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Identification of a New Glycerol-3-phosphate Acyltransferase Isoenzyme, mtGPAT2, in Mitochondria*

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Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the initial and rate-limiting step of glycerolipid synthesis. Two distinct GPAT isoenzymes had been identified in mammalian tissues, an *N*-ethylmaleimide (NEM)-sensitive isoform in the endoplasmic reticulum membrane (microsomal GPAT) and an NEM-resistant form in the outer mitochondrial membrane (mtGPAT). Although only mtGPAT has been cloned, the microsomal and mitochondrial GPAT isoforms can be distinguished, because they differ in acyl-CoA substrate preference, sensitivity to inhibition by dihydroxyacetone phosphate and polymixin B, temperature sensitivity, and ability to be activated by acetone. The preponderance of evidence supports a role for mtGPAT in synthesizing the precursors for triacylglycerol synthesis. In mtGPAT$^{-/-}$ mice, PCR genotyping and Northern analysis showed successful knockout of mtGPAT; however, we detected a novel NEM-sensitive GPAT activity in mitochondrial fractions and an anti-mtGPAT immunoreactive protein in liver mitochondria, but not in microsomes. Rigorous analysis using two-dimensional gel electrophoresis revealed that the anti-mtGPAT immunoreactive proteins in wild type and mtGPAT$^{-/-}$ liver mitochondria have different isoelectric points. These results suggested the presence of a second GPAT in liver mitochondria from mtGPAT$^{-/-}$ mice. Characterization of this GPAT activity in liver from mtGPAT null mice showed that, unlike the mtGPAT activity in wild type samples, activity in mtGPAT knockout mitochondria did not prefer palmitoyl-CoA, was sensitive to inactivation by NEM, was inhibited by dihydroxyacetone phosphate and polymixin B, was temperature-sensitive, and was not activated by acetone. We conclude that a novel GPAT (mtGPAT2) with antigenic epitopes similar to those of mtGPAT is detectable in mitochondria from the livers of mtGPAT$^{-/-}$ mice.

The initial and rate-limiting step of glycerolipid synthesis is the acylation of glycerol 3-phosphate with long-chain fatty acyl-CoA to form 1-acyl-glycerol 3-phosphate (LPA).[1] This reaction is catalyzed by two glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15) isoenzymes that are encoded by different genes (1). One isoform is present in the endoplasmic reticulum membrane (microsomal GPAT), and the other is located in the outer mitochondrial membrane (mtGPAT). Although the microsomal GPAT has not been cloned or purified, its activity is easily distinguished, because, unlike mtGPAT, the microsomal isoform is inhibited by sulfhydryl reagents (2). In most tissues, the microsomal GPAT activity is 10 times higher than that found in the mitochondrial fraction, but in liver, mtGPAT contributes 30–50% of the total activity (1).

Microsomal GPAT and mtGPAT also differ in their acyl-CoA substrate preference. The microsomal isoform esterifies both saturated and unsaturated long-chain acyl-CoAs equally well, but mtGPAT prefers C16:0-CoA (2). In rat liver, kidney, and heart, mtGPAT activity is 3–10-fold higher with C16:0-CoA than with other long-chain saturated or unsaturated acyl-CoA substrates (3–6). The activity of recombinant mouse mtGPAT purified from an insect cell expression system is 2-fold higher with C16:0-CoA than with C18:1-, C18:2n6-, C18:3n3-, or C20:4n6-CoA (7). Because most naturally occurring glycerolipids contain saturated fatty acids at the *sn*-1 position and unsaturated fatty acids at the *sn*-2 position, it was proposed that mtGPAT establishes the initial asymmetric distribution of fatty acids (4). This hypothesis was borne out by studies with GPAT$^{-/-}$ mice showing that, compared with the wild-type mice, their liver phosphatidylethanolamine and phosphatidylcholine have 20% less palmitate in the *sn*-1 position and 36–40% more arachidonate in the *sn*-2 position (8). These results confirm both the important role mtGPAT plays in the positioning of 16:0 at the *sn*-1 position of phospholipids and its indirect role in positioning 20:4 at the *sn*-2 position.

Although the microsomal GPAT is present in the same subcellular membrane fraction as the terminal enzymes for TAG synthesis, physiological data suggest that TAG formation is regulated by the mitochondrial isoform (1, 8–10). Because only the mitochondrial GPAT isoform has been cloned (11, 12), changes in activity of the two isoenzymes can be compared, but not changes in mRNA abundance or protein expression. In general, mtGPAT mRNA, protein, and activity in liver and adipose tissue increase with carbohydrate feeding and with insulin stimulation, whereas microsomal GPAT activity does not change (11, 13–15). In addition, mtGPAT mRNA is up-regulated by sterol regulatory element-binding protein-1c, a potent activator of lipogenesis (16). The role for mtGPAT in up-regulating TAG synthesis is also supported by studies of overexpressed rat mtGPAT in Chinese hamster ovary cells. Expression of recombinant rat mtGPAT in Chinese hamster ovary cells results in a 3.8-fold increase in NEM-resistant GPAT activity, a 4-fold increase in [^{14}C]oleate incorporation into TAG, and a 30% decrease in [^{14}C]oleate incorporation into phospholipids (10). To confirm the critical role of mtGPAT in the regulation of TAG synthesis, mtGPAT null mice were generated (8). The knockout mice weigh less than controls and

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[1] The abbreviations used are: LPA, lysophosphatidic acid; DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; G3P, glycerol 3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; NEM, *N*-ethylmaleimide; TAG, triacylglycerol; nt, nucleotides.

have reduced gonadal fat pad weights, lower hepatic TAG content, a lower plasma TAG and very low density lipoprotein TAG, and decreased secretion of TAG from liver (8). These data suggest that mtGPAT is required for normal synthesis of TAG in both fat cells and hepatocytes but show that the microsomal GPAT also contributes.

We were surprised to find that mitochondria isolated from liver of mtGPAT$^{-/-}$ retained considerable GPAT activity but that this activity, unlike that of mtGPAT, was inactivated by NEM. Further, an anti-GPAT immunoblot analysis of GPAT$^{-/-}$ liver mitochondria detected an immunoreactive protein with approximately the same molecular mass as mtGPAT. Rigorous analysis using two-dimensional gel electrophoresis revealed that the anti-mtGPAT immunoreactive proteins in wild type and mtGPAT$^{-/-}$ liver mitochondria had different isoelectric points. These results suggested the presence of a second mtGPAT isoform. Further characterization showed that, unlike the mtGPAT activity in wild type samples, the NEM-sensitive GPAT activity in mitochondria from liver of knockout animals did not prefer C16:0-CoA, was inhibited by DHAP and polymixin B, was temperature-sensitive, and was not activated by acetone. We conclude that liver mitochondria from mtGPAT$^{-/-}$ mice express a novel GPAT activity.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were purchased from Sigma if not otherwise indicated. [2-^{3}H]Glycerol was from PerkinElmer Life Sciences. Lipid standards were from Doosan Serdary Research Laboratories.

Animals—Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Male mtGPAT$^{-/-}$ and wild type mice were genotyped by PCR as described previously (8). Mice were housed on a 12-h/12-h light/dark cycle with free access to water and Prolab RMH 3000 SP76 chow.

Preparation of Mouse Liver Mitochondrial and Microsomal Fractions—Two-month-old male mtGPAT deficient mice and male wild type mice were anesthetized with Avertin (0.01 ml/g of body weight) and killed by cervical dislocation. Liver was removed immediately, minced, and placed in Medium 1 + DTT (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT). Tissue was homogenized with 10 up and down strokes in a Teflon-glass homogenizing vessel and centrifuged at 600 × *g* for 5 min to remove large debris and nuclei. Mitochondria were obtained by centrifuging the supernatant at 10,300 × *g* for 10 min. The microsomal fraction was acquired by centrifuging the supernatant for 1 h at 100,000 × *g*. Liver mitochondrial and microsomal fractions were stored in aliquots at −80 °C. Protein concentrations were determined by the bicinchoninic acid method (Pierce) using bovine serum albumin as the standard.

Purity of the Mitochondrial and Microsomal Fractions—Purity of the liver subcellular fractions was established by measuring the activity of marker enzymes, NADH cytochrome *c* reductase (17), and cytochrome *c* oxidase (cytochrome *c* oxidase kit; Sigma), for endoplasmic reticulum and mitochondria, respectively.

Immunoblotting—Proteins were separated on an 8% polyacrylamide gel containing 1% SDS, transferred to a polyvinylidene fluoride membrane (Bio-Rad), and incubated with antibody against recombinant rat mtGPAT expressed in bacteria and gel-purified from inclusion bodies. For chemiluminescent detection, the immunoreactive bands were visualized by incubating the membrane with horseradish peroxidase-conjugated goat anti-rabbit IgG and PicoWest reagents (Pierce). For two-dimensional analyses, mitochondria (1 mg of protein) were added to 600 μl of rehydration buffer containing 7 M urea, 2 M thiourea, 0.5% (v/v) Biolytes 3–10 (Bio-Rad), 50 mM Tris-HCl, 1% ASB-14 (Calbiochem) and 1% Triton X-100, and 2 mM tributyl phosphine. The sample was mixed and sonicated in a water bath sonicator for 5 min. The sample was incubated for an additional 40 min at room temperature and centrifuged at 13,000 × *g* to pellet the insoluble particulate. Immobilized pH gradient gel strips (Bio-Rad) were rehydrated overnight (12–16 h) at 50 V with rehydration buffer containing the protein sample. Proteins were separated on a protein isoelectric focusing cell (Bio-Rad) for 60,000 V-h. The immobilized pH gradient gel strip was conditioned for 15 min with 250 V, and then the voltage was raised to 30,000 V over 3 h. After isoelectric focusing, the immobilized pH gradient gel strips were incubated for 10 min with shaking in equilibration buffer (6 M urea, 2% SDS, 20% glycerol, 0.15 M Tris-HCl) containing 130 mM DTT and subsequently in rehydration buffer containing 135 mM iodoacetamide for an additional 10 min with shaking. The immobilized pH gradient gel strips were placed on an 8% polyacrylamide gel containing 1% SDS to separate proteins in the second dimension. Immunoblotting was performed as described above.

TABLE I
Activities of ER and mitochondrial marker enzymes

	NADPH cytochrome *c* reductase		NADPH cytochrome *c* oxidase	
	Total activity[a]	Percentage[b]	Total activity[a]	Percentage[b]
	mol/min	%	*mol/min*	%
Mitochondria				
+/+	0.03	3.6	14.69	94.2
−/−	0.05	2.0	15.33	98.3
Microsomes				
+/+	0.70	96.4	0.90	5.8
−/−	2.5	98.0	1.72	1.7

[a] Total activity in each fraction.
[b] Percentage of total activity in all fractions.

Glycerol 3-Phosphate Synthesis and GPAT Assay—*sn*-[2-^{3}H]Glycerol 3-phosphate was synthesized enzymatically from [2-^{3}H]glycerol (1 mCi/ml), purified, and assayed as described (18). GPAT activity was assayed in a 200-μl mixture containing 75 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mg/ml bovine serum (essentially fatty acid-free), 1 mM DTT, 8 mM NaF, 800 μM [^{3}H]glycerol 3-phosphate, and 80 μM palmitoyl-CoA (19). The reaction was initiated by adding 15–30 μg of mitochondrial or microsomal protein to the assay mix. All assays were performed for 10 min at room temperature unless indicated. Assay modifications are indicated in the figures. All experiments were performed at least twice.

Lipid Analysis—Products from the GPAT assay were pooled, dried, and resuspended in chloroform. The lipids were spotted on a Silica G plate (Whatman) together with standards for LPA, phosphatidic acid, and diacylglycerol. The chromatograms were developed in $CHCl_3$/pyridine/formic acid (88%) (50:30:7). The plates were exposed to iodine vapor, and the bands corresponding to LPA, phosphatidic acid, and diacylglycerol were scraped into scintillation vials containing Cyoscint (ICN) for scintillation counting.

RESULTS

Mitochondrial GPAT$^{-/-}$ Liver Mitochondria Contain an NEM-sensitive GPAT Activity—Mitochondrial GPAT (mtGPAT) knockout mice were generated by replacing a 0.5-kb genomic sequence encoding part of the active site with *neo* and confirmed by PCR genotyping (8). To make certain that we had truly eliminated mtGPAT in the knockout mouse, we probed RNA from mtGPAT +/+, +/−, and −/− mice with two probes for rat mtGPAT, Probe A (nt 785–1582, 0.8 kb) and Probe B (nt 1583–2194, 0.6 kb). The active site of mtGPAT is composed of homology regions I (nt 679–717), II (nt 814–834), III (nt 934–969), and IV (nt 1039–1062) (20). Regions II–IV are contained within Probe A. The targeting construct deletes a 0.5-kb region of genomic DNA that contains homology regions II and III. Neither Probe A nor Probe B, which together encode 53% of the open reading frame, identified an RNA band in liver from mtGPAT$^{-/-}$ mice (8). These previously published Northern analyses demonstrate that mtGPAT$^{-/-}$ mice lack expression of the mtGPAT transcript.

Therefore, we were surprised to find that the mitochondria of mtGPAT$^{-/-}$ mice contained a GPAT activity that was sensitive to NEM inhibition. Thus, we undertook a careful study of the novel GPAT activity in highly pure mitochondrial fractions (Table I) from livers of wild type and mtGPAT null mice. Historically, the GPAT activity in mitochondria has been measured as NEM-resistant glycerol-3-phosphate acyltransferase activity, because sulfhydryl reagents only inhibit the GPAT activity present in microsomes (2). In our initial characterization of the mtGPAT null mice, we assayed liver total membrane fractions in the presence and absence of NEM. In these total membrane fractions, GPAT activity in the presence of NEM

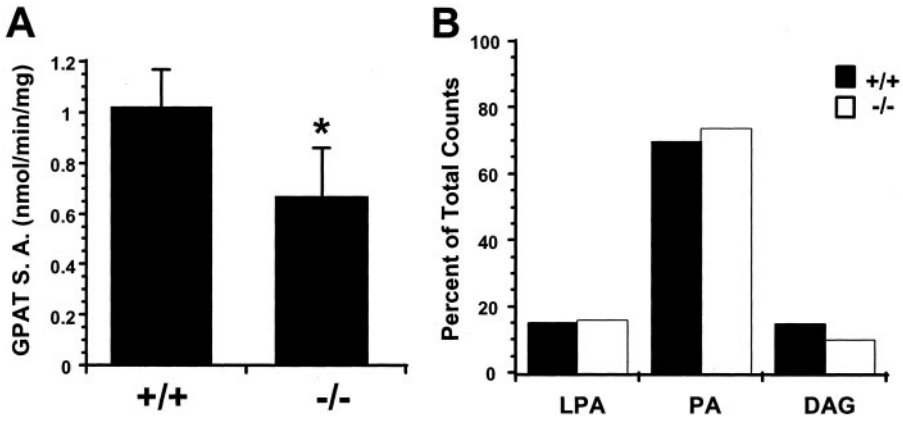

FIG. 1. **Purified mitochondria from mtGPAT−/− mice retain GPAT activity.** GPAT-specific activity was determined in liver mitochondria from wild type (+/+) and mtGPAT knockout (−/−) mice as described under "Experimental Procedures." *A*, the *bars* represent GPAT-specific activity. Values are means ± S.D. of eight independent mitochondria isolations and were analyzed by paired Student's *t* test (*, $p = 0.002$). *B*, LPA, phosphatidic acid, and diacylglycerol were separated by TLC as described under "Experimental Procedures." The *bars* represent the percentage of total counts (+/+, 3028 cpm; −/−, 2210 cpm) collected from the plate and are the average of two independent experiments that varied by less than 10%.

was only 8% of the NEM-resistant activity observed in wild type mice (8), consistent with the amount of residual microsomal GPAT activity that is usually observed after NEM treatment (21, 22). The NEM-sensitive GPAT activities were similar in wild type and mtGPAT knockout mice. Highly purified mitochondria from mtGPAT$^{-/-}$ mice, however, contained substantial GPAT activity that was 60% of the activity present in mitochondria from wild type mice (1.0 nmol/min/mg) (Fig. 1*A*). When the labeled glycerolipid products formed in the assays for GPAT activity in mitochondria were analyzed by TLC, a similar distribution of LPA, phosphatidic acid, and diacylglycerol was observed in samples from wild type and mtGPAT null mice (Fig. 1*B*). We reasoned that the GPAT activity still present in mitochondria from the knockout must be an NEM-sensitive enzyme. To confirm this hypothesis, liver mitochondria from wild type and mtGPAT null mice were assayed for GPAT activity with increasing concentrations of NEM (Fig. 2). GPAT activity in wild type mitochondria was stable in the presence of 0.4 mM NEM as previously reported (2); however, as little as 0.05 mM NEM inhibited 75% of activity in mitochondria from mtGPAT$^{-/-}$ mice. These data clearly distinguish GPAT activity in wild type and knockout animals and show that the GPAT activity in liver mitochondria from mtGPAT null mice is NEM-sensitive, similar to the microsomal GPAT (Table II).

Mitochondria from mtGPAT$^{-/-}$ Mice Contain a GPAT Immunoreactive Protein—Liver mitochondria from the mtGPAT$^{-/-}$ mice contained an anti-mtGPAT immunoreactive protein with a molecular mass (90 kDa) similar to that of mtGPAT (Fig. 3*A*). The mitochondria were not contaminated with endoplasmic reticulum (Table I), and no immunoreactive protein was detected in the microsome fraction (Fig. 3*A*), consistent with previous reports that the microsomal and mitochondrial GPAT isoforms are distinct (1, 2, 11) and that their amino acid sequences are very different. A two-dimensional Western blot analysis was performed in order to distinguish the anti-GPAT immunoreactive proteins in wild type and mtGPAT$^{-/-}$ liver mitochondria. The immunoreactive protein detected in mtGPAT knockout mouse liver had a different isoelectric point than the protein detected in wild type mice (Fig. 3*B*). In the *upper panel*, two major bands (labeled *1* and *2*) and several minor bands were detected in a mixed sample of liver mitochondria from wild type and mtGPAT null mice. In samples containing liver mitochondria from mtGPAT$^{-/-}$ mice (*lower panel*), band 1 (presumably mtGPAT) is absent, whereas band 2 remains. Since the antibody used was raised against full-length recombinant rat mtGPAT protein, we suspected that the novel immunoreactive protein was a novel GPAT isoenzyme with considerable amino acid sequence similarity to mtGPAT.

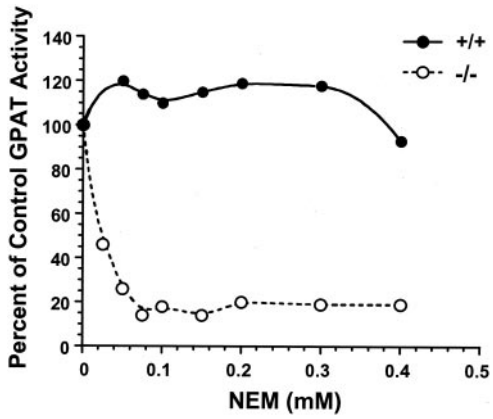

FIG. 2. **GPAT activity in mtGPAT$^{-/-}$ liver mitochondria is NEM-sensitive.** GPAT-specific activity was determined in liver mitochondria from wild type (●) and mtGPAT knockout (○) mice. Samples were preincubated on ice at the indicated NEM concentrations for 15 min. The GPAT assay was performed at room temperature with 20 μg of protein. Each value represents the average of two determinations. GPAT-specific activities at 0 mM NEM (100%) were 0.80 nmol/min/mg for wild type and 0.50 nmol/min/mg for mtGPAT knockout liver mitochondria. Data are from a representative experiment repeated three times.

Kinetic Characterization of GPAT Activity in GPAT$^{-/-}$ Liver Mitochondria—The NEM sensitivity of GPAT activity in knockout liver mitochondria indicated the presence of a new GPAT isoenzyme. To further characterize this isoenzyme, we determined the K_m for the two substrates, glycerol 3-phosphate (G3P) and acyl-CoA. The apparent K_m for G3P in wild type mitochondria was 400 μM (Fig. 4*A*) and in the same range (670 μM) as previous studies of purified recombinant mouse mtGPAT (7). The apparent K_m for G3P in mitochondria from the mtGPAT null mice was similar (300 μM). Microsomal GPAT had an apparent K_m for G3P of 140 μM (Fig. 4*B*).

Palmitoyl-CoA is the preferred substrate for mtGPAT (2); unsaturated fatty acyl-CoA species, like oleoyl-CoA, give a maximum velocity that is 2–9-fold lower (3–6). The only comparative study of mouse mtGPAT showed that recombinant enzyme purified from insect cells had a 2-fold preference for C16:0-CoA compared with C18:1 at 25 μM (7). In mtGPAT$^{-/-}$

TABLE II

Mitochondrial GPAT activity from wild type and mtGPAT$^{-/-}$ mice responds differently to temperature, NEM, polymixin B, acetone, and salts

GPAT-specific activity was determined in liver mitochondria from wild type (+/+) and mtGPAT knockout (−/−) mice and in microsomes from wild type (+/+) mice. All values represent the average of duplicates from a representative experiment repeated at least twice. Control activities in the different preparations ranged from 2.5–7 nmol/min/mg for microsomes, 0.7–1.5 nmol/min/mg for wild type mitochondria, and 0.3–0.9 nmol/min/mg for knockout mitochondria.

Assay modifications		Percentage of control GPAT-specific activity		
		Mitochondria[a]		Microsomes[a] (+/+)
		+/+	−/−	
pH	7–7.5	100	100	100
$MgCl_2$	4 mM[b]	100	100	100
Bovine serum albumin	1 mg/ml[b]	100	100	100
NaF	8 mM[b]	100	100	100
DTT	1 mM[b]	100	100	100
NaCl	125 mM	100	100	ND[d]
KCl	125 mM	100	100	ND
Oleoyl-LPA	200 μM	13	20	ND
Palmitoyl-LPA	100 μM	24	23	ND
40.5 °C	4 min[c]	79	39	72
NEM	0.075 mM	114	14	7
Acetone	20 μl	195	95	ND
$CaCl_2$	1 mM	70	33	ND
EDTA	1 mM	90	90	171
EGTA	1 mM	90	61	151

[a] Mitochondrial and microsomal fractions were highly purified (Table I).
[b] The indicated concentrations gave the highest NEM-sensitive and NEM-resistant GPAT-specific activities.
[c] Preincubation time.
[d] ND, not determined.

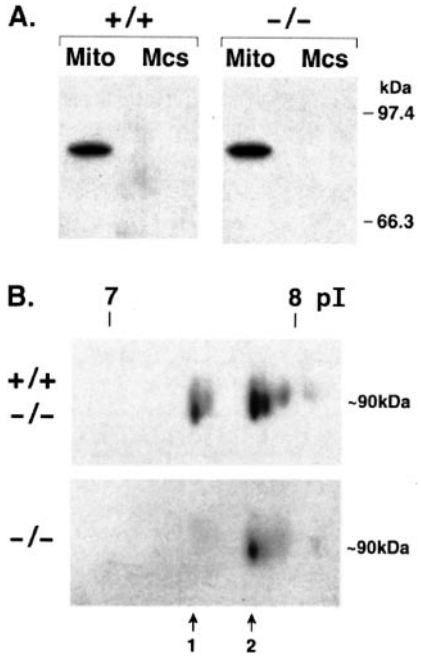

FIG. 3. **Liver mitochondria from mtGPAT$^{-/-}$ mice contain a novel anti-GPAT immunoreactive protein.** Liver mitochondria and microsomes were isolated from wild type and mtGPAT null mice. *A*, protein (100 μg) was analyzed by Western blot with mtGPAT antiserum. *Lanes 1* and *2*, wild type mitochondria and microsomes, respectively. *Lanes 3* and *4*, knockout mitochondria and microsomes, respectively. Molecular mass markers are indicated on the *right side*. *B*, mitochondrial proteins (1 mg) were first subjected to isoelectric focusing and then separated by SDS-PAGE and immunoblotted with mtGPAT antiserum. *Upper panel*, 1:1 mixture of mitochondria from wild type and mtGPAT$^{-/-}$ mice. *Lower panel*, mitochondria from mtGPAT$^{-/-}$ liver only. pI is indicated at the *top*, calculated molecular mass is indicated on the *right*, and *arrows 1* and *2* indicate the position of major species recognized by the mtGPAT antibody.

liver mitochondria, no difference in apparent V_{max} was observed between C18:1- and C16:0-CoA substrates, but wild type liver mitochondria showed a 30% preference for C16:0-CoA as compared with C18:1-CoA at 75 μM (Fig. 4*C*). In wild type and mtGPAT$^{-/-}$ mice, the apparent K_m values for C16:0- and C18:1-CoA could not be calculated because of inhibition at concentrations higher than 75 μM.

The lack of a preference for C16:0-CoA for activity from mtGPAT null mitochondria closely resembles that of the microsomal GPAT isoenzyme (2). Because the microsomal GPAT from rat fat cells and liver also acylates DHAP, which acts as a competitive inhibitor (19, 21), we measured the ability of DHAP to inhibit GPAT activity. Increasing concentrations of DHAP inhibited [^{3}H]G3P acylation in mitochondria from mtGPAT$^{-/-}$ liver but not in wild type (Fig. 5*A*). The K_i for DHAP was calculated to be 545 μM (Fig. 5*B*), similar to previous reports for rat microsomal GPAT (19, 23). Mouse liver microsomal GPAT activity was not inhibited by DHAP at 1.75 mM when 800 μM [^{3}H]G3P was present in the assay (Fig. 5*A*), but at 200 μM [^{3}H]G3P, both mouse and rat liver microsomal GPAT were inhibited 30% (data not shown), similar to previous assays that used lower G3P concentrations (19). Thus, we conclude that DHAP is a competitive inhibitor of the novel GPAT activity present in knockout liver mitochondria. The susceptibility to inhibition by DHAP is similar to microsomal GPAT, whereas in wild type mitochondria, DHAP does not inhibit mtGPAT activity.

GPAT Activity in mtGPAT$^{-/-}$ Liver Mitochondria Is Temperature-sensitive, Inhibited by Polymixin B, and Unresponsive to Acetone Activation—Microsomal and mtGPAT activities are differently affected by temperature, polymixin B, acetone, salts, and chelators (2), so we examined the effects of these on mitochondrial GPAT activities from wild type and GPAT$^{-/-}$ liver mitochondria (Table II). GPAT activities in microsomes, wild type mitochondria, and knockout mitochondria were maximal under similar assay conditions (pH 7–7.5, 4 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 8 mM NaF, and 1 mM DTT). The addition of monovalent salts (NaCl, KCl) and chelators (EDTA, EGTA) did not differently affect GPAT activities. Malonyl-CoA, a potent inhibitor of carnitine palmitoyl transferase 1, did not inhibit or activate GPAT activity in wild type or knockout mitochondria. LPA, the product of the GPAT reaction, inhibits GPAT activity equally well in mitochondria from wild type and mtGPAT$^{-/-}$ mice, similar to previous reports (24).

Since microsomal GPAT activity is inhibited 50% after preincubation for 7 min at 40.5 °C (19), we tested the temperature

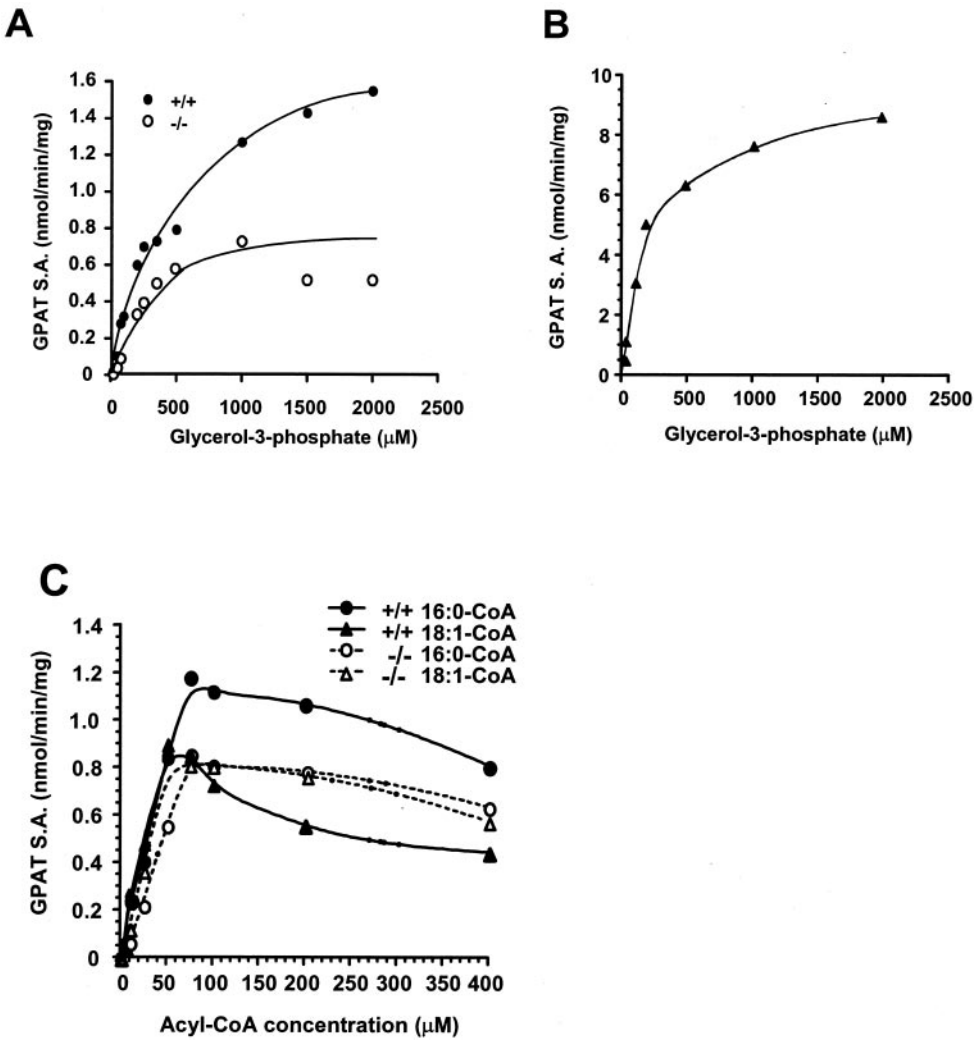

FIG. 4. **GPAT dependences on glycerol 3-phosphate, palmitoyl-CoA, and oleoyl-CoA in wild type and mtGPAT$^{-/-}$ liver.** GPAT-specific activity was determined in liver mitochondria (*A*) and microsomes (*B*) from wild type (●) and mtGPAT knockout (○) mice. The glycerol 3-phosphate concentration varied as indicated. Palmitoyl-CoA was held constant at 80 μM. The assay was initiated with 20 μg of protein. *C*, GPAT-specific activity was determined in liver mitochondria from wild type (*solid symbols*) and mtGPAT knockout (*open symbols*). Each reaction mixture contained 20 μg of protein and palmitoyl-CoA (●) or oleoyl-CoA (▲) concentrations as indicated. Glycerol 3-phosphate was held constant at 800 μM. Data are from representative experiments repeated three times.

sensitivity of mtGPAT. In contrast to GPAT activity in wild type mitochondria, GPAT activity from mtGPAT$^{-/-}$ liver mitochondria was sensitive to heating, with 80% of the glycerol-3-phosphate acyltransferase activity lost after incubation for 4 min at 40.5 °C (Fig. 6*A*, Table II). Polymixin B, an antibiotic that interacts with membranes, inhibits microsomal GPAT and activates mtGPAT (25, 26). In liver mitochondria from GPAT$^{-/-}$ mice, 0.1 mg/ml Polymixin B inhibited GPAT activity 80%, similar to microsomal GPAT, whereas mtGPAT from wild type mice was only inhibited 20% (Fig. 6*B*, Table II). In contrast to previous reports, we did not observe activation of wild type mtGPAT, because our assay contained 10-fold lower concentrations of polymixin B (25, 26). Because both elevated temperature and polymixin B perturb the phospholipid environment (27), we cannot rule out the possibility that the membrane composition of wild type and knockout liver mitochondria might differ enough to alter sensitivity to temperature and polymixin B (8).

Additional differences between GPAT activity in wild type and knockout liver mitochondria were observed with the addition of acetone or $CaCl_2$ to the acyltransferase assay. Previous studies have reported that 5–10% acetone in the assay mix increases rat mtGPAT activity 200%, whereas the microsomal activity is inhibited or unaffected (2). Acetone increased GPAT activity 195% in wild type mitochondria, but no change was observed in mitochondria from mtGPAT$^{-/-}$ mice (Fig. 6*C*). The addition of 1 mM $CaCl_2$ inhibited GPAT activity in mitochondria from wild type and mtGPAT null mice, 33 and 70%, respectively (Fig. 6*D*). These data further strengthen our hypothesis that a novel GPAT isoenzyme is present in liver mitochondria from mtGPAT null mice.

DISCUSSION

GPAT is a critical enzyme in glycerolipid synthesis because it catalyzes the initial and committed step required for the formation of triacylglycerol and all of the glycerophospholipids. Historically, two distinct GPAT isoenzymes have been identified, an NEM-sensitive isoform present in the endoplasmic reticulum membrane and an NEM-resistant enzyme present in the outer mitochondrial membrane. It now appears that an additional mitochondrial GPAT isoform exists, bringing the total to three. We propose the following nomenclature for the GPAT isoenzymes: erGPAT for the microsomal enzyme, mtGPAT1 for the originally cloned mitochondrial GPAT, and mtGPAT2 for the new activity characterized in this paper.

Southern and Northern analyses showed absence of the tar-

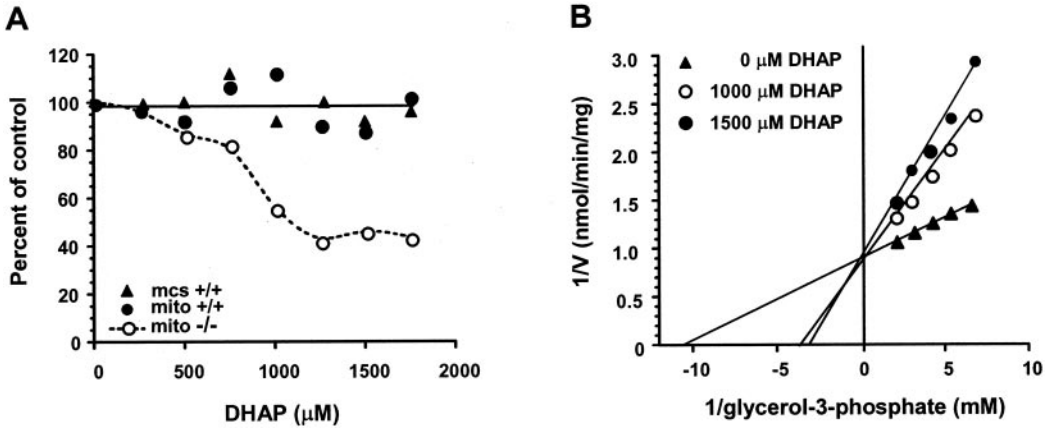

FIG. 5. **DHAP inhibits GPAT activity in mtGPAT$^{-/-}$ liver mitochondria.** *A*, liver microsomes (▲) and mitochondria from wild type (●) and mtGPAT knockout (○) mice were assayed at 800 μM [^{3}H]G3P with increasing concentrations of DHAP (0–1750 μM) as indicated. GPAT-specific activities at 0 μM DHAP (100%) were 3.2 nmol/min/mg for microsomes, 1.6 nmol/min/mg for wild type mitochondria, and 1.1 nmol/min/mg for knockout mitochondria. Data are from a representative experiment repeated three times. *B*, liver mitochondria from mtGPAT$^{-/-}$ mice were assayed with increasing concentrations of [^{3}H]G3P and fixed concentrations of DHAP, 0 μM (▲), 1000 μM (○), and 1500 μM (●). Data are from a representative experiment repeated three times.

geted mtGPAT in the mtGPAT$^{-/-}$ mice, and the phenotype of these animals included decreases in liver TAG and alterations in the acyl composition of the glycerophospholipids (8). However, when we assayed liver mitochondria and microsomes from mtGPAT1$^{-/-}$ mice, we discovered that unlike mitochondria from wild type liver, the knockout liver mitochondria contained an NEM-sensitive GPAT activity (mtGPAT2) (Fig. 2). The existence of an NEM-sensitive GPAT in knockout liver mitochondria coincides with the presence of an anti-mtGPAT immunoreactive protein (mtGPAT2) with a similar molecular mass (90 kDa), but a different isoelectric point than GPAT from wild type liver mitochondria (Fig. 3). It is not surprising that the rat mtGPAT antiserum recognized another glycerol-3-phosphate acyltransferase, since the antibody was raised against full-length recombinant rat liver mtGPAT, and GPAT and 1-acyl-glycerol acyltransferase isoforms share a high degree of amino acid similarity, although the low molecular masses of the 1-acyl-glycerol acyltransferases (30–40 kDa) preclude misidentification of these isoforms (9). In a similar fashion, antibody raised against full-length rat long-chain acyl-CoA synthetase-1 recognizes three isoforms, acyl-CoA synthetase-1, -4, and -5 (28). The mtGPAT antiserum did not detect any protein in liver microsomes (Fig. 3*A*), suggesting that the erGPAT differs significantly from the mitochondrial isoforms. We believe that mtGPAT2 is compensatorily up-regulated in mtGPAT1 null mice, because the appearance of a new anti-mtGPAT1 immunoreactive protein coincides with the detection of a mitochondrial NEM-sensitive GPAT activity whose characteristics are distinct from those of mtGPAT1.

An alternative explanation for these data would be that mtGPAT has two splice variants, that our targeting construct eliminated only one of the variants, and that the increased GPAT activity in mtGPAT1$^{-/-}$ liver mitochondria is due to a compensatory increase in the amount of the alternate mRNA. We do not believe this is the case, because Northern analyses (8), performed with probes that encompassed 53% of the open reading frame including two critical motifs that have been proven essential for acyltransferase activity (20), failed to detect any message in mtGPAT1$^{-/-}$ mice. In addition, the enzymatic characteristics of GPAT in knockout liver mitochondria differ considerably from wild type.

Although mtGPAT2 must contain one or more epitopes recognized by antibodies raised against mtGPAT1, its enzymatic properties differ substantially from those of mtGPAT1. mtGPAT2 activity (measured in liver mitochondria from mtGPAT1-deficient mice) is inhibited by NEM, clearly differentiating this activity from the NEM-resistant mtGPAT1. In addition, these data refute the possibility that mtGPAT2 is a reported inner mitochondrial membrane GPAT, because this (as yet uncloned) GPAT activity was NEM-resistant (29).

Further attributes also distinguish mtGPAT2 from mtGPAT1 and other acyltransferases. mtGPAT2 uses saturated and unsaturated acyl-CoAs equally well and is competitively inhibited by DHAP, properties exhibited by erGPAT but not mtGPAT1. Also, unlike mtGPAT1, mtGPAT2 is temperature-sensitive, inhibited by polymixin B, and not stimulated by acetone. Although the properties of mtGPAT2 are reminiscent of erGPAT, the activity cannot reflect the microsomal isoform, because our mitochondrial fractions contained less than 4% of the endoplasmic reticulum marker NADPH cytochrome *c* reductase (Table I), and the immunoblot analysis showed no anti-mtGPAT immunoreactive protein in microsomal fractions under any conditions (Fig. 3*A*). In addition, mtGPAT2 activity is not likely to be a previously described mitochondrial DHAP acyltransferase activity, because this (uncloned) activity is NEM-resistant (30). Taken together, the appearance of a unique anti-mtGPAT immunoreactive protein in mtGPAT$^{-/-}$ liver mitochondria corresponds with a distinct GPAT activity, similar in its enzymatic properties to those of erGPAT, but not detectable in wild type mitochondria.

Identification of a second mtGPAT isoform may shed some light on previously reported discrepancies in GPAT protein expression *versus* activity. Previous studies in rats show that, compared with liver and adipose tissue, mtGPAT protein expression is high in heart and adrenal gland but that NEM-resistant GPAT activity is low (20). It is likely that mtGPAT2 is expressed in these tissues and recognized by the antibody. An NEM-sensitive mtGPAT2 activity in mitochondria would not have been detected, because (*a*) activity was assayed in a total membrane preparation, and (*b*) the GPAT activity was measured in the presence of NEM (8). To determine whether the heart and adrenal proteins are really mtGPAT2 will require measurement of NEM-sensitive and -resistant GPAT activity in highly purified mitochondrial and microsomal fractions from each tissue. Although the function of mtGPAT2 is not known at this juncture, it is clearly distinct from mtGPAT1, because mtGPAT1$^{-/-}$ mice weigh less and have reduced gonadal fat pad weights, lower hepatic TAG content, lower plasma TAG, low density lipoprotein TAG, and decreased hepatic TAG secretion (8). Since the novel mtGPAT2 cannot sub-

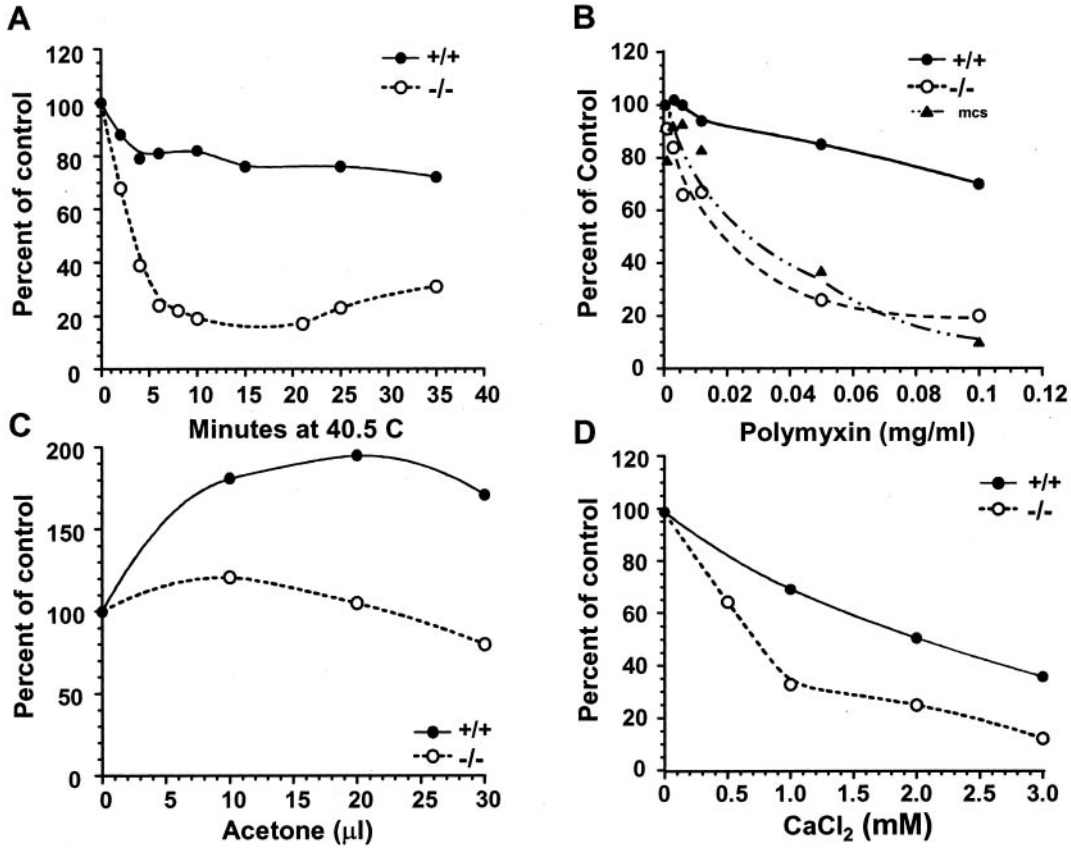

FIG. 6. **GPAT activity in mtGPAT$^{-/-}$ liver mitochondria is temperature-sensitive, inhibited by polymixin B, and unresponsive to acetone activation.** *A*, liver mitochondria from wild type (●) and mtGPAT knockout (○) mice were preincubated at 40.5 °C for the time indicated. The GPAT assay was performed at room temperature using 20 μg of protein. GPAT-specific activities at time 0 (100%), were 0.78 nmol/min/mg for wild type and 0.32 nmol/min/mg for mtGPAT knockout liver mitochondria. Data are from a representative experiment repeated three times. *B*, liver microsomes (▲) and mitochondria from wild type (●) and mtGPAT knockout (○) mice were preincubated with the indicated concentrations of polymixin B. The assay was initiated with 20 μg of protein. GPAT-specific activities at 0 mg/ml (100%) were 2.3 nmol/min/mg for microsomes, 1.4 nmol/min/mg for wild type mitochondria, and 0.8 nmol/min/mg for knockout mitochondria. Data are from a representative experiment repeated twice. *C*, liver mitochondria from wild type (●) and mtGPAT knockout (○) mice were assayed with increasing amounts (5–30 μl or 2.5–15% of the 200-μl assay mix) of acetone as indicated. GPAT-specific activities at 0 μl of acetone (100%) were 0.79 nmol/min/mg for wild type and 0.55 nmol/min/mg for mtGPAT knockout liver mitochondria. Data are from a representative experiment repeated twice. *D*, liver mitochondria from wild type (●) and mtGPAT knockout (○) mice were assayed with increasing concentrations (0.5–3 mM) $CaCl_2$ as indicated. GPAT-specific activities at 0 mM $CaCl_2$ (100%) were 0.70 nmol/min/mg for wild type 0.60 nmol/min/mg for mtGPAT knockout liver mitochondria. Data are from a representative experiment repeated twice.

stitute for the ability of mtGPAT1 to synthesize TAG in fat cells and hepatocytes, we propose that mtGPAT2 may function in catalyzing the initial step in the synthesis of mitochondrial phospholipids (see below). This hypothesis is consistent with the presence of mtGPAT2 activity in wild type and mtGPAT1$^{-/-}$ heart,[2] a tissue rich in mitochondria.

Since none of the unique properties of mtGPAT2 (NEM sensitivity, temperature sensitivity, lack of acetone stimulation, and DHAP inhibition) were observed in wild type liver mitochondria, it appears that mtGPAT2 may be up-regulated in mtGPAT1$^{-/-}$ mouse liver, possibly to maintain a critical level of mitochondrial glycerolipids. mtGPAT1 is highly homologous to the sole bacterial GPAT (PlsB) (9, 20). By extension from the immunoblot data (Fig. 1), mtGPAT2 is also likely to be similar to PlsB, which initiates the synthesis of the phospholipids prominent in mitochondria (phosphatidylglycerol, cardiolipin, and phosphatidylserine). Many mitochondrial proteins have a bacterial origin, and mtGPAT1 and -2 are both probably derived from *plsB*. Functionally, mtGPAT1 contributes to the synthesis of TAG, a product absent from most bacteria; therefore, mtGPAT2 could be important for synthesizing mitochondrial phospholipids, similar to PlsB. Full-length mtGPAT1 protein is 21% identical to *Escherichia coli* PlsB, and mtGPAT amino acid residues 153–444, which contain the motifs required for substrate binding and catalysis (9), is 42% identical to *E. coli* PlsB (14). A BLAST search of the mouse data base using acyltransferase homology regions I–IV (20) reveals the presence of many potential acyltransferases, one of which may be mtGPAT2. Several glycerolipid and cholesterol ester synthetic enzymes (acyl-CoA:cholesterol acyltransferases 1 and 2, 1-acyl-glycerol acyltransferases 1–6, cytidylyltransferases α and β, diacylglycerol acyltransferases 1 and 2, monoacylglycerol acyltransferase 1–3, phosphatidylethanolamine methyltransferases 1 and 2, and phosphatidylserine synthases 1 and 2) have two or more isoforms that probably have distinct functions (9, 31–45). Although characterization of mtGPAT2 function awaits the completion of molecular cloning, it is fascinating to contemplate that endoplasmic reticulum phospholipid synthesis, mitochondrial phospholipid synthesis, and TAG synthesis may be initiated by distinct GPAT isoenzymes.

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[2] T. M. Lewin, unpublished data.

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