathway for de novo synthesis of PC in the *gpt2* strain, and a reduced flux in the *sct1* strain (34). However, an alternative explanation would be that Gpt2, but not Sct1, can reacylate GPC due to its similarity to the GPAT substrate G3P. Because yeast (or eukaryotes in general) has not been shown to possess a GPC-acylating activity, we first tested whether extracts from WT yeast cells are able to acylate GPC. Yeast extracts were incubated with either 16:0-CoA or 18:1-CoA as acyl donor and ^{14}C-labeled GPC as an acceptor. Lipids were then extracted and analyzed by TLC. We found that labeled GPC is indeed incorporated into lipids, and that the two major radioactive products formed are LPC and PC (**Fig. 2A**). In the absence of acyl-CoA, there was only a

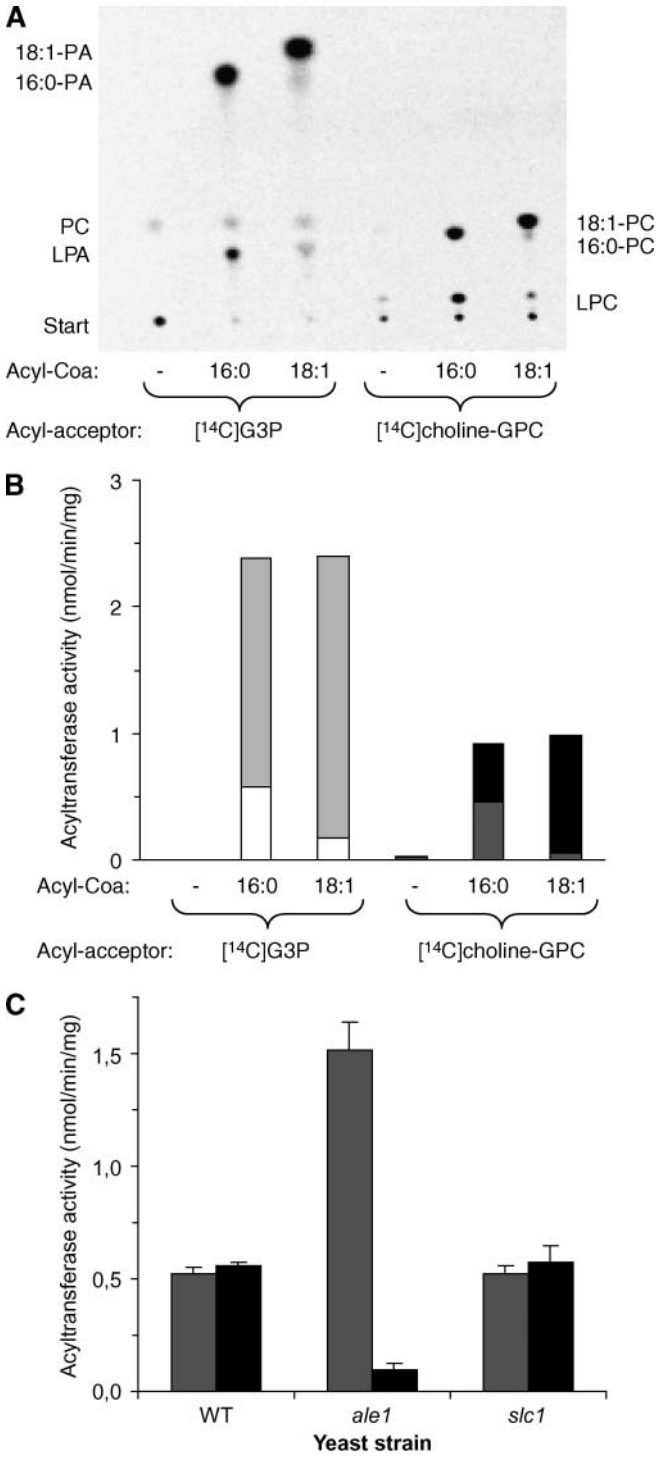

Fig. 2. GPCAT and glycerophosphocholine acyltransferase (GPAT) activities in yeast extracts. A: TLC image of separated radioactive lipids derived from yeast extracts (50 μg) incubated with 2 mM (10,000 dpm/nmol) of either [^{14}C]G3P or [^{14}C]choline-GPC, either in the absence of acyl group donors or in the presence of 0.1 mM 16:0-CoA or 18:1-CoA, as indicated in the figure. B: Quantification of the acyltransferase activities detected in A by scintillation counting of removed radioactive lipid spots. C: GPCAT activity in extracts (50 μg) of wild-type (WT), *ale1*, and *slc1* knockout strains incubated with 2 mM GPC and 0.1 mM [^{14}C]16:0-CoA (5,000 dpm/nmol) at 30°C for 10 min. Different phospholipids are indicated as follows: white, LPA; gray, PA; dark gray, LPC; black, PC. Error bars indicate SD (n = 3).

minor background incorporation of radioactivity into the lipid fraction (Fig. 2A). As a positive control, we also performed a GPAT assay using the same yeast extracts and ^{14}C-labeled G3P as acyl group acceptor. As expected, radioactivity was then incorporated into LPA and PA, with no detectable radioactivity being found in LPC or PC (Fig. 2A). Quantification of the radioactivity incorporated into LPC and PC suggested that the GPC-acylating activity is around 1 nmol/min/mg under the conditions used, which is approximately 40% of the GPAT activity in the same extracts (Fig. 2B). We conclude that yeast cells possess an enzymatic activity that efficiently can acylate GPC in an acyl-CoA dependent reaction, an activity that we refer to as GPCAT.

The gene encoding the LPLAT enzyme, which is responsible for the acyl-CoA dependent acylation of LPC to PC, was recently cloned in yeast and now has the standard gene name *ALE1*. To test whether the *ALE1* gene product is responsible for the conversion of LPC to PC seen in the GPCAT assay shown in Fig. 2A, the GPCAT assay was performed on extracts from the WT, an *ale1* knockout strain, and, for comparison, also from an *slc1* knockout strain, using [^{14}C]16:0-CoA and GPC as substrates (Fig. 2C). The extracts from the *slc1* knockout strain and the WT strain both synthesized approximately equal amounts of LPC and PC. This is in good agreement with the data in Fig. 2A. The extract from the *ale1* knockout strain, however, accumulated large amounts of radiolabeled LPC, but only minor amounts of radiolabeled PC (Fig. 2C). This shows that the Ale1 protein is the major enzyme responsible for the further acylation of the LPC that is formed by the GPCAT enzyme in yeast extracts.

The GPCAT activity in yeast extracts is not dependent on the yeast GPATs Gpt2 or Sct1

To test our hypothesis that one of the two GPATs in yeast, Gpt2, might possess GPCAT activity, we proceeded to assay GPCAT in yeast strains deleted for either the *GPT2* gene or the *SCT1* gene. As a control, we assayed GPAT activity in the same strains. There was no significant effect on the GPCAT activity in either the *gpt2* or *sct1* knockout strain, regardless of whether the cells were grown on glucose or glycerol (**Fig. 3A**). In contrast, the GPAT activity was significantly affected in both deletion strains, and as expected, the magnitude of the effect was dependent on the carbon source. Thus, a similar reduction in GPAT activity was observed for both deletion strains when the cells were grown on glucose, whereas the *gpt2* deletion had a much stronger effect than the *sct1* deletion when the cells were grown on glycerol (Fig. 3A). We conclude that neither of the two GPAT enzymes contributes significantly to the GPCAT activity, which is therefore likely to be mediated by a different, yet to be identified enzyme.

To further verify that the GPCAT activity is not mediated by one of the GPATs, we also studied the effect of overexpressing either gene from a multicopy plasmid using the strong galactose-inducible *GAL1* promoter. This experiment was done in the *gpt2* deletion strain in order to reduce the background GPAT activity. As a negative control,

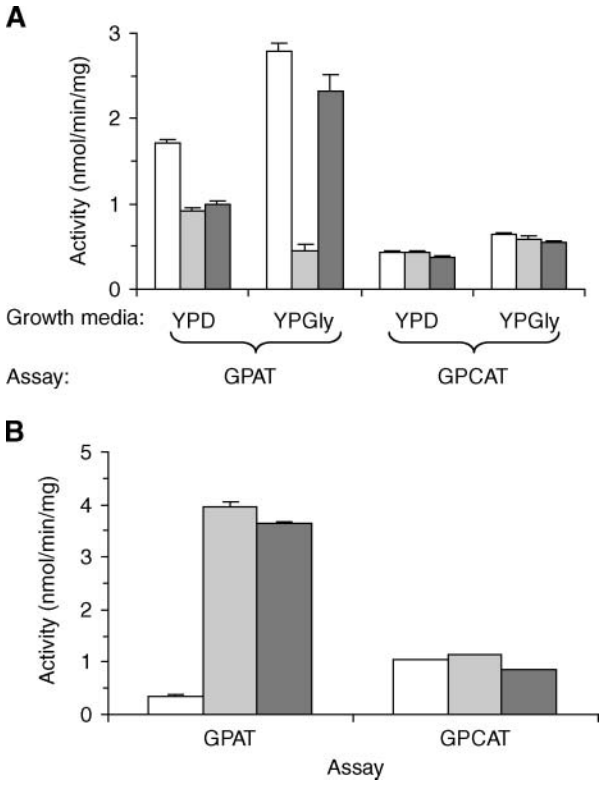

Fig. 3. GPAT and GPCAT activities in WT yeast and in yeast strains with either deletions or overexpression of the GPAT-encoding genes. A: GPAT and GPCAT activities were assayed in yeast extracts (50 μg) from *gpt2*, *sct1*, and WT strains grown in rich media supplemented with either glucose (YPD) or glycerol (YPGly), by adding 2 mM [^{14}C]G3P (2,000 dpm/nmol) or [^{3}H]choline-GPC (6,000 dpm/nmol) and 0.1 mM 16:0-CoA to the reaction mixtures. B: GPAT and GPCAT activities in yeast extracts (50 μg) from the *gpt2* knockout strain transformed with the p*GAL1-GPT2* (light gray) or p*GAL1-SCT1* (dark gray) GPAT overexpression plasmids or with the empty vector as a control (white). Error bars indicate SD (n = 3).

we included *gpt2* cells transformed with the empty expression vector. We found that although overexpression of either *GPT2* or *SCT1* causes a 7- to 8-fold increase in the GPAT activity, neither gene has any significant effect on the GPCAT activity (Fig. 3B). From these experiments, we can exclude Gpt2 and Sct1 as GPCAT candidate genes, at least under the conditions tested in our experiment.

Several other known or predicted yeast acyltranferases are also dispensable for GPCAT activity

In an attempt to identify the gene(s) coding for the new enzymatic activity, we assayed GPCAT activity in a number of yeast strains deleted for various known or predicted acyltranferase genes. The genes tested in this way included genes coding for known acyltransferases, such as Gpt2 and Sct1 (27), Taz1 (35, 36), Slc1 (37), Dga1 (38), Lro1 (39), Are1 and Are2 (40), Gup1 (41), and Ale1 (11), and also several genes without known functions: Mum3, Cst26, YDR018c, Vps66, YJR098c, Gup2, Ybp1, Ybp2, Ygr042w, Pmc1, and Pep7, all of which are predicted to encode acyltransferases, based on the presence of acyltransferase motifs within their protein sequences. As shown in **Fig. 4**,

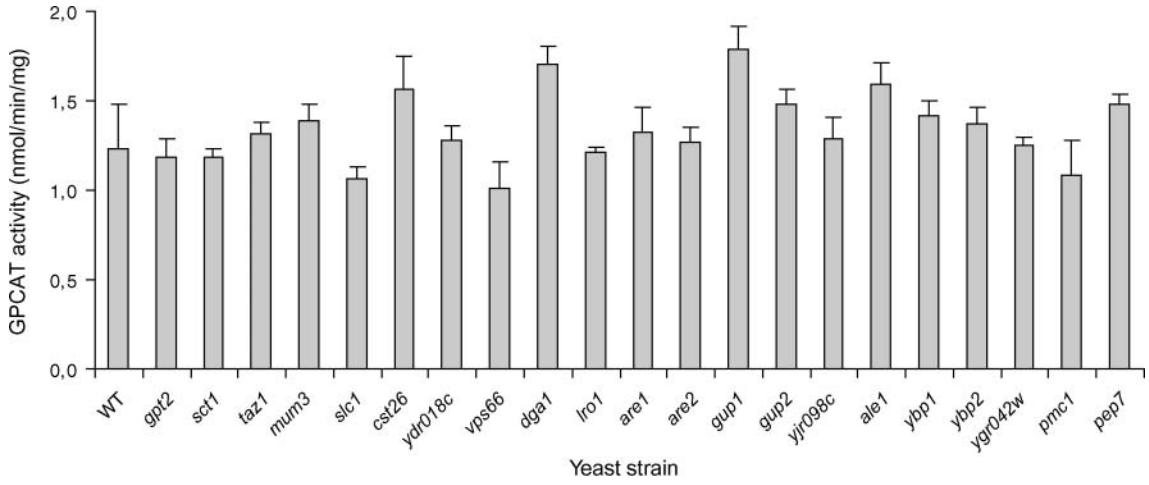

Fig. 4. GPCAT assay on extracts from different yeast knockout strains. Strains with deletions of genes coding for known or suspected acyltransferases were assayed for GPCAT activity. GPC (2 mM) and 0.1 mM [^{14}C]16:0-CoA (5,000 dpm/nmol) were incubated with extracts from different yeast strains (50 μg) at 30°C for 10 min. Error bars indicate SD (n = 3-8).

we found that none of the deletion mutants tested had any significant effect on GPCAT activity. We conclude that none of these genes are required for GPCAT activity.

We cannot exclude that two or several of the tested genes encode GPCATs that are completely redundant, in which case single gene deletions would have no effects. However, we consider this an unlikely possibility, because complete redundancy in gene function is rare in yeast and usually limited to cases where there are two almost identical copies of the same gene. Furthermore, even in such cases, a partial reduction in enzymatic activity is usually seen in either deletion mutant. A more common situation in yeast is where two related enzymes with different expression patterns and/or enzymatic properties contribute to the total activity, as exemplified by the two GPATs (27). For this reason, we consider it likely that the GPCAT activity is encoded by one or more genes that were not included in our experiment, and which therefore remain to be identified.

Acyl group donor and acceptor specificity of yeast GPCAT

There is no significant difference in the GPCAT acylation rates of yeast extracts using either 16:0-CoA or 18:1-CoA as acyl group donors, because approximately equal amounts of radiolabeled lipids are formed in each case (Fig. 2B). This is also true for the GPAT activity in the same yeast extracts, which was used as control reaction (Fig. 2B). Interestingly, the subsequent acylations of LPC into PC, and of LPA into PA, which are catalyzed by the LPCAT and LPAAT enzymes, respectively (42, 43), show a clear preference for 18:1-CoA as acyl chain donor (Fig. 2B). Thus, most of the radioactivity ended up in PC or PA in the presence of 18:1-CoA, indicating an efficient further acylation of LPC and LPA that is formed by acylation of GPC or G3P. In the presence of 16:0-CoA, the rate of conversion appears to be much slower; approximately one-half of the GPC-derived radioactivity is found in LPC, and one-quarter of the G3P-derived radioactivity is found in LPA (Fig. 2B).

The ability of yeast extract to acylate glycerophosphodiesters other than GPC was tested by incubating extracts with unlabeled GPE, GPS, GPI, and GPG in combination with ^{14}C-labeled 16:0- or 18:1-CoA (**Fig. 5A, B**, respectively) using assay conditions similar to those shown in Fig. 2. We saw no significant formation of labeled lipid in the presence of either GPS or GPI. However, as shown in Fig. 5, there was a minor synthesis of labeled phosphatidylethanolamine (PE) when GPE was added together with 18:1-CoA (11% of the PC formed in the presence of GPC) and also a small but detectable synthesis of phosphatidylglycerol when GPG was added together with 18:1-CoA (1% of the PC formed in the presence of GPC). From this we conclude that GPCAT shows at least some acyl group acceptor specificity in that it does not acylate GPS or GPI under the conditions tested. Furthermore, if the GPE- and GPG-acylating activities are mediated by the same enzyme as the GPC-acylating activity (which remains to be determined), then there is a strong preference for GPC as acyl group acceptor.

To further verify that a GPE-acylating activity is present in yeast extracts, we synthesized [^{14}C]ethanolamine-GPE and incubated it with yeast extract in the presence of either unlabeled 16:0-CoA or unlabeled 18:1-CoA (Fig. 5B). Interestingly, we found that the product formed differed for the two acyl group donors. Thus, in the presence of 16:0-CoA, mainly lysophosphatidylethanolamine (LPE) was formed, whereas only PE was formed in the presence of 18:1-CoA. Specifically, in the presence of 16:0-CoA, two faint spots were visible at a location corresponding to PE, which might represent labeled PE with different acyl compositions, but in the presence of 18:1-CoA, no trace of radioactive LPE was visible. From this we conclude that all LPE formed in the presence of 18:1-CoA is efficiently further acylated to PE, whereas further acylation of LPE formed in the presence of 16:0-CoA occurs only very slowly, if at all. This in turn suggests a remarkable acyl group specificity for the LPEAT activity in yeast extract.

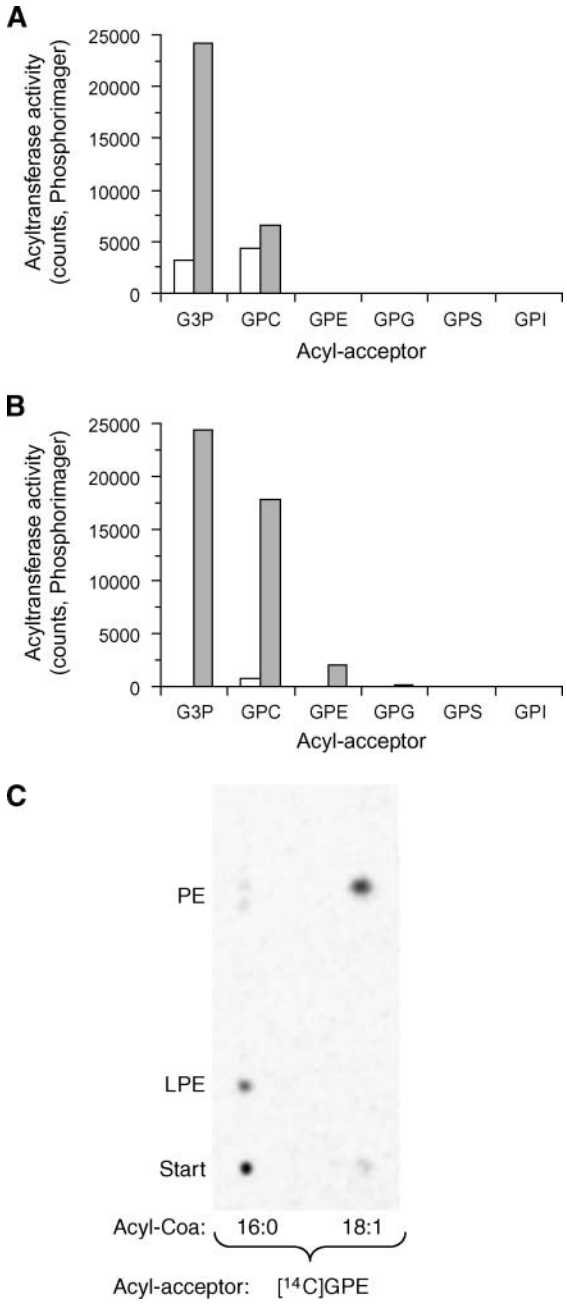

Fig. 5. Acyl-acceptor specificity of GPCAT. Yeast extract (50 μg) was incubated for 10 min at 30°C with 0.75 mM of different acyl acceptors, as indicated in the figure, together with 0.1 mM of (A) [^{14}C] 16:0-CoA (5,000 dpm/nmol) or (B) [^{14}C]18:1-CoA (5,000 dpm/nmol). Extracted lipids were separated on TLC and analyzed using a phosphorimager. White bars indicate lysophospholipids, and gray bars diacylphospholipids. C: TLC image of separated radioactive lipids from assays with yeast extract (50 μg) incubated with 2 mM (4,000 dpm/nmol) [^{14}C]ethanolamine-GPE either in the absence of acyl group donors or in the presence of 0.1 mM of 16:0-CoA or 18:1-CoA.

Biochemical characterization of the GPCAT activity in yeast microsomes

Fractionation of the yeast extract by ultracentrifugation led to a quantitative recovery of the GPCAT activity in the microsomal fraction, indicating that GPCAT is a membrane-bound or membrane-associated enzyme. The linearity of the GPAT and GPCAT activities as a function of both microsomal protein added and incubation time were studied. The GPCAT activity showed good linearity up to 25 μg of added protein and for an incubation time of at least 10 min (**Fig. 6A, B**). The GPAT activity showed a fairly good linearity for up to 20 μg of added protein and for incubation times of up to 5 min (Fig. 6A, B). For further characterization of the enzymatic activities, we used 20 μg of microsomal protein in both assays, with an incubation time of 5 min in the GPAT assay and 10 min in the GPCAT assay.

In Fig. 6C, the effects of the GPC and G3P concentrations on the GPCAT and GPAT activities are compared. The Lineweaver-Burk plot of the data (Fig. 6D) gives an apparent K_m value of 2.5 mM and an apparent V_{max} value of 8.7 nmol/min/mg for the GPCAT activity, whereas the GPAT activity in the same microsomal preparation had an apparent K_m value of 0.57 mM and an apparent V_{max} of 38 nmol/min/mg. Both the maximum rate and the substrate affinity are about four times higher in the GPAT reaction than in the GPCAT reaction. We further found that the GPCAT reaction has a broad pH optimum, with maximum activity between pH 6.5 and pH 8.5 (Fig. 6E). Below pH 6.5, the activity drops quickly. This is fairly similar to the effects of the pH on the GPAT activity (Fig. 6F). Finally, we found that both the GPCAT and the GPAT activity have an optimal acyl group donor concentration of 0.1 mM of 16:0-CoA (Fig. 6G, H), and similar results were obtained with the other three major acyl-CoAs present in yeast: 16:1-CoA, 18:0-CoA, and 18:1-CoA (data not shown). It should be noted, however, that 18:1-CoA appears to be somewhat preferred as acyl group donor, yielding a GPCAT activity that is 30% higher than with the other three acyl-CoAs tested (**Table 2**).

GPCAT is more heat stable than the two GPAT enzymes

Deacylation of PC to GPC in yeast is strongly enhanced at elevated temperatures (13), which suggests a possible role for phospholipid turnover in environmental adaptation of membrane lipid composition. If the GPCAT enzyme(s) is involved in such turnover, one should therefore expect it to be heat stable. Accordingly, we proceeded to test the heat stability of the GPCAT activity and also, as a control, the GPAT activity. As shown in **Table 3**, we found that a preincubation of yeast microsomes for 30 min at 50°C actually increases the GPCAT activity by around 30%, as compared with microsomes preincubated on ice. The GPAT activity, on the other hand, was almost completely destroyed by the same treatment. In fact, a 30 min preincubation at 40°C was sufficient to cause a 50% inactivation of GPAT. In order to obtain a similar inactivation of the GPCAT activity, a preincubation at 60°C was necessary (Table 3). From these experiments we conclude that GPCAT is clearly more heat stable than GPAT, which is consistent with a role for GPCAT in phospholipid remodeling at elevated temperatures. The difference in heat stability also suggests that the GPCAT and GPAT reactions are catalyzed by different enzymes, consistent with the knockout and overexpression results.

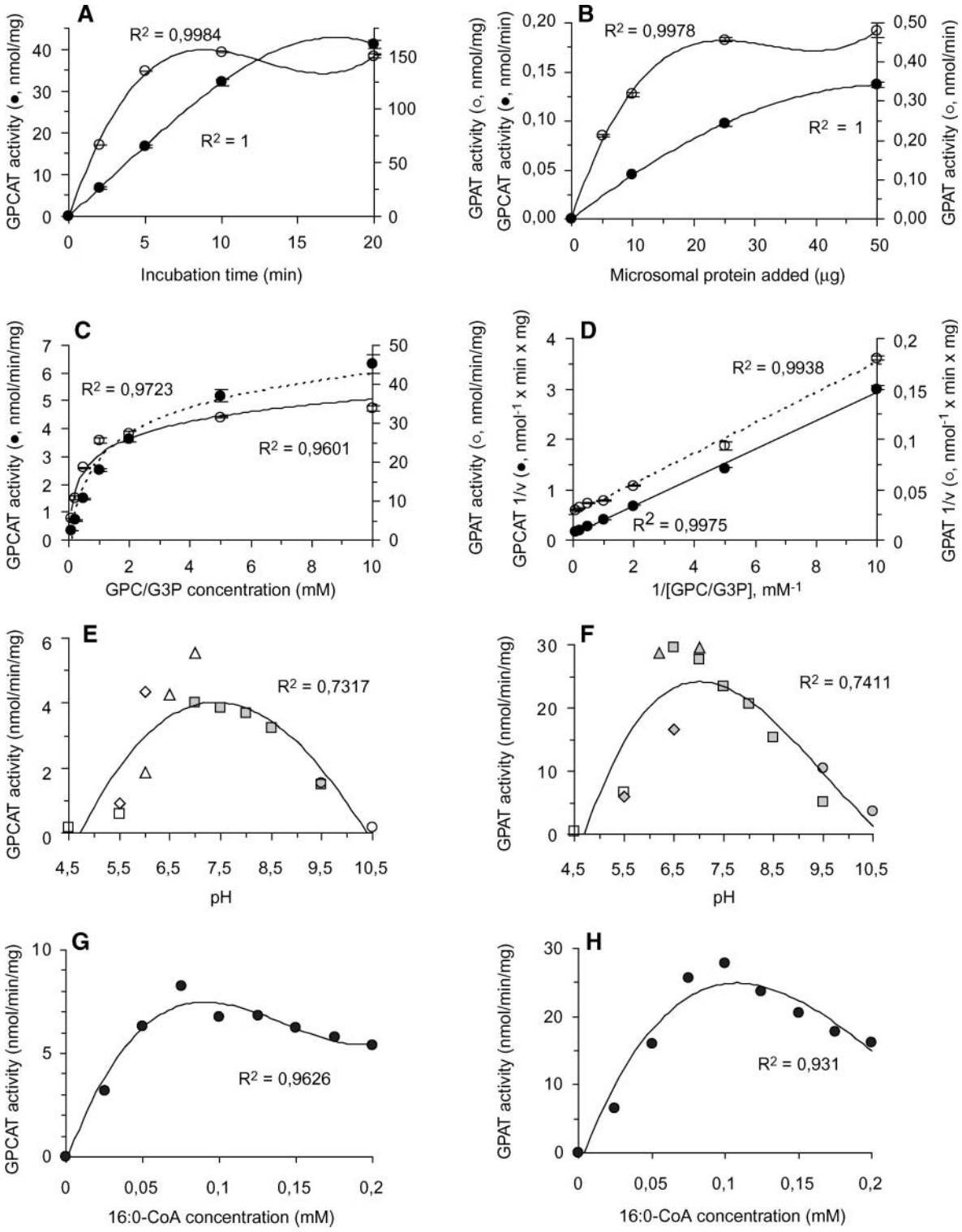

Fig. 6. Kinetic characterization of the GPCAT activity in yeast microsomes and comparison with the GPAT activity. GPCAT and GPAT activities were measured as a function of incubation time (A), amount of microsomal protein added (B), and the effects of added GPC and G3P on the assays (0.1 to 10 mM) (C). The GPCAT and GPAT activity data from C were used for the Lineweaver-Burk plot in D. The effect of pH on the GPCAT activity (E) was determined by performing the assay with 0.1 M of the following buffers: pH 4.5 and 5.5, acetic acid (open squares); pH 5.5 and 6.0, citrate (diamonds); pH 6.0, 7.0, and 7.5, malate (triangles); pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5, bis-Tris propane (closed squares); pH 9.5 and 10.5, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (circles). The effect of pH on the GPAT activity (F) was determined by using 0.1 M of the following buffers: pH 4.5 and 5.5, acetic acid (open squares); pH 5.5 and 6.5, MES [diamonds, 2-(*N*-morpholino)ethanesulfonic acid]; pH 6.2 and 7.0, imidazole (triangles); pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5, bis-Tris propane (closed squares); pH 9.5–10.5, CAPS (circles). Finally, GPCAT and GPAT activities were also measured as a function of the acyl-CoA concentration (0.025–0.15 mM 16:0-CoA) as shown in G and H, respectively. Error bars in A–D represent the average of the absolute deviations, n = 2.

TABLE 2. Acyl-CoA specificity of GPCAT activity

Acyl-CoA Substrate	Concentration	Relative Activity (n = 3)
	mM	% ± *SD*
16:0-CoA	0.1	100 ± 3.4
16:1-CoA	0.1	99 ± 1.7
18:0-CoA	0.1	106 ± 5.3
18:1-CoA	0.1	130 ± 3.4

GPCAT, glycerophosphocholine acyltransferase.

Sensitivity of GPCAT to divalent cations, chelating agents, and sulfhydryl agents

To test the effect of different divalent cations on GPCAT activity, 2 mM Mg^{2+}, Ca^{2+}, Mn^{2+}, or Zn^{2+} was added to incubation mixtures containing buffer, yeast microsomes, [^{3}H]GPC, and 16:0-CoA. We found that the GPCAT activity is very sensitive to divalent cations. Thus, Mg^{2+} and Ca^{2+} both inhibit the GPCAT activity by approximately 50%, Mn^{2+} causes a nearly complete inhibition, and Zn^{2+} causes a complete inhibition (**Table 4**). We proceeded to test the effect of different cation chelators on GPCAT activity. The addition of EDTA had no significant effect, but phenanthroline completely inhibited the GPCAT activity (Table 4). Attempts to identify the cation that is required for GPCAT activity by adding different cations to phenanthroline-treated yeast microsomes were not successful. Taken together, our data suggest that GPCAT is dependent on a divalent cation that remains to be identified.

Finally, we also tested the effect of preincubating the microsomes with the sulfhydryl reagent *N*-ethylmaleimide (NEM). As shown in Table 4, we found that this treatment inhibits GPCAT by 95%. We conclude that sulfhydryl groups also are important for GPCAT activity.

Inhibition of GPCAT by G3P

We proceeded to test the extent of inhibition that substrate analogs have on GPCAT activity. Accordingly, G3P, DHAP, and GPE were added to the reaction mixtures in both equimolar amounts and 5-fold excess as compared with the ^{3}H-labeled GPC. As a positive control for competitive inhibition, we also added unlabeled GPC in equimolar amounts and 5-fold excess. We found that the addition of unlabeled DHAP or GPE had no significant effect on the GPCAT activity (**Table 5**). Addition of equimolar amounts of G3P, on the other hand, inhibits the activity even more (44%) than the same amount of unlabeled GPC (34%). To test whether the observed effect of G3P is due to a true inhibition of the GPCAT enzyme rather than substrate competition between GPAT and GPCAT activities, microsomes were preheated at 50°C for 30 min in order to inactivate the GPAT activity, and then tested for G3P inhibition. As shown in Table 3, this treatment does not inactivate GPCAT, but instead causes a 30% increase in GPCAT activity. We found that the effect of G3P addition on the GPCAT activity was strongly reduced by the heat pretreatment but not completely eliminated. Thus, addition of equimolar amounts of G3P had only a minor effect after heat treatment, reducing the GPCAT activity by about 10%, whereas addition of a 5-fold excess of G3P led to a 40% reduction in activity (Table 5). These results suggest that most of the inhibitory effect of G3P on GPCAT activity may be due to competition by GPAT (which is stimulated by the addition of G3P) for acyl-CoA, but also that some of the effect may be mediated by GPCAT itself. Possible mechanisms could be that G3P acts as an antagonist that blocks GPC binding to GPCAT, or that G3P is a substrate of GPCAT that competes with GPC. However, because the GPAT double knockout *gpt2 sct1* is not viable, it is unlikely that GPCAT possesses a significant GPAT activity.

TABLE 3. Temperature tolerance of GPCAT and GPAT activities

Preincubation Temperature	Preincubation Time	Relative GPCAT Activity (n = 3)	Relative GPAT Activity (n = 3)
°*C*	*min*	% ± *SD*	% ± *SD*
0	30	100 ± 2.4	100 ± 3.4
40	30	112 ± 0.9	48 ± 1.1
50	30	131 ± 5.1	7 ± 0.3
60	30	59 ± 3.2	12 ± 0.4

GPAT, glycerol-3-phosphate acyltransferase.

TABLE 4. Effects of divalent cations, reducing, and sulfhydryl agents on GPCAT activity

Addition	Concentration	Relative Activity (n = 3)
	mM	% ± *SD*
None (substrates and buffer)	—	100 ± 1.0
$MgCl_2$	2	49 ± 2.5
$CaCl_2$	2	58 ± 4.9
$MnCl_2$	2	3 ± 0.3
$ZnCl_2$	2	0 ± 0.0
EDTA	20	100 ± 1.4
Phenanthroline	5	0 ± 0.6
NEM (preinc. 15 min on ice)	2	5 ± 0.9

NEM, *N*-ethylmaleimide.

In vivo evidence of a GPCAT-dependent pathway for PC synthesis in yeast

In order to test whether a GPCAT-dependent pathway for biosynthesis of PC can be shown to be active in vivo,

TABLE 5. Substrate analog inhibition of GPCAT activity

Added Substrate/Substrate Analog	Concentration	Relative Activity (n = 3)
	mM	% ± *SD*
None	—	100 ± 7.8
GPC	2	66 ± 1.5
GPC	10	17 ± 0.2
G3P	2	56 ± 1.8
G3P	10	23 ± 1.9
DHAP	2	96 ± 2.3
DHAP	10	90 ± 1.8
GPE	2	100 ± 6.6
GPE	10	110 ± 1.7
Preinc. 50°C no addition	—	135 ± 0.3
Preinc. 50°C + G3P	2	124 ± 0.3
Preinc. 50°C + G3P	10	81 ± 1.4

GPC, glycerophosphocholine; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; GPE, glycerophosphoethanolamine.

we added [^{3}H]choline-GPC to growing yeast cells. The incorporation of exogenously added choline into PC uses the CDP-choline pathway, which is dependent on the *PCT1* gene. Incorporation of radioactivity from exogenously added [^{3}H]choline-GPC into PC in a *pct1* strain would therefore indicate the presence of an alternative GPCAT-dependent pathway for PC biosynthesis.

When [^{3}H]choline-GPC is fed to WT yeast cells grown in synthetic complete media supplemented with glucose, radioactivity is incorporated into the chloroform-extractable lipid fraction (**Fig. 7A**). As expected, the radioactivity was identified as PC by TLC (data not shown). We further found that uptake of radioactivity from exogenously added [^{3}H]choline-GPC is dependent on the Hnm1 choline transporter, because no uptake was seen in an *hnm1* strain (Fig. 7A). In the *pct1* strain, uptake was not affected, but all radioactivity taken up by the cells was found in the methanol-water fraction, consistent with further incorporation of [^{3}H]choline into lipids through the CDP-choline pathway being blocked in *pct1* cells. Deletion of *GIT1* or *GDE1* had no significant effect on either uptake of radioactivity or its incorporation into lipids. Taken together, these data indicate that yeast cells use exogenously added GPC as a choline source by degrading it outside the cell, after which the free choline is taken up through the Hnm1 choline transporter. In contrast, direct uptake of GPC (and its further metabolism inside the cell) does not seem to be important under these conditions.

Significantly, it was recently shown that yeast can utilize GPC as a phosphate source under phosphate-limiting conditions, and this process is dependent on uptake of GPC by Git1, and its subsequent hydrolysis inside the cell by Gde1 (21, 44). We therefore repeated the [^{3}H]choline-GPC feeding experiment under phosphate limitation according to Fisher et al. (21). As seen in Fig. 7B, we found that WT yeast cells incorporate radioactivity into the chloroform-extractable lipid fraction also under these conditions. However, unlike cells grown in the absence of phosphate starvation, the *hnm1* strain incorporated almost the same amount of radioactivity as the WT strain (data not shown). Incorporation was severely reduced in a *git1* strain, consistent with direct uptake of GPC (20) being important under these conditions (Fig. 7B). Unlike the first experiment, we found that under phosphate limitation, the *pct1* strain incorporates considerable amounts of radioactivity into the chloroform-extractable lipid fraction (Fig. 7B, C). This clearly demonstrates that GPC-derived radioactivity can be incorporated into PC independently of the CDP-choline pathway, provided that the cells are grown under conditions (phosphate starvation) that permit direct uptake of GPC, and thus provides evidence of a GPCAT-dependent pathway for biosynthesis of PC.

We further found that the *gde1* strain incorporates radioactivity into both the chloroform- and methanol-water-extractable fractions under phosphate limitation (Fig. 7B). This result clearly differs from the first experiment, where most of the radioactivity in the *gde1* strain was found in the lipid fraction, similar to the WT (Fig. 7A). Because the Gde1 protein is responsible for intracellular degradation of GPC, we conclude that hydrolysis of GPC is not necessary for incorporation of radioactivity into PC under these conditions, which is consistent with a GPCAT-dependent acylation of GPC being involved. We proceeded to test four sister

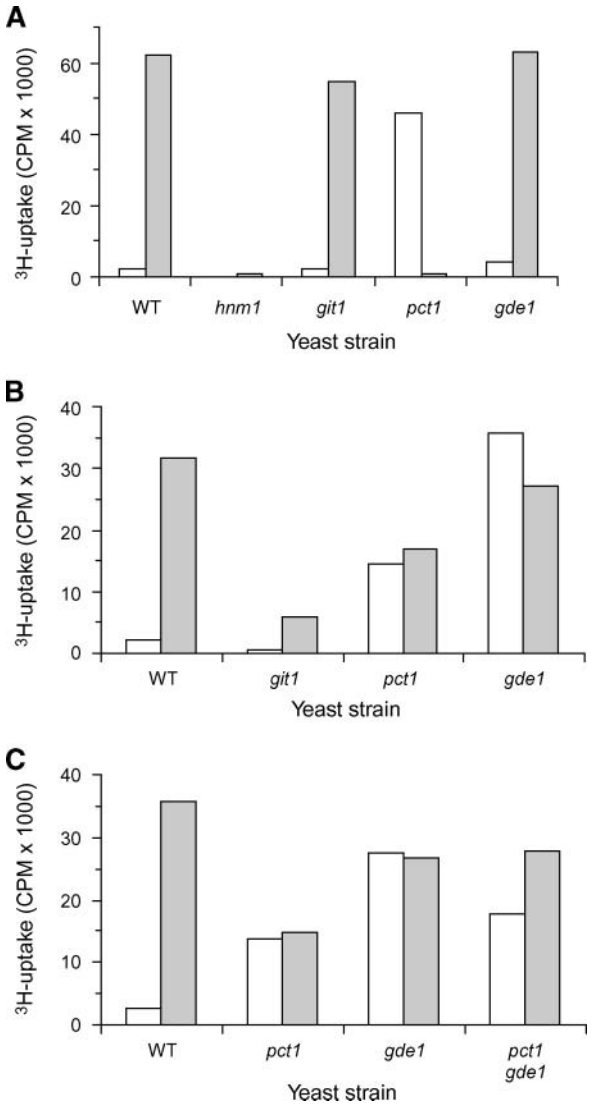

Fig. 7. In vivo labeling of yeast cells with [^{3}H]choline-GPC. A: WT and knockout yeast strains were grown to mid-log phase in YPD, washed, inoculated to an OD_{600} of 1.5 in complete liquid synthetic media supplemented with 2% glucose and 33 mM (30,000 dpm/nmol) [^{3}H]choline-GPC, and then grown for 80 min at 30°C with rotary shaking. Cells were harvested by centrifugation and washed extensively, after which the incorporation of radioactivity into the water-methanol-soluble (white) and chloroform-soluble (gray) fractions was determined. B, C: WT and knockout yeast strains as indicated in the figure were inoculated at an OD_{600} of 0.2 into phosphate-free media supplemented with 2% glucose, 200 μM KH_2PO_4, and 75 μM inositol, and incubated for 5 h. These cultures were then used to inoculate 5 ml of phosphate-free media supplemented with 2% glucose, 200 μM KH_2PO_4, 75 μM inositol and 10 mM (30,000 dpm/nmol) [^{3}H]choline-GPC at an OD_{600} of 0.1. The cultures were grown for 18 h at 30°C with rotary shaking to an OD_{600} of about 3.4, after which they were harvested. Incorporation of radioactivity into the water-methanol-soluble (white) and chloroform-soluble lipid (gray) cellular fractions was determined as in A. Both A and B show representative data from experiments that were repeated at least two times.

spores from a single tetratype tetrad in a *pct1* × *gde1* cross, one of which carried a double *pct1 gde1* deletion, under phosphate-limited conditions (Fig. 7B). We found that the incorporation of radioactivity from exogenously added [^{3}H]choline-GPC into the chloroform-extractable lipid fraction was not reduced in this double knockout strain as compared with the *pct1* single knockout strain, but rather was enhanced. The radioactivity in the chloroform fractions of both the *gde1* and the *pct1 gde1* strains was identified as PC by TLC (data not shown). These results support the notion that degradation of internalized [^{3}H]choline-GPC into [^{3}H]choline is not necessary for incorporation of radioactivity into PC, but rather inhibits this process by competing with GPCAT for internalized GPC.

DISCUSSION

In this study, we have identified a novel acyltransferase activity in yeast microsomal membranes that carries out acylation of the glycerophosphodiester GPC. We call this novel enzymatic activity GPCAT, for GPC acyltransferase. We further present evidence (Fig. 7) that the GPCAT activity together with LPCAT 5–11,42 creates a novel in vivo pathway for reacylation of GPC, produced by phosphoplipid hydrolysis, into new phospholipids (Fig. 1). Interestingly, this pathway for recycling of phosphoplipds is less energy-demanding and involves fewer enzymatic steps than the recently described glycerophosphodiesterase pathway (18).

There was no acylation activity in yeast extract that was specific for GPS or GPI and only a minute activity specific for GPG. GPE was, however, acylated at a significant rate, 11% of the GPCAT activity, indicating that a PE deacylation/reacylation cycle also may occur in yeast (Fig. 1). The acylation of GPC by yeast extracts is clearly not a minor reaction, because its specific activity is about one-fourth of the highly active GPAT reaction. The affinity of GPCAT for GPC was also about a fourth of the affinity of GPAT for its substrate, G3P. We infer from this that a rather high GPC concentration is required in order to have a significant GPCAT activity. It should be noted, however, that the intracellular GPC levels in yeast can be dramatically increased in vivo both during heat stress and in the presence of free choline in the media (13). Furthermore, GPC can accumulate to high intracellular concentrations in certain animal tissues such as the kidney (23).

The GPCAT activity showed no strong acyl-CoA preference, though a somewhat (30%) higher activity was seen with 18:1-CoA as acyl group donor, as compared with 16:0-CoA. The biochemical characterization of the GPCAT activity further revealed a remarkable heat stability as compared with GPAT. This is particularly interesting because an elevated temperature is known to increase deacylation of PC into GPC. Our results, together with the fact that the acyl composition of membranes is known to be remodeled as a response to changes in the temperature in many eukaryotic cells (45), suggest a possible in vivo function of the GPCAT activity in phospholipid remodeling and/or recycling.

We further found that the GPCAT activity is sensitive to the sulfhydryl reagent NEM, indicating that one or several cysteine residues are important for the enzyme activity. The strong Zn^{2+} chelator, *o*-phenanthroline, which previously has been shown to inhibit the LPCAT enzyme in yeast (42), also efficiently inhibited the GPCAT activity. In contrast, EDTA, a strong general divalent ion chelator but a weaker Zn^{2+} chelator than *o*-phenanthroline, did not have any significant effect on the activity. This suggests that a Zn^{2+} ion may be important for the activity, but we cannot rule out that some other cation that remains to be identified is responsible for the *o*-phenanthroline sensitivity. A role for Zn^{2+} in GPCAT is also suggested by the fact that high (millimolar) concentrations of Zn^{2+} ions severely reduce the GPCAT activity, an effect that was seen also for LPCAT in yeast (42), and for zinc-dependent proteases that contain high-affinity Zn^{2+} binding sites (46).

We found that the addition of [^{3}H]choline-GPC to yeast cells during phosphate starvation results in incorporation of radioactivity into PC in a *pct1* strain, where the CDP-choline pathway for PC biosynthesis is blocked (Fig. 7B). A likely explanation for this incorporation is direct acylation of GPC to PC, mediated by the GPCAT activity. This interpretation is further supported by the fact that the *pct1 gde1* double knockout strain shows no reduction but rather an increase in its ability to incorporate [^{3}H]choline-GPC into PC, as compared with the *pct1* single knockout strain, which suggests that the alternative Pct1-independent pathway for PC biosynthesis is not dependent on a prior hydrolysis of GPC into choline (Fig. 7B). An alternative explanation for our finding that exogenously added [^{3}H]choline-GPC can be incorporated into PC in a Pct1-independent manner could be that the Muq1 enzyme, which activates phosphoethanolamine to CDP-ethanolamine (47), also can use phosphocholine as a substrate, thereby partially complementing the *pct1* deletion. However, this is an unlikely explanation, because a *cho2 pct1*^{ts} yeast strain is dead at the nonpermissive temperature (48), presumably due to an inability to synthesize CDP-choline. Another possible explanation would be that a PC synthase exists in yeast that provides an alternative route for choline incorporation into PC. Both the fact that all incorporation of radioactive choline into PC is dependent on the *PCT1* gene when cells are grown in synthetic complete media (Fig. 7A) and the fact that incorporation of radioactive GPC into PC takes place in the *pct1 gde1* double knockout strain when grown on phosphate starvation media argue against such a reaction. These findings instead support the notion that direct acylation of GPC to PC, mediated by GPCAT, is responsible for the observed incorporation of GPC-derived radioactivity into PC.

In this paper, we have described the initial characterization of a novel yeast acyltransferase activity that is involved in lipid metabolism and more specifically in PC biosynthesis, turnover, and recycling. Further studies, in particular the identification of the gene(s) coding for this activity, will reveal whether GPCAT also exists in organisms other than yeast and will make it possible to study the cellular function(s) of GPCAT in more detail.

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