Expression and Identification of p90 as the Murine Mitochondrial Glycerol-3-phosphate Acyltransferase†

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Received February 9, 1993; Revised Manuscript Received June 1, 1993®

ABSTRACT: Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the initial and committed step in glycerolipid biosynthesis. Mitochondrial GPAT, unlike the microsomal isozyme, prefers saturated fatty acids as a substrate. We have recently reported cloning of a cDNA to an unidentified 6.8-kb mRNA by a differential hybridization. The mRNA contains an open reading frame of 827 amino acids (p90) with 30% sequence homology in a 300 amino acid stretch to *Escherichia coli* GPAT. The 6.8-kb mRNA was induced dramatically when fasted mice were refed a high-carbohydrate diet. Here, we have expressed the open reading frame as trpE fusion proteins and used them to generate antibodies. The antibodies recognized a polypeptide of 90 kDa (p90) when the 6.8-kb cDNA sequence was used for in vitro transcription and translation. By Western blot analysis using these antibodies, we detected p90 in mitochondrial fractions of liver, and the p90 level was increased by refeeding. The increase in the p90 level correlated with the increase in mitochondrial GPAT activity. Moreover, p90 was not detectable in 3T3-L1 preadipocytes but markedly increased during adipose conversion. This increase was consistent with the 11-fold increase we observed in *N*-ethylmaleimide (NEM)-resistant mitochondrial GPAT activity during adipocyte differentiation. In addition, we have expressed p90 in CHO cells by stable transfection. The transfected genes in both correct and reverse orientations produced distinct 3.9-kb transcripts owing to the truncation of a part of the noncoding regions of the endogenous 6.8-kb mRNA before insertion into the pMSXND vector. The transfected CHO cells were treated with 2-aminopurine, an agent that increases expression of exogenous genes. There was a 6-fold increase in the p90 level in mitochondria of the CHO cells transfected with the p90 sequence in the correct orientation, and the activity of the NEM-resistant mitochondrial GPAT also increased accordingly. Moreover, the mitochondrial GPAT of the transfected cells preferred palmitoyl CoA as a substrate over oleoyl CoA. The above correlation between the level of p90 expression and GPAT activity in mitochondria and the substrate specificity of the expressed enzyme show evidence that p90 is the murine mitochondrial GPAT. We have also demonstrated here that the changes in mitochondrial GPAT activity during different nutritional and developmental conditions are due primarily to the changes in enzyme concentration, and not to modulation of the catalytic activity of the existing enzyme.

The initial and committed step of glycerolipid synthesis is the acylation of *sn*-glycerol 3-phosphate to form 1-acyl-*sn*-glycerol 3-phosphate. The reaction is carried out by glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15). This step may be rate-limiting, and GPAT, therefore, plays a pivotal role in regulation of triacylglycerol and phospholipid biosynthesis (Bell & Coleman, 1983). However, mammalian GPAT has never been purified or characterized. 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). In most tissues, the GPAT activity in microsomal fractions is about 10 times higher than that found in mitochondrial fractions (Bell & Coleman, 1983). Although its functional role is unclear, mitochondrial GPAT activity is relatively high in liver and GPAT activity is found in equal levels in mitochondria and microsomes (Bremer et al., 1976). Unlike microsomal GPAT, the activity of mitochondrial GPAT is known to be insensitive to sulfhydryl group reagents, such as *N*-ethylmaleimide (NEM), and this difference in NEM sensitivity has been used to distinguish the two isozymes (Bates et al., 1977; Coleman & Haynes, 1983). It has also been reported that mitochondrial GPAT prefers saturated fatty acyl CoA over unsaturated fatty acids as an acyl donor, whereas the microsomal enzyme does not show this substrate specificity (Kelker & Pullman, 1979; Stern & Pullman, 1978). It has been postulated that the predominance of saturated fatty acids found in the sn-1 position in naturally occurring acylglycerols, in contrast to unsaturated fatty acids at the sn-2 position, is the result of this fatty acyl CoA preference of the mitochondrial GPAT.

The partitioning of fatty acids between esterification and oxidation pathways is partly carried out by glycerol-3-phosphate acyltransferase, and GPAT activity is thought to be under nutritional and hormonal regulation (Brindley, 1991). GPAT activity has been reported to be low during starvation and increased by insulin administration (Saggerson & Carpenter, 1987). Moreover, liver mitochondrial GPAT activity was thought to be influenced more by the dietary and hormonal manipulations (Bates & Saggerson, 1979). However, it is not known whether the changes in mitochondrial GPAT activity are due to the allosteric regulation, covalent modification, or changes in enzyme concentration. GPAT is also under developmental control in that GPAT activity, as other enzymes involved in lipid metabolism, has been reported to increase during adipocyte differentiation (Coleman et al., 1978; Grimaldi et al., 1978; Kuri-Harcuch & Green, 1977). The increase was reported to be mainly in NEM-sensitive mi-

† 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*, September 1, 1993.

0006-2960/93/0432-9486$04.00/0

crosomal GPAT (Coleman et al., 1978). Reports suggest that microsomal GPAT activity may be regulated by phosphorylation–dephosphorylation mechanisms (Bell & Coleman, 1983; Nimmo, 1980). Neither the characterization of GPAT nor the mechanisms underlying regulation of GPAT activity have been studied due to the lack of the purified mammalian GPATs. Purification of mammalian GPATs proved to be very difficult because of their membrane association and the hydrophobic nature of the acyl CoA substrate and product. The structural gene for *Escherichia coli* GPAT (*plasB*), on the other hand, has been identified, and its 83-kDa polypeptide product has been studied (Lightner et al., 1983; Green et al., 1983). Since GPAT may play a pivotal role in the regulation of triacylglycerol and phospholipid biosynthesis, it would be important to understand regulation of GPAT at the molecular level.

During the course of cloning specific genes which are induced in livers of mice in a lipogenic state, we isolated cDNA sequences to an unidentified, 6.8-kb mRNA (Paulauskis & Sul, 1988). The 6.8-kb mRNA contained an open reading frame of 827 amino acids which we previously designated as p90. A homology search of Genbank revealed that p90 has 30% identity and 42% similarity in a 322 amino acid stretch to *Escherichia coli* GPAT (Shin et al., 1991). We report here the positive identification of p90 as the murine mitochondrial glycerol-3-phosphate acyltransferase by employing antibodies we have generated against p90–trpE fusion proteins and by transfecting the p90 sequence to CHO cells and thereby correlating GPAT activity with the p90 protein expression. By identifying p90 as the mitochondrial GPAT, the amino acid sequence and the molecular size of this enzyme are now known. This information along with the available antibodies will be helpful for future studies. We also show here that, during fasting/refeeding, mitochondrial and not the microsomal GPAT activity is increased due to an increase in the mitochondrial GPAT protein level. In addition to the previously reported increae in the microsomal GPAT activity, we found that the mitochondrial GPAT is also induced during adipocyte differentiation. Mitochondrial GPAT is not expressed in 3T3-L1 preadipocytes, and its activity is increased with the corresponding increase in the mitochondrial GPAT protein level during 3T3-L1 adipocyte differentiation. Therefore, the above changes in mitochondrial GPAT activity are due to the changes in enzyme concentration.

EXPERIMENTAL PROCEDURES

Fusion Protein Preparation and Antibody Production. To produce trpE–p90 fusion proteins, fragments spanning nt 980–2251 and 1583–2251 were inserted into the pATH-10 vector between *Sma*I and *Pst*I sites and between *Eco*RI and *Pst*I sites, respectively. Expression of the fusion proteins was initiated by tryptophan starvation, and the cells were extracted as described (Dieckmann & Tzagoloff, 1985). Following fractionation on 7.5% SDS–PAGE, the fusion protein band was located by Coomassie blue staining of flanking lanes, excised, frozen in liquid nitrogen, and pulverized in water. Approximately 100 μg of fusion protein in water was emulsified with an equal volume of Freunds complete adjuvant and used to immunize 6-week-old female NZW rabbits. Booster injections in Freunds incomplete adjuvant were given at approximately 3-week intervals, and serum was collected 7 and 14 days postinjection.

In Vitro Transcription, Translation, and Immunoprecipitation. Full-length p90 cDNA was inserted between the *Bam*HI and *Not*I sites in plasmid pcDNAI (Invitrogen) and was linearized by *Apa*I digestion. Capped, full-length p90 sense RNA was synthesized utilizing T7 RNA polymerase (Stratagene). A 1-μg sample of synthesized transcript was used for in vitro translation using nuclease-treated rabbit reticulocyte lysates (Promega) with the incorporation of [^{35}S]-cysteine (NEN). The translation products were immunoprecipitated with antiserums against trpE–p90 fusion protein and heat-killed, formalin-fixed *Staphylococcus aureus* (Pansorbin, Behring Diagnostics). The washed samples were heated at 100 °C for 2 min in the presence of 2% (v/v) β-mercaptoethanol, and Pansorbin was removed by centrifugation. Proteins were analyzed by subjecting them to 7.5% SDS–PAGE, fluorography (Entensify, NEN), and exposure to Fuji RX film.

Western Blot Analysis. The cultured cells or livers from previously fasted mice fed a high-carbohydrate, fat-free diet 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, and subsequently washed once with the same buffer. The postmitochondrial supernatant was centrifuged at 100000*g* for 1 h to pellet the microsomal fraction. Both mitochondrial and microsomal fractions were subjected to SDS–PAGE and electroblotted onto Immobilon PVDF membranes (Millipore) using 10 mM CAPS, pH 11, 10% methanol transfer buffer. For immunodetection of proteins, membranes were blocked for 1 h at room temperature in 1X 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 antiserums. Detection of the antigen–antibody complexes was accomplished via goat antirabbit 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.

Cell Culture Conditions. 3T3-L1 and stably transfected CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Adipocyte differentiation was initiated by treating confluent 3T3-L1 cells with dexamethasone and 1-methyl-3-isobutylxanthine as previously described (Shin et al., 1991; Rubin et al., 1978).

Construction of Plasmids and Stable Transfection of p90. The putative mitochondrial glycerol-3-phosphate acyltransferase cDNA sequence (*Eco*RV–*Fsp*I fragment which corresponds to nt −209 to +2668) was inserted at the *Xho*I site of the pMSXND vector (Lee & Nathans, 1981) by blunt end ligation and used for transfection in CHO cells. Cells were plated at 1 × 10^6/100-mm dish 24 h prior to transfection and media changed 3 h before transfection. Cells were transfected with 20 μg of plasmid DNA by the calcium phosphate coprecipitation method and selected for 2–3 weeks in 400 μg/mL G418. Independent G418-resistant clones with the GPAT sequence in correct and reverse orientations were isolated. The cells were grown and treated with 10 mM 2-aminopurine for 16 h to increase expression from the transfected sequence before harvesting (Kalvakolanu et al., 1991).

Northern Blot Analysis. Cell monolayers were rinsed twice with PBS and harvested by scraping in guanidium isothiocyanate followed by centrifugation over CsCl (Chirgwin et al., 1979). A 20-μg sample of RNA was size-fractionated on 1% agarose gels in 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, and 1 mM EDTA, stained with ethidium bromide, and transferred to a Hybond membrane (Amersham). RNA blots were hybridized with the *Eco*RV–*Fsp*I fragment labeled with ^{32}P by random-priming (Feinberg & Vogelstein, 1983).

Measurements of Glycerol-3-phosphate Acyltransferase Activity. The assay mixture contained 75 mM Tris–HCl, pH 7.5, 4 mM $MgCl_2$, 1 mg/mL bovine serum albumin, 8 mM NaF, 50 μM palmitoyl CoA, 3 mM glycerol 3-phosphate, and 3–5 μCi of [^{14}C]glycerol 3-phosphate. The reaction was started by the addition of extracts and incubated for 20 min at 25 °C. A 25-μg sample of mitochondrial and microsomal fractions prepared by the conventional differential centrifugation as described above were used for GPAT activity measurements in the presence and absence of NEM. In some cases the mitochondrial fraction of cultured cells was further purified using a Percoll–metrizamide gradient (Storrie & Madden, 1990). For the determination of NEM-resistant GPAT activity, the mitochondrial and microsomal fractions were 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).

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

RESULTS AND DISCUSSION

Generation of Antibodies Against p90. To initiate studies of identification and characterization of p90, we decided to generate antibodies against the open reading frame. Regions of p90 covering the C-terminal half, as described in the Experimental Procedures, were expressed as trpE fusion proteins in *E. coli*. The expressed fusion proteins, unlike the anthranilate synthase which is made as a soluble protein, were found in insoluble fractions. Therefore, the insoluble fractions were solubilized in SDS–PAGE sample buffer containing 10% SDS and subjected to electrophoresis. The corresponding fusion proteins with the calculated molecular weights were easily recognizable due to overexpression as shown in lanes 8 and 9 in panel A of Figure 1. The pulverized gel fragments were used for injection in rabbits for antibody generation. To determine if p90 was recognized by the antibodies, the p90 open reading frame was inserted into the pcDNAI vector and used for in vitro transcription. The transcript was subjected to in vitro translation using [^{35}S]cysteine and nuclease-treated rabbit reticulocyte lysates. As shown in panel B of Figure 1, the major polypeptide synthesized was 90 kDa and was specifically immunoprecipitated by the antibodies generated using the trpE–p90 fusion protein but not by nonimmune serum. These results indicated that the antibodies generated specifically recognize the p90 protein.

Identification of p90 as a Mitochondrial Protein. To identify the endogenous murine protein recognized by the antibodies we generated, the Western blot was carried out using mouse liver extracts. We have previously reported that the p90 sequence has 30% homology and 40% similarity in a 300 amino acid stretch to *E. coli* glycerol-3-phosphate acyltransferase. Since in mammalian tissues GPAT activity has been found in both mitochondrial and microsomal fractions, we carried out the Western blot analysis of liver mitochondrial and microsomal preparations which show different patterns of Coomassie blue stained proteins as shown in Figure 2A. The antibodies generated against p90 specifically recognized a polypeptide, with a correct molecular

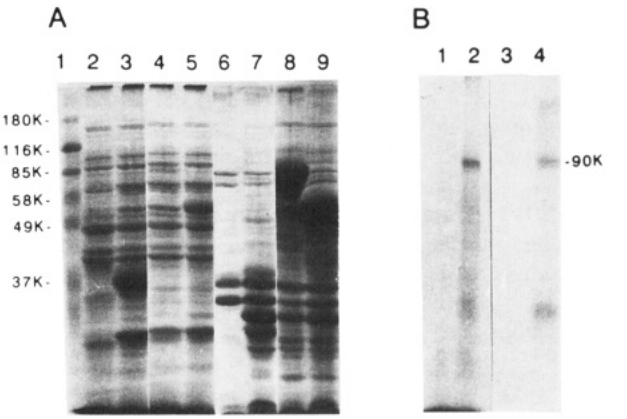
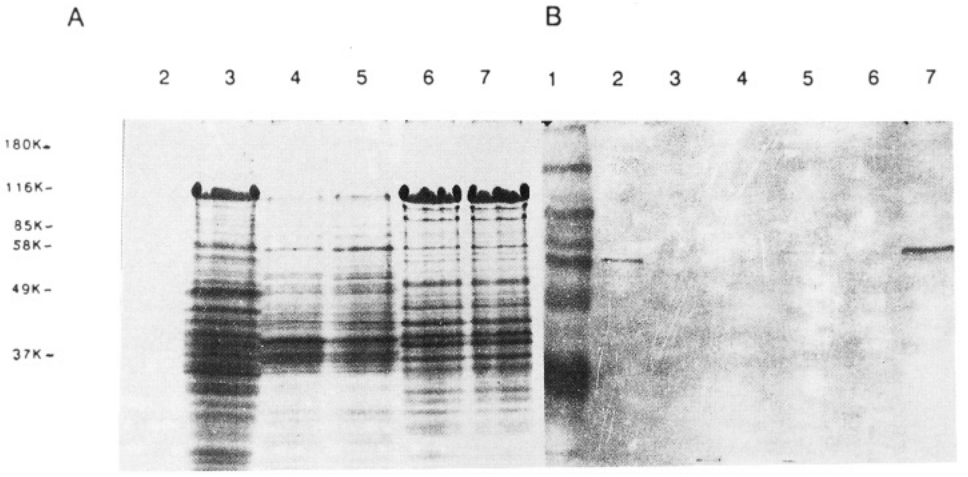


FIGURE 1: Overexpression of the p90 sequence in *E. coli* as trpE fusion proteins and the recognition of p90 by the antibodies generated against the fusion proteins. Panel A: p90 sequences covering the C-terminal half were inserted into the pATH-10 vector as described in the Experimental Procedures. The expression of trpE fusion proteins was initiated by tryptophan starvation. The soluble and insoluble fractions (50 μg of protein/lane) in SDS sample buffer were subjected to SDS–PAGE and stained with Coomassie blue: lane 1, molecular weight standards; lane 2, soluble fraction of nontransformed RR1 cells; lane 3, soluble fraction of cells transformed with the pATH-10 vector only; lane 4, soluble fraction from cells transformed with pATH-10 containing amino acids 328–751 of p90; lane 5, soluble fraction from cells transformed with pATH-10 containing amino acids 528–751 of p90; lanes 6–9, the same cell extracts as lanes 2–5 but insoluble fractions. Panel B: the p90 sequence in pcDNAI was in vitro transcribed using T7 polymerase, translated using [^{35}S]cysteine and reticulocyte lysates, and immunoprecipitated using antibodies against trpE fusion protein: lane 1, total in vitro translated product with no exogenous RNA; lane 2, total translation product using 1 μg of in vitro transcribed RNA; lane 3, immunoprecipitation of the in vitro translation product using normal serums; lane 4, immunoprecipitation of the translation product using antiserums against trpE fusion protein.

weight, from mitochondria (Figure 2B). The faint band observed in the microsomal fraction was probably due to contamination by the mitochondrial fraction. Moreover, we barely detected p90 in mitochondria prepared from fasted mice but found it was markedly increased in the refed mouse liver. This observation correlates with our previous report that the 6.8-kb mRNA is induced in the livers of previously fasted mice which were refed a high-carbohydrate, fat-free diet. These results, therefore, suggest that p90 may be the mitochondrial GPAT. When we measured the GPAT activity in mitochondrial and microsomal fractions, the NEM-sensitive GPAT activity in the microsomal fraction was relatively constant. On the other hand, the mitochondrial GPAT activity was mostly NEM-resistant and increased approximately 6-fold in refed mouse liver as compared to that in fasted mice (Figure 2C). These data indicate that p90 is a mitochondrial protein and probably represents GPAT.

Increase in the p90 Protein Level and Mitochondrial GPAT Activity during 3T3-L1 Adipocyte Differentiation. We have previously reported that the 6.8-kb mRNA which codes for p90 is increased during differentiation of 3T3-L1 cells to adipocytes. However, previous reports indicated that the increase in GPAT activity observed during adipose conversion is due to an increase in microsomal GPAT activity (Coleman et al., 1978). On the other hand, we have observed an increase in the p90 mRNA level during adipogenesis, and we have detected p90 in the mitochondrial fraction. To resolve this discrepancy, we examined the level of p90 in preadipocytes and in adipocytes after differentiation. In agreement with the Northern blot data, p90 was not detectable in preadipocytes, but was markedly increased in the mitochondria of cells which had undergone adipose conversion (Figure 3). We have

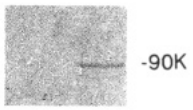


C

	Mitochondria		Microsomes	
	(nmol/mg/min)			
	-NEM	+NEM	-NEM	+NEM
Fasted	1.23	1.35	5.39	0.32
Refed	8.12	8.28	8.21	0.51

FIGURE 2: Western blot analysis of the mouse liver mitochondrial and microsomal fractions and mitochondrial and microsomal GPAT activities in fasted and refed mouse liver. The extracts (40 μg) from each fraction were subjected to SDS–PAGE and stained with Coomassie blue. A duplicate gel without staining was used for Western blot using antiserums against trpE–p90 fusion protein as described in the Experimental Procedures. Panel A shows Coomassie staining of the gel, and Panel B shows Western blot analysis: lane 1, molecular weight standards; lane 2, trpE fusion protein used for antibody generation; lane 3, total liver extracts from refed mice; lanes 4 and 5, microsomal fractions from fasted and refed mouse liver, respectively; lanes 6 and 7, mitochondrial fractions from fasted and refed mouse liver, respectively. Panel C shows GPAT activities in liver microsomal and mitochondrial fractions of fasted and refed mice in the presence and absence of NEM. Essentially the same results were obtained in three separate experiments.

A



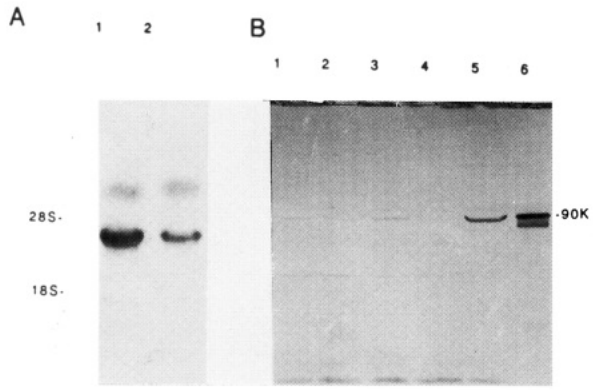
B

	Mitochondria	Microsomes
	nmol/mg/min	
preadipocytes (d0)	0.46	4.85
adipocytes (d7)	5.11	74.22

FIGURE 3: Expression of endogenous p90 during 3T3-L1 adipocyte differentiation. The mitochondrial fraction was prepared from cells at confluence (d0) and after adipose conversion (d7) and subjected to Western blot analysis. Microsomal GPAT activity and NEM-resistant mitochondrial activity were determined as described in the Experimental Procedures using the same extracts employed in Western blots. The activities are averages of duplicate measurements. Essentially identical results were obtained in two separate experiments.

also compared mitochondrial GPAT activities in preadipocytes and adipocytes. Since microsomal GPAT activity was an order of magnitude higher than mitochondrial activity in preadipocytes, we utilized *N*-ethylmaleimide to measure only the mitochondrial GPAT and not the contaminating microsomal activity. Unlike microsomal GPAT, mitochondrial GPAT is resistant to the sulfhydryl group reagent *N*-ethylmaleimide (Bates et al., 1977; Coleman & Haynes, 1983). The mitochondrial GPAT activity increased 11-fold measured as NEM-resistant GPAT activity of the mitochondrial fraction (Figure 3B). These results demonstrate that not only the more abundant microsomal GPAT but also the mitochondrial GPAT is induced during 3T3-L1 adipocyte differentiation. Previous reports on changes in GPAT activity during adipocyte differentiation utilized the total particulate fraction (Coleman et al., 1978; Grimaldi et al., 1978; Kuri-Harcuch & Green, 1977). The high microsomal GPAT activity probably made the mitochondrial activity difficult to determine. Our results, however, are in agreement with the findings of Coleman and Bell (1980) who showed low but increased NEM-resistant GPAT activity during differentiation of 3T3-L1 cells. The corresponding increases in mitochondrial GPAT activity, the p90 protein, and the 6.8-kb mRNA levels further indicate that p90 corresponds to the mitochondrial GPAT. A point to be noted is that the microsomal GPAT activity was found to be an order of magnitude higher in fully differentiated adipocytes than in liver.

Expresssion of p90 and Mitochondrial GPAT Activity by Stable Transfection to CHO Cells. To further demonstrate the identity of the p90 as the mitochondrial GPAT, CHO cells were stably transfected with the p90 open reading frame under the control of the metallothionein I promoter in the pMSXND vector. CHO cells were chosen because they possess relatively low GPAT activities in both mitochondrial and microsomal compartments. The Northern blot in panel A of Figure 4 shows a low but detectable endogenous 6.8-kb mRNA in CHO cells. In addition, the transfected DNA produced a shorter 3.9-kb transcript which is expressed at a higher level than the endogenous 6.8-kb mRNA. The shorter transcript for p90 was produced because regions of the long 5′- and 3′-noncoding regions were truncated before the insertion into the vector. As a control, the open reading frame has also been inserted in the reverse orientation, which is also expressed as a 3.9-kb transcript. The amounts of the p90 protein in these cells were determined by Western blot (Figure 4B). The p90 protein level was very low in both nontransfected CHO cells and the cells transfected with the p90 sequence in reverse orientation. The p90 protein level in mitochondria was 2–3-fold higher in cells transfected with p90 ORF in the correct orientation. This indicates that the transfected DNA



C

	Mitochondria		Mitochondria*		Microsomes	
	(nmol/mg/min)				(nmol/mg/min)	
	-NEM	+NEM	-NEM	+NEM	-NEM	+NEM
CHO	5.23	0.84			1.20	0.02
p90(-)	5.02	0.83	0.87	0.29	1.49	0.03
p90(+)	6.09	1.37			1.35	0.02
p90(-)/2-AP	5.12	0.66			1.70	0.05
p90(+)/2AP	6.65	2.85	2.10	2.57	1.74	0.08

FIGURE 4: Expression of p90 in stably transfected CHO cells. Panel A: independent CHO clones stably transfected with either the correct (lane 2) or the reverse orientation (lane 1) of the p90 ORF were cultured. Total RNA was prepared and subjected to Northern blot analysis. Panel B: the mitochondrial fractions prepared from the cells transfected with the correct (lanes 3 and 5) or the reverse orientation (lanes 2 and 4) of the p90 ORF were treated with (lanes 4 and 5) or without 2-aminopurine (lanes 1–3) and subjected to Western blot analysis using antibodies against trpE-p90 fusion protein. Nontransfected CHO cells (lane 1) and mouse liver mitochondrial fractions (lane 6) were used as controls. Panel C: the microsomal and mitochondrial GPAT activities were determined in the presence and absence of NEM using the same fractions employed in Western blots. * The GPAT activities were determined in mitochondrial fractions which were further purified using a Percoll–metrizamide gradient after differential centrifugation. The activities are expressed as averages of duplicate measurements. Essentially identical results were obtained in two separate experiments. The minus sign indicates the reverse orientation and the plus sign the correct orientation of the p90 sequence in the pMSXND vector.

generated a protein identical to the endogenous p90 protein, when compared to GPAT in liver mitochondria (lane 6 which contained the liver mitochondrial fraction also shows a smaller proteolytic degradation product generated during storage). Although RNA transcribed from the transfected gene was significantly higher than the endogenous 6.8-kb mRNA, the increase in p90 protein produced by stable transfection was not very high. CHO cells transfected with the correct orientation of the p90 sequence showed approximately 2-fold NEM-resistant mitochondrial GPAT activity as compared to that of nontransfected cells or cells transfected with p90 in reverse orientation (Figure 4C). This may be due to the less than optimal efficiency in translation and targeting to mitochondria. Therefore, we treated the cells with 2-aminopurine, a reagent recently reported to enhance expression of exogenous genes via a posttranscriptional mechanism (Kalvakolanu et al., 1991). The p90 protein level significantly increased by 3-fold after 2-aminopurine treatment only in cells transfected with the p90 ORF in the correct orientation. No protein band was detected in cytosolic or microsomal fractions of the transfected cells (data not shown). The increase in the p90 protein level was correlated to the enzyme activity in that the NEM-resistant mitochondrial GPAT activity was 5-fold higher in cells transfected with p90 in the correct orientation as compared to that in the reverse orientation, when cells were treated with 2-aminopurine (Figure 4B and column 3 of Figure 4C). However, by

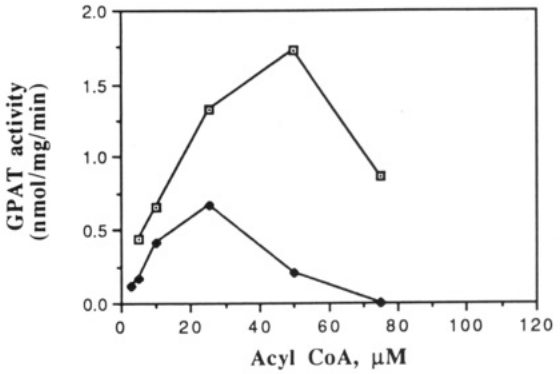


FIGURE 5: Fatty acyl CoA specificity of glycerol-3-phosphate acyltransferase from mitochondria of CHO cells stably transfected with p90. Mitochondrial fractions were used in the determination of the NEM-resistant GPAT activity using either palmitoyl (□) or oleoyl (◆) CoA as a substrate as described in the Experimental Procedures.

comparing GPAT activity in the presence and absence of NEM, it appeared that, unlike the preparation from mouse liver, mitochondrial fractions from CHO cells contain high amounts of the microsomal fraction. Therefore, we further purified the mitochondria by differential centrifugation using a Percoll–metrizamide gradient. As shown in Figure 4C, GPAT activity in purified mitochondria from cells transfected with p90 in the correct orientation was approximately 8-fold higher than in the reverse orientation in the presence of NEM. The NEM-sensitive microsomal GPAT activity remained unchanged in control cells or cells transfected with either constructs (Figure 4C). These results are consistent with the fact that the transfected p90 open reading frame is that of the mitochondrial GPAT. Moreover, the transfected sequence contains sufficient information for proper localization of p90 to mitochondria.

Previous reports showed preference for saturated fatty acyl CoA as a substrate of mitochondrial GPAT and postulated that, although the principal site for glycerolipid synthesis is the endoplasmic reticulum, this substrate preference of mitochondrial GPAT may explain the asymmetric distribution of saturated fatty acids at the sn-1 position (Kelker & Pullman, 1979; Stern & Pullman, 1978; Haldar et al., 1979). Therefore, to ascertain that the transfected p90 shows the characteristic of the mitochondrial GPAT, we compared palmitoyl CoA to oleoyl CoA as a substrate for mitochondrial GPAT from transfected cells. Since mitochondrial GPAT in cells transfected with p90 ORF constitutes more than 80% of the total mitochondrial GPAT activity, the studies on mitochondrial GPAT probably reflect primarily the transfected GPAT. As shown in Figure 5, the mitochondrial GPAT prefers palmitoyl CoA as a substrate over oleoyl CoA. The mitochondrial GPAT displayed higher maximal activity when palmitoyl CoA was used as a substrate compared to oleoyl CoA. The GPAT activity declined when palmitoyl CoA and oleoyl CoA were at concentrations greater than 50 and 25 μM, respectively. Nevertheless, we used a double reciprocal plot of the data obtained with fatty acyl CoAs below inhibitory concentrations to estimate the apparent affinity. The apparent K_m values for palmitoyl CoA and oleoyl CoA were similar, approximately 20 μM under our assay conditions. When microsomal fractions were used to measure GPAT activity, the preference for saturated fatty acyl CoA as a substrate was not observed (data not shown). These results reinforce the identity of p90 as the mitochondrial GPAT.

It is unclear as to the mechanisms underlying targeting of the mitochondrial GPAT. However, the transfection of p90 resulting in targeting to mitochondria. It has been previously

reported, using protease sensitivity, that GPAT spans the transverse plane of the outer mitochondrial membrane (Hesler et al., 1985). We did not observe, at least in 7.5% SDS–PAGE, a gross size difference in molecular weights between the in vitro translated product and the mitochondrial p90 protein. Unlike most polypeptides that are imported into the mitochondrial matrix, the translocation machinery for outer mitochondrial membrane proteins is unclear (Glick et al., 1992). One or more of the same components that are used by matrix-targeted precursors may be involved in targeting to the outer membrane. It is interesting to speculate on the putative signal in p90 for the mitochondrial outer membrane localization. The amino acid sequence of p90 reveals that the first 50–60 amino acid residues are rich in amino acids containing a hydroxyl group (Shin et al., 1991), a characteristic of some mitochondrial matrix proteins. In addition, the C-terminal amino acid residues are rich in hydrophobic amino acids, whicy may function as a membrane anchor. Further studies will be necessary to understand the targeting process of the mitochondrial GPAT.

In conclusion, we correlated the level of p90 protein measured by immunodetection with the mitochondrial GPAT activity not only in liver and adipocytes but also in stably transfected CHO cells. Transfected p90 was expressed in mitochondria, and the expressed GPAT revealed a substrate preference for saturated fatty acids, a characteristic of the mitochondrial enzyme. The evidence presented here, although indirect, clearly identified p90 as the mitochondrial GPAT. We have also demonstrated here that the changes in mitochondrial GPAT activity during different nutritional and developmental conditions are due to the changes in enzyme concentration. Our cloning and its identification as the mitochondrial GPAT will help in studying this difficult enzyme, which was not successfully purified by conventional techniques. With the specific polyclonal antibodies, we will also be able to examine if the mitochondrial GPAT is regulated by posttranslational modification.

ACKNOWLEDGMENTS

We wish to thank Se-Jin Lee (Carnegie Institution of Washington) for providing the plasmid pMSXND and A. Tzagoloff (Columbia University) for the pATH vectors. Dr. Ann Jerkins is gratefully acknowledged for critically reading the paper.

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