Functional characterization of human 1-acylglycerol-3-phosphate-O-acyltransferase isoform 9: cloning, tissue distribution, gene structure, and enzymatic activity

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Abstract

Most cells synthesize their glycerophospholipids and triglycerides (TG) to maintain the cellular integrity and to provide energy for cellular functions. The phospholipids are synthesized *de novo* in cells through an evolutionary conserved process involving serial acylations of glycerol-3-phosphate. Several isoforms of the enzyme 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) acylate lysophosphatidic acid at the *sn*-2 position to produce phosphatidic acid. We cloned a cDNA predicted to be an AGPAT isoform and designated it AGPAT9. The human *AGPAT9* gene spans across 14 exons and encodes for a polypeptide of 534 amino acids. AGPAT9 is highly expressed in the lung and spleen, followed by leukocyte, omental adipose tissue, and placenta. In the Chinese Hamster Ovary (CHO), cell lysates overexpressing AGPAT9, we observed AGPAT activity but not the lysophosphatidylcholine acyltransferase activity. When AGPAT9 is coexpressed with

AGPAT1 in CHO cells, both the isoforms localize to the endoplasmic reticulum (ER) and occupy the same ER domain as AGPAT1. Despite substitution of asparagine with proline in the NHX₄D motif and arginine with cysteine in the EGTR motif, AGPAT9 retains AGPAT activity suggesting that residues asparagine and arginine in the NHX₄D and EGTR motifs respectively are not essential for the enzymatic activity. Based on the X-ray crystallographic structure of a related acyltransferase, squash gpat, a model is proposed in which a hydrophobic pocket in AGPAT9 accommodates fatty acyl chains of both substrates in an orientation, whereas the HX₄D motif participates in catalysis. Based on the activity and expression pattern of AGPAT9 in the lung and spleen, this novel isoform could be implicated in the biosynthesis of phospholipids and TG in these tissues.

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Introduction

Glycerophospholipids and triglycerides (TG) constitute an important pool of phospholipids and neutral lipids, involved in several cellular functions. For example, phosphatidic acid (PA) and phosphatidyl inositol are involved in signal transduction; phosphatidylethanolamine and phosphatidylserine in membrane integrity and TG in storing energy. Intracellular requirements of these phospholipids and TG are met by the *de novo* biosynthesis through several pathways (Coleman & Lee 2004, McMaster & Jackson 2004). Among these, the more widely studied pathway involves generation of phospholipids and TG from glycerol-3-phosphate. In this pathway, there is sequential esterification of the glycerol chain catalyzed by a variety of acyltransferases. The initial esterification at the *sn*-1 position of glycerol-3-phosphate is catalyzed by glycerol-3-phosphate acyltransferases (GPATs), followed by esterification at the *sn*-2 position by 1-acylglycerol-3-phosphate-O-acyltransferases (AGPATs,

also known as lysophosphatidic acid acyltransferase (LPAAT)) and finally at the *sn*-3 position by diacylglycerol acyltransferases (Agarwal & Garg 2003). The other members of the acyltransferase family include enzymes involved in acylation of proteins (palmitoyl acyltransferase) transferring fatty acid to amino acids or glyceronephosphate-O-acyltransferase (Webber & Hajra 1993).

Recently, through positional cloning, we discovered that mutations in *AGPAT2* cause congenital generalized lipodystrophy (CGL; Agarwal *et al.* 2002, Agarwal & Garg 2003), a syndrome with almost complete loss of adipose tissue and predisposition to develop diabetes, hepatic steatosis, and hyperlipidemia (Garg 2004). Besides AGPAT2, there are seven other known isoforms, each of which is encoded by independent genes (Leung 2001, Li *et al.* 2003, Ye *et al.* 2005, Agarwal *et al.* 2006). A multiple sequence alignment of these eight known AGPAT isoforms indicates that several are duplicated, suggesting that several AGPATs are still to be discovered. Furthermore, given the availability of numerous structurally distinct lysophosphatidic

acids (LPAs) and fatty acids for acylation intracellularly, we hypothesized that there could be additional AGPAT isoforms which still remain to be identified. These new AGPAT isoforms may differ in substrate specificity, i.e. may prefer specific LPAs and specific fatty acids for *sn*-2 acylation to perform distinct cellular functions. With the sequencing of the human and mouse genomes and the bioinformatics tools available, we cloned and characterized an additional AGPAT isoform encoding human AGPAT9. This isoform retains AGPAT activity very similar to another isoform AGPAT2.

Materials and Methods

Bioinformatics

The National Center for Biotechnology Information (NCBI) and Celera databases were used for the sequence analysis. Multiple protein alignment and phylogenetic tree were created using Vector NTi suites (InforMax, Bethesda, MD, USA).

Cloning of human AGPAT9

The search for additional gene encoding for an acyltransferase was performed by homolog comparison with known acyltransferases deposited in the various databases including NCBI, Celera and expressed sequence tags (ESTs). One of the hits, AK090444, showed significant homology to the known acyltransferases, including the conserved motifs, suggesting that this sequence may have the acyltransferase activity.

Amplification of human AGPAT9 cDNA

To determine if the sequences are expressed in human tissues, we initially designed primers 1 and 2 (Table 1) within the coding region to amplify the mRNA from the adipose tissue. The amplified PCR product was purified and the sequence was found to be identical to the predicted open reading frame (ORF).

Table 1 Primers for human AGPAT9 cDNA amplification with restriction sites underlined

5'–3' primer sequences	
Primer #	
1	GCCATCCCTGTGACCATGACGAT
2	ATACAGCGCGGGGTTCTCTTCT
3	CGCACCATGTGGTTCGCCGGCGGGCTT
4	AGTCAGTCACGGAGACACCCAAGGC
5	GCCTTGGGTGTCTCCGTGACTGACT
6	CAGACGTCACCTCGCAAAGAAGCTCA
7	CGAGACCCGCGCCCAAGCTAG
8	CGGGATCCATGCGCACCATGTGGTTCGCCGG
9	GGACTGCGCCCGTCTCTTGA
10	CGCTCGAGCTAATCCAGCTTCTTGCGAACAGG
11	CGGGATCCATGAGGCTGCGGGGAGGATGCGGAC
12	CGCTCGAGATGAGGCTGCGGGGAGGATGCGGAC
13	CGGGATCCATCCAGCTTCTTGCGAACAGGCTTC

To amplify the entire ORF, additional primers were then designed in the 5' and 3' untranslated regions and amplified in two overlapping fragments using primer pairs 3 and 4, and primer pairs 5 and 6 (Table 1) and the human adipose tissue Marathon Ready cDNA (Clontech). The PCR products were gel purified and sequenced directly using the amplification primers and cloned in TA cloning vector (pCR2.1; Invitrogen). These overlapping PCR fragments were then combined and reamplified using only the 5' and 3' flanking primer pairs 3 and 6 (Table 1). The PCR product was gel purified and cloned in pDrive vector (Qiagen) for further sequencing and cloning steps.

Generation of wild-type AGPAT9 expression vector (short form)

To construct the AGPAT9 expression plasmid, the pDrive-AGPAT9 was used as the template and primer pair designed to only amplify the ORF (primers 8 and 10; Table 1). The restriction sites *Bam*HI and *Xho*I were incorporated into the primers for ease in subsequent cloning into the mammalian expression vector; pcDNA3.1(+)neo. The amplified product and the vector were digested with *Xho*I and *Bam*HI and ligated to the same sites. The expression plasmid was restricted with appropriate enzymes to ascertain the orientation of the insert in relation to the promoter. This expression plasmid was named as pAGPAT9-SF.

Generation of wild-type AGPAT9 expression vector (long form)

Initially, we amplified and generated mammalian expression construct based on available nucleotide sequences (GenBank entry AK090444), however, on further alignment of human AGPAT9 protein with homologous protein sequences from several other species revealed that there are additional amino terminal protein sequences which were missed in the human. Based on this information, we amplified these 5'-sequences such that an overlapping fragment with the short form of AGPAT9 was generated using primer pairs 7 and 9 (Table 1). The generation of the mammalian expression plasmid was the same as for the short form except primer pairs 10 and 11 (Table 1). This expression plasmid was named as pAGPAT9-LF.

Generation of V5- epitope-tagged wild-type AGPAT9 expression vector (long form)

The construction strategy for the V5 epitope tagged AGPAT9 expression vector was the same as described above, except the AGPAT9-LF was amplified with primer containing the V5 epitope sequences with the removal of the first ATG of the AGPAT9 protein (in order to remove the spurious protein translation). (5'-CGGGATCCATGGGTAAGCCTATCCC-TAACCCCTCTCCTCGGTCTCGATTCTACGAGGCTG-CGGGGATGCGGACCCCG-3'; the *Bam*HI restriction site is in bold and the V5 epitope is underlined) and the primer 10 (Table 1).

Generation of EGFP-tagged wild-type AGPAT9 expression vector (long form)

The construction of the wild-type fusion protein with GFP was carried out using the AGPAT9 wild-type plasmid as the template and primer pairs 12 and 13 (Table 1). The amplified product and the plasmid pEGFP-N3 (Clontech) were restricted with *XhoI* and *BamHI* and cloned in the similar site. The expression plasmid was sequenced to ascertain the orientation and the correctness for the junction sequences. The human AGPAT1-REP was generated by excising the AGPAT1 coding region from the pDrive-hAGPAT1 (unpublished) to the KpnI and XhoI and cloning in the compatible restriction site of expression vector dsRed-T4-N1 (Clontech).

Genomic localization of human AGPAT9 and gene structure

The human cDNA for AGPAT9 was blasted against the genomic databases (NCBI) which identified the gene located on chromosome 5p15.5 (contig: NT_006576.15, nt 1 526 408–1 426 586 and Hs5_6733, nt 1 589 408–1 489 586).

Northern blot analysis

To determine the tissue expression pattern and transcript size, a multiple tissue total RNA blot was obtained from Clontech and was hybridized with the probe generated with primer pairs 5 and 6 (274 bp fragment). Briefly, the blot was hybridized with ³²P-labeled probe generated from the above fragment overnight at 60 °C. The blot was washed with decreasing concentration of sodium salt at 65 °C. The final wash was at 0.1 × SSC; 0.1% SDS for 30 min at 65 °C. The blot was exposed either overnight or for 7 days at –80 °C. The blot was stripped and reprobed with the β-actin probe as a control as suggested by the manufacturer. The scanned images were quantified using LabWorks 4.0 software (UVP Inc., Upland, CA, USA).

Quantitative real-time PCR in human tissue panel

Quantitative PCR was performed using the TaqMan primer and probes, designed using primer express software, ABI PRISM 7700 sequence detection system and primer express analysis software (Livak 1997; ABI user bulletin 2). The human cDNA panel was from Clontech. Briefly, 100 pg cDNA was added to the forward (5'-TCGCTGACTTCCA-CAGGTTTG-3') and reverse (5'-GGTGAGGTCTCTGCA-CAGCTTT-3') primers along with fam-labeled probe (AATACCTGTACCCGGATCA) and universal mix containing AmpliTaq and appropriate buffers. The PCR was followed for 40 cycles of 94 °C for 15 s and 60 °C for 30 s. The cDNA was amplified in duplicate with *G3PDH* as an internal control. The ΔC_t value for each tissue was calculated as $\Delta C_t = (C_t \text{ (tissue)} - C_t \text{ (G3PDH)})$.

Generation of stably expressing wild-type AGPAT9 in Chinese Hamster Ovary (CHO) cells

The CHO cells stably expressing AGPAT9 were generated as described earlier (Haque *et al.* 2005). Stable-transfected CHO cell lines were selected in 500 µg/ml G418 (Sigma). The G418 resistant cells in each well were scored as a single pool. Such pools were expanded for further studies. Cells transfected with pcDNA 3.1(+) vector alone were used as controls. The heterologous expression of human AGPAT9 was verified by reverse-transcriptase polymerase chain reaction (RT-PCR) as described before.

AGPAT enzymatic activity of AGPAT9 in the cell lysate

Three to four independent pools of cells were expanded after selection in G418. The cells were plated in 60 mm dishes and grown to confluence. The medium was then removed and the cells were rinsed twice with PBS and harvested by scraping in 200 µl of 20 mM Tris-HCl (pH 7.5) and 5 mM NaCl containing protease inhibitor cocktail (Roche). Cells were subjected to a single freeze thaw step and then sonicated three times with 7 s burst, followed by a 1 min cooling on ice (Vibracell High Intensity Ultrasonic Liquid Processor; Sonics & Materials Inc., Newtown, CT, USA). Cellular debris and nuclei were removed by centrifugation (1000 g for 10 min at 4 °C), and the post nuclear supernatant was used for the enzymatic activity. Protein concentration was determined by a commercially available colorimetric assay (Bio-Rad Laboratories).

The enzyme activity was determined by measuring the conversion of [³H]LPA to [³H]PA as described previously (Haque *et al.* 2005, Agarwal *et al.* 2006). Briefly, the enzymatic reaction was assembled in 200 µl of 100 mM Tris-HCl (pH 7.4), containing the following: 10 µmol/l LPA (oleoyl-*sn*-1-glycerol-3-phosphate, Avanti Polar Lipids, Alabaster, AL, USA), 50 µmol/l oleoyl CoA (Sigma), 1 µl [³H]oleoyl-LPA (specific activity 30–60 Ci/mmol; Perkin-Elmer Life and Analytic Sciences, Boston, MA, USA), and 1 mg/ml fatty acid free bovine serum albumin. The reaction was started by adding 20 µg cell lysate followed by incubation for 10 min at 37 °C. The reaction was terminated by adding 0.5 ml 1-butanol containing 1N-HCl and to extract phospholipids. The butanol extract was dried under vacuum and the LPA and PA were resolved using the solvent system chloroform-methanol-acetic acid-water (25:10:3:1). Radioactive spots were identified by comigration with unlabeled LPA and PA standard and visualized in iodine vapors. The [³H]LPA and [³H]PA spots were scraped and counted for radioactivity (Tri-Carb Liquid Scintillation Counter 3100TR; Perkin-Elmer Life Sciences). The enzyme activity of each cell pool were determined as described before (Haque *et al.* 2005, Agarwal *et al.* 2006).

Lysophosphatidylcholine acyltransferase (LPCAT1) enzymatic activity of AGPAT9-LF in the cell lysate and tissue homogenate

LPCAT1 activity in cell lysate was determined as described previously (Chen *et al.* 2006, Nakanishi *et al.* 2006). Cell lysates were obtained from the V5-tagged expressing cells and incubated with [¹⁴C]palmitoyl lysophosphatidylcholine in the presence of palmitoyl-CoA. The incubation was terminated by adding chloroform: methanol (2:1 v/v) mixture. Organic phase was dried and resolved on thin layer chromatography (TLC) using solvent system chloroform: methanol:acetic acid:water (50:25:8:4 v/v). Bands corresponding to standard LPC and PC were visualized by iodine, eluted, and quantitated. The tissue homogenate was prepared in the same buffer as for (AGPAT) assay, centrifuged to remove cell debris, and assayed using 20 µg total protein.

Acyl-CoA specificity

specificity of esterification of *sn*-2 position of [³H]LPA (*sn*-1-oleoyl-2-hydroxy-*sn*-glycerol-3-phosphate) was determined by substituting the fatty acids with the following acyl-CoAs. Decanoyl (C10:0)-, lauroyl (C12:0)-, myristoyl (C14:0)-, palmitoyl (C16:0)-, stearoyl (C18:0)-, oleoyl (C18:1)-, linolenoyl (C18:3; *n*-3)-, γ-linolenoyl (C18:3; *n*-6)-, arachidoyl (C20:0)-, arachidonoyl (C20:4; *n*-6)-, behenoyl (C22:0)-, docosahexaenoyl (C22:6; *n*-3)-, tricosanoyl (C23:0)-, ligneceroyl (C24:0)-, nervonoyl (C24:1; *n*-9)-, and hexacosanoyl (C26:0)-CoA. The assay conditions remain the same as described for the acyltransferase activity.

sn-1-acyl-lysophosphatidic acid specificity

To determine the specificity of various LPAs for the enzymatic activity, the following LPAs with various fatty acids at *sn*-1 position were used for the assay. Myristoyl (C14:0)-, palmitoyl (C16:0)-oleoyl (C18:1), arachidonoyl (C20:4)-, arachidoyl (C20:0)-, linoleoyl (C18:2)- and linolenoyl (C18:3). The enzymatic assay was assembled as before except that unlabeled LPAs and [¹⁴C]oleoyl-CoA were used as substrates, and activity was determined by formation of [¹⁴C]PA.

Western blot analysis

Total cell lysate (40 µg) from the V5-AGPAT9-LF and vector alone were resolved on 10% SDS-PAGE followed by transfer on to nylon membrane (Millipore, Billerica, MA, USA). The membrane blocked with 5% non fat milk containing 0.2% Tween -20 and then incubated with V5 antibody conjugated to horse radish peroxidase (HRP; Invitrogen) for 2 h at room temperature, the membrane was washed and then incubated ECL reagent (GE Healthcare Piscataway, NJ, USA) to visualize the immunoreactive protein.

Immunofluorescence microscopy

Stable expressing AGPAT9-EGFP CHO cells were grown on cover slip 1 day before the experiment. Cells were fixed/permeabilized by incubation with methanol (−20 °C) for 20 min. Cells were washed 3×5 min with PBS and then incubated with primary antibody (sec 61 β; Upstate, Lake Placid, NY, USA) diluted in the ratio of 1:200 for 30 min at 37 °C in a humidified chamber. Cells were then washed 3×5 min with PBS and incubated with AlexaFluor 555 coupled fluorescent secondary antibody (Invitrogen) for 30 min at 37 °C in a humidified chamber. After incubation, cells were washed 3×5 min with PBS and mounted on a glass slide with Mowiol. Cells were observed with a Zeiss Axioplan 2E, and pictures were captured with a Hamamatsu monochrome digital camera (Hamamatsu City, Japan). Images were analyzed with Openlab (Improvision, Lexington, MA, USA).

For live cell imaging, the cells were seeded in dishes and transfected with equal amount of AGPAT1-REP and AGPAT9-GEP expression plasmids. Cells were imaged directly 72 h after transfection using a DeltaVision RT Deconvolution Microscope (Applied Precision, LLC; Issaquah, WA, USA). The obtained images were deconvoluted to remove the reflective fluorescence using softWoRx software.

Results

Cloning of the human AGPAT9

Based on *in silico* sequence assembly, the human AGPAT 9 was amplified from the omental adipose tissue RNA. The amplified sequences showed that the start of the ORF for human AGPAT9 was in good context for the Kozak rule in initiating protein translation (Kozak 1984) predicting a protein of 432 residues. However, based on protein sequence homology, only a truncated form had been cloned, missing an N-terminal sequence (Fig. 1). Thus, based on human genomic sequences, we then designed additional primers to amplify the 5'-region sequences. The assembled sequences for AGPAT9-long form (AGPAT9-LF) had an in-frame stop codon located 60 nucleotides upstream in the 5'-untranslated region, suggesting that this ATG codon is most likely used as a translational start codon. The predicted protein consists of 534 residues and molecular weight of 59 147 Da. Alignment of AGPAT9-LF from several species showed that this isoform was highly conserved and is also present in invertebrates like fruit fly (*Drosophila melanogaster*). The putative conserved motifs, NHX₄D and EGTR, seen in almost all proteins of the glycerophospholipid acyltransferase family, were modified to PHX₄D and EGTC respectively in both the AGPAT-SF and the AGPAT9-LF.

Figure 1 Alignment of protein sequences of AGPAT9 for various species. Multiple protein alignment of human, mouse, rat, opossum, zebrafish, and stickleback and fruit fly AGPAT9 amino acid sequences. The conserved motifs PHX4D and EGTC are boxed. The identical and conserved residues are shaded gray. The start of the protein sequence for the short form of AGPAT9 is shown by ▼. GeneBank accession numbers: Human AGPAT9; this study and NM_024830, NP_0791061; mouse: NM_145376, NP_663351; rat: XM_341747, XP_341748; opossum: AAFR02008846; stickleback; from trace database and zebrafish: XM_701606, XP_706698; fly: NP_001036265.

Human AGPAT9 genomic organization

A search against the human genomic databases localized the human gene on chromosome 5p15.5. Based on the predicted sequences and the amplified sequences, the exon–intron boundaries were determined. The gene contains 14 exons with the last exon having a long untranslated region (Table 2). All the exon and intron junctions obeyed the AG–GT dinucleotide acceptor–donor rule. Visual inspection of the sequences revealed two regions of repeat sequences located in introns 1 and 2. As shown in Fig. 2, the first repeat consisted of a sequence of 17 nucleotides and was located 130 nucleotides from exon 2. The other repeat sequence located in intron 2 was nearly 3.5 kb from the splice site junction of exon 3. This repeat sequence, although not a perfect dinucleotide repeat, can still form a stretch of the alternating purine–pyrimidine polynucleotides. It is interesting to note that the short form of AGPAT9 starts in the exon 3 and could be the result of these repeat sequences.

Tissue distribution and quantitative expression in human tissue panel

Northern blot analysis of human tissues showed that the AGPAT9 mRNA was close to 4.4 kb and expressed in several tissues, with the lung and spleen expressing high levels, followed by the brain, heart, and placenta (Fig. 3). We did not observe any alternative transcript, even upon long exposure. In the rodents, a single transcript of about 4 kb has been identified in the lung and spleen (Chen *et al.* 2006). In the rat, AGPAT9 is also expressed in the stomach, but not in the mouse (Chen *et al.* 2006) indicating species variation. In our human northern blot, the stomach RNA was not represented. As reported earlier, the human AGPAT2 isoform is highly expressed in the liver and pancreas (Eberhardt *et al.* 1997) and in the adipose tissue (Agarwal *et al.* 2004) with relatively low expression in the lung and spleen. These RNA analyses indicate differential pattern of RNA expression of AGPAT2 and AGPAT9 isoforms in human tissues. Whether there are

Table 2 The exon–intron boundary sequences for human *AGPAT9*. Intronic sequences are in lower case whereas exonic sequences are in uppercase letters. The splice site acceptor and donor dinucleotide are in bold

	Length (bp)	cDNA position	Acceptor	Donor	Intron
Exon number					
1	164	1–264		GAAGGCCAGgtgggtgccag	1
2	143	265–407	ctctgtctcagCTGGCCCTCAT	TGTGGAGGAgtagttgcgcg	2
3	215	408–622	ccctcttttcagGGTTGTGGACTT	ATCTGGGGAgtgagtgagaca	3
4	113	623–735	ccctcttccagCTCTGATCCAGT	GTGGCCACAGtaatgcacgta	4
5	61	736–796	ccacttcagATAATGATTTT	TTCAAACCTGgtatgtagttt	5
6	59	797–855	cctttctgcagGTGCATTCAT	AAATAAACTGgtgagttgt	6
7	35	856–890	cttctccccagGACACCATCACA	GACCTGGAGCgtagttgaacg	7
8	55	891–945	tctgttttaagGCTGGAAATCCT	GGAAATCGAGgttcgtatgat	8
9	83	946–1028	tctgtgtctcagTTCCTTCCTGTG	TCATGGCCGAgtaagtgcagc	9
10	126	1029–1154	gccctccttagGGCCTTGGGT	GGGGCTCGgtgagtcctggt	10
11	154	1155–1308	ttttttgttagGCTAAACCA	GTTCGACGAgtagggcctgtcg	11
12	99	1309–1407	tctctccccagAGCGGCAGCG	GGCTTTCAAGgtgagtcagagtt	12
13	142	1408–1549	ttgccttcagATGTACGGAGC	ATCACATTCgtgagcccgagg	13
14	185	1550	ttgtcttcagCTGACTTCCACA		

functional redundancies among these isoforms remains unclear.

We expanded our search for expression in the human tissues by using quantitative real-time PCR. Shown in Table 3

are the ΔC_t values for various tissues after normalization to the housekeeping gene, glycerol-3-phosphate dehydrogenase (*G3PDH*). Quantitative real-time PCR revealed that AGPAT9 had about twofold higher expression in the lung

Figure 2 Nucleotide repeat sequences located in the intron of *AGPAT9* gene: Shown is the partial gene structure for *AGPAT9* gene where filled boxes represent exons, not drawn to scale. The 17 nucleotide repeat sequence, GGTCAGCCTTGGTCACT, located in intron 1 are shown in bold which are located 130 nucleotides upstream of exon 2. The alternating pyrimidine (cytosine and thymine) and purine (guanine and adenine) sequences in intron 2 are located 3499 nucleotide upstream of exon 3 and are shown in bold. The two putative transcriptional start sites are marked with arrows.

Figure 3 Northern blot analysis. (A) Human multiple tissue blot containing 10 µg poly(A)⁺ RNA for each tissue was hybridized with ³²P-cDNA for human AGPAT9 sequences. The blot was hybridized and washed as described in methods. The blot was exposed for 4 days at –80 °C to detect any weak signal. The blot was stripped and reprobed with β-actin cDNA as an internal control and exposed for 16 h. (B) Shown are the ratio for the AGPAT9 and β-actin expressed as an arbitrary units.

than in the spleen. AGPAT9 was also expressed in the omental adipose tissue and placenta, but about sevenfold less than the lung. AGPAT9 is expressed poorly in the skeletal muscle.

Comparison of amino acid sequence with other known acyltransferases

Multiple sequence alignment of the known human acyltransferases revealed that AGPAT9 belongs to the family of acyltransferases (Fig. 4A). Previous studies have revealed that AGPATs 1–5 and 8 have the same two conserved motifs, NHX₄D and EGTR, as shown in Fig. 4B. As more of these acyltransferases are cloned and sequenced there appears to be flexibility in these conserved motifs. The conserved proline in AGPAT9 from several species indicates that the asparagine residue in the NHX₄D motif is not an absolute requirement

Table 3 Tissue distribution of human AGPAT9 as quantified by TaqMan real-time PCR. Shown are the mean ΔC_t values, performed in duplicate and corrected for internal control, G3PDH. Fold increase between isoforms in various tissues were calculated 2 – ΔC_t according to ABI bulletin # 2. Expression in the lung and spleen are about the same, but about 3.27-fold less in the leukocyte when compared with the lung. Placenta and adipose tissues express at similar levels but are about 6.96-fold less than the lung. Our TaqMan probe does not distinguish between the AGPAT9-LF and the AGPAT9-SF

	C _t values	Tissue	C _t values
Tissue			
Heart	10.01	Prostate	8.00
Brain	7.53	Testis	7.99
Placenta	6.14	Ovary	7.86
S. muscle	13.41	S. intestine	8.23
Kidney	8.39	Colon	8.41
Pancreas	6.87	Leukocyte	4.91
Lung	3.20	Adipose	6.00
Spleen	3.95	Liver	8.54
Thymus	7.37		

for the enzymatic activity. Likewise, substitution of arginine to cysteine in EGTR motif is also tolerated (Fig. 1). In *Synechocystis* sp. PCC6803, an unrelated acyltransferase had the EGAT sequence instead of the motif EGTR (Weier *et al.* 2005). In a recent study, using *in vitro* translated mouse acyltransferases, it was shown that Agpat 3, 4, and 5 possessed acyltransferase activity, which was only 41–48% of mouse Agpat2 (Lu *et al.* 2005).

Subcellular localization of AGPAT9-LF-GFP

In stably expressing CHO cells, the AGPAT9-LF-GEP protein showed an endoplasmic reticulum (ER) like expression pattern. This was confirmed by colocalizing AGPAT9-LF-GFP with the ER protein Sec 61 β (Fig. 5A). AGPAT9-LF-GFP also colocalized with the ER proteins disulfide isomerase and the ER-chaperone GRP78/BIP (data not shown). Since previous studies have shown that AGPAT1, 2, 6, and 8 are all localized to the ER, we tried to determine whether, the AGPAT9-LF is localized to the same or different subdomain of ER. We transiently transfected CHO cells with AGPAT9-LF-GFP and AGPAT1-REP and captured images for both the proteins using live cells imaging. Shown in Fig. 5B are the fluorescence for AGPAT9-LF and AGPAT1 indicating that the two proteins appear to colocalize to similar ER domains. However, further colocalization studies with higher resolution will further confirm this observation.

AGPAT activity

The human AGPAT9-SF has moderate acyltransferase activity, only 22.6% when compared with the other isoform, human AGPAT2, using oleoyl-CoA as the acyl donor and 1-oleoyl glycerol-3-phosphate as the acceptor (data not

Figure 4 Evolutionary dendrogram and conserved protein motifs among AGPATs. (A) The dendrogram was computed from the aligned protein sequences using the ClustalW algorithm and VectorNTi's phylogenetic tree program with default settings. (B) Alignment of the partial amino acid sequences of AGPAT isoforms 2, 4, 6, 8, and 9 showing the conserved (N/P)HX₄D and EGT(R/D/C) motifs. Conserved residues are shown in bold.

shown). We then created a V5- epitope tagged AGPAT9-LF and determined the acyltransferase activity as above. This long form has much higher activity (72·8% when compared with AGPAT2) than the AGPAT9-SF, indicating that the amino terminus residues increase its activity over threefold (Fig. 6A and B).

Substrate specificity of AGPAT9-LF in CHO cell lysates

To assess the substrate specificity of AGPAT9-LF, LPAs with various fatty acids at the *sn*-1 position were incubated with lysates of CHO cells over expressing wild-type AGPAT9 and [¹⁴C]oleoyl-CoA. Wild-type AGPAT9 accommodated myristoyl, linoleoyl, and palmitoyl fatty acids at the *sn*-1 position of the LPA, synthesizing PAs at about the same rate as the preferred substrate, *sn*-1-oleoyl-LPA (Table 4). Other LPAs, such as *sn*-1-linolenoyl, and *sn*-1-arachidonoyl were esterified at 50–70% the rate of *sn*-1-oleoyl-LPA. However, *sn*-1-arachidoyl form of LPA was not esterified.

To assess the specificity of AGPAT9-LF for the other substrate, the fatty acyl-CoA esterified to the *sn*-2 position, cell lysates were incubated with [³H]LPA with various unlabeled acyl-CoAs. Wild-type AGPAT9 used palmitoyl-CoA, myristoyl-CoA, and stearoyl-CoA at the rate of about 25% of the preferred substrate oleoyl-CoA, (Fig. 8B). All other acyl-CoAs were poor substrates when compared with oleoyl-CoA (Table 5).

LPCAT1 activity in stable cell lines and mouse tissue homogenates

While this study was in progress, two independent studies showed that the mouse homolog has acyl-CoA:LPCAT1 activity and that the enzyme is highly expressed in the lung (Chen *et al.* 2006, Nakanishi *et al.* 2006). Although, the mouse clone is 88% identical to our human AGPAT9, in our

hands stable CHO lines expressing AGPAT9 lacked LPCAT activity, whether determined using whole cell lysate (Fig. 7A) or in cell fractions (data not shown). Such LPCAT activity, even though small, could be observed in the mouse lung and spleen tissue homogenates (Fig. 7B).

Discussion

We have cloned the cDNA for a human acyltransferase, AGPAT9, which has much higher expression in the lung and spleen, followed by the placenta, and leukocytes. In omental adipose tissue, the expression of AGPAT9 is moderate. In cell lysates obtained from cultured cells over-expressing AGPAT9, both the short and the long forms of AGPAT9 catalyze the synthesis of PA. However, the enzymatic activity of SF was markedly lower when compared with that of AGPAT2, whereas the LF was only slightly lower than that of AGPAT2. Thus, AGPAT9 resembles other AGPAT isoforms, such as AGPAT3, 4, 5, and 8 which also have lower activity than AGPAT2 (Lu *et al.* 2005). As this study was in progress, a cDNA for a mouse acyl-CoA:LPCAT1 was reported with the similar sequence as our human AGPAT9 cDNA (Chen *et al.* 2006, Nakanishi *et al.* 2006). This same sequence has also been reported as phosphonoformate immuno-associated protein 3 (PFAAP3; UniProtKB/Swiss-Prot entry Q8NF37). However, we did not observe any LPCAT activity in whole cell lysate obtained from CHO cells overexpressing AGPAT9. The reasons why the two identical sequences (mouse and human clones are 88% identical) show a different spectrum of enzymatic activities than previously reported are not known. One possibility could be the different host cells used for the protein expression. While we employed CHO cells, other investigators used COS-7 (monkey kidney cell) and HEK-293 (human embryonic kidney cells; Chen *et al.* 2006). Differences in enzymatic activity might result from

Figure 5 Colocalization of AGPAT9-GFP to endoplasmic reticulum and AGPAT1-RFP in stably or transiently expressing CHO cells: (A) Overexpressing AGPAT9-GFP (long form) CHO cell were fixed in methanol and incubated with antibody, Sec 61 β , specific for endoplasmic reticulum and imaged for green and red fluorescence using fluorescence microscope. Shown is a representative cell for section 61 (red fluorescence), AGPAT9 (green fluorescence) and the merge. (B) For live cell imaging, cells were grown in culture dishes and transfected with equal quantities of both the constructs, AGPAT9-LF-GFP, and AGPAT1-REP. Fluorescence images were acquired 72 h after transfection using fluorescence microscope equipped with incubation chamber with temperature and CO₂ controls. Shown is a representative cell for AGPAT9 (green fluorescence), AGPAT1 (red fluorescence) and the merge. Scale bar=10 μ m.

improper post-translational processing of the recombinant protein. A robust expression system needs to be established to resolve this issue.

The AGPAT9 protein is highly conserved among species, and homologs are found in rodents, vertebrate fishes and invertebrate fruit fly (GenBank entry as a hypothetical protein). Our search did not show this protein to be present in plants, but this may be due to incomplete genome annotation in many of these species. The high degree of

homology (Fig. 1) and conservation among species suggests that the *AGPAT9* gene evolved very early during evolution suggesting that this enzyme serves an important function in cellular physiology in tissues other than the lung as indicated by previous investigators (Chen *et al.* 2006, Nakanishi *et al.* 2006). However, unlike mouse LPCAT1 (Chen *et al.* 2006, Nakanishi *et al.* 2006), AGPAT9 does not appear to use LPC as substrate in our *in vitro* studies, which would argue against its sole role in lung physiology. Our data show that the

Figure 6 The enzymatic activities of wild-type human AGPAT2, and long forms of AGPAT9 in stably expressing CHO cells. (A) Shown is the Western blot for the cell pool, used for determining the acyltransferase activity, for the V5-epitope tagged AGPAT9-long form as probed with V5-antibody. G3PDH is included to demonstrate equal loading. (B) The enzymatic activities were determined as percentage conversion of ³H-LPA to ³H-PA in the presence of oleoyl-CoA. The LPA to PA conversion in control cells was subtracted from the values as described before (9). Shown are the mean values from 3–4 independent cell pools assayed in triplicate. Values are computed against the wild-type AGPAT2 activity as 100%.

enzyme is present in the spleen as well, suggesting its role in the immune system. Secondly, fruit flies lack tissue equivalent to the lung. Finally, presence of the LPCAT activity in the lung and spleen tissues represents the total LPCAT activity,

Table 4 Specificity of human wild type recombinant AGPAT9 long form for *sn*-1-lysophosphatidic acid acceptor. The enzymatic activities were determined using oleoyl-CoA as donor and various medium and long chain fatty acids at *sn*-1 position of lysophosphatidic acid as an acceptor. All enzymatic activities were determined in two independent experiments in triplicate. The enzymatic activities are compared with *sn*-1-oleoyl-lysophosphatidic acid

Carbon chain: number of double bonds		% Activity
Fatty acid at position of <i>sn</i>-1-LPA		
Oleoyl	C18:1	100
Myristoyl	C14:0	84
Palmitoyl	C16:0	86
Linoleoyl	C18:2	78
Linolenoyl	C18:3	68
Arachidoyl	C20:0	34
Arachidonoyl	C20:4	36

Table 5 Specificity of human wild type recombinant AGPAT9 long form for acyl-CoA donor. The enzymatic activities were determined using *sn*-1-oleoyl-lysophosphatidic acid as an acceptor and various medium and long chain fatty acids as donor. All enzymatic activities were determined in two independent experiments in triplicate. The enzymatic activities are compared with oleoyl-CoA which is computed as 100%. Where: *n*-3, *n*-6, *n*-9 indicate the position of the first double bond from the CH₃ end of the molecule

Length of carbon chain: number of double bonds		% Activity
Acyl-CoA		
Oleoyl	C18:1	100.0
Decanoyl	C10:0	9.5
Lauroyl	C12:0	9.8
Myristoyl	C14:0	26.7
Palmitoyl	C16:0	29.3
Stearoyl	C18:0	16.8
Linolenoyl	C18:3, <i>n</i> -3	7.0
γ-linolenoyl	C18:3, <i>n</i> -6	5.6
Arachidoyl	C20:0	11.4
Arachidonoyl	C20:4, <i>n</i> -6	6.2
Behenoyl	C22:0	9.1
Docosahexaenoyl	C22:6, <i>n</i> -3	11.7
Tricosanoyl	C23:0	12.9
Lignoceroyl	C24:0	7.2
Nervonoyl	C24:1, <i>n</i> -9	9.1
Hexacosanoyl	C26:0	7.9

which may or may not be due to the LPCAT1 activity. Lack of LPCAT activity in the recombinant protein will require further studies with more robust expression system.

The gene structure has revealed some interesting features. The presence of intergenic 17 nucleotide repeat sequence in intron 1 and alternating purine pyrimidine (APP) sequences in the intron 2 could influence the alternate splicing with shorter transcript originating in exon 3 of the *AGPAT9* gene (Hui *et al.* 2005). However, not directly demonstrated, these nucleotide repeat sequences, have been shown to influence the expression of a number of genes including 11β-hydroxysteroid dehydrogenase 2 (Agarwal 2001). The fact that the short form of the AGPAT9 is also enzymatically active and the APP regulates the

Figure 7 Lysophosphatidylcholine acyltransferase enzymatic activity of AGPAT9-LF in the stably expressing cell lysate and mouse tissue homogenate: (A) Shown is the LPCAT activity for the CHO cells, vector alone, and AGPAT9-long form in CHO cells determined by conversion of [¹⁴C]LPC to [¹⁴C]PC. Values are mean of 2–3 independent experiments in triplicate. (B) LPCAT enzymatic activity in the mouse spleen, lung, and liver is included as a control where tissue homogenate was omitted. Values are from two independent experiments in triplicate.

expression of several genes, might suggest why we initially cloned the short form for AGPAT9. The study of the enzymatic activities of the long and short forms of AGPAT9 also reveal that the first 102 residues at the amino terminus of the protein increase the enzymatic activity, suggesting a physiological role of alternative splicing in this gene.

This study corroborates the previous study that AGPAT9 localizes to the ER using different set of ER-resident proteins, Sec 61 β . This study also shows that AGPAT1, another ER-resident acyltransferase, and AGPAT9 partially colocalize, i.e. they are present in the similar ER domain.

The two signature motifs are highly conserved amongst the acyltransferase superfamily, NHX₄D, and EGTR (Agarwal & Garg 2003, Leung 2001), but these motifs have amino acid substitutions in AGPAT9. The NHX₄D motif has asparagine substituted with proline while in the EGTR motif arginine is replaced with cysteine, EGTC. Thus, asparagine in NHX₄D and arginine in EGTR may be conserved amongst

acyltransferases, but are not required for absolute enzymatic activity, at least in AGPAT9 (this study) and in AGPAT8, as reported previously (Agarwal *et al.* 2006). Thus, the conserved NHX₄D motif can now be represented as (N/P)HX₄D where asparagine is not critical for the enzymatic activity.

We have previously reported the role of hydrophobic residues downstream to the NHX₄D motif to be important for the enzymatic activity (Agarwal *et al.* 2006). Similar, strongly hydrophobic region is obvious in the AGPAT9 as well (Fig. 8); suggesting that these hydrophobic residues might similarly fold to form a hydrophobic tunnel to accommodate the acyl chain, as observed with the tertiary structure of squash (*Cucurbita moschata*) chloroplast gpat (Turnbull *et al.* 2001a,b, Slabas *et al.* 2002, Tamada *et al.* 2004). Analysis of the predicted secondary structure of gpat and AGPATs show that the positioning of the HX₄D domain is in the loop region between a beta strand (β_c) and alpha helix

Figure 8 Partial protein sequence of human AGPAT2 and AGPAT9, and squash (*Cucurbita moschata*) chloroplast gpat showing the predicted secondary structure: Based on *gpat* crystal structure, the region between residues 91 and 270 was selected, which includes the enzyme's active site, NHX₄D. Based on sequence homology between various isoforms of AGPAT1 through AGPAT9 (data not shown), protein region between amino acids, 61 and 270, were selected for secondary structure prediction of AGPAT2 and AGPAT9. Secondary structure prediction was carried out using Predict protein server (www.predictprotein.org; PredictProtein: Rost *et al.* 2004) W321–W326). Shown are the partial protein sequences flanking the conserved (N/P)HX₄D motif boxed in red and EGT(R/C) motif in blue. All hydrophobic residues are shown in green, including the weakly hydrophobic residues glycine, (G), and serine, (S). Below each protein sequence is shown the predicted secondary structure. α -Helix (shaded barrel), β -sheet (shaded arrow), and unstructured or random coil as a straight line. In all the three protein sequences, the (N/P)HX₄D is flanked by beta-sheet and alpha helix and predicted to fold as determined for *gpat* creating the active site of the enzymes.

(α_6). The positioning of the other secondary structure elements also fall in a similar way for AGPAT2 and AGPAT9, which is comparable to that of the gpat. It is important to note that even though AGPATs share ~ 12 – 15% sequence homology with gpat, these proteins are similar in the secondary structure prediction and hydrophobicity distribution, which would be the key features in determining the fold of the protein and also the active site hydrophobic tunnel. This indicates that the entire hydrophobic region is critical for the enzymatic activity and not the EGT(R/D/C) motif as observed in acyltransferases cloned till now. As shown in Fig. 8, the secondary structure prediction also indicates that the active site of the proteins, AGPAT2 and AGPAT9, should fold similar to the gpat as determined by X-ray crystal structure (Turnbull *et al.* 2001a,b, Slabas *et al.* 2002, Tamada *et al.* 2004). This would strongly suggest that the cloned enzyme should have sn-2 acyltransferase activity similar to AGPAT2 whose activity is now established (Fig. 9).

The physiological function of AGPAT2 has emerged from the mutational analysis of patients with CGL in humans (Agarwal *et al.* 2002). The loss of adipose tissue might result, from defects either in adipocyte development or in triglyceride biosynthesis. Inactivation of another isoform, *Acp6*, in mice displays resistance to diet-induced obesity and subdermal loss of adipose tissue. Furthermore, the *Acp6*^{−/−} mice fail to lactate suggesting a role of AGPAT6 in diacylglycerol and triacylglycerol synthesis in mammary epithelium (Beigneux *et al.* 2006, Vergnes *et al.*

2006). Interestingly, *Acp6* mRNA is expressed at highest levels in interscapular brown adipose tissue, visceral, and subcutaneous adipose tissue as well as in mammary epithelium of breast tissue (Beigneux *et al.* 2006, Vergnes *et al.* 2006). AGPAT9, however, has a distinct pattern of expression, being highly expressed in the lung and spleen instead of in adipose tissue. Thus, it can be speculated that the deficiency of AGPAT9 might affect lung physiology and may affect immunological response (due to high expression in the spleen) instead of affecting adipocyte differentiation and development.

It is interesting to observe that so many AGPATs are now cloned. Isoforms 1–5, 8, and 9 all appear to have varying degree of acyltransferase activity. Most, isoforms 1, 2, and 6–9 are all localized to ER. This raises an important question: why would cells express all of these AGPATs localized to the ER? Are the activities redundant? Answers to these questions will emerge when detailed investigation of these AGPATs are carried out, in terms of hormonal and nutritional regulation, both at the transcriptional and translational level, tissue expression, substrate specificities and subcellular localization. To understand the physiological function of each of these AGPATs either by knocking down the activities in cultured cells or by developing the knockout mouse model shall reveal the function for each of these in specific cell types or in an integrated animal system. It is quite likely that each of these AGPATs perform a unique function within the cells, which maintain cellular membrane structure and/or produce

Figure 9 Space filling model for the partial squash (*Cucurbita moschata*) gpat protein as shown in Fig. 8. (A) The active site residues, histidine 139 in red and aspartate 144 in yellow are buried deep at the end of the hydrophobic tunnel which accommodates the acyl-CoA shown as a black arrow. The hydrophobic residues are shown in green where the green color gradient indicates the hydrophobic residues from strong (in dark green) to weakly hydrophobic residues shown in light green. (B) The same model shown at a different angle such that only the histidine (red) is visible. In this view, the glycerol-3-phosphate-binding site is exposed shown as a blue curved arrow. A straight black arrow represents the fatty acid positioned in the hydrophobic tunnel. The space filling model was created using PYMOL (DeLano Scientific LLC, San Francisco, USA). The hydrophobic scale is as follows dark green: Leu, Ile, Met, Phe, Trp; medium green: Val, Pro, Lys and light green: Ala, Tyr, His, Thr, Arg.

specific phospholipids signaling molecule. Identification and characterization of AGPAT9 is a step towards understanding the role of these acyltransferases in human physiology.

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