Agpat6—a novel lipid biosynthetic gene required for triacylglycerol production in mammary epithelium

Anne P. Beigneux,[1,]* Laurent Vergnes,† Xin Qiao,* Steven Quatela,§ Ryan Davis, Steven M. Watkins,** Rosalind A. Coleman,†† Rosemary L. Walzem,** Mark Philips,§ Karen Reue,† and Stephen G. Young***

Division of Cardiology,* Department of Internal Medicine, University of California, Los Angeles, CA 90095; Departments of Medicine and Human Genetics,† David Geffen School of Medicine, University of California, Los Angeles, CA 90095, and Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 90073; Department of Medicine, Cell Biology, and Pharmacology,§ New York University School of Medicine, New York, NY 10016; Lipomics Technologies,** West Sacramento, CA 95691; and Department of Nutrition,†† University of North Carolina, Chapel Hill, NC 27599

Abstract **In analyzing the sequence tags for mutant mouse embryonic stem (ES) cell lines in BayGenomics (a mouse gene-trapping resource), we identified a novel gene, 1-acylglycerol-3-phosphate *O*-acyltransferase (*Agpat6*), with sequence similarities to previously characterized glycerolipid acyltransferases. *Agpat6*'s closest family member is another novel gene that we have provisionally designated *Agpat8*. Both *Agpat6* and *Agpat8* are conserved from plants, nematodes, and flies to mammals. AGPAT6, which is predicted to contain multiple membrane-spanning helices, is found exclusively within the endoplasmic reticulum (ER) in mammalian cells. To gain insights into the in vivo importance of *Agpat6*, we used the *Agpat6* ES cell line from BayGenomics to create *Agpat6*-deficient (*Agpat6*$^{-/-}$) mice. *Agpat6*$^{-/-}$ mice lacked full-length *Agpat6* transcripts, as judged by northern blots. One of the most striking phenotypes of *Agpat6*$^{-/-}$ mice was a defect in lactation. Pups nursed by *Agpat6*$^{-/-}$ mothers die perinatally. Normally, *Agpat6* is expressed at high levels in the mammary epithelium of breast tissue, but not in the surrounding adipose tissue. Histological studies revealed that the aveoli and ducts of *Agpat6*$^{-/-}$ lactating mammary glands were underdeveloped, and there was a dramatic decrease in the size and number of lipid droplets within mammary epithelial cells and ducts. Also, the milk from *Agpat6*$^{-/-}$ mice was markedly depleted in diacylglycerols and triacylglycerols. Thus, we identified a novel glycerolipid acyltransferase of the ER, AGPAT6, which is crucial for the production of milk fat by the mammary gland.**—Beigneux, A. P., L. Vergnes, X. Qiao, S. Quatela, R. Davis, S. M. Watkins, R. A. Coleman, R. L. Walzem, M. Philips, K. Reue, and S. G. Young. ***Agpat6*—a novel lipid biosynthetic gene required for triacylglycerol production in mammary epithelium.** ***J. Lipid Res.*** **2006.** 47: **734–744.**

Supplementary key words acyltransferase • transacylase • milk fat

BayGenomics is a genomics program that uses gene-trapping vectors to produce mutant lines of mouse embryonic stem (ES) cells (http://baygenomics.ucsf.edu/) (1). The gene inactivated by the insertion of the gene-trapping vector can easily be identified with a unique DNA sequence tag. To date, BayGenomics has inactivated >3,000 unique genes in ES cells and has distributed >2,500 different cell lines to the research community for the purpose of creating knockout mice. Aside from producing mutant ES cell clones, BayGenomics also produces a few knockout mice from the gene-trap ES cell lines, with the goal of identifying genes relevant to lipid metabolism and cardiopulmonary disease.

While analyzing the sequence tags for BayGenomics ES cell clones, we encountered a novel gene with sequence similarities to 1-acylglycerol-3-phosphate *O*-acyltransferases [AGPATs, which convert lysophosphatidic acid to phosphatidic acid (2, 3)] and other members of the glycerolipid acyltransferase family. Because of amino acid sequence similarities to AGPAT1, AGPAT2, and other putative AGPATs (AGPAT3, AGPAT4, and AGPAT5), the novel gene was provisionally designated *Agpat6*. Although this provisional name was based on similarities to other AGPATs, it should be noted that AGPAT6 also has sequence similarities to a variety of "non-AGPAT" glycerolipid acyltransferases. At about the same time, another report drew attention to the existence of *AGPAT6* in the human cDNA databases (4); however, there have been no published data on the intracellular localization of the enzyme or its in vivo importance.

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Abbreviations: AGPAT, 1-acylglycerol-3-phosphate *O*-acyltransferase; DGAT, diacylglycerol acyltransferase; ECFP, enhanced cyan fluorescent protein; ER, endoplasmic reticulum; ES, embryonic stem; GNPAT, glyceronephosphate *O*-acyltransferase; GPAM, glycerol-3-phosphate acyltransferase; LYCAT, lysocardiolipin acyltransferase.

[1] To whom correspondence should be addressed.
e-mail: abeigneux@mednet.ucla.edu

The glycerolipid acyltransferase protein family, which has been defined largely on the basis of amino acid sequence similarities, includes glycerol-3-phosphate acyltransferase (GPAM), glyceronephosphate *O*-acyltransferase (GNPAT), tafazzin, lysocardiolipin acyltransferase (LYCAT), 2-acylglycerophosphoethanolamine acyltransferase, the AGPATs, and a number of novel enzymes of unknown function. Amino acid sequence alignments of GPAM, AGPAT1, AGPAT2, and GNPAT have defined four regions of homology (motifs I–IV) that represent signatures for glycerolipid acyltransferases (5–7). Site-directed mutagenesis, followed by expression studies in *Escherichia coli*, have indicated that motifs I and IV are involved in catalysis, whereas motifs II and III are required for glycerol-3-phosphate binding (5, 8, 9). Recent studies of naturally occurring mutations in *AGPAT2* (10, 11) have revealed a fifth important sequence motif (V), but its function has not yet been identified.

The biochemical properties, subcellular localization, and biological relevance of GPAM, GNPAT, tafazzin, LYCAT, AGPAT1, and AGPAT2 have been at least partially characterized either in humans or mice (2, 3, 12–15). GPAM is located in mitochondria and catalyzes the acylation of glycerol-3-phosphate at the *sn-1* position to generate lysophosphatidic acid, and studies with *Gpam*-deficient mice have revealed that this enzyme plays a major role in triacylglycerol synthesis (12). GNPAT is located in peroxisomes and catalyzes the acylation of glyceronephosphate to generate 1-acyl-glycerone-3-phosphate, a precursor in plasmalogen synthesis (16). Mutations in GNPAT cause rhizomelic chondrodysplasia punctata type 2, characterized by shortening of the upper extremities, mental retardation, and cataracts (13). The activity of tafazzin, which is located in mitochondria, is not known with certainty, but it could be involved in transferring acyl groups from phosphatidylcholine or phosphatidylethanolamine to monolysocardiolipin (17). Mutations in tafazzin cause Barth syndrome, an X-linked disease associated with dilated cardiomyopathy, skeletal myopathy, neutropenia, and growth retardation (14). Another cardiolipin biosynthetic enzyme, LYCAT, was identified recently (15).

The biochemical roles for AGPAT1 and AGPAT2 in generating phosphatidic acid are well documented (2, 3). Mutations in *AGPAT2*, which is expressed largely in adipose tissue, cause congenital generalized lipodystrophy (10), a disease characterized by a striking absence of subcutaneous and abdominal fat, hypertriglyceridemia, and severe insulin resistance. The other putative AGPATs (AGPAT3, AGPAT4, AGPAT5, and AGPAT7) (18, 19) were identified by sequence homology, and little is known about their biochemical properties or physiologic importance. AGPAT activity was attributed to AGPAT3, AGPAT4, and AGPAT5 in one study (18), but the activity levels were very low and in no way comparable to that observed for AGPAT2 (18). The physiological relevance of the novel BayGenomics gene, *Agpat6*, had never been investigated.

We sought to determine the intracellular localization of AGPAT6 and to define its relatedness, in terms of amino acid sequence, to known glycerolipid acyltransferases. In addition, we sought to ascertain the biological importance of AGPAT6 by examining the phenotypes of *Agpat6*-deficient (*Agpat6*$^{-/-}$) mice. Here, we report that AGPAT6 is located exclusively in the endoplasmic reticulum (ER) and that its absence leads to underdeveloped mammary epithelium and the production of milk depleted in diacylglycerols and triacylglycerols. In addition, we report the identification of another novel gene, provisionally designated *Agpat8*, which is the closest homolog of *Agpat6* within the glycerolipid acyltransferase family. Remarkably, AGPAT6 and AGPAT8 are conserved from plants, flies, and worms to mammals.

MATERIALS AND METHODS

Agpat6$^{-/-}$ mice

A mouse ES cell line (DTM030, strain 129/OlaHsd) containing an insertional mutation in *Agpat6* was identified by BayGenomics, a gene-trapping resource (1). The gene-trap vector used (pGT1dTMpfs) contains a splice-acceptor sequence upstream of the reporter gene *βgeo* (a fusion of β-galactosidase and neomycin phosphotransferase II) (1). As judged by 5′ rapid amplification of cDNA ends (20), the insertional mutation in DTM030 was located in the second intron of *Agpat6*. Thus, the mutation results in the production of an in-frame fusion transcript consisting of exons 1 and 2 from *Agpat6* and *βgeo*. Another BayGenomics ES cell line, RRF360, was used to create *Agpat4* knockout mice.

We determined the exact site of insertion of the vector within intron 2, which allowed us to design a PCR strategy to genotype the mice. The following primers were used for genotyping: primer 1, 5′-ACAGGCTTTTGTGGTTTGGTTTGCT-3′; primer 2, 5′-AGAAATCCTCCCCAACAGTGGGACT-3′; and primer 3 (from vector sequences), 5′-CGTGTCCTACAACACACACTCCAACC-3′. The wild-type allele was detected with primers 1 and 2 (located in sequences flanking the insertion, yielding a 458 bp product), whereas the mutant allele was detected with primers 1 and 3, yielding a 378 bp fragment.

ES cell line DTM030 was injected into C57BL/6 blastocysts to generate chimeric mice, which were bred to establish *Agpat6* knockout mice. All mice had a mixed genetic background (C57BL/6 and 129/OlaHsd). The mice were weaned at 21 days of age, housed in a barrier facility with a 12 h light/12 h dark cycle, and fed a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO).

Northern blots

Total RNA was isolated from 50–150 mg of mouse tissue with Tri-Reagent (Sigma, St. Louis, MO). Total RNA (5 μg) was separated by electrophoresis on 1% agarose/formaldehyde gels and transferred to a Nytran SuPerCharge membrane (Schleicher and Schuell, Keene, NH). A mouse multiple-tissue poly(A)$^+$ RNA blot and a mouse embryo poly(A)$^+$ RNA blot (Clontech, Palo Alto, CA) were used to determine the tissue pattern of *Agpat6* expression in adult mice and to examine the temporal expression of *Agpat6* during embryogenesis. Bands on Northern blots were visualized by autoradiography (Fuji Super RX films; Fujifilm, Tokyo, Japan) and quantified by densitometry (Molecular Imager FX; Bio-Rad, Hercules, CA). A cDNA probe comprising sequences within the 3′ untranslated region of *Agpat6* (a region not conserved in *Agpat8*) was amplified by RT-PCR with 5′-GTGGCAGGACAAGGTCAGAGCTACA-3′ and 5′-TCCCTCCTGACTCACCAGTTCTTCC-3′. The *lacZ* probe was prepared as described previously (21). β-Actin and 18S cDNA probes were used as controls for RNA integrity and loading. $[^{32}P]$dCTP-labeled cDNA probes were prepared with All-in-One random prime labeling mixture (Sigma). Standard prehybridization, hybridization, and washing procedures were used (21).

Western blots

The complete open reading frame of *Agpat6* was amplified from a mouse embryo cDNA library by RT-PCR with primers 5′-ACCATGTTCCTGTTGCTACCT-3′ and 5′-GGACCGGCTGCGG TCCTCATGGTTTCC-3′. For expression in mammalian cells, the *Agpat6* coding sequence was cloned in-frame with a C-terminal V5-His tag into pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen, Carlsbad, CA). For expression in insect cells, *Agpat6* was amplified by PCR from pcDNA3.1/V5-His TOPO with the C-terminal V5-His tag with primers 5′-ATCGGAATTCATGTTCC TGTTGCTACCT-3′ and 5′-CGATGAATTCTCAATGGTGATG GTGATGATGACC-3′, subcloned into pCR2.1 (Invitrogen), and then subsequently cloned into the *Eco*RI site into pBacPAK8 (Clontech). The integrity of the *Agpat6* sequence within each construct was verified by sequencing. High-Five insect cells infected with C-terminal V5-tagged *Agpat6*, *Agpat2*, or *Gpam*-recombinant baculovirus, or COS-7 cells transfected with the *Agpat6*-pcDNA3.1/V5-His construct, were collected in 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS containing a mixture of protease inhibitors (Complete Mini EDTA-free; Roche Applied Science, Indianapolis, IN). Samples were sonicated on ice and clarified by centrifugation at 500 *g* for 10 min at 4°C. The protein content of the supernatant fluid (whole-cell extract) was determined with a Bradford assay (Bio-Rad). Denatured proteins (5 ng for insect cell extracts, 1 μg for COS-7 cell extracts) were size-fractionated on 4–12% Bis-Tris NuPAGE gels (Invitrogen). After electrotransfer to a sheet of nitrocellulose membrane (Invitrogen), the blots were blocked with phosphate-buffered saline containing 0.2% Tween and 5% nonfat powdered milk overnight at 4°C, then incubated for 1 h at room temperature with a horseradish peroxidase-conjugated mouse monoclonal antibody against the V5 tag (Invitrogen) (1:5,000). Antibody binding was detected with the ECL Plus Western blotting kit (Amersham Biosciences, Piscataway, NJ) and Fuji Super RX X-ray film (Fujifilm).

Histology and immunohistochemistry

Mice were anesthetized intraperitoneally with a ketamine/xylazine cocktail and perfusion-fixed with normal saline followed by 4% paraformaldehyde. For β-galactosidase staining, tissues were fixed and stained as described previously (21). For hematoxylin/eosin staining, mammary glands were fixed in 10% buffered formalin for 24 h, dehydrated in ethanol, transitioned into xylene, embedded in paraffin, sectioned (1 μm thick), and stained.

For fat staining, mice were deeply anesthetized intraperitoneally with a ketamine/xylazine cocktail and perfusion-fixed with 0.1 M cacodylate followed by 2.5% glutaraldehyde in 0.1 M cacodylate. Mammary glands were dissected out and cleaved into thin slices to ensure thorough fixation. After fixation, tissues were stained in 1% osmium tetroxide, dehydrated in ethanol, transitioned into acetonitrile, and then embedded in Epon resin.

For some immunohistochemistry studies, the *Agpat6*-pcDNA3.1/V5-His construct described above was transfected into HeLa cells. AGPAT6 subcellular localization was compared with that of protein disulfide isomerase (an ER marker) and of manganese superoxide dismutase (a mitochondrial marker). The following antibodies were used: affinity-purified rabbit anti-6×Histine (1:6,000; Immunology Consultants Laboratory, Newberg, OR); Alexa Fluor 568-labeled goat anti-rabbit IgG (1:800; Molecular Probes, Eugene, OR); mouse monoclonal anti-protein disulfide isomerase (1:700; Abcam, Cambridge, MA); Alexa Fluor 488-labeled goat anti-mouse IgG (1:800; Molecular Probes); FITC-conjugated mouse anti-V5 tag (1:1,500; Invitrogen); rabbit polyclonal anti-manganese superoxide dismutase (1 μg/ml; Stressgen, Victoria, Canada); Cy3-labeled goat anti-rabbit IgG (1:800; Abcam).

In addition, the complete coding sequences for *Agpat2*, *Agpat6*, and *Gpam* were inserted in-frame with a C-terminal enhanced cyan fluorescent protein marker in pECFP-N1 (Clontech). The ECFP-tagged constructs were expressed in COS-1 cells along with M1-YFP, a yellow fluorescent protein-tagged ER marker (22). To assess mitochondrial localization, ECFP-tagged constructs were expressed in COS-1 cells labeled with a MitoTracker dye (Molecular Probes). Cells were imaged alive with an inverted Zeiss 510 laser scanning confocal microscope (63× lens).

Analysis of lipids in milk

Three hours after removing newborn pups, lactating females were given two successive intraperitoneal injections of oxytocin (5 μl/g body weight of a 20 U/ml solution; injections separated by 20 min). At the time of the second oxytocin injection, mice were deeply anesthetized with a ketamine/xylazine cocktail, and milk was collected from the mammary glands and stored at −80°C for analysis. For all experiments, the milk was collected 24 h postpartum, when pups were still alive and suckling.

Milk lipids were extracted in the presence of authentic internal standards by the method of Folch, Lees, and Sloane Stanley (23) with chloroform-methanol (2:1, v/v). Twenty microliters of milk was used for each analysis. In some experiments, neutral lipids were separated by thin-layer chromatography in hexane-ethyl ether-acetic acid (80:20:2), subsequently visualized with iodine vapor, and identified by comigration with lipid standards.

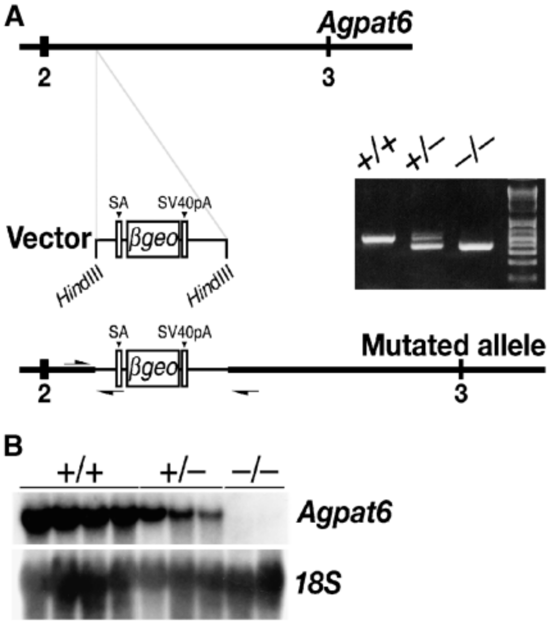

Fig. 1. An insertional mutation in 1-acylglycerol-3-phosphate *O*-acyltransferase (*Agpat6*). A: Scheme of the insertion event, and detection of the insertional mutation by PCR with genomic DNA from *Agpat6*$^{+/+}$, *Agpat6*$^{+/-}$, and *Agpat6*$^{-/-}$ mice. Numbers indicate exons. Primer orientation and location are indicated with arrows. SA, splice acceptor; SV40pA, poly(A) tail. B: Northern blot with total RNA showing the expression of *Agpat6* in *Agpat6*$^{+/+}$, *Agpat6*$^{+/-}$, and *Agpat6*$^{-/-}$ embryos. An 18S cDNA was used for normalization.

In other experiments, individual lipid classes within each extract were separated by preparative HPLC. Each isolated lipid class fraction was transesterified in 3 N methanolic-HCl in a sealed vial under nitrogen at 100°C for 45 min. The fatty acid methyl esters were extracted from the mixture with hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography by sealing the hexane extracts under nitrogen. Fatty acid methyl esters were then separated and quantified by capillary gas chromatography with a gas chromatograph (model 6890; Hewlett-Packard, Wilmington, DE) equipped with a 30-m DB-

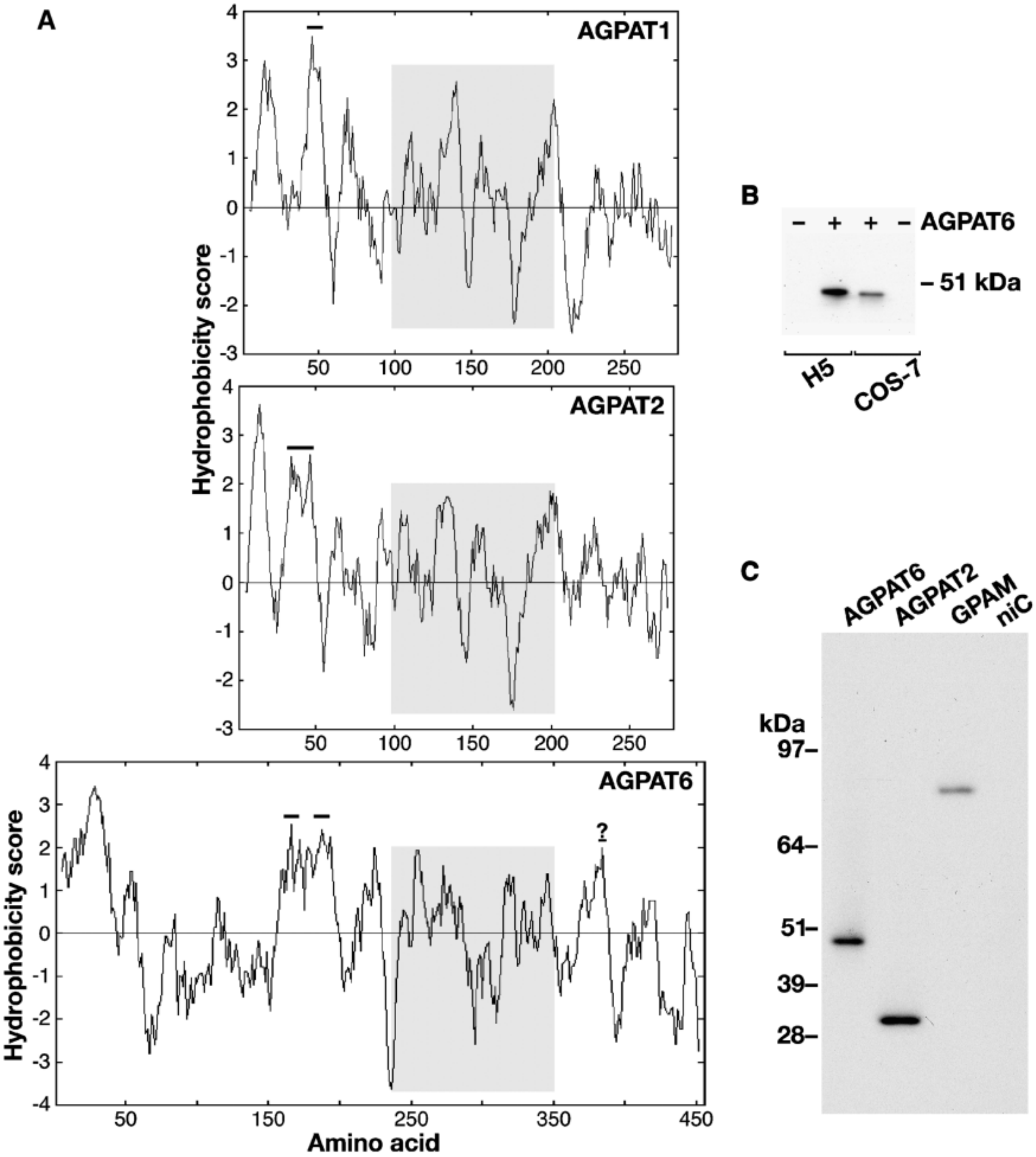

Fig. 2. Structural features of AGPAT6. A: Kyte-Doolittle hydrophobicity profiles for mouse AGPAT6, AGPAT1, and AGPAT2. Numbers on the x axis refer to amino acid residues. AGPAT6 (456 amino acids) is larger than AGPAT1 (285 amino acids) and AGPAT2 (278 amino acids), mainly because AGPAT6 contains 140 extra amino acid residues upstream of the region containing the signature glycerolipid acyltransferase sequence motifs (5–9) (highlighted in gray). Bold horizontal lines indicate predicted transmembrane domains (as judged by http://www.cbs.dtu.dk/services/TMHMM-2.0/, http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0E.html, and http://smart.embl-heidelberg.de/). The question mark in the AGPAT6 profile indicates a potential transmembrane domain that is predicted by one of the three sequence analysis programs (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0E.html). B: Western blot, with an anti-V5 antibody, showing that the molecular mass of the tagged version of AGPAT6 is 48 kDa, both in extracts from COS-7 cells transfected with V5-tagged *Agpat6* (+) and in High-Five cells infected with a V5-tagged *Agpat6* recombinant baculovirus (+). The 48 kDa band was absent in extracts from nontransfected COS-7 cells (−) and noninfected High-Five cells (−). C: Western blot of total membrane fractions (5 ng each) from High-Five cells infected with V5-tagged AGPAT6 (48 kDa), AGPAT2 (31 kDa), or glycerol-3-phosphate acyltransferase (GPAM; 93.7 kDa) baculoviruses. The molecular mass of AGPAT2 was identical to that predicted for the full-length open reading frame. Membranes from noninfected cells (niC) were included as a control.

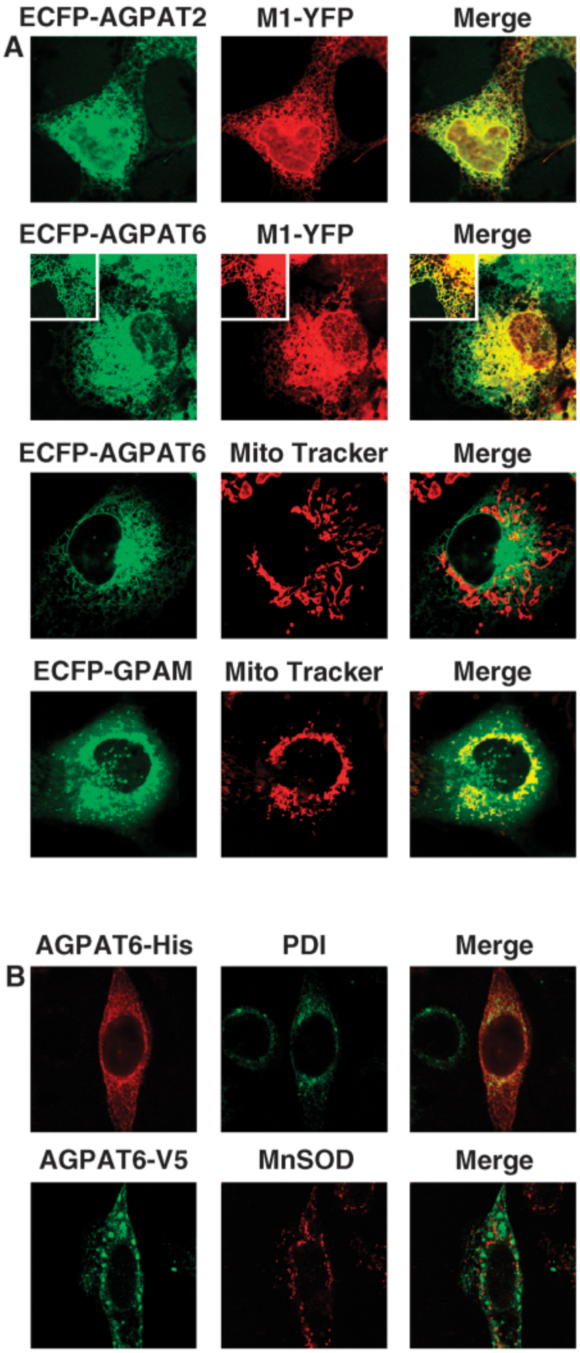

Fig. 3. AGPAT6 localizes to the endoplasmic reticulum (ER). A: Enhanced cyan fluorescent protein (ECFP)-tagged *Agpat2*, *Agpat6*, or *Gpam* constructs were cotransfected into COS-1 cells with M1-YFP (a yellow fluorescent protein-tagged ER marker) (22). To assess mitochondrial localization, ECFP-tagged constructs were expressed in COS-1 cells labeled with a MitoTracker dye. ECFP-AGPAT6 colocalized with M1-YFP but not with the MitoTracker dye, indicating an ER membrane localization for AGPAT6. Representative enlargements are shown as inserts in the panels for AGPAT6. AGPAT2 and GPAM were used as controls for ER and mitochondrial localization, respectively. B: HeLa cells transfected with an expression vector for a V5-His-tagged *Agpat6*. The subcellular localization of AGPAT6 was compared with that of an ER marker, protein disulfide isomerase (PDI), and a mitochondrial marker, manganese superoxide dismutase (MnSOD). Merged images show colocalization of AGPAT6 and PDI but not MnSOD.

225MS capillary column (J&W Scientific, Folsom, CA) and a flame-ionization detector, as described previously (24). Lipid metabolome data were expressed as nanomoles per gram and were assembled in tables as means ± SD for each group.

RESULTS

Agpat6 knockout mice

The BayGenomics library of mutant ES cells contained a mouse ES cell line with an insertional mutation in *Agpat6*, which was used to generate *Agpat6*$^{-/-}$ mice. The site of insertion was identified within intron 2, making it possible to design a PCR strategy to distinguish heterozygous (*Agpat6*$^{+/-}$) from homozygous (*Agpat6*$^{-/-}$) mice (**Fig. 1A**). As expected, full-length *Agpat6* transcripts were absent in *Agpat6*$^{-/-}$ embryos (Fig. 1B).

Fig. 4. *Agpat6* expression during development. A: β-Galactosidase staining of an *Agpat6* knockout embryo (E15). Arrowheads point to expression in brain (*b*), dorsal fat pad (*df*), lung (*lg*), liver (*lv*), and mesenchyme (*m*) of the guts. B: A mouse embryo poly(A)$^{+}$ RNA blot showing the expression of *Agpat6* throughout embryogenesis in wild-type mice. β-Actin was used for normalization.

AGPAT6 structure

AGPAT6 is predicted to contain at least two transmembrane helices (**Fig. 2A**) as well as a peptide signal and a peptide cleavage site after residue 38 (http://www.cbs.dtu.dk/services/SignalP/). When expressed in COS-7 or insect cells, the C-terminal V5-tagged version of AGPAT6 migrates at 48 kDa, smaller than the predicted size (52.2 kDa) for the full open reading frame (Fig. 2B, C). However, the predicted size of AGPAT6 after cleavage of the 38 amino acid signal peptide is 48 kDa, the size that we observed by Western blotting. In contrast, the molecular mass of AGPAT2 by Western blotting, 31 kDa, was the same as the predicted size of its entire open reading frame.

AGPAT6 is located in the ER

To assess the subcellular localization of AGPAT6, we constructed a cyan fluorescent protein-tagged AGPAT6 and transfected it into COS-1 cells. These studies revealed that AGPAT6 is located in the ER, and none was in the mitochondria (**Fig. 3A**). As expected, GPAM was located entirely in the mitochondria, and AGPAT2 was located entirely in the ER (Fig. 3A). Immunofluorescence studies with a V5-His-tagged AGPAT6 in HeLa cells also indicated that AGPAT6 is an ER protein (Fig. 3B).

AGPAT6 expression patterns in mice

β-Galactosidase staining of tissues from *Agpat6*$^{-/-}$ embryos revealed that *Agpat6* is expressed in the brain, dorsal fat pad, lung, liver, and a wide variety of mesenchymal tissues, including the mesenchyme of the gut and lung (**Fig. 4A**). Northern blots revealed that *Agpat6* is expressed at high levels throughout embryogenesis (Fig. 4B).

In adult wild-type mice, *Agpat6* is expressed in a wide variety of tissues, including kidney, liver, brain, the ovarian fat pad, and testes, as judged by Northern blots (**Figs. 5A, B**). *Agpat6* is expressed at particularly high levels in brown adipose tissue (Fig. 5B). β-Galactosidase staining confirmed high-level *Agpat6* expression in brown adipose tissue (Fig. 5C) and showed that, in the testis, *Agpat6* is expressed primarily in spermatids, with lower levels of expression in Sertoli cells (Fig. 5D). In the adult brain, *Agpat6* is expressed predominantly in cerebellum (Fig. 5E) and hippocampus (Fig. 5F). In the kidney, *Agpat6* is expressed in tubular cells (Fig. 5G).

A role for AGPAT6 in milk production

Agpat6$^{-/-}$ mice were born at the expected Mendelian frequency from crosses between heterozygous mice, indicating that, despite prominent expression in embryos, *Agpat6* is not required for survival. However, offspring derived from *Agpat6*$^{-/-}$ females die within 48 h unless they are transferred to a foster mother. Very rarely, pups that are nursed by *Agpat6*$^{-/-}$ females survive the early postnatal period, but those mice are invariably runts and die by 3–4 weeks of age. These observations led us to suspect that *Agpat6* could have a role in lactation.

Agpat6 is expressed in nonlactating mammary gland, and the expression levels are upregulated during lactation, as judged by Northern blot analysis. This upregulation was evident both for the full-length *Agpat6* transcript in wild-type mice and for the *Agpat6*-βgeo fusion transcript in *Agpat6*$^{-/-}$ mice (**Fig. 6A**). β-Galactosidase staining revealed that *Agpat6* is expressed predominantly in epithelial cells of the mammary gland; no staining was detected in the surrounding white adipose tissue (Fig. 6B). Hematoxylin and eosin-stained sections revealed that the alveoli and ducts of the mammary glands of *Agpat6*$^{-/-}$ lactating

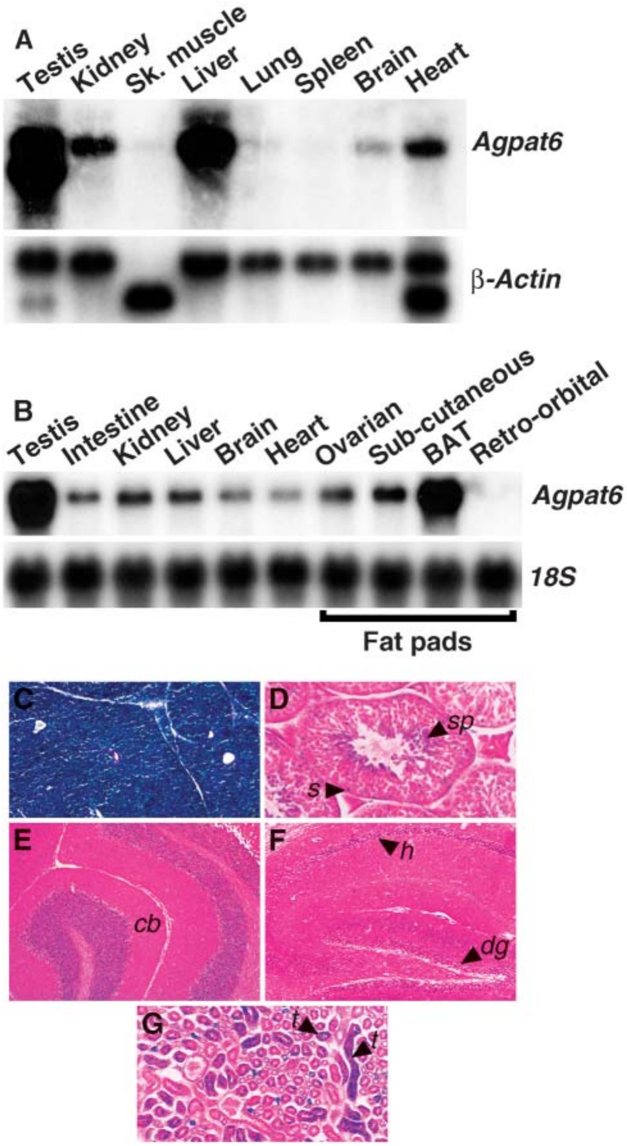

Fig. 5. *Agpat6* expression in adult mice. A: A mouse poly(A)$^{+}$ RNA blot showing the expression of *Agpat6* in adult tissues. β-Actin cDNA was used for normalization. Sk., skeletal. B: A mouse total RNA blot showing prominent expression of *Agpat6* in testis and brown adipose tissue in adult mice. 18S cDNA was used for normalization. BAT, brown adipose tissue. C–G: β-Galactosidase staining of brown adipose tissue (C), testis (D), cerebellum (E), hippocampus (F), and kidney (G) from a 6 week old *Agpat6*$^{-/-}$ male. Arrowheads point to expression in spermatids (*sp*), Sertoli cells (*s*), cerebellar lobule (*cb*), hippocampus (*h*), dentate gyrus (*dg*), and tubular cells (*t*).

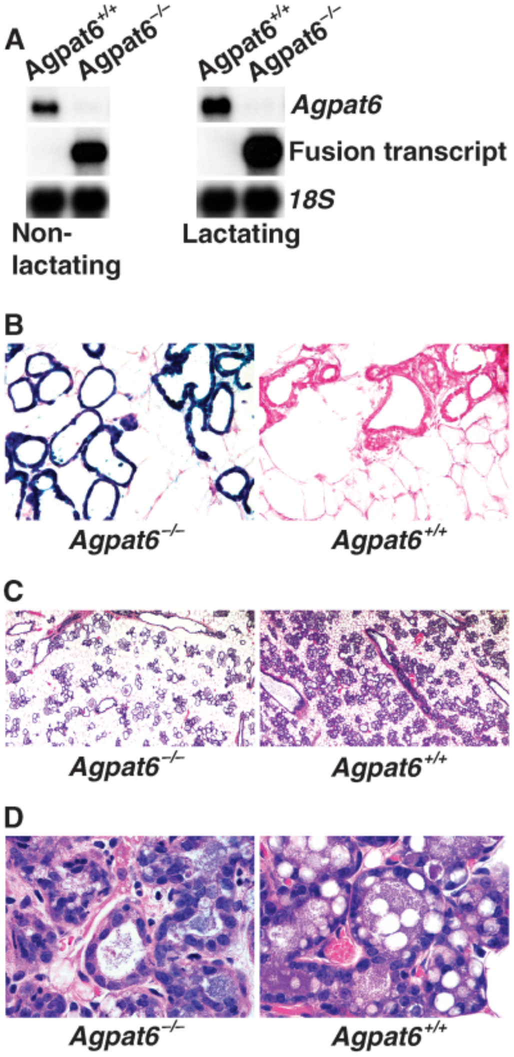

Fig. 6. Expression of *Agpat6* in mammary gland. A: Northern blot analysis of total RNA from *Agpat6*$^{+/+}$ and *Agpat6*$^{-/-}$ mammary glands. An *Agpat6* cDNA was used to detect the full-length *Agpat6* mRNA in wild-type tissues, and a *lacZ* probe was used to detect the fusion transcript in the knockout mice. An 18S cDNA was used for normalization. B: β-Galactosidase staining of mammary gland from *Agpat6*$^{-/-}$ and *Agpat6*$^{+/+}$ lactating females, revealing a high level of *Agpat6* expression in epithelial cells of the mammary gland. C, D: Hematoxylin and eosin staining of *Agpat6*$^{-/-}$ and *Agpat6*$^{+/+}$ mammary glands, showing reduced size and number of alveoli in the mammary glands of lactating *Agpat6*$^{-/-}$ mice (C) as well as reduced numbers of fat droplets in *Agpat6*$^{-/-}$ epithelial cells (D). In all experiments, the mammary glands were dissected 24 h postpartum, when pups were still alive and suckling.

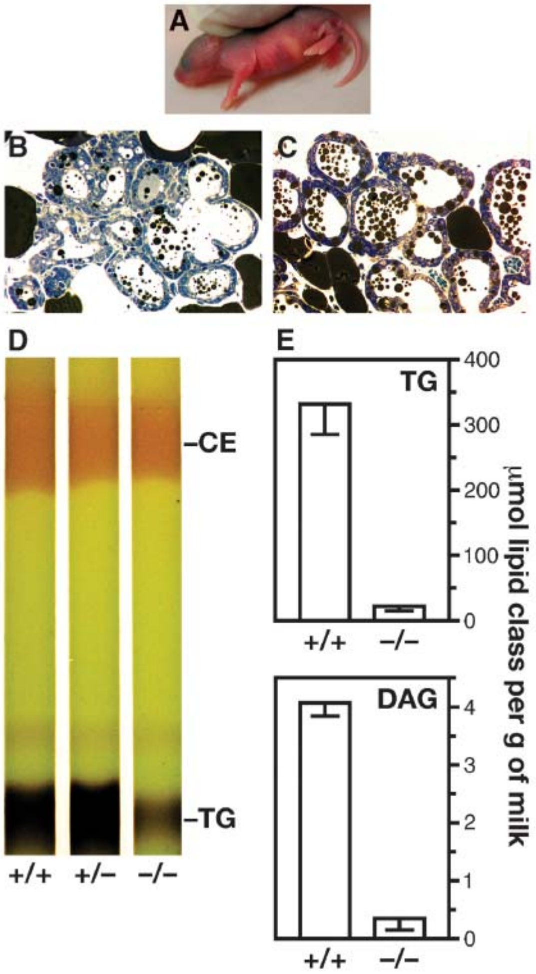

Fig. 7. Influence of *Agpat6* deficiency on the composition of milk. A: Milk streak in a pup nursed by an *Agpat6*$^{-/-}$ female. B, C: Osmium tetroxide-stained sections of mammary glands from *Agpat6*$^{-/-}$ (B) and *Agpat6*$^{+/+}$ (C) lactating females, showing decreased lipid droplets in the alveoli and ducts of mammary glands from the *Agpat6*$^{-/-}$ female. D: Reduced triacylglycerol (TG) content of milk from an *Agpat6*$^{-/-}$ female, compared with heterozygous and wild-type controls, as assessed by thin-layer chromatography. The intensity of the cholesteryl ester (CE) band was not significantly reduced in milk from *Agpat6*$^{-/-}$ females. E: Reduced diacylglycerol (DAG) and triacylglycerol content of milk from *Agpat6*$^{-/-}$ females, as assessed by gas chromatography. In all experiments, the milk was collected (or the mammary glands were dissected) 24 h postpartum, when pups were still alive and suckling. Diacylglycerol and triacylglycerol content of milk is expressed as mean ± SD for each group.

females were underdeveloped compared with those of wild-type mice (Fig. 6C). Furthermore, reduced numbers of fat droplets were evident in the epithelial cells of *Agpat6*$^{-/-}$ mammary glands compared with wild-type controls (Fig. 6D).

Agpat6$^{-/-}$ mothers had some capacity to make milk, evident by a milk stripe in newborn mouse pups (**Fig. 7A**). However, the amount of fat in the milk was reduced. Osmium tetroxide-stained sections of mammary gland revealed a decrease in the size and amount of lipid droplets in the alveoli and ducts of *Agpat6*$^{-/-}$ mammary glands (Fig. 7B) compared with the alveoli of wild-type females (Fig. 7C). In addition, thin-layer chromatography revealed that the milk from homozygous lactating females was depleted in triacylglycerols (Fig. 7D). Gas chromatography-based measurements revealed an ~90% reduction in diacylglycerols and triacylglycerols in the milk of *Agpat6*$^{-/-}$ mice (Fig. 7E).

The fact that a minimal amount of milk fat was evident in the mammary epithelium of *Agpat6*$^{-/-}$ mice suggests the possibility that another glycerolipid acyltransferase

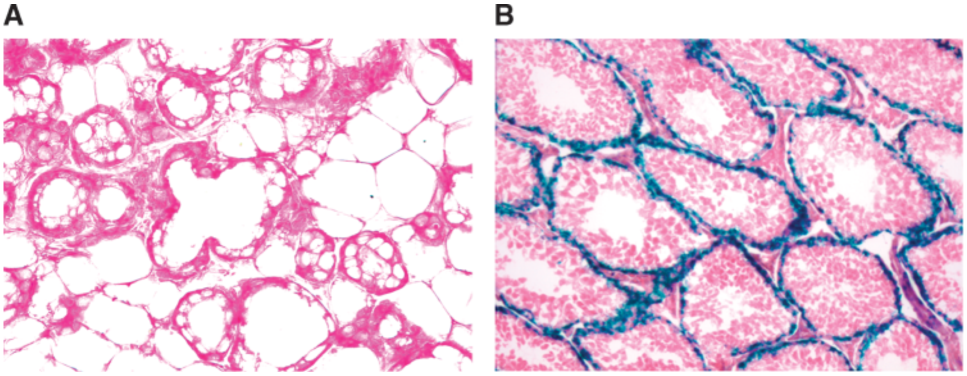

Fig. 8. *Agpat4* expression in mammary gland (A) and testis (B), as judged by β-galactosidase staining. *Agpat4* expression in the mammary gland was undetectable, whereas it was robust in the Sertoli cells of the testis.

might have a redundant role in producing triglycerides in mammary epithelium. Ultimately, understanding lipid synthesis in mammary epithelium will probably require the development of reporter alleles for all of the different glycerolipid acyltransferase enzymes. To date, we have developed a reporter allele for *Agpat4* (BayGenomics cell line RRF360); that gene is clearly not expressed in mammary epithelium or in the surrounding adipose tissue (**Fig. 8A**), whereas it is expressed strongly in the Sertoli cells of the testis (Fig. 8B).

DISCUSSION

In this study, we report the discovery, within the BayGenomics gene-trapping resource, of *Agpat6*, a new member of the glycerolipid acyltransferase family. AGPAT6 is 48 kDa in size (with the V5-His tag) and is found exclusively within the ER. *Agpat6* is expressed predominantly in brown adipose tissue and mammary epithelium. The milk from *Agpat6*$^{-/-}$ mice is depleted in diacylglycerols and triacylglycerols, and the mammary epithelium from *Agpat6*$^{-/-}$ mice is underdeveloped and depleted in intracellular fat droplets. In an accompanying article (25), we show that the triacylglycerol content of brown and white adipose tissue in *Agpat6*$^{-/-}$ mice is also reduced significantly. These experimental findings, together with sequence similarities between AGPAT6 and other glycerolipid acyltransferases (**Table 1**), suggest that AGPAT6 is a bona fide acyltransferase with an important role in the synthesis of triacylglycerols.

The mammary gland abnormalities in *Agpat6*$^{-/-}$ mice are reminiscent of those in mice lacking acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1), an enzyme that adds an acyl group to diacylglycerol to generate triacylglycerols. *Dgat1*$^{-/-}$ nursing mothers have underdeveloped mammary glands and lack the ability to produce triacylglycerol-rich milk droplets (26). The fact that the mammary glands from *Agpat6* and *Dgat1* knockout females appeared underdeveloped suggests that defective lipid biosynthetic pathways interfere, directly or indirectly, with mammary gland development.

This identification of *Agpat6* within BayGenomics illustrates the utility of gene trapping for inactivating a broad spectrum of genes, including novel genes that had escaped scientific scrutiny. Once the sequence tag for the *Agpat6* ES cell line was in hand, we were able to classify *Agpat6* as a member of the glycerolipid acyltransferase family and then move on to examine the mouse and human genomes for additional *Agpat6*-like sequences. By searching the DNA sequence databases, we quickly identified a novel, never previously reported gene resembling *Agpat6*, which we have provisionally designated *Agpat8* (Table 1). Both AGPAT6 and AGPAT8 contain the classic sequence motifs (I–IV) characteristic of glycerolipid acyltransferases (5–7). When the sequences spanning motifs I–IV were analyzed for relatedness, it was apparent that AGPAT6 and AGPAT8 were most related to each other (**Fig. 9**) (66% identical at the amino acid level, but only ~2–15% identical to the other family members). More importantly, we found that both AGPAT6 and AGPAT8 contain sequences within motifs I–IV that distinguish them from other members of the family (Table 1). Domain III is strikingly conserved between AGPAT6 and AGPAT8, representing a signature sequence for these two proteins (Table 1). Also, the arginine in the VPEGTR consensus sequence within motif III is changed to a cysteine in AGPAT6 and AGPAT8, a feature shared only by AGPAT7 and the unknown protein at locus 270084. Remarkably, orthologs for *Agpat6* and *Agpat8* appear to exist in the genomes of plants, worms, and flies (Table 1), suggesting that, together, AGPAT6 and AGPAT8 probably play a unique, fundamental, and conserved function in lipid biosynthesis.

By analyzing conserved sequences (motifs I–IV) from multiple glycerolipid acyltransferases in four species (human, mouse, *Drosophila melanogaster*, and *Caenorhabditis elegans*), we divided the family into eight subgroups (as indicated in Table 1). AGPAT1 and AGPAT2, which have been proven to carry out the AGPAT reaction (conversion of lysophosphatidic acid to phosphatidic acid) (2, 3), belong to the same subgroup. AGPAT3, AGPAT4, and AGPAT5, which have been reported to have very weak AGPAT activities (18), constitute two distinct subgroups.

TABLE 1. Proposed glycerolipid acyltransferase subgroups as assessed by alignment of amino acid sequences of putative orthologues from human, mouse, *C. elegans*, and *D. melanogaster*

Protein Subgroup	Motif I Catalysis	Motif II Glycerol-3-Phosphate Binding	Motif III Glycerol-3-Phosphate Binding	Motif IV Catalysis	Motif V	Accession Number
Human AGPAT1 (283 amino acids)	^{101}VSNHQSSLDLLGM	^{142}AGVIFIDRKR	^{175}VFPEGTRNHNGSMLPFKRGAF	^{203}VPIVPIVMSS	^{233}VLPPVPTEGLTPDD	NP_006402
Mouse AGPAT1 (285 amino acids)	^{98}VSNHQSSLDLLGM	^{139}AGIIFIDRKR	^{172}VFPEGTRNHNGSMLPFKRGAF	^{200}VPIIPIVMSS	^{231}VLPPVSTEGLTPDD	NP_061350
Human AGPAT2 (278 amino acids)	^{95}VSNHQSILDMMGL	^{136}GGVFFINRQR	^{169}IYPEGTRNDNGDLLPFKKGAF	^{197}VPIVPVVYSS	^{227}VLEAIPTSGLTAAD	NP_006403
Mouse AGPAT2 (278 amino acids)	^{95}ISNHQSILDMMGL	^{136}GGVYFINRQQ	^{169}IYPEGTRNDNGDLLPFKKGAF	^{197}VPIIPVVYSS	^{227}VLDAVPTNGLTDAD	NP_080488
Acl-1[a] (262 amino acids)	^{91}IANHQSALDVLGM	^{132}CDSVYINRFS	^{155}IYPEGTRNAEPELLPFKKGAF	^{193}IPIVPCVFSS	^{223}IIPEVDSSKFDSID	NP_510606
Acl-2 (282 amino acids)	^{96}ICNHQSSLDILSM	^{136}SNTIFIDRYN	^{169}VFPEGTRNREGGFIPFKKGAF	^{197}IPIIPVVFSD	^{228}VLDAIPTKGLTLDD	NP_505578
CG3812-PA[b] (343 amino acids)	^{98}VANHQSSLDVLGM	^{141}AGLIFIDRVR	^{174}VFPEGTRRNTGALHPFKKGAF	^{202}IPILPVVFSS	^{232}TIPPVSTEGLTKDD	NP_572828
CG17608-PB (271 amino acids)	^{93}IMNHQSAVDLCVL	^{136}WGTLYIDRSR	^{169}IFPEGTRNSKDSLLPFKKGSF	^{197}SPVQPVVISK	^{226}IIPEVSTEKYKRED	NP_723398
Human AGPAT5 (364 amino acids)	^{90}LANHQSTVDWIVA	^{135}QHGGIYVKRS	^{170}IFPEGTRYNPEQTKVLSAAQA	^{202}HVLTPRIKAT		NP_060831
Mouse AGPAT5 (365 amino acids)	^{90}LANHQSTVDWIVA	^{135}QHGGIYVKRS	^{170}IFPEGTRYNATYTKLLSASQA	^{202}HVLTPRIKAT		NP_081068
Acl-11 (368 amino acids)	^{96}ISNHQSNVDWIIP	^{144}QHGYIYVRRF	^{179}IFPEGTRNSAKKKHLLESSNR	^{211}NVLCPRSGGL		NP_491479
Human GPAM (828 amino acids)	^{227}LPVHRSHIDYLLL	^{271}LGGFFIRRRL	^{312}IFIEGTRSRSGKTSCARAGLL	^{347}ILIIPVGISY		AAH30783
Mouse GPAM (827 amino acids)	^{227}LPVHRSHIDYLLL	^{271}LGGFFIRRRL	^{312}IFIEGTRSRSGKTSCARAGVL	^{347}ILVIPVGISY		AAA37647
Acl-6 (718 amino acids)	^{164}LPLHRSHLDYLLI	^{208}TGAFFIRRRV	^{249}FFIEGTRSFFGKALTPKNGLI	^{284}CYLVPVSYTY		NP_001023769
CG5508-PA (850 amino acids)	^{260}VPLHRSHLDYIMV	^{304}LGAFFIKRKI	^{344}FFIEGGRTRTGKPCMPKGGIL	^{379}ALLVPVSVNY		NP_651597
Human GNPAT (680 amino acids)	^{159}LPSHRSYIDFLML	^{204}SGAFFMRRTF	^{240}FFIEGTRSRSAKTLTPKFGLL	^{275}TYLVPISISY		NP_055051
Mouse GNPAT (678 amino acids)	^{158}LPSHRSYIDFLML	^{203}SGAFFMRRTF	^{239}FFIEGTRSRAAKTLTPKFGLL	^{274}TYFVPISISY		NP_034452
Acl-7 (671 amino acids)	^{145}MPSHKTYFDFLLL	^{190}SGAFFMRRSF	^{226}FFVEATRSRVGKSLHPKYGML	^{261}IVIVPVSMNY		NP_496725
CG4625-PA (724 amino acids)	^{158}LPSHRSYMDFILM	^{203}TGAFFMRRSF	^{239}FFIEGTRSRNFKALVPKIGLL	^{274}VMIVPVSVAY		NP_525010
Human AGPAT7 (524 amino acids)	^{126}AAPHSTFFDPIVL	^{165}NQAILVSRHD	^{200}FFPEGTCSNKKALLKFKPGAF	^{224}VPVQPVLIRY		AAU34184
Mouse AGPAT7 (524 amino acids)	^{126}AAPHSTFFDPIVL	^{165}NQAILVSRHD	^{200}FFPEGTCSNKKALLKFKPGAF	^{224}VPVQPVLIRY		NP_997089
Human LOC54947 (544 amino acids)	^{143}AAPHSTFFDGIAC	^{182}VQPVLVSRVD	^{217}VFPEGTCTNRSCLITFKPGAF	^{241}VPVQPVLLRY		NP_060309
Mouse LOC270084 (544 amino acids)	^{143}VAPHSTFFDGIAC	^{182}LQPVLVSRVD	^{217}VFPEGTCTNRSCLITFKPGAF	^{241}VPVQPVLLRY		NP_766602
Human AGPAT3 (376 amino acids)	^{93}ILNHNFEIDLFCG	^{139}LEIVFCKRKW	^{173}LYCEGTRFTETKHRVSMEVAA	^{202}YHLLPRTKGF		NP_064517
Mouse AGPAT3 (376 amino acids)	^{93}ILNHNFEIDFLCG	^{139}LEIVFCKRKW	^{173}LYCEGTRFTETKHRISMEVAA	^{202}YHLLPRTKGF		NP_443747
CG4753-PA (380 amino acids)	^{90}LMNHTYEIDWLTA	^{136}AEFIFLDRNF	^{170}LNAEGTRFTPAKHELSVKFAE	^{199}HHLIPRTKGF		NP_730158
Human AGPAT4 (378 amino acids)	^{93}VLNHKFEIDFLCG	^{139}TEMVFCSRKW	^{173}IHCEGTRFTEKKHEISMQVAR	^{202}HHLLPRTKGF		AAF80338
Mouse AGPAT4 (378 amino acids)	^{93}VLNHKFEIDFLCG	^{139}VEMIFCTRKW	^{173}IHCEGTRFTEKKHQISMQVAQ	^{202}HHLLPRTKGF		NP_080920
CG4729-PB (386 amino acids)	^{95}IMNHKYEIDWLNG	^{141}AEFVFLNRNF	^{175}LNAEGTRFTPAKHEASVKFAQ	^{204}HHLIPRTKGF		NP_730160
Human AGPAT6 (456 amino acids)	^{245}VANHTSPIDVIIL	^{286}PHVWFERSEV	^{319}IFPEGTCINNTSVMMFKKGSF	^{343}ATVYPVAIKY	^{385}VWYLPPMTREADED	NP_848934
Mouse AGPAT6 (456 amino acids)	^{245}VANHTSPIDVIIL	^{286}PHVWFERSEV	^{319}IFPEGTCINNTSVMMFKKGSF	^{343}ATVYPVAIKY	^{385}VWYLPPMTREKDED	BAC32273
Acl-4 (617 amino acids)	^{334}VANHTSPIDALIL	^{375}SHIWFERSEA	^{408}IFPEGTCINNTSVMMFKKGSF	^{432}TTIYPIAMKY	^{474}VWYLPPMTRRDGED	NP_508379
CG3209-PA (537 amino acids)	^{331}VANHTSPIDVLVL	^{372}PHIWFERGEA	^{405}IFPEGTCINNTSVMQFKKGSF	^{429}GVIYPVAIKY	^{471}VWYLPPMYRQEGES	NP_611880
Human AGPAT8 (434 amino acids)	^{226}VANHTSPIDVLIL	^{267}PHVWFERSEM	^{300}IFPEGTCINNTSVMMFKKGSF	^{324}GTIHPVAIKY	^{366}VWYMPPMTREEGED	NP_116106
Mouse AGPAT8 (438 amino acids)	^{226}VANHTSPIDVLIL	^{267}PHVWFERSEI	^{300}IFPEGTCINNTSVMMFKKGSF	^{324}GTIYPVAIKY	^{366}VWYMPPMTREEGED	NP_766303
Acl-5 (512 amino acids)	^{241}VANHTSPIDVMVL	^{282}HHIWFERGEA	^{315}IFPEGTCINNTSVMMFKKGSF	^{339}STIYPIAVKY	^{381}VWYLPAMTRGENED	NP_509732
CG15450-PA (407 amino acids)	^{217}VCNHTSPLDVLVL	^{258}HHMWFDRKEL	^{290}IFPEGTCINNTAVMQFKKGSF	^{315}DVVHPVAIRY	^{358}VWYMPALSRCNDES	NP_608409
At5g60620[c] (376 amino acids)	^{168}VANHTSMIDFIVL	^{209}GCIWFNRSEA	^{242}IFPEGTCVNNNYTVMFKKGAF	^{266}CTVCPIAIKY	^{308}VWYLEPQTIRPGET	NP_568925
Human LYCAT (414 amino acids)	^{120}IMNHRTRMDWMFL	^{166}AAYIFIHRKW	^{200}IFPEGTDLTENSKSRSNAFAE	^{229}YVLHPRTTGF		NP_872357
Mouse LYCAT (376 amino acids)	^{82}IMNHRTRVDWMFL	^{128}AAFIFIHRKW	^{162}IFPEGTDLTENNKARSNDFAE	^{191}YVLHPRTTGF		XP_128781
Acl-8 (344 amino acids)	^{82}IMNHRTRLDWLFS	^{130}GSYIFLDRNF	^{164}IFAEGTDKGERATRLSDAFAD	^{193}YVLHPRTTGF		NP_504643
Acl-10 (439 amino acids)	^{46}VMNHRTRLDWMYM	^{94}AQFVFLERNA	^{128}IFPEGTDKSEWTTLKSREFAK	^{157}YVLYPRTTGF		NP_505971
Acl-9 (399 amino acids)	^{98}IMNHRTRLDWLFF	^{146}ASYIFLDRSF	^{180}IFPEGTDKCPKATERSRIHSE	^{209}YVLHPRVTGF		NP_504644
Human tafazzin (292 amino acids)	^{66}VSNHQSCMDDPHL	^{87}IWNLKLMRWT	^{177}IFPEGKVNMS-SEFLRFKWGIG	^{207}PIILPLWHVG	^{237}VLIGKPFSALPVLE	NP_000107
Mouse tafazzin (262 amino acids)	^{66}VSNHQSCMDDPHL	^{87}IWNLKLMRWT	^{147}IFPEGKVNMS-SEFLRFKWGIG	^{177}PIILPLWHVG	^{207}VLIGKPFSTLPVLE	NP_852657
Acl-3 (248 amino acids)	^{41}VSNHRSNIDDPLM		^{123}IFPEGKVCTLESEPLRFKWGIG	^{154}PVILPVWCKE		NP_502202
CG8766-PA (378 amino acids)	^{185}VSNHYSCFDDPGL	^{205}VCNTYKIRWS	^{266}VFPEGKVNMDKEE-LRLKWGVG	^{296}PIILPMWHEG		NP_477432

AGPAT, 1-acylglycerol-3-phosphate *O*-acyltransferase; GNPAT, glyceronephosphate *O*-acyltransferase; GPAM, glycerol-3-phosphate acyltransferase; LYCAT, lysocardiolipin acyltransferase. Numbers refer to amino acid residue positions within each protein sequence. Boldface characters show consensus motifs that define the glycerolipid acyltransferase family. Underlined characters represent amino acid identity that helped discern eight different subgroups within the glycerolipid acyltransferase family.

[a] Acl-1 through Acl-11 represent putative orthologues in *C. elegans* according to best possible match.

[b] Gene names starting with CG represent putative orthologues in *D. melanogaster* according to best possible match.

[c] Putative orthologue in *Arabidopsis thaliana* according to best possible match.

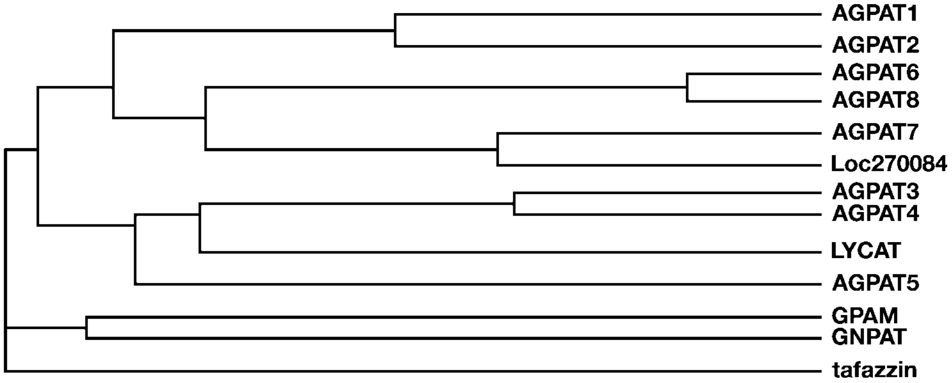

Fig. 9. Dendrogram illustrating the amino acid sequence-relatedness of mouse glycerolipid acyltransferases within the region of the proteins spanning functional domains I–IV (5–7). Alignments were performed with the Clustal W algorithm (http://www.ebi.ac.uk/clustalw/).

GPAM and GNPAT, two enzymes that add acyl groups to the *sn-1* position (16, 27–29), are more closely related to each other than to any other member of the family. LYCAT (15) falls into a fifth subgroup. Interestingly, we identified three putative *C. elegans* orthologs for mammalian LYCAT (15). Tafazzin falls into a sixth subgroup; tafazzin is involved in cardiolipin remodeling, but its precise biochemical role remains to be established (17). AGPAT7 (19) and a closely related novel protein (locus 270084) form a seventh subgroup; their biological importance and biochemical function remain to be determined. AGPAT6 and AGPAT8 form the eighth subgroup.

For the entire glycerolipid acyltransferase family, we hypothesize that sequence-relatedness within domains I–IV will ultimately be shown to correlate with biochemical function. For example, in the case of AGPAT6 and AGPAT8, we hypothesize that these two enzymes will ultimately be shown to have acyl acceptor and/or donor preferences that are similar to each other and distinct from those of other AGPATs, GPAMs, GNPATs, or LYCATs.

The identification of enzymatic activities for putative lipid biosynthetic enzymes can be straightforward (2, 3, 30, 31), but in some cases it has been very challenging. For example, despite sequence motifs suggesting an acyltransferase activity (32) and despite years of biochemical studies by multiple groups, the biochemical role for tafazzin in cardiolipin remodeling has not yet been identified with certainty. This has certainly been the case for several of the AGPATs.

We expressed AGPAT2, GPAM, and AGPAT6 in insect cells with sequence-verified plasmids and then tested the membrane fractions for GPAM or AGPAT activities under a variety of reaction conditions. Although the biochemical activities of our experimental controls, GPAM and AGPAT2, were invariably extremely robust (>5–10× background), we have not observed any AGPAT or GPAM activities in insect cell membranes overexpressing AGPAT6 (at least no activity above background) (A. Beigneux and S. G. Young, unpublished results). Similarly, we have not identified AGPAT or GPAM activities in *E. coli* membranes overexpressing AGPAT6.

One way to explain these results, of course, is to postulate that the reaction conditions were not appropriate for AGPAT6, although they were perfectly suitable for the AGPAT2 and GPAM controls. This is definitely possible. For example, DGAT1 and DGAT2 carry out the same reaction but have different requirements for magnesium (31), so it would be a mistake to assume that AGPAT2 and AGPAT6 would share identical in vitro reaction conditions. Given the phenotypes of the mice, an AGPAT or GPAM activity for AGPAT6 would make a lot of sense. A reduced capacity for synthesizing lysophosphatidic acid or phosphatidic acid would probably explain the reduced amounts of diacylglycerols and triacylglycerols in the milk and in the brown fat (25) of *Agpat6*$^{-/-}$ mice.

On the other hand, it is possible that AGPAT6 catalyzes a distinct biochemical activity. One reason for suspecting a different activity is that we have been unable, to date, to detect even a small increase in AGPAT or GPAM activity in AGPAT6-enriched membranes under a variety of assay conditions with various *sn*-1-acylglycerols and acyl-CoA species as substrates. A second reason for suspecting a different enzymatic function is that it would be astonishing if mammals (and lower organisms such as worms and flies) truly require seven or more distinct AGPAT enzymes. Enzymes for crucial steps in lipid biosynthesis are frequently redundant (6), but the need for seven or more different AGPATs would be quite remarkable, particularly because the fatty acyl chain specificities of AGPAT1 and AGPAT2 are broad (2, 3) and the loss of *AGPAT2* causes such striking disease phenotypes (33).

In the end, biochemical studies, as well as the characterization of a knockout mouse for each of the glycerolipid acyltransferases, will be critical for understanding lipid synthesis and lipid storage in different tissues and for understanding the potential relevance of this family of enzymes to health and disease.

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