Purification and Reconstitution of Murine Mitochondrial Glycerol-3-phosphate Acyltransferase. Functional Expression in Baculovirus-Infected Insect Cells†

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Received January 13, 1995; Revised Manuscript Received March 29, 1995⊗

ABSTRACT: Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the intial step in glycerolipid biosynthesis. We recently cloned a cDNA to a 6.8-kb mRNA, a message that can be induced dramatically by feeding a high-carbohydrate diet [Paulauskis & Sul (1988) *J. Biol. Chem. 263*, 7049−7054; Shin et al. (1991) *J. Biol. Chem. 266*, 23834−23839], and identified the open reading frame, p90, as mitochondrial GPAT [Yet et al. (1993) *Biochemistry 32*, 9486−9491]. To initiate characterization of mitochondrial GPAT, we purified and reconstituted the GPAT activity using phospholipids after expressing functional enzyme in Sf9 insect cells. Infection with recombinant virus containing p90 sequence resulted in high levels of GPAT expression in mitochondria, compared to noninfected cells or cells infected with the reverse orientation insertion baculovirus. There was a dramatic increase in *N*-ethylmaleimide-resistant mitochondrial GPAT activity. The GPAT protein was not detectable by Western blot in noninfected Sf9 cells or in cells infected with the GPAT sequence in the reverse orientation. However, in cells infected with GPAT in the correct orientation, there was a dramatic increase in the GPAT protein that was readily detectable by Coomassie staining both in total extracts and in the mitochondrial fraction. To ease the purification, we next expressed GPAT as a polyhistidine fusion protein in insect cells. The polyhistidine tag did not interfere with targeting to mitochondria or with the catalytic activity of GPAT. After solubilization of the mitochondrial fraction with the nonionic detergent $C_{12}E_8$, we purified the GPAT fusion protein using a Ni^{2+} matrix column. The purified p90 protein was not enzymatically active, but the GPAT activity could be reconstituted by adding crude soybean phosphatidylcholine. Other phospholipids in decreasing order of effectiveness in reconstituting GPAT activity were phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine. Cardiolipin, a major mitochondrial membrane phospholipid, was least effective. Using GPAT expressed in mitochondria of the Sf9 insect cells, we determined the apparent K_m value for glycerol 3-phosphate to be 0.67 mM. When various fatty acyl-CoAs were compared as acyl donors, GPAT showed preference for saturated fatty acyl-CoAs from carbon lengths of 8 to 16 as substrate, and unsaturated fatty acyl-CoAs tested were only 20% as effective. We also demonstrated that the catalytic activity of GPAT was lost when a stretch of 78 amino acids (aa 250−327), a region that has sequence homology to *Escherichia coli* GPAT, was deleted. GPAT, expressed at a high level in mitochondria employing a baculovirus system, can be purified in a single step and will be useful in the future for the structure−function studies.

The initial step of glycerolipid synthesis is the acylation of the *sn*-1 hydroxyl of glycerol 3-phosphate to form 1-acyl-*sn*-glycerol 3-phosphate (lysophosphatidic acid) which is carried out by glycerol-3-phosphate acyltransferase (GPAT)[1] (EC 2.3.1.15). This step may be rate-limiting, and the partitioning of fatty acids between esterification and oxidation pathways is partly carried out by GPAT. GPAT, therefore, may play a pivotal role in the regulation of phospholipid and glycerolipid biosynthesis (Bell & Coleman, 1983). GPAT activity is thought to be under nutritional and hormonal regulation (Brindley, 1991) and has been reported to be low during starvation and to increase on insulin administration (Saggerson & Carpenter, 1987). However, it is not known whether the changes in GPAT activity are due to allosteric regulation, covalent modification, or changes in enzyme concentration. Reports suggest that GPAT activity may be regulated by phosphorylation−dephosphorylation mechanisms (Bell & Coleman, 1983; Nimmo, 1980).

There are two major forms of GPAT in mammalian tissues—mitochondrial and microsomal (Bell & Coleman, 1983). Mitochondrial GPAT has been postulated to traverse the outer membrane, whereas the microsomal enzyme faces the cytoplasm (Coleman & Bell, 1983; Hesler et al., 1985). The activity of mitochondrial GPAT, unlike that of microsomal GPAT, is known to be insensitive to sulfhydryl group reagents such as *N*-ethylmaleimide (NEM). This difference in NEM sensitivity has been used to distinguish the two isozymes (Bates et al., 1977; Coleman & Haynes, 1983).

† This work was supported by National Institutes of Health Grant DK 36264 and Juvenile Diabetes Foundation Grant 191510.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

[1] Abbreviations: GPAT, glycerol-3-phosphate acyltransferase; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SV40, simian virus 40; PCR, polymerase chain reaction; hRP, horseradish peroxidase; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ORF, open reading frame.

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Mitochondrial GPAT activity is relatively high in liver and found at equal levels in mitochondria and microsomes (Bremer et al., 1976). Liver mitochondrial GPAT activity is thought to be more sensitive to dietary and hormonal manipulations (Bates & Saggerson, 1979). In most tissues, however, the GPAT activity in microsomal fractions is about 10 times higher than that found in mitochondrial fractions (Bell & Coleman, 1983). The mitochondrial and microsomal fractions were used for characterization of the two isoforms. Mitochondrial GPAT was shown to prefer saturated to unsaturated fatty acyl-CoA as an acyl donor, whereas the microsomal enzyme showed no substrate specificity (Kelker & Pullman, 1979; Stern & Pullman, 1978). The predominance of saturated fatty acids at the *sn*-1 position in naturally occurring acylglycerols, in contrast to unsaturated fatty acids at the *sn*-2 position, has been postulated to be the result of the fatty acyl-CoA preference of mitochondrial GPAT (Brindley, 1991). Some of the tumor cell lines with very low mitochondrial GPAT activity contain phospholipids with a higher abundance of unsaturated fatty acids in the *sn*-1 position (Haldar et al., 1979). Mammalian GPATs, however, have not been successfully purified nor have their characteristics been compared in detail due to their membrane association and their low abundance in tissues.

While cloning specific genes which are induced in the livers of mice in a lipogenic state, we isolated cDNA sequences to an unidentified, 6.8-kb mRNA (Paulauskis & Sul, 1988). We showed that the 6.8-kb mRNA is regulated by nutrients and hormones at the transcriptional level (Shin et al., 1991). The 6.8-kb mRNA contains an open reading frame of 827 amino acids which we previously designated p90 (Shin et al., 1991). A homology search of GenBank revealed that p90 has 30% identity and 42% similarity to a 322 amino acid stretch of *Escherichia coli* GPAT (Lighter et al., 1983; Green et al., 1983). Subsequently, we positively identified p90 as the murine mitochondrial GPAT by correlating the increase in mitochondrial GPAT activity with that of p90 protein level in stably transfected CHO cells (Yet et al., 1993). By identifying p90 as the mitochondrial GPAT, we have made available the cDNA and amino acid sequence of the mitochondrial GPAT for studying this important enzyme.

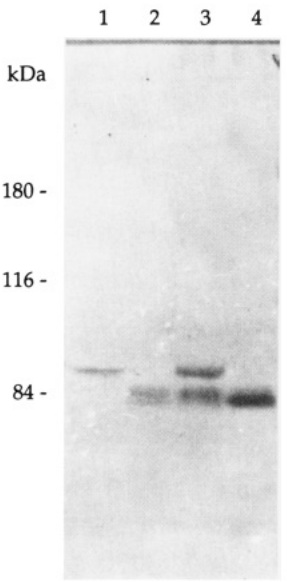
Here, we describe functional expression and characterization of mitochondrial GPAT in insect Sf9 cells infected with a recombinant baculovirus containing the complete p90 ORF. The expressed GPAT protein was targeted to mitochondria of the infected cells. We purified the p90 protein, demonstrated reconstitution of the GPAT activity with phospholipids, and determined the enzyme characteristics. We have also shown that a stretch of 78 amino acids (aa 250−327), with the highest sequence homology to *E. coli* GPAT, is necessary for catalytic activity of the mitochondrial GPAT. Our expression system may be utilized to study structure−function relationships employing *in vitro* mutagenesis in the future.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. The 2.87-kb GPAT *Eco*RV−*Fsp*I fragment from p3513 (Yet et al., 1993) was subcloned into the *Eco*RV site of the pcDNAI vector by blunt-end ligation to generate pcDNA.GPAT/C (correct orientation) and pcDNA.GPAT/R (reverse orientation) constructs. To make the deletion construct, plasmid pcDNA.GPAT/C was digested with *Nar*I, treated with mung bean nuclease, and digested with *Sma*I. The digested larger fragment was purified after agarose gel electrophoresis by Gene Clean (Bio 101). The eluted fragment was then religated to generate plasmid with a 234-bp in-frame deletion in the GPAT ORF. As a result of cloning, amino acid 328, Arg, was converted into Gly. Three constructs were generated for baculovirus expression. First, we inserted the GPAT ORF into pVL1392 and -1393 (PharMingen) in correct and reverse orientations, respectively (Gruenwald & Heitz, 1993). To make GPAT transfer constructs, we amplified by PCR the sequence from 930 to 1736 bp using p3513 as a template, 5′GCGGCCGCCTGCCATGGAGGAGTCT3′ (including a *Not*I site) as 5′ primer, and 5′TGAAAAAGCCCCCAAGC3′ as 3′ primer. The purified 800-bp fragment was inserted into a PCRII vector (Invitrogen). The resultant PCRII containing a GPAT 5′-coding sequence was digested with *Not*I and *Bam*HI present at multicloning sites flanking the insert and subsequently digested with *Nar*I. The 800-bp *Not*I−*Nar*I fragment was first purified by agarose gel electrophoresis followed by Gene Clean and was used to replace the 1000-bp *Not*I−*Nar*I fragment in GPAT sequence in pcDNA.GPAT/R. The largest *Not*I−*Nar*I fragment of the pcDNA.GPAT/R was ligated with the 800-bp *Not*I−*Nar*I fragment. The resultant plasmid was digested with *Not*I and *Bam*HI. The 2.4-kb *Not*I−*Bam*HI fragment containing GPAT sequence was then inserted into *Not*I and *Bam*HI-digested pVL1392 and pVL1393. We also inserted the GPAT sequence into the pAcSG.His NT vector (PharMingen) that contains six histidine residues at the N-terminus in-frame with the GPAT sequence. A polyhistidine-containing fusion protein was generated to ease the purification of the recombinant protein because polyhistidine fusion proteins bind with high affinity to metal chelate matrices. The 2.87-kb *Eco*RV−*Fsp*I fragment was excised from plasmid p3513 and purified after agarose gel electrophoresis using Gene Clean. pAcSG.His NT-B was digested with *Sma*I and purified after agarose gel using Gene Clean. The 2.87-kb GPAT *Eco*RV−*Fsp*I fragment and the *Sma*I-digested vector were blunt-end-ligated using T4 DNA ligase. The resultant plasmid with GPAT sequence inserted in the correct orientation was digested with *Nco*I. The largest *Nco*I fragment which contained 3′-GPAT and vector sequences was gel-purified and ligated with the 889-bp *Nco*I GPAT fragment. The 244-bp *Nco*I fragment that contained the 5′-noncoding region of the GPAT sequence was, thus, removed. GPAT was also inserted into pET14b (Novagen) for expression in *E. coli* (Studier et al., 1990). p3513 was digested with *Nco*I, filled-in using the Klenow fragment, and digested with *Xho*I. The 632-bp fragment corresponding to the 5′-GPAT sequence was then inserted into the pET14b, which was first digested with *Nde*I, treated with mung bean nuclease, digested with *Xho*I, and gel-purified. pET14b also contained a histidine tag for purification of the protein. Next, pcDNA.GPAT was digested with *Xho*I, and the 1872-bp 3′-GPAT sequence was gel-purified and inserted into *Xho*I site of the above pET14b containing 5′ 632 bp of the GPAT sequence.

DNA Sequencing. To verify correct insertion of the GPAT sequence into various vectors, regions of insertion sites were sequenced by double-stranded DNA sequencing of plasmids utilizing Sequenase (USB) and synthetic oligonucleotide primers by the chain termination method (Sanger et al., 1977).

A



B

	GPAT activity nmol/min·mg
NT	1.34
GPAT-p90	4.14
GPAT-Del	1.35

FIGURE 1: Western blot analysis of CMT cells transfected with GPAT, or GPAT with a 78 amino acid deletion. Mitochondrial fractions were prepared according to Experimental Procedures and subjected to Western blot (panel A). The SDS−PAGE was carried out longer than usual to clearly separate the deleted and wild-type GPAT. Antibodies against murine mitochondrial GPAT and horesradish peroxidase-conjugated rabbit anti-mouse IgG were used. Lane 1, murine mitochondrial fraction; lane 2, nontransfected CMT cell extract; lane 3, CMT cells transfected with the full-length GPAT; lane 4, CMT cells transfected with the GPAT deletion construct. Mitochondrial GPAT activity was also measured. Panel B shows comparison of mitochondrial GPAT activities from nontransfected control CMT cells or from cells transfected with full-length GPAT or mutated GPAT. The activity represents the average of duplicate determinations. Similar results were obtained from two separate experiments.

Expression of GPAT in CMT Cells. The pcDNAI contructs containing the SV40 origin of replication were transfected into CMT cells (Gerard & Gluzman, 1985), which are COS cells stably transformed with the SV40 large T antigen under the control of the mouse metallothionein promoter. We chose the CMT line for its inducible expression of SV40 large T antigen, which drives replication of pcDNAI, leading to high expression of the transfected gene. CMT cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% Serum Plus, that contains a low level of fetal bovine serum supplemented with proteins for growth, transport, and attachment (JRH Biosciences), and were split 1:10 every other day. Confluent plates of CMT cells were split 1:2 and transiently transfected by the DEAE-dextran method followed by chloroquine treatment (Ausubel et al., 1987). One day after transfection, cells were transferred to medium containing 100 μM $ZnSO_4$. Cells harvested 3 days after transfection and the extracts subjected to Western blot analysis and GPAT activity measurement.

Production of GPAT Using the Baculovirus System. All procedures involving Sf9 cell cultures, including routine subculturing, infections, and viral titering of supernatant, were performed as described by Summers and Smith (1987). The Sf9 cells (PharMingen), typically 3×10^6 cells, were seeded on a T25 flask in serum-free medium (Sf-900 II SFM, GIBCO/BRL); when cells attached, medium was changed to TMN-FH insect medium (PharMingen). The recombinant transfer vectors and lethally deleted linearized BaculoGold DNA (Pharmingen) were cotransfected into Sf9 insect cells to generate a recombinant baculovirus with a recombination efficiency of almost 100%. The resulting recombinant virus stock was further amplified in Sf9 cells for recombinant protein expression. The cells were typically harvested after 3 days and used for GPAT preparation.

SDS−PAGE and Western Blot Analysis. The cultured cells were homogenized in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF with 10 up-and-down strokes in a motor-driven Teflon−glass homogenizer at a moderate speed and centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 8000*g* for 10 min to pellet the mitochondrial fraction. The remaining supernatant which contained the microsomal fraction was used without further centrifugation. Both fractions were subjected to SDS−PAGE and electroblotted onto Immobilon PVDF membranes (Millipore) using 10 mM CAPS/10% methanol transfer buffer. For immunodetection of proteins, membranes were blocked for 1 h at room temperature in 1× NET (145 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100, and 50 mM Tris-HCl, pH 7.4) followed by incubation for 1 h at room temperature with primary antisera [antisera generation was described in Yet et al. (1993)]. Detection of the antigen−antibody complexes was accomplished via goat anti-rabbit IgG−hRP (Bio-Rad) conjugate and developed with 0.015% H_2O_2, 16% methanol, 8.3 mM Tris-HCl, pH 7.4, and 0.05% w/v 4-chloro-1-naphthol.

Purification and Reconstitution of GPAT. The infected Sf9 cells were homogenized as described above and centrifuged at 13000*g* for 10 min to pellet the membrane fraction. The membrane particles were solubilized by shaking in loading buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) containing 0.1% $C_{12}E_8$ (w/v) and 10% glycerol for 30 min and centrifuged at 13000*g* for 30 min. The supernatant was loaded on a Ni^{2+} matrix column (His.Bind, Novagen) and equilibrated with loading buffer containing 0.1% $C_{12}E_8$ and 10% glycerol. The column was washed with buffer (20 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) containing 10% glycerol. The enzyme was eluted with elution buffer (300 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) containing 10% glycerol. GPAT activity was reconstituted by addition of phospholipid vesicles. Before GPAT activity was measured, purified protein was added to phospholipid vesicles prepared as described by Haldar and Vancura (1992) and incubated for 10 min at 4 °C. Briefly, phospholipids (20 mg/mL, Sigma) in 10 mM Tris, pH 7.4, and 1 mM EDTA were sonicated 0.5 s with 0.5-s intervals for 6 min using Ultrasonics (Branson Ultrasonics). The samples were then centrifuged at 100000*g* for 13 min at 4 °C. The following phospholipids were used at increasing concentrations without additional detergent: phosphatidylethanolamine (contains primarily linoleic acid), phosphatidylinositol (contains primarily linoleic and palmitic acids), phosphatidylserine (crude preparation from bovine brain), cardiolipin (contains prima-

Table 1: GPAT Activity of Recombinant Virus-Infected Sf9 Cells[a]

		GPAT activity (nmol mg^{-1} min^{-1})	
		−NEM	+NEM
noninfected	mitochondria	5.48 ± 0.32	0.24 ± 0.03
	supernatant	0.98 ± 0.20	0.21 ± 0.14
reverse orientation	mitochondria	1.68 ± 0.26	0.47 ± 0.44
	supernatant	0.73 ± 0.72	0.84 ± 0.64
correct orientation	mitochondria	30.42 ± 0.24	30.92 ± 3.16
	supernatant	5.78 ± 0.07	4.38 ± 0.76
His.TAG-GPAT	mitochondria	43.90 ± 1.32	42.70 ± 1.71
	supernatant	1.26 ± 0.21	1.38 ± 0.38

[a] Sf9 cells were infected by recombinant baculovirus containing GPAT in reverse and correct orientation, and His.TAG-GPAT as described under Experimental Procedures. The cells were homogenized and centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 8000*g* for 10 min to separate the mitochondrial fraction. The mitochondrial fraction and supernatant fractions were assayed for GPAT activity in the presence or absence of 0.4 mM *N*-ethylmaleimide (NEM). Values are means ± SE of duplicate measurements from two independent experiments.

rily linoleic acid), and phosphatidylcholine (crude preparation from soybean).

Measurements of GPAT Activity. The assay mixture contained 75 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mg/mL bovine serum albumin (essentially fatty acid-free, Sigma), 8 mM NaF, 50 μM palmitoyl-CoA, 3 mM glycerol 3-phosphate, and 0.25−5 μCi of [^{14}C]glycerol 3-phosphate (157 mCi/mmol) (Amersham). The reaction was started by the addition of the enzyme and incubated for 10 min at 25 °C. To determine the mitochondrial GPAT activity from cultured cells, the mitochondrial fraction was preincubated with 0.4 mM *N*-ethylmaleimide for 15 min at 4 °C. The assay conditions were chosen so that GPAT activities were linear with time and rates were proportional to enzyme concentrations. The reaction mixture was extracted with 1-butanol, and the labeled lipids were counted as previously described (Haldar & Vancura, 1992). The protein concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

RESULTS AND DISCUSSION

Expression of GPAT in Mitochondria by Transient Transfection of CMT Cells. We previously reported p90 expression correlating with GPAT activity by stable transfection of CHO cells. However, the expression level was not high, and 2-aminopurine treatment for the increased expression of transfected protein was necessary to detect the GPAT protein by Western blot (Yet et al., 1993). Therefore, we utilized CMT cells (COS cells stably transformed with SV40 T antigen) for transient transfection in an attempt to establish a relatively rapid and high-level expression system. We also generated a plasmid containing a deletion of 78 amino acids, from amino acids 250 to 327 of the GPAT ORF; because there was no known structural information on this enzyme, we selected a region with the highest homology to *E. coli* GPAT. Our hypothesis was that the conserved region may serve an important role in catalysis. We then compared the mitochondrial GPAT activity of CMT cells transiently transfected with full-length and deleted GPAT. The cells transfected with either of the constructs expressed corresponding GPAT protein in mitochondria, indicating that the information for targeting does not reside in this region of deletion. As shown in the Western blot in Figure 1, the nontransfected CMT cells expressed an immunoreactive

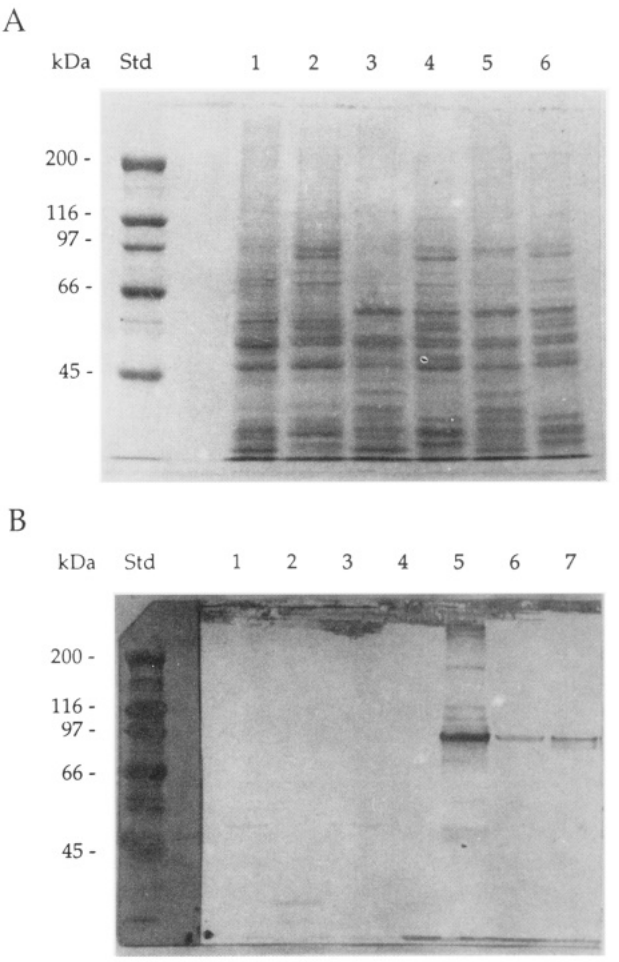


FIGURE 2: Expression of the murine mitochondrial GPAT in Sf9 insect cells infected with the recombinant baculovirus. Panel A: Insect cells, noninfected (lanes 1 and 2) or infected with either the reverse (lanes 3 and 4) or the correct orientation (lanes 5 and 6) of the GPAT ORF, were cultured as described under Experimental Procedures. The mitochondrial fractions (lanes 1, 3, and 5) and supernatant (lanes 2, 4, and 6) prepared from these cells were subjected to SDS−PAGE. Panel A shows Coomassie R250 staining, and panel B shows Western blot analysis carried out as described in Figure 1 and under Experimental Procedures. Lane 7 in panel B contains the control murine liver mitochondrial fraction.

protein in mitochondria. The protein was smaller than the murine GPAT by about 7 kDa. The protein may represent endogenous GPAT, but we do not have definitive identity of this smaller protein. Transfection of the full-length GPAT resulted in the expression of the p90 protein that was immunoreactive to the GPAT antibodies, with a size identical to that of the liver mitochondrial protein. In agreement with the increase in GPAT protein levels, there was a 3-fold increase in mitochondrial GPAT activity in CMT cells transfected with the full-length GPAT (Figure 1). However, when the mutated GPAT was transfected, although the protein was expressed in mitochondria, there was no concomitant increase in the GPAT activity in mitochondria of these cells. As predicted, the protein was smaller than the wild-type GPAT by 8 kDa. (The GPAT with an internal deletion was slightly smaller than the endogenous protein detected by GPAT antibodies in CMT cells.) These results indicate that removal of the stretch of 78 amino acids, a region with the highest homology to the *E. coli* GPAT, eliminates the catalytic activity of GPAT. This may indicate a potential catalytic site for the GPAT; it is also possible that, due to a large deletion, protein folding was altered and made the protein nonfunctional. We need a system where

Table 2: Purification of GPAT Expressed in Sf9 Cells Infected with His.TAG-GPAT[a]

	total protein (mg)	total activity (mol/min)	specific activity (nmol mg^{-1} min^{-1})	yield (%)	purification (fold)
membrane fraction	16.10	185.0	11.40	100.0	1.00
$C_{12}E_8$ extracts[b]	14.50	107.0	7.36	58.0	0.64
Ni^{2+} matrix-eluted[b]	0.05	9.6	192.00	5.2	16.80

[a] Membrane fraction preparation and purification steps are described under Experimental Procedures. [b] Crude phosphatidylcholine from soybean was added for reconstitution of GPAT activity.

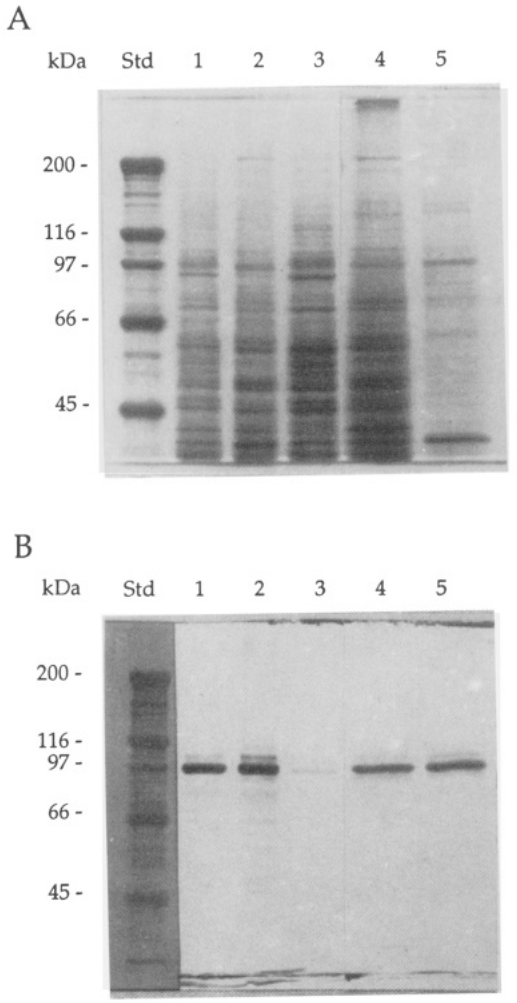


FIGURE 3: Expression of murine mitochondrial GPAT with the polyhistidine tag in Sf9 insect cells. Cells infected with His.TAG-GPAT recombinant baculovirus were grown, and the mitochondrial fraction was prepared. His.TAG-GPAT fusion protein was purified by Ni^{2+} matrix chromatography as described under Experimental Procedures. Total extract (lane 1), mitochondrial fraction (lane 2), supernatant (lane 3), the detergent-solubilized mitochondrial fraction that was subsequently loaded on the Ni^{2+} column (lane 4), and the eluted fraction from the Ni^{2+} column (lane 5) were subjected to SDS−PAGE. The polyacrylamide gel was either stained with Coomassie R250 or subjected to Western blot analysis as described in Figure 1 and under Experimental Procedures.

we can culture a large amount of cells and easily purify the expressed protein to examine the mitochondrial GPAT *in vitro*.

Functional Expression of GPAT in Insect Cells Using the Baculovirus System. In an attempt to establish high levels of expression that would permit purification of GPAT, we initially expressed mitochondrial GPAT in *E. coli* using the T7 RNA polymerase system. A high level of GPAT as a polyhistidine fusion protein was expressed in this system. The GPAT protein was present in inclusion bodies that could be solubilized with urea in the presence of reducing agents. We subsequently purified the GPAT protein to homogeneity

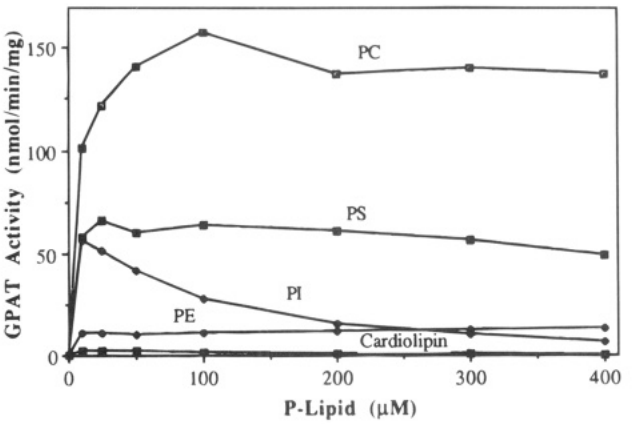


FIGURE 4: Comparison of various phospholipids for their ability to reconstitute GPAT activity. Phospholipid vesicles were prepared by sonication and incubated with purified protein for 10 min on ice before addition to mixture for GPAT enzyme activity measurements as described under Experimental Procedures. The GPAT activities were reconstituted with crude soybean phosphatidylcholine (PC), phosphatidylserine (PS) phosphatidylinositol (PI), phosphatidylethanolamine (PE), and cardiolipin. Essentially the same results were obtained in three separate experiments.

by subjecting the solubilized fraction to Ni^{2+} matrix chromatography (data not shown). However, we were unable to reconstitute the GPAT activity of this purified protein with phospholipids.

We, therefore, employed the baculovirus system to express functional GPAT in Sf9 insect cells. Because the GPAT ORF contains information for appropriate targeting to mitochondria, we predicted that GPAT would be expressed in the mitochondrial membrane of Sf9 insect cells, as is the case for the endogenous protein in murine hepatocytes. We hypothesized that targeting GPAT to the mitochondrial membrane might increase the probability of expressing functional enzyme. Initially we expressed GPAT as a nonfused protein under the polyhedrin promoter. The NEM-resistant GPAT activity was measured in extracts of Sf9 cells infected with the GPAT recombinant virus. Upon infection of the recombinant virus that contained the GPAT sequence in the correct orientation (4.77 ± 0.33 nmol mg^{-1} min^{-1}), there was approximately a 32-fold increase in GPAT activity as compared to the noninfected cells (0.15 ± 0.16 nmol mg^{-1} min^{-1}). However, infection of the recombinant virus containing the GPAT sequence in the reverse orientation (0.03 ± 0.01 nmol mg^{-1} min^{-1}) caused a 5-fold decrease in the activity as compared to the noninfected cells. This decrease may have been caused by significant expression of the antisense GPAT sequence from the reverse orientation plasmid. We separated by centrifugation crude mitochondrial fraction from supernatant that contained microsomal fraction. The GPAT activity present in these fractions was determined in the absence and presence of NEM. Mitochondrial GPAT is known to be resistant to this sulfhydryl agent while microsomal GPAT is not (Bates et al., 1977; Coleman & Haynes, 1983). As shown in Table 1, the GPAT activity in

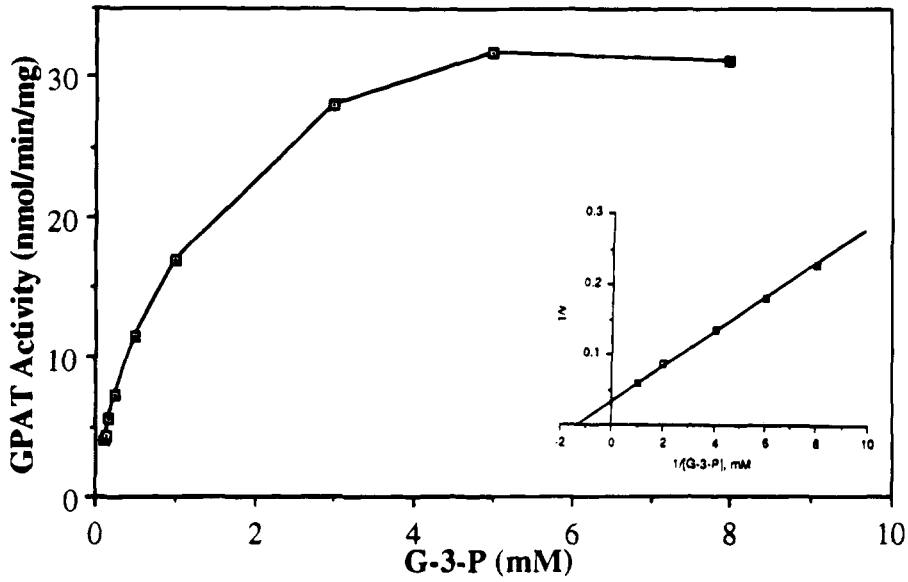


FIGURE 5: Glycerol 3-phosphate dependence of mitochondrial GPAT expressed in Sf9 insect cells. Initial velocities were determined as described under Experimental Procedures. Palmitoyl-CoA at 50 μM concentration was used in these assays. A double-reciprocal plot is shown in the inset. Essentially the same results were obtained in three separate experiments.

mitochondria from Sf9 cells, infected with recombinant virus containing the GPAT sequence, was NEM-resistant and increased 2 orders of magnitude when compared to that from cells infected with recombinant virus containing the GPAT sequence in the reverse orientation. The GPAT activity in the supernatant of cells infected with the GPAT sequence in the correct orientation was also increased somewhat. This is probably due to contaminating mitochondria in the supernatant, because we used the *8000g* supernatant and not the microsomal pellet. The probable mitochondrial contamination was further evidenced by the fact that the GPAT activity in the supernatant was NEM-resistant. We also subjected these fractions to SDS−PAGE (Figure 2). The 90-kDa GPAT protein was easily detected by Coomassie staining in Sf9 cells infected with GPAT in the correct orientation in the mitochondrial pellet, but not in the supernatant. The noninfected cells or cells infected with vector containing the GPAT sequence in the reverse orientation did not show the p90 protein. The identification of the GPAT protein was carried out by Western blot using antibodies we generated with the GPAT-TrpE fusion protein expressed in *E. coli*. These data clearly indicate that we have expressed functional mitochondrial GPAT in Sf9 cells at a high level, properly targeted to mitochondria.

Purification and Reconstitution of GPAT Expressed in Sf9 Cells. Because of its low abundance and membrane association, mitochondrial GPAT was not successfully purified and reconstituted. Our goal was to purify the protein in order to study characteristics of the purified enzyme *in vitro*. As shown in Figure 2, GPAT is readily detectable by Coomassie staining of the SDS−PAGE in insect cells infected with the GPAT recombinant baculovirus. To ease the purification of recombinant GPAT, however, we generated a construct that contains a 6-histidine tag in-frame with the GPAT protein, resulting in a polyhistidine fusion protein. After infection with the constructed recombinant baculovirus, the mitochondrial fraction from the infected Sf9 cells was prepared by differential centrifugation. The GPAT activity in the mitochondrial fraction from insect cells infected with the His.TAG-GPAT recombinant baculovirus was very high and NEM-resistant, similar to that shown in cells infected with the nonfusion GPAT baculovirus (Table 1). These results indicate that the His tag did not interfere with either the targeting to mitochondria or the catalytic activity of GPAT. The mitochondrial pellet was subsequently solubilized with *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), a nonionic detergent, and the supernatant was subjected to a Ni^{2+} matrix column. We originally employed Triton X-100 as previously used for *E. coli* GPAT to solubilize the mitochondrial pellet, but GPAT activity was lost during purification (data not shown) (Scheideler & Bell, 1986). We then tested $C_{12}E_8$, a detergent successfully used by Bell and co-workers for solubilization and reconstitution of *E. coli* GPAT (Scheideler & Bell, 1989). We found that solubilization of the mitochondrial fraction with $C_{12}E_8$ and inclusion of 10% glycerol during chromatography stabilized the GPAT activity. The summary of purification is presented in Table 2. There was a 40% decrease in the specific activity of GPAT when the mitochondrial pellet was solubilized with detergent. This may be due to the inhibitory effect of detergent on GPAT activity. A decrease in GPAT activity by various detergents such as Triton X-100, Lubrol, and Brij 35 and an increase in activity by subsequent removal of detergent were previously reported (Monroy et al., 1973; Green et al., 1981). It is also possible that addition of exogenous phospholipids does not fully restore GPAT activity. The yield of GPAT was only 10% from Ni^{2+} matrix chromatography. Nonetheless, as shown in Table 2, we were successful in purifying and reconstituting GPAT activity by addition of phospholipids. As compared to the specific activity of detergent extract, the specific activity of the eluted fraction from the Ni^{2+} matrix column increased approximately 25-fold, reaching 200 nmol mg^{-1} min^{-1}. We subjected various fractions during purification to SDS−PAGE. The p90 band, which was recognized by the antibodies against the mitochondrial GPAT by Western blot, was readily detectable by Coomassie staining in both the total extract and the mitochondrial fraction (Figure 3). The GPAT protein was a major band of the fraction eluted from the Ni^{2+} column, although another unidentified protein band of smaller molecular weight was present at a higher level (Figure 3). The fact that p90 was the only band whose level

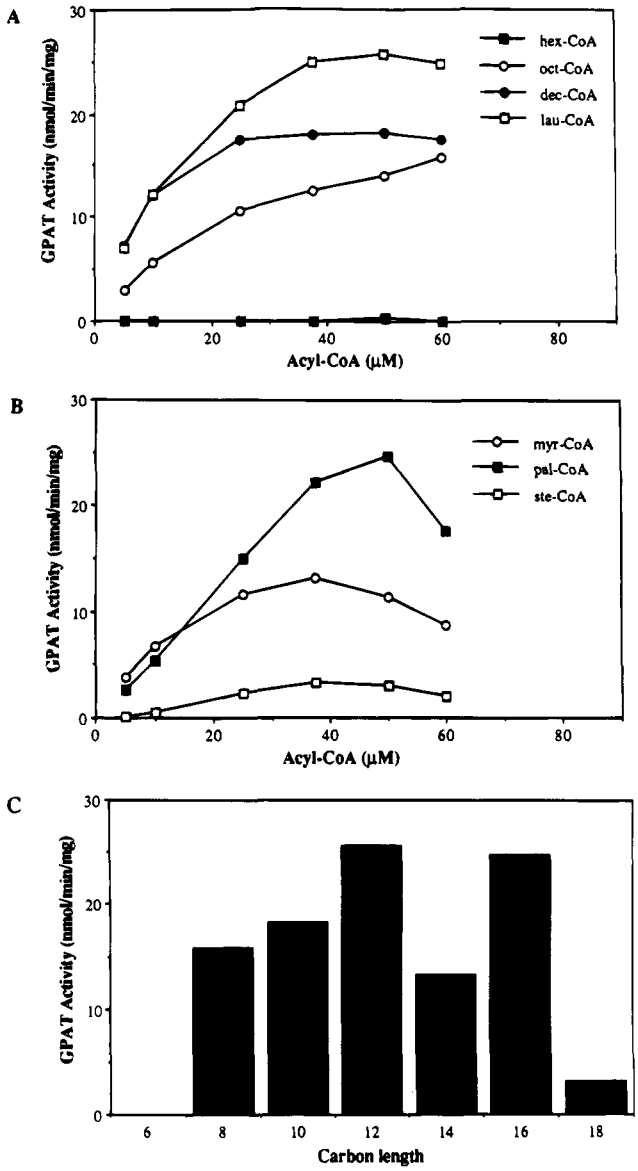


FIGURE 6: Comparison of various saturated fatty acyl-CoA as acyl donors in GPAT activity in Sf9 cells infected with the baculovirus containing the GPAT coding sequence. GPAT activity was measured using increasing concentrations of hexanoyl-CoA (■), laural-CoA (□), octanoyl-CoA (○), and decanoyl-CoA (●) in panel A and myristoyl-CoA (○), palmitoyl-CoA (■), and stearoyl-CoA (□) in panel B as substrates as described under Experimental Procedures. Panel C shows a comparison of maximal activity obtained with various saturated fatty acyl-CoAs. The values represent the average of duplicate measurements. Essentially the same results were obtained in three separate experiments.

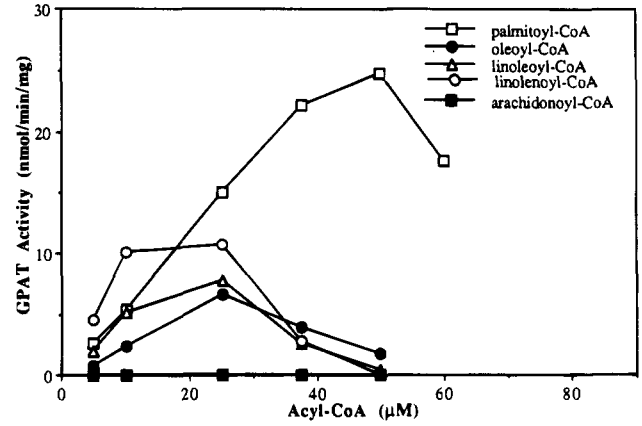


FIGURE 7: Effectiveness of various unsaturated fatty acyl-CoAs as acyl donors in GPAT activity expressed in Sf9 cells infected with the baculovirus containing the GPAT coding sequence. The GPAT activity was measured using increasing concentrations of oleoyl-CoA (●), linoleoyl-CoA (△), linolenoyl-CoA (○), and arachidonoyl-CoA (■) as substrate as described under Experimental Procedures. For comparison, GPAT activity obtained with palmitoyl-CoA (□) as a substrate was shown also. The values represent the average of duplicate measurements. Essentially the same results were obtained in two separate experiments.

was increased along with GPAT activity upon GPAT recombinant baculovirus infection further enforces the conclusion that the reconstituted GPAT activity observed by the addition of phospholipids was due to the p90 protein. An increase in the GPAT activity of the detergent-solubilized mitochondrial fraction by the addition of exogenous phospholipids was known previously. Here we provide definitive evidence of the reconstitution of the mitochondrial GPAT activity with p90 protein. Involvement of other protein components for reconstitution of GPAT is unlikely, because the protein was purified from insect cells in which the endogenous GPAT activity was extremely low and only the p90 was overexpressed by baculovirus infection. High expression level (2 orders of magnitude, as described above) and a single-step purification of the expressed GPAT, whose activity can be subsequently reconstituted, provide us an excellent system for studying this important enzyme in the phospholipid and triacylglycerol biosynthetic pathway.

Properties of the Mitochondrial GPAT Expressed in Insect Cells. The purified wild-type GPAT protein was employed to examine the enzyme characteristics. First, to determine the phospholipid specificity, we also compared various phospholipids in reconstitution of GPAT activity using the purified protein. The purified preparation was not enzymatically active, but GPAT activity was reconstituted by the addition of soybean phosphatidylcholine. As shown in Figure 4, phosphatidylserine and phosphatidylinositol were effective in reconstituting GPAT activity but to a lesser degree than crude phosphatidylcholine prepared from soybean. However, cardiolipin, a major phospholipid in mitochondrial membrane, was not effective in reconstituting GPAT activity to any appreciable level. In fact, it has been previously reported that caridolipin is a potent inhibitor of GPAT activity (Kelker & Pullman, 1979). Although less than 10% as effective compared to crude phosphatidylcholine, phosphatidylethanolamine yielded low GPAT activity when added to the reaction mixture in reconstituting GPAT activity. The order of effectiveness of phospholipids at comparable concentrations was crude soybean phosphatidylcholine > phosphatidylserine > phosphatidylinositol > phosphatidylethanolamine > cardiolipin. Further detailed studies are necessary for phospholipid and head group requirement in reconstituting the mitochondrial GPAT activity. The differences in reconstitution of GPAT activity shown here may simply demonstrate a requirement for a fluid phase bilayer and may not reflect the actual phospholipid specificity.

We also examined properties of GPAT using a mitochondrial fraction from the Sf9 cells infected with recombinant virus that contains the GPAT sequence. A broad pH optimum for the enzyme was observed, maximal activities being noted over a pH range from 6.0 to 8.0, without any dramatic decrease in activity (data not shown). The substrate specificities for glycerol 3-phosphate and fatty acyl-CoAs were determined. Figure 5 is a reciprocal plot of the reaction

velocity of GPAT at varying concentrations of glycerol 3-phosphate from 0.1 to 8 mM with 50 μM palmitoyl-CoA, at pH 7.4. The apparent K_m value for glycerol 3-phosphate was 0.67 mM. We then compared saturated fatty acyl-CoAs from 8 to 18 carbon length at varying concentrations of fatty acyl-CoA from 5 to 60 μM in the presence of saturating 3 mM glycerol 3-phosphate (Figure 6A,B). However, we could not determine the apparent affinity for fatty acyl-CoAs due to the inhibitory effects of fatty acyl-CoAs at higher concentrations. The maximal GPAT activity observed with various saturated fatty acyl-CoAs was compared in Figure 6C. Saturated fatty acyl-CoAs from carbon lengths of 8 to 16 were effective acyl donors for GPAT. However, GPAT activity was not detectable when a shorter hexanoyl-CoA was used. GPAT activity with a longer stearoyl-CoA as a substrate was less than 20% of that with palmitoyl-CoA. The effectiveness of various unsaturated fatty acyl-CoAs as acyl donors for GPAT activity was also examined. As shown in Figure 7, all of the unsaturated fatty acyl-CoAs, oleoyl-CoA, linoleoyl-CoA, linolenoyl-CoA, and arachidonoyl-CoA, were not efficient acyl donors, and the maximal GPAT activity with any of these unsaturated fatty acyl-CoAs was less than 30% of that obtained with palmitoyl-CoA. The substrate specificity determined with our preparation is similar to that reported with crude rat liver mitochondrial extracts. Overall, mitochondrial GPAT prefers saturated fatty acyl-CoA as a substrate (Monroy et al., 1973; Kelker & Pullman, 1979; Monroy et al., 1972). The preference for saturated fatty acyl donors was also demonstrated in *E. coli* GPAT, consistent with the known positional distribution of saturated fatty acids (Green et al., 1981). Although the endoplasmic reticulum is the principal site for the synthesis of glycerolipids and the mammalian microsomal GPAT is a widely distributed enzyme in a variety of tissues, microsomal GPAT does not show substrate preference (Stern & Pullman, 1978; Lands & Hart, 1965).

In summary, we purified and reconstituted murine mitochondrial GPAT for the first time by expressing functional enzyme using a baculovirus system. The expressed protein was targeted to the mitochondria of Sf9 cells. We purified the p90 protein using a Ni^{2+} matrix column and were able to reconstitute GPAT activity with phospholipids. We have also shown that a stretch of 78 amino acids that has sequence homology to *E. coli* GPAT was required for the catalytic activity of this enzyme. Infection of baculovirus containing GPAT sequence targets the protein to mitochondria at a high expression level that can be purified easily. During submission of this paper, purification of rat liver mitochondrial GPAT using several conventional purification steps was reported. Our purified preparation from baculovirus expression (this report) and the purified endogenous mitochondrial GPAT (Vancura & Haldar 1994) showed a similar specific activity of approximately 200 nmol mg^{-1} min^{-1} and faty acyl-CoA specificity. Both preparations showed high GPAT activity when phosphatidylcholine was used for reconstitution. The similar characteristics of the mitochondrial GPAT expressed in insect cells to the native enzyme clearly validate our system, and, unlike that from liver, the protein structure can be modified via *in vitro* mutagenesis. This provides an excellent system that can be employed in detailed structure–function studies in the future.

ACKNOWLEDGMENT

We thank Dr. Mu-En Lee (Harvard School of Public Health) for help with CMT cell transfection and careful reading of the manuscript. We also than Chung-Ming Hsieh for artistic work.

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BI950090Y