Expression, regulation, and triglyceride hydrolase activity of Adiponutrin family members

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Abstract **Adiponutrin and a related protein, adipocyte triglyceride lipase (ATGL; also known as Desnutrin), were recently described as adipocyte-specific proteins with lipid hydrolase activity. Using bioinformatics, we identified three additional Adiponutrin family members (GS2, GS2-Like, and PNPLA1). Here, we report on the expression, regulation, and activity of GS2 and GS2-Like compared with Adiponutrin and Desnutrin/ATGL. GS2-Like is expressed and regulated in a manner similar to Adiponutrin; however, the absolute levels of mRNA are significantly lower than those of Adiponutrin or Desnutrin/ATGL. GS2 transcripts were identified only in humans and are highly expressed in adipose as well as other tissues. All four proteins show lipase activity in vitro, which is dependent on the presence of the active site serine for Adiponutrin, Desnutrin/ATGL, and GS2. Overexpression of Desnutrin/ATGL, GS2, and GS2-Like, but not Adiponutrin, decreases intracellular triglyceride levels. This is consistent with a function for Desnutrin/ATGL, GS2, and GS2-Like in lipolysis, but not for Adiponutrin. Consistent with previously reported data, Desnutrin/ATGL is upregulated by fasting in adipose tissue, whereas Adiponutrin is downregulated. Additionally, Adiponutrin and GS2-Like, but not Desnutrin/ATGL, are strongly induced in the liver of *ob/ob* mice. Our data support distinct functions for Adiponutrin and Desnutrin/ATGL and raise the possibility that GS2 may contribute significantly to lipolysis in human adipose tissue.**—Lake, A. C., Y. Sun, J-L. Li, J. E. Kim, J. W. Johnson, D. Li, T. Revett, H. H. Shih, W. Liu, J. E. Paulsen, and R. E. Gimeno. **Expression, regulation, and triglyceride hydrolase activity of Adiponutrin family members.** ***J. Lipid Res.*** **2005.** 46: **2477–2487.**

Supplementary key words adipose tissue • adipocyte • lipolysis • Desnutrin • triglyceride lipase • patatin domain

Adipose tissue is a key regulator of energy balance, not only as a storage depot for fat but also as an important source of paracrine and endocrine factors (1–4). Fatty acid storage (lipogenesis) and release (lipolysis) from adipose tissue is tightly regulated (5), and dysregulation of these processes has been implicated in the pathophysiology of obesity, insulin resistance, dyslipidemia, and cardiovascular disease (6–9).

Lipolysis is mediated by intracellular lipases that act sequentially to remove fatty acid groups from the glycerol backbone of triglycerides to ultimately form glycerol and free fatty acids. Until recently, the major triglyceride lipase was thought to be hormone-sensitive lipase (HSL), a lipid droplet-associated protein whose activity and subcellular localization are regulated by lipogenic and lipolytic stimuli (5, 10). The presence of significant residual lipolysis in adipose tissue of HSL-null mice suggested the existence of an additional triglyceride lipase, and recently, a candidate for this activity, adipocyte triglyceride lipase (ATGL; also known as Desnutrin), was identified and shown to be responsible for most, if not all, lipolysis remaining in HSL-null mice (11, 12).

Interestingly, Desnutrin/ATGL is most closely related to Adiponutrin, an adipocyte-specific protein of unknown function that recently was also shown to have lipid hydrolase activity (13). Both Adiponutrin and Desnutrin/ATGL contain an N-terminal patatin-like domain that includes a conserved catalytic dyad [Gly-X-Ser-X-Gly and Asp-X-Gly/Ala (14)]. In contrast to Desnutrin/ATGL, whose expression is upregulated under conditions of increased lipolysis (i.e., fasting), Adiponutrin mRNA dramatically decreases in adipose tissue during fasting (15–18). Two additional Adiponutrin-related genes have been identified in the literature (13, 19); however, their expression pattern in adipose tissue and function have not yet been examined, and the existence of other Adiponutrin family members is unclear.

We performed a comprehensive bioinformatic analysis of the Adiponutrin family and identified five family mem-

Abbreviations: ATGL, adipocyte triglyceride lipase; DGGR, 1,2-*o*-dilauryl-*rac*-glycero-3-glutaric acid-(6′-methylresorufin) ester; EST, expressed sequence tag; HMM, hidden Markov model; HSL, hormone-sensitive lipase; OD, optical density; Q-PCR, quantitative reverse transcription polymerase chain reaction.

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bers: Adiponutrin, Desnutrin/ATGL, GS2, GS2-Like, and PNPLA1. Our data demonstrate that Adiponutrin family members are expressed and regulated in a manner consistent with a role in energy homeostasis. We also report lipid hydrolase activity and the effects of overexpression of Adiponutrin, Desnutrin/ATGL, GS2, and GS2-Like on triglyceride storage in cells. Our data support Desnutrin/ATGL as the major Adiponutrin family lipase in mouse adipocytes, but they raise the possibility that an Adiponutrin homolog, GS2, may contribute to lipolysis in human adipocytes. In addition, our data support a possible role for Adiponutrin and GS2-Like in lipid metabolism in the liver.

MATERIALS AND METHODS

Bioinformatic analysis

A profile hidden Markov model (profile HMM) was generated using Desnutrin/ATGL (accession number NP_080078), Adiponutrin (accession number NP_473429), and the patatin family Pfam alignment as a seed alignment. The profile HMM was used to search public databases to generate a list of potential family members (20). CLUSTAL W (21) was used to align the patatin-like domain-containing sequences identified by the profile HMM, which in turn were added to the profile HMM for subsequent searches (22). The final alignment, which included predicted as well as confirmed sequences, was used as the input for the phylogenetic tree using the neighbor-joining method and 1,000 bootstrap trials. The resulting files were processed to create the diagram shown in **Fig. 1**. Public accession numbers and the amino acid or nucleotide ranges used for the alignment are indicated in **Table 1**. Where indicated, protein sequences were corrected using available genomic and expressed sequence tag (EST) sequences; nucleotide sequences were translated before alignment.

Northern blot analysis

Human tissue Northern blots containing 1 μg of poly(A)-enriched mRNA were purchased from Clontech. Blots were prehybridized in Quickhyb (Stratagene) for 30 min at 68°C followed by hybridization in Quickhyb for 1.5 h at 68°C with gene-specific ^{32}P-labeled probes. Blots were washed to high stringency in 0.1× SSC, 1% SDS at 65°C. Human total RNA Northern blots (United States Biological) were incubated in hybridization buffer (6× SSC, 5× Denhardt's solution, 10 mg of denatured salmon sperm DNA, 50% formamide, and 0.5% SDS) for 3 h at 42°C followed by hybridization with gene-specific probes for 16–24 h at 42°C and washes in 0.1× SSC, 0.1% SDS at 50°C. Random-primed, ^{32}P-

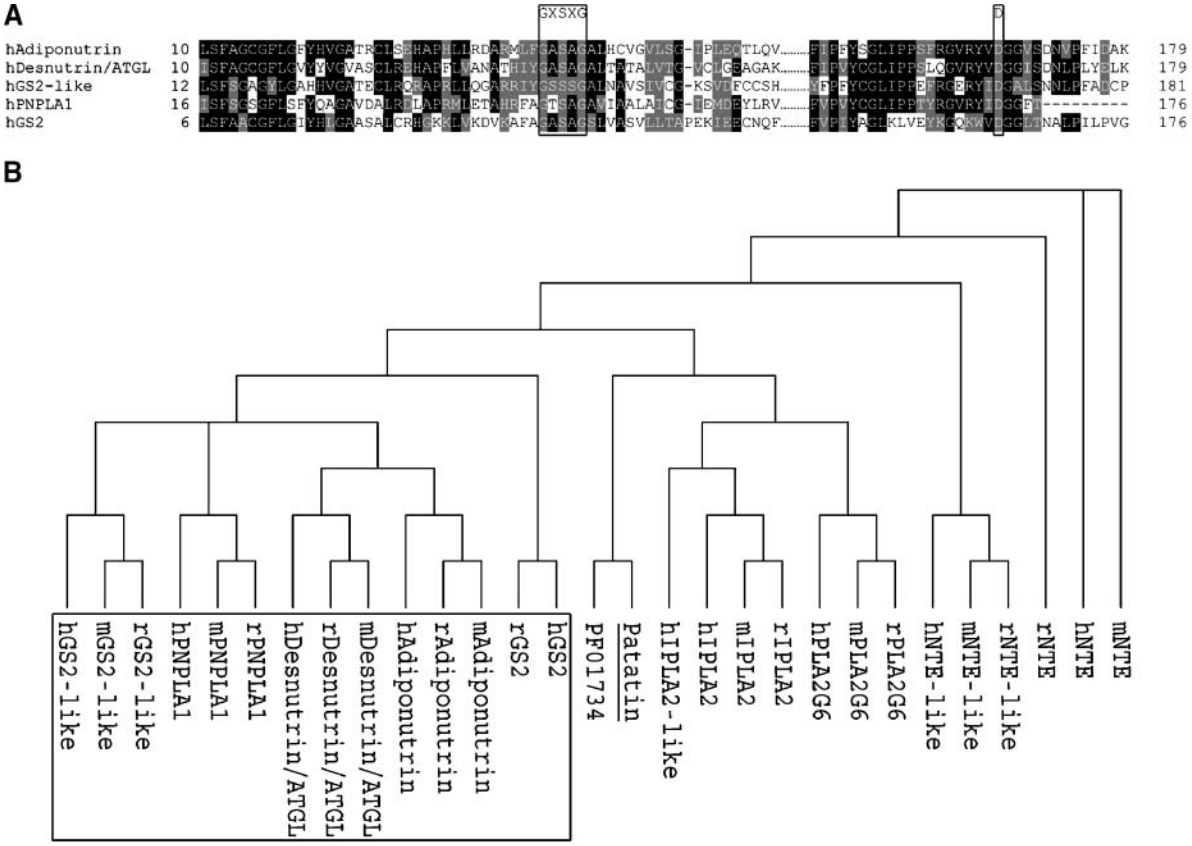

Fig. 1. Adiponutrin family members contain a patatin-like domain and are closely related to other lipid acyl hydrolases. A: Amino acid alignment of the patatin-like domains of human (h) adipocyte triglyceride lipase (ATGL; also known as Desnutrin/ATGL), GS2, GS2-Like, and predicted PNPLA1 proteins. The conserved motifs (Gly-X-Ser-X-Gly, the conserved Asp of the Asp-X-Gly/Ala motif) are boxed. B: Bootstrap neighbor-joining phylogenetic tree showing the evolutionary relationship between the patatin-like domains of the patatin gene sequence and human (h), mouse (m), and rat (r) gene sequences. The box indicates the close evolutionary relationship between the amino acid sequences of the patatin-like domains of GS2, Adiponutrin, Desnutrin/ATGL, PNPLA1, and GS2-Like proteins. The range of amino acids used to create the sequence alignments and the tree, along with the accession numbers for each protein sequence, are listed in Table 1. PF01734 is the Pfam model alignment.

TABLE 1. Accession numbers and range of amino acids used for alignments

Protein	Accession Number	Patatin Domain
Potato patatin	AAK56395	Amino acids 32–196
hADPN	NP_079501	Amino acids 10–179
mADPN	NP_473429	Amino acids 10–179
rADPN	XP_343303	Amino acids 10–176 with three amino acids (DHI) inserted after amino acid 62
hGS2	NP_004641	Amino acids 6–176
rGS2	XP_343791	Amino acids 6–175
hGS2-like	NP_620169	Amino acids 12–181
mGS2-like	XP_128189	Amino acids 12–181
rGS2-like	XP_235538	Amino acids 12–178
hIPLA2	XP_374515, XP_291241	Amino acids 473–668
mIPLA2	NP_080440	Amino acids 439–634
rIPLA2	XP_234092	Amino acids 444–639
hIPLA2-like	ENST00000329749	Amino acids 443–604
hNTE	NP_006693	Amino acids 933–1,099
mNTE	NP_056616	Amino acids 933–1,099
rNTE	XP_341026	Amino acids 987–1,148
hNTE-like	NP_689499	Amino acids 928–1,094
mNTE-like	NP_666363	Amino acids 924–1,090
rNTE-like	AAM44077	Amino acids 280–446
hPLA2G6	NP_003551	Amino acids 481–665
mPLA2G6	NP_058611	Amino acids 427–611
rPLA2G6	NP_446344	Amino acids 426–610
hPNPLA1 (HUM_CHR6-DNA:51465675_271; HUM_CHR6-DNA:51465675_272)	NT_007592	Nucleotides 96,532–96,690, 17,346–17,581, 19,090–19,153, 20,217–20,240
mPNPLA1 (MUS_CHR17-DNA:63705158_054)	NT_039649	Nucleotides 85,712–85,868, 103,609–103,852, 104,198–104,249, 105,163–105,198
rPNPLA1	XP_342106	Amino acids 16–68, 189–288, 343–351
hDesnutrin/ATGL	NP_065109	Amino acids 10–179
mDesnutrin/ATGL	NP_080078	Amino acids 10–179
rDesnutrin/ATGL	XP_341961	Amino acids 10–179

ATGL, adipocyte triglyceride lipase.

labeled, double-stranded cDNA probes were generated using the Prime-It kit and protocol (Stratagene) and purified using the NICK column and protocol from Amersham Biosciences. Probes were made from gel-purified restriction fragments of I.M.A.G.E. Consortium cDNA clones as follows: human Desnutrin/ATGL, ~1,200 bp *Apa*I, clone 6598433; human Adiponutrin, ~800 bp *Eco*RI/*Xho*I, clone 6081351; human GS2, ~800 bp *Eco*RV/*Sal*I, clone 6109547; human GS2-Like, ~2,300 bp *Sal*I/*Not*I, clone 4778605; and human PNPLA1, ~700 bp *Sal*I/*Not*I, clone 5123005. Autoradiography was performed at −80°C using Optex L-plus intensifying screens. Blots were subsequently hybridized with a β-actin probe.

Sample acquisition and RNA isolation

Normal mouse tissues were collected from 9 week old male C57Bl/6J mice fed ad libitum and euthanized by CO_2 asphyxiation. For all tissues with the exception of liver, two pools containing tissues from two individual animals each were analyzed. For liver, two individual animals were analyzed. The mouse preadipocyte line 3T3-L1 was obtained from the American Type Culture Collection. For differentiation, cells were grown in basal medium (Zen-Bio) for 4 days, transferred to adipocyte differentiation medium (Zen-Bio) for 4 days, followed by culture in adipocyte medium (Zen-Bio) for 2 days before harvest. Undifferentiated control cells were harvested after 2 days in basal medium only. Three independent samples were analyzed for both undifferentiated and differentiated cells. Primary adipocytes were obtained from epididymal adipose tissue of ad libitum-fed male C57Bl/6J mice at 8–12 weeks of age. Adipose tissue was cut into small pieces, rinsed in isolation buffer (120 mM NaCl, 0.5 mM KCl, 1.2 mM KH_2PO_4, 0.6 mM $MgSO_4{\cdot}7H_2O$, 0.9 mM $CaCl_2{\cdot}6H_2O$, 10 mM HEPES, 200 nM adenosine, and 2.5% BSA), and digested with collagenase type I (1 mg/ml/g fat; Worthington Biochemical Corp.) for 30–60 min with gentle shaking at 37°C. The digested material was passed through a 400 μm nylon mesh (Tetko), and stromal cells and adipocytes were separated by centrifugation, with adipocytes floating on the surface. Adipocytes were transferred to clean tubes and washed four times with the isolation buffer. The pellet containing stromal vascular cells was resuspended in red blood cell lysis buffer (0.83% NH_4Cl, 0.05 mM Na_2EDTA, and 0.1% $KHCO_3$, pH 7.3) and then pelleted for RNA extraction. One pool of stromal cells and three pools of primary adipocytes representing at least 15 mice each were analyzed. Liver and adipose tissue were obtained from 10 week old, ad libitum-fed, male *ob/ob* or control (C57Bl/6J) mice [n = 6; average body weight at 9 weeks of age, 25 ± 0.5 g (wild type) and 49 ± 0.5 g (*ob/ob*); fasting blood glucose at 9 weeks of age, 60 ± 1.1 mg/dl (wild type) and 140 ± 9.2 mg/dl (*ob/ob*)] and from 8 week old male C57Bl/6J mice either fed ad libitum or fasted for 24 h before euthanasia. Samples from individual animals (n = 6) were analyzed, and data are expressed as means ± SEM. All mouse tissues were flash-frozen in liquid nitrogen and stored at −80°C until RNA isolation.

RNA was extracted from cells and tissues using Trizol (Invitrogen) and purified using the RNeasy kit and protocol, including DNaseI treatment (Qiagen). RNA quantity and quality were assessed by optical density (OD) 260/280 and by visualization on an ethidium bromide-stained agarose gel.

TaqMan real-time quantitative reverse transcription PCR analysis

Murine Adiponutrin, Desnutrin/ATGL, GS2-Like, and PNPLA1 mRNA expression was measured by quantitative reverse transcription polymerase chain reaction (Q-PCR) analysis on panels of cDNAs from a variety of murine tissues and cell lines, which are described above. Oligonucleotide primers and fluorescently

labeled TaqMan probes were designed using Primer Express 2.0 software (Applied Biosystems). Sequences for primers and probes were as follows. Adiponutrin: forward, 5′-CGAGGCGAGCGGTACGT-3′; reverse, 5′-GTGACACCGTGATGGTGGTTT-3′; probe, 5′-FAM-ACGGAGGAGTGAGCGACAACGTCC-TAMRA-3′. Desnutrin/ATGL: forward, 5′-CAGCACATTTATCCCGGTGTAC-3′; reverse, 5′-AAATGCCGCCATCCACATAG-3′; probe, 5′-FAM-TGGCCTCATTCCTCCTACCCTCCAA-TAMRA-3′. GS2-Like: forward, 5′-CTCTGATCATGGTATTGACTGCTCTAA-3′; reverse, 5′-TCCTCACTATCACAGGGATCAATC-3′; probe, 5′-FAM-TGGGCTCCTTTCTCTGACCCACACTTATCT-TAMRA-3′. PNPLA1: forward, 5′-ACTGAATGCAGCGTACCTTGACT-3′; reverse, 5′-GGCGACCTCTATCTGGCAGTATAC-3′; probe, 5′-FAM-TCCCAGCAAGAGAGTGATTTTCCCGA-TAMRA-3′.

Q-PCR analysis was performed in an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Reactions were performed in a 25 μl volume with a final concentration of 1× TaqMan PCR master mix (PE Applied Biosystems), 450 nM forward primer, 450 nM reverse primer, 250 nM probe primer, 10 ng of reverse-transcribed total RNA (TaqMan Reverse Transcription Reagents kit first-strand cDNA synthesis system protocol; Roche), and 1× Eukaryotic 18S rRNA Endogenous control (VIC/TAMRA; PE Applied Biosystems). The thermal cycler conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 s and 60°C for 1 min. Threshold cycle values were obtained for mouse Adiponutrin, Desnutrin/ATGL, GS2-Like, and PNPLA1, and the values were normalized relative to the 18S internal control. Q-PCR was performed in duplicate, and average values were used for quantification. Data analysis was performed as recommended by the manufacturer, and values were assigned based on standard curves, which were generated for each probe-primer set. Plasmid DNA containing the gene of interest was serially diluted and amplified by Q-PCR as described above. The following image clones were used for the Q-PCR standard curves: Desnutrin/ATGL, clone 225573; Adiponutrin, clone 1265861; and GS2-Like, clone 1150147. PCR efficiency was evaluated for each gene by examining the slope, which was ~3.0 for all genes in this study.

Expression vectors

Human Adiponutrin, Desnutrin/ATGL, GS2, and GS2-Like expression constructs encoding only the open reading frame with or without epitope tags were constructed by subcloning PCR amplification products into the mammalian expression vector pAdori (CMV promoter) or into the Gateway entry vector pDONRr (Invitrogen) followed by recombination into the Gateway destination vector pDEST40 (Invitrogen). PCR amplification was performed using Invitrogen's Platinum Taq DNA polymerase, I.M.A.G.E. Consortium cDNA clones 5243623 (Adiponutrin), 6598433 (Desnutrin/ATGL), 6109547 (GS2), and 4778605 (GS2-Like), and the following primers: hDesnutrin/ATGL Forward, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTTTCCCCGCGAGAAGACG-3′; hDesnutrin/ATGL no tag Reverse, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACAGCCCCAGGGCCCCGAT-3′; hDesnutrin/ATGL carboxy V5-6His fusion Reverse, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCAGCCCCAGGGCCCCGAT-3′; hAdiponutrin Forward, 5′-TATATACTAGTACTAGTCGGACCATGTACGACGCAGAGCGCGGCTGGAGC-3′; hAdiponutrin no tag Reverse, 5′-ATATAAAGCTTAAGCTTTCATCACAGACTCTTCTCTAGTGAAAAACT-3′; hAdiponutrin carboxy-V5 tag Reverse, 5′-ATATAAAGCTTTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCAGACTCTTCTCTAGTGAAAAACT-3′; hGS2 Forward, 5′-ATATAGAATTCCGGACCATGAAGCACATCAACCTATCATTTGCA-3′; hGS2 no tag Reverse, 5′-ATATAAAGCTTTCATTCAAACCAATTTTCTTTAAGTAA-3′; hGS2 carboxy V5-tag Reverse, 5′-ATATAAAGCTTTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTTCAAACCAATTTTCTTTAAGTAA-3′; hGS2-Like Forward, 5′-ATGGGCTTCTTAGAGGAGGAGG-3′; hGS2-Like no tag Reverse, 5′-GGACTAGTTCAATGGTGATGGTGATGATGGGTACGCGTAGAGTCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGGCCTGGTGGGTGG-3′; hGS2-Like carboxy V5-6His tag Reverse, 5′-GGACTAGTTCAATGGTGATGGTGATGATGGGTACGCGTAGAGTCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGGCCTGGTGGGTGGGCCC-3′.The resulting plasmids were sequence confirmed. Each expression vector was used to transiently transfect HEK293 cells, and expression was confirmed by Western analysis.

Patatin-like domain mutant generation

The C-terminal, V5-tagged hAdiponutrin, hDesnutrin/ATGL, hGS2, and hGS2-Like expression plasmids were used to generate site-directed patatin-like domain mutants. The mutant design was based on previously reported mutants for patatin (14). Using the QuikChange® XL Site-Directed Mutagenesis Kit and protocol (Stratagene), the serine of the Gly-X-Ser-X-Gly motif (Fig. 1A) was changed to an alanine for each family member. The resulting plasmids were sequence confirmed.

Cell culture and transfection

HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2. Ten centimeter tissue culture dishes with 90% confluent HEK293 cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Seventy-two hours after transfection, cells were washed with ice-cold TBS and harvested. Cells were pelleted by centrifugation at 1,000 *g* for 5 min. Supernatant was discarded, and pellets were stored at −80°C until used for assays.

Preparation of cell lysates and immunoprecipitations

Frozen cells, as described above, were resuspended in 1 ml of lipase reaction buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.53% sodium taurodeoxycholate, and 1.33 mM $CaCl_2$) containing complete mini protein inhibitor tablets (one tablet per 7 ml; Invitrogen). The resulting suspensions were then sonicated on ice with four bursts of 10 s from a probe sonicator. Homogenized lysate was centrifuged at 1,000 *g* for 10 min to remove cell debris. A 50 μl aliquot of the resulting lysate was saved for analysis, and the remaining lysate was used for immunoprecipitation. Anti-V5 mouse monoclonal antibody (7.2 μg; Invitrogen) was added to each lysate and then placed at 4°C overnight with tumbling. Next, 20 μl of protein A beads (Repligen) was added to each sample and tumbled at 4°C for 2 h. Beads were then pelleted with gentle centrifugation, and 900 μl of supernatant was saved for analysis. The beads were washed four times with 900 μl of lipase reaction buffer followed by resuspension in 100 μl of lipase reaction buffer.

Lipase assay

The lipase assay uses 1,2-*o*-dilauryl-*rac*-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as a substrate (23). DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester that is spontaneously hydrolyzed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 581 nm. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Ten microliters of whole cell lysate or immunoprecipitation beads was added to the wells of a 96-well plate. After diluting the samples up to 125 μl in lipase reaction buffer, 125 μl of reaction buffer containing DGGR was added (final concentration of 36 μg/ml in a final assay volume of 250 μl per well). After mixing, OD 581 was monitored at 5 min intervals for 2 h to assess lipase activity. Lipase activity is plotted as ΔOD 581/s. All samples were assayed in triplicate.

[1-^{14}C]oleic acid incorporation into triglyceride

HEK293 cells were cultured in DMEM and 10% FBS at 37°C with 5% CO_2. HEK293 cells grown on 12-well plates to 60–70% confluence were transfected with 0.5 μg of previously described expression plasmid DNA using FuGene (Roche). Empty vector (pAdori) was used as a control. After 48 h, cells were treated with 4 μM [1-^{14}C]oleic acid (204 μCi/ml total; Perkin-Elmer) and 16 μM cold oleic acid (Sigma) in serum-free DMEM. After 4 h, cells were washed twice and incubated further with DMEM supplemented with 10% FBS. After 16 h, lipids were extracted and separated by TLC using hexane-ether-acetic acid (80:20:1). Radioactive lipids were detected and quantitated by Molecular Imager FX (Bio-Rad).

RESULTS

Identification of Adiponutrin-related proteins as a subfamily of patatin-like domain-containing proteins

Adiponutrin, Desnutrin/ATGL, GS2, and GS2-Like are characterized by the presence of a patatin-like domain [Gly-X-Ser-X-Gly and Asp-X-Gly/Ala motifs (14)]. To identify additional patatin-like domain-containing proteins, we generated a profile HMM and used it to search public protein, EST, and genomic databases. Ten ortholog patatin families were identified (Fig. 1). Phylogenetic analysis using patatin as the base sequence clustered Adiponutrin, Desnutrin/ATGL, GS2-Like, and GS2 on one branch of the phylogenetic tree. We term this the Adiponutrin family (Fig. 1B). A newly identified gene, PNPLA1, also clustered with the Adiponutrin family. N-terminal patatin-like domains and variable C-terminal domains characterize all of the Adiponutrin family members. In contrast to the Adiponutrin family, other patatin-like domain-containing proteins, including the phospholipases iPLA2, PLA2G6, and NTE, clustered on separate branches of the phylogenetic tree (Fig. 1B). In addition, these proteins are characterized by a C-terminal patatin-like domain and by a variable region at the N terminus of the protein.

With the exception of GS2, we were able to identify human, mouse, and rat orthologs for all members of the Adiponutrin family. Searches of EST and genomic databases failed to identify a mouse ortholog of GS2. Syntenic analysis of mouse, human, and rat genomic regions shows that the region expected to contain the GS2 gene is absent in published mouse genomic sequences, allowing for the possibility that a gene corresponding to mouse GS2 remains to be discovered. The absence of any EST or cDNA sequences corresponding to mouse GS2 in public or internal databases makes it unlikely that this gene is expressed at significant levels in major tissues.

Gene and protein prediction algorithms support the existence of PNPLA1 in the genome. However, very few ESTs for human or mouse PNPLA1 exist in the database, and our Northern analysis of multiple human tissues using a PNPLA1 EST (BI257213) or Q-PCR analysis of mouse tissues using multiple primer-probe sequences for the predicted mouse PNPLA1 failed to detect any appreciable levels of transcripts. The expression and physiological significance of PNPLA1 remains to be determined.

Expression analysis of Adiponutrin family members

To elucidate the expression patterns of the Adiponutrin gene family, Northern analysis was performed using gene-specific probes. Both Desnutrin/ATGL and GS2 transcripts are easily detectable by Northern blotting, and both genes show highest expression in metabolically active tissues, such as adipose tissue, heart, skeletal muscle, and portions of the gastrointestinal tract (**Fig. 2A**), consistent with a role in lipid metabolism. As reported previously, two Desnutrin/ATGL transcripts (~2.2 and ~4.3 kb) (11) and two GS2 transcripts (~1.4 and ~4.4 kb) (24) are detectable by Northern analysis and appear to be coordinately expressed in most, but not all, tissues (Fig. 2A). We were unable to detect human Adiponutrin or GS2-Like transcripts by Northern analysis, presumably because of the lower levels of expression (data not shown). It is important to note, however, that Adiponutrin has been shown to be expressed in human adipose tissue using Q-PCR analysis (16).

To assess the mRNA expression of mouse Adiponutrin family members, we used Q-PCR analysis. As expected, we found that both Adiponutrin and Desnutrin/ATGL are most abundant in both brown and white adipose tissue. In addition, we found that GS2-Like is expressed predominantly in adipose tissue, but in addition it is also present in the lung. We noticed that multiple probes for GS2-Like consistently required very high cycle numbers, suggesting poor expression of GS2-Like mRNA in the tissues examined. To verify that this was indeed the case, we quantitated our Q-PCR analysis using standard curves generated on known quantities of plasmid containing the indicated genes (Fig. 2B, insets). These standard curves were used to convert cycle time into copies of cDNA normalized to the amount of starting RNA. Figure 2B shows that GS2-Like cDNA is much less abundant (~10,000-fold) than Adiponutrin or Desnutrin/ATGL cDNA. Identical data were generated with an independent set of primers/probes (data not shown). We conclude that GS2-Like is expressed at very low levels, likely explaining our inability to detect expression by Northern analysis in either mouse or human (data not shown).

To further examine the expression of Adiponutrin, Desnutrin/ATGL, and GS2-Like, we examined expression during 3T3-L1 adipocyte differentiation and in primary adipocytes separated from the stromal fraction of mouse adipose tissue. As expected, both Adiponutrin and Desnutrin/ATGL were highly upregulated during adipocyte differentiation and were detected almost exclusively in the adipocyte fraction (**Fig. 3**, columns 1, 2). Similarly, GS2-Like was found to be an adipocyte-expressed gene that is upregulated during differentiation (Fig. 3, columns 1, 2).

Regulation of Adiponutrin family member expression

Desnutrin/ATGL has been reported to be downregulated in the adipose tissue of genetically obese (*ob/ob*) mice (19), whereas Adiponutrin is strongly induced in the adipose tissue of obese (*fa/fa*) rats (15). We examined the expression of Adiponutrin family members in adipose and liver tissue from *ob/ob* mice and from fasted mice. As shown

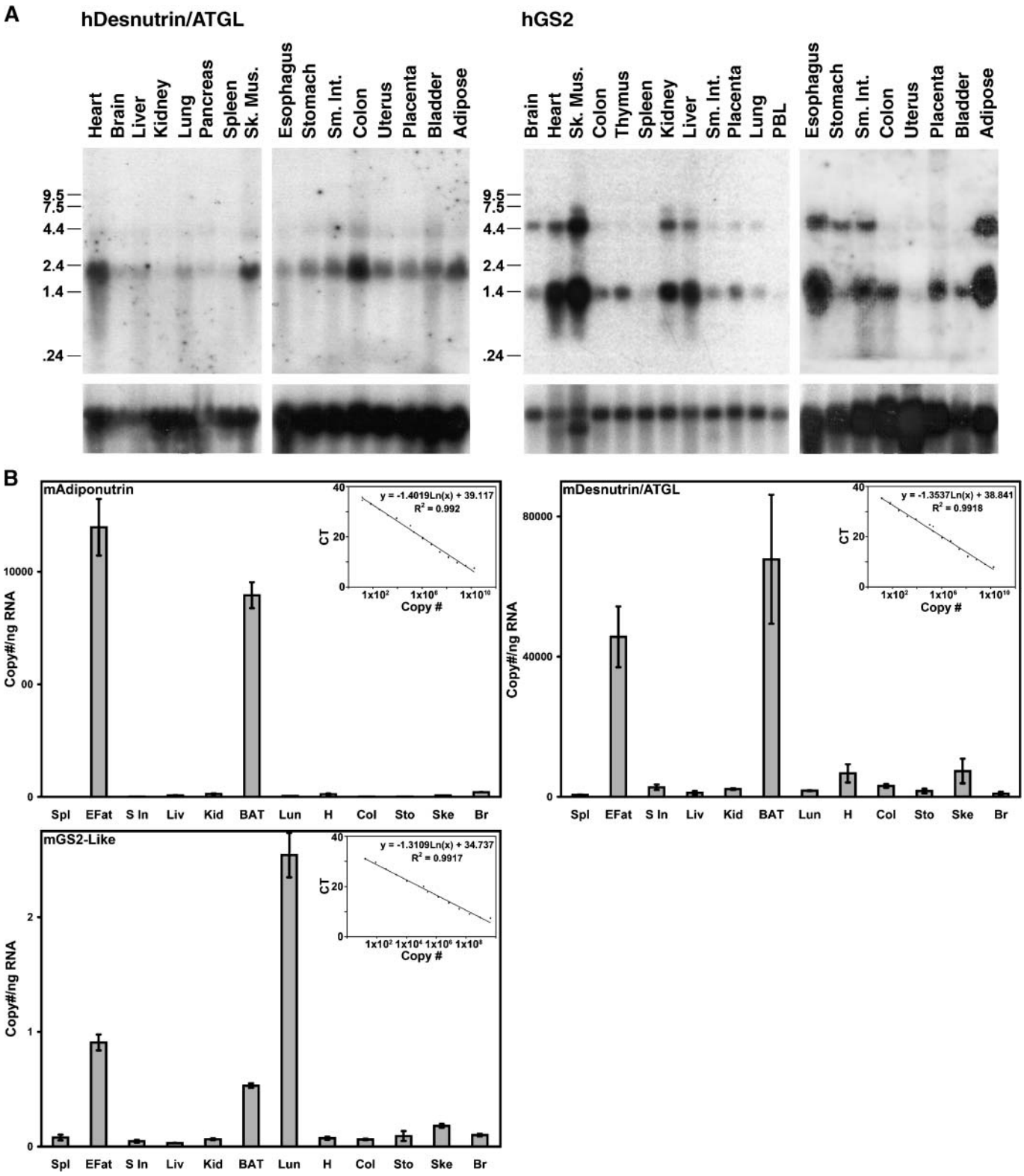

Fig. 2. RNA tissue distribution of Adiponutrin family genes. A: Northern blots of human poly(A) (third panel) or total RNA (first, second, and fourth panels) derived from the tissues indicated and probed with Desnutrin/ATGL or GS2 gene-specific probes. The lower panels show the corresponding blots probed with β-actin. Sk. Mus., skeletal muscle; Sm. Int., small intestine. B: Quantitative reverse transcription polymerase chain reaction (Q-PCR) analysis of mouse Adiponutrin, Desnutrin/ATGL, and GS2-Like RNA in multiple mouse tissues: spleen (Spl), epididymal fat (Efat), small intestine (S In), liver (Liv), kidney (Kid), brown adipose (BAT), lung (Lun), heart (H), colon (Col), stomach (Sto), gastrocnemius muscle (Ske), and brain (Br). Inset graphs are standard curves generated for each gene and were used to determine copy number/ng RNA. Error bars depict standard deviation of the mean copy number. CT, cycle time.

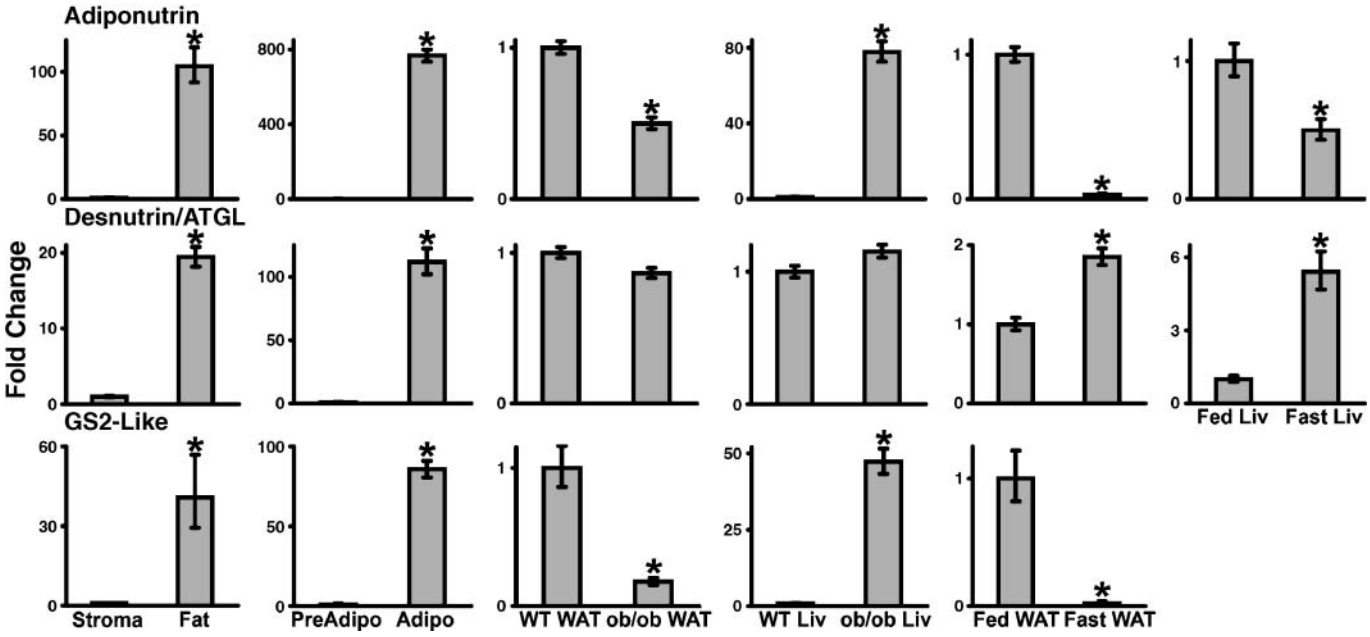

Fig. 3. Regulation of mouse Adiponutrin family members in metabolic models. RNA quantitation of mouse Adiponutrin, Desnutrin/ATGL, and GS2-Like from samples as detailed below. Q-PCR amplification was performed with gene-specific probe/primer sets, values were normalized to 18s rRNA, and data are expressed as relative fold change. Error bars represent the fold change range for the standard error of the mean. * $P < 0.05$ relative to control. Column 1: Stromovascular (Stroma) or adipocyte (Fat) fraction of epididymal adipose tissue. Separations were carried out as described in Materials and Methods. Column 2: Undifferentiated 3T3-L1 cells and differentiated 3T3-L1 adipocytes. Columns 3, 4: Epididymal white adipose tissue (WAT) or liver tissue (Liv) isolated from either wild-type control (WT) or genetically obese (*ob/ob*) mice. Columns 5, 6: Epididymal white adipose tissue (WAT) or liver tissue (Liv) isolated from mice that were either fed ad libitum or fasted for 24 h.

in Fig. 3, column 3, we were unable to detect any significant changes in Desnutrin/ATGL mRNA expression in adipose tissue of *ob/ob* mice but found significant decreases in Adiponutrin (~2-fold). Consistent with previous data, we found that Adiponutrin expression is strongly suppressed upon fasting (~80-fold), whereas Desnutrin/ATGL expression is increased ~2-fold in adipose tissue from fasted mice (Fig. 3, column 5). It is interesting that tissues from our *ob/ob* mice were collected under fed rather than fasting conditions. It is possible, therefore, that the previously observed decrease of Desnutrin/ATGL in fasted *ob/ob* adipose tissue (19) reflects a lack of upregulation of Desnutrin/ATGL upon fasting rather than a general downregulation.

Interestingly, GS2-Like regulation closely parallels the expression of Adiponutrin in both animal models. GS2-Like decreases significantly in *ob/ob* adipose and in adipose from fasted animals (Fig. 3, columns 3, 5). The regulation of Adiponutrin and GS2-Like in adipose tissue is reminiscent of the regulation found for genes involved in lipogenesis rather than lipolysis. Because genes expressed in lipogenesis [e.g., SCD1 (25–27)] are upregulated in the livers of animals with hepatic steatosis, such as *ob/ob* mice, and downregulated in livers from fasting animals, we decided to examine the expression of Adiponutrin family members in the livers of these animals. Both Adiponutrin and GS2-Like expression are strongly induced in *ob/ob* livers compared with control mice, whereas Desnutrin/ATGL expression is unchanged (Fig. 3, column 4). During fasting, Desnutrin/ATGL expression increases significantly, whereas Adiponutrin expression decreases below starting levels (Fig. 3, column 6). GS2-Like expression is undetectable in the liver of C57Bl/6J mice under both fasting and fed conditions. The upregulation of both Adiponutrin and GS2-Like in the liver of *ob/ob* mice suggests a role for these proteins in hepatic lipogenesis.

Adiponutrin family members have lipase activity

Zimmermann et al. (11) and Jenkins et al. (13) recently demonstrated lipase and transacylase activity for a subset of Adiponutrin family members. We examined the ability of Adiponutrin family members to hydrolyze a commonly used lipase substrate, DGGR (23). V5-tagged human Adiponutrin, Desnutrin/ATGL, GS2, and GS2-Like proteins were expressed in HEK293 cells and partially purified by immunoprecipitation. Green fluorescent protein or untagged versions of the same protein, processed in an identical manner, were used as controls. Western analysis confirmed that the proteins were expressed and present in the immunoprecipitate (**Fig. 4**, insets). All four Adiponutrin family members tested showed a significantly higher rate of hydrolysis compared with immunoprecipitates from cells expressing green fluorescent protein or untagged controls (Fig. 4). To verify that the observed increase in activity is attributable to the overexpressed protein, we mutated the predicted active-site serine within the Ser-Asp catalytic dyad motif to an alanine. Equivalent amounts of wild-type and mutant proteins were assayed as confirmed

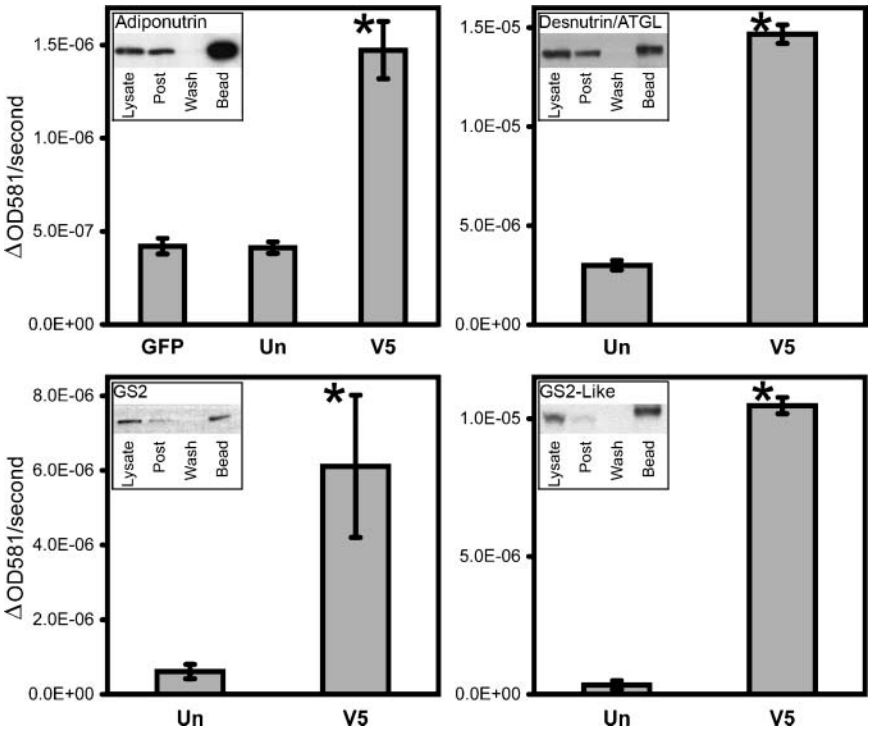

Fig. 4. Immunoprecipitated Adiponutrin family members have lipase activity. The graphs show lipase activity (ΔOD581/s) of immunoprecipitation beads from cells transfected with untagged (Un) or V5-tagged (V5) Adiponutrin (top left), Desnutrin/ATGL (top right), GS2 (bottom left), or GS2-Like (bottom right) or with green fluorescent protein (GFP; top left graph only). * $P < 0.05$ relative to control. The insets show Western blots probed with anti-V5 antibody demonstrating V5-tagged protein expression in cell lysates from transfected cells (Lysate), lysate after immunoprecipitation (Post), the first wash (Wash), and protein A beads after immunoprecipitation (Bead). Error bars indicate standard deviation.

by Western analysis (**Fig. 5**, insets). For three family members, Adiponutrin, Desnutrin/ATGL, and GS2, the rate of lipid hydrolysis was significantly higher when wild-type rather than mutant protein was used, demonstrating that the lipase activity is attributable to the overexpressed protein and depends upon an intact active-site serine (Fig. 5). The activity of preparations containing GS2-Like was not affected by mutating the active-site serine. GS2-Like contains two serine residues immediately adjacent to the predicted active-site serine (Fig. 1B), and it is possible that one of these two serines can substitute for serine 49; alternatively, another lipase may coimmunoprecipitate with GS2-Like.

Overexpression of Adiponutrin family proteins modulates triglyceride incorporation in cells

To further assess the ability of Adiponutrin family members to function as lipases, we examined whether the overexpression of these proteins affects intracellular triglyceride levels. It has been shown that the overexpression of Desnutrin/ATGL in mammalian cell lines decreases the incorporation of radiolabeled fatty acids into triglycerides by increasing the release of fatty acids during a pulse-chase experiment (19). We examined Adiponutrin, Desnutrin/ATGL, GS2, and GS2-Like in a similar experiment using overexpression in HEK293 cells. Consistent with previous results, overexpression of Desnutrin/ATGL caused a significant decrease in intracellular triglycerides compared with control cells (**Fig. 6**). GS2 and GS2-Like also decreased intracellular triglycerides significantly. In contrast, we were unable to demonstrate a decrease upon Adiponutrin overexpression; rather, in several experiments, Adiponutrin showed a trend toward increased triglyceride incorporation (data not shown). Thus, Desnutrin/ATGL, GS2, and GS2-Like all function to decrease intracellular triglycerides, at least when overexpressed, whereas Adiponutrin does not.

DISCUSSION

Controlled storage and release of fatty acids is a central function of the adipocyte. A novel patatin-like domain-containing protein, Desnutrin/ATGL, was recently shown to mediate lipolysis in mouse adipocytes (11). Two homologs of Desnutrin/ATGL, Adiponutrin and GS2, have also been described (13, 15, 16, 24); however, only limited information is available about their function. We conducted a systematic analysis of the Adiponutrin family to determine which proteins belong to this family and to investigate expression, regulation, and function in enzymatic and cell-based assays of Adiponutrin family members. Our bioinformatic analysis shows that the Adiponutrin family is composed of five members: Adiponutrin, Desnutrin/

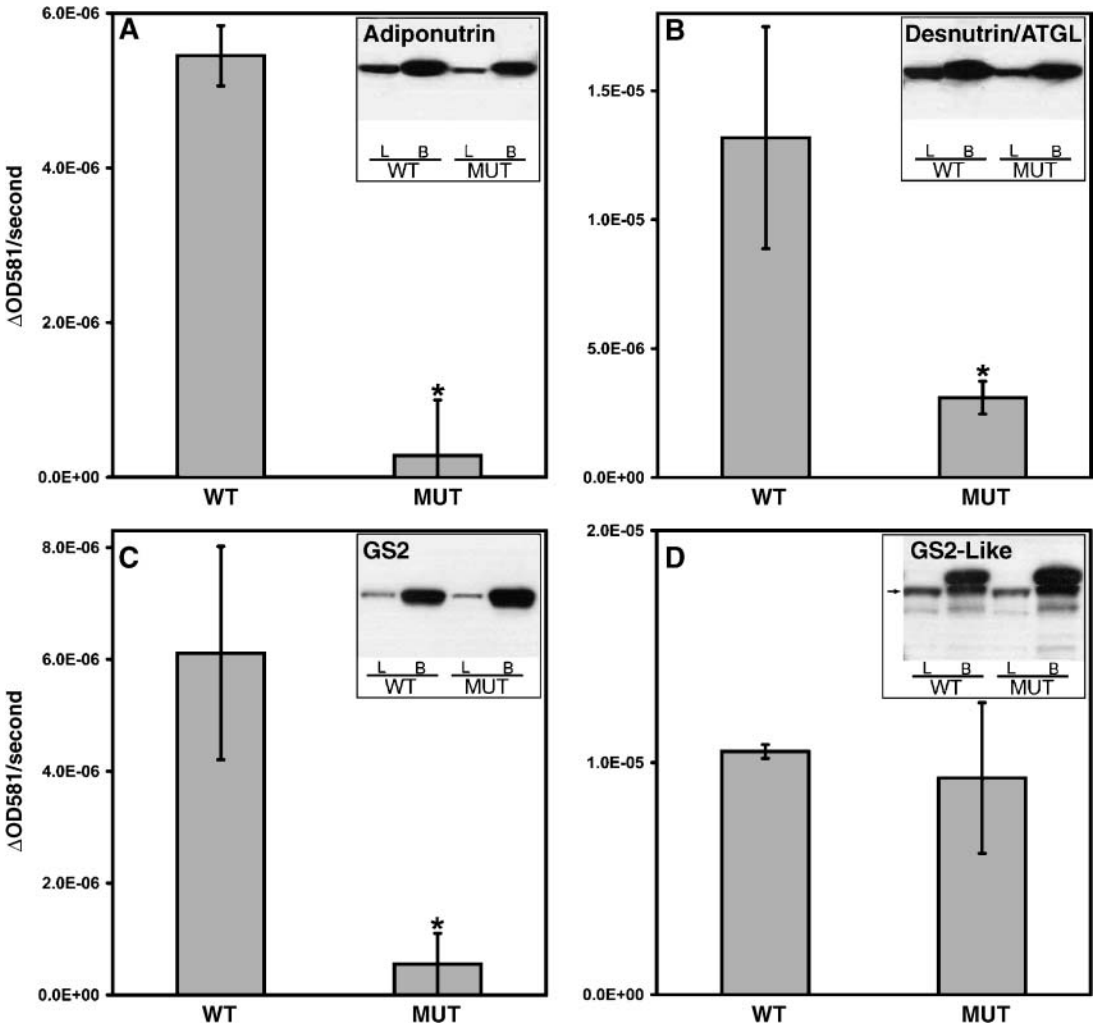

Fig. 5. Patatin-like domain mutants have lower lipase activity. The graphs show lipase activity (ΔOD581/s) of immunoprecipitation beads from cells transfected with V5-tagged wild-type (WT) or mutant (MUT) hAdiponutrin (top left), hDesnutrin/ATGL (top right), hGS2 (bottom left), or hGS2-Like (bottom right) expression constructs. * $P < 0.05$ relative to control. The insets show Western blots probed with anti-V5 antibody. The first two lanes (WT) in each blot were loaded with either cell lysates (L) or immunoprecipitation beads (B) from cells transfected with V5-tagged wild-type family members, whereas the second two lanes (MUT) were loaded with either cell lysates (L) or immunoprecipitation beads (B) from cells transfected with V5-tagged mutant family members. For the GS2-Like inset, the uppermost band in the Western blot corresponds to immunoglobulin, and an arrow denotes the V5-tagged GS2-Like. Error bars indicate standard deviation.

ATGL, GS2, GS2-Like, and PNPLA1. We were unable to obtain physical clones of PNPLA1 or evidence of PNPLA1 expression in any of the tissues examined; therefore, we focused our experiments on Adiponutrin, Desnutrin/ATGL, GS2, and GS2-Like.

Consistent with previous reports (11, 15, 19), we found high levels of expression of Adiponutrin and Desnutrin/ATGL in adipose tissue and primary adipocytes, upregulation during adipocyte differentiation, and differential regulation of these two genes upon fasting. Contrary to previously reported results (19), we did not observe a downregulation of Desnutrin/ATGL in *ob/ob* mice. Because our study used fed animals and the previous study relied on fasted animals, it is possible that this difference reflects a dysregulation of Desnutrin/ATGL mRNA in *ob/ob* mice specifically upon fasting. We were also surprised to find that Adiponutrin mRNA was downregulated in the adipose tissue of leptin-deficient, obese *ob/ob* mice. It had been reported previously that Adiponutrin mRNA is upregulated dramatically in adipose tissue of *fa/fa* rats (15), a related obesity model caused by a mutation in the leptin receptor (28). It is possible that differences in species, diet, or physiology between the two models account for this discrepancy.

The regulation of Adiponutrin family members in the liver had not been examined previously. Similar to adipose tissue, the liver undergoes cycles of lipogenesis and lipolysis, and increased triglyceride accumulation in the liver accompanied by increased expression of genes involved in lipid storage [e.g., peroxisome proliferator-activated receptor γ (29) and SCD1 (27, 30)] is commonly observed in genetic and diet-induced obesity (28, 31). Because we did find lower, but detectable, levels of both Adiponutrin and Desnutrin/ATGL in the liver of wild-type mice, we in-

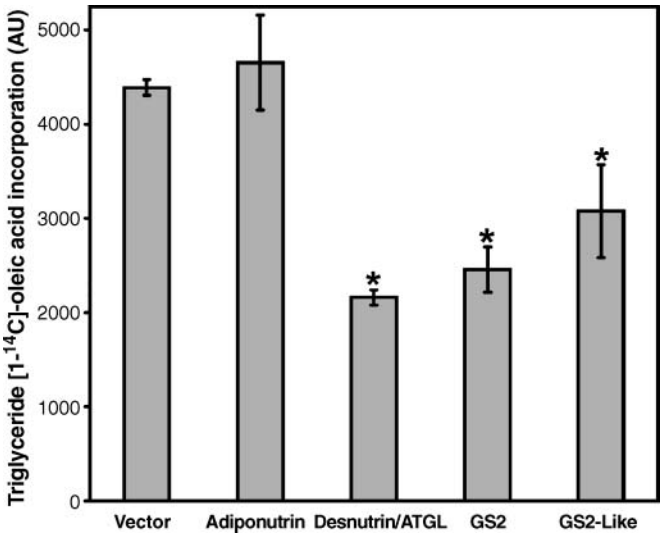

Fig. 6. Overexpression of Adiponutrin family members modulates [1-^{14}C]oleic acid incorporation into triglyceride. HEK293 cells transfected with the indicated expression constructs were treated with [1-^{14}C]oleic acid for 4 h followed by a 16 h chase period. Lipids were extracted and separated by TLC, and radiolabeled triglycerides were quantitated using a Molecular Imager. * $P < 0.05$ relative to control (Vector). AU, absorbance units. Error bars indicate standard deviation.

vestigated the regulation of these genes upon fasting and in *ob/ob* mice. We found that regulation of Adiponutrin and Desnutrin/ATGL in the liver during fasting mirrored their regulation in adipose tissue. In addition, we observed a dramatic upregulation of Adiponutrin, but not Desnutrin/ATGL, in liver of *ob/ob* mice, further accentuating the distinct regulation of Desnutrin/ATGL and Adiponutrin. Overall, the regulation of Adiponutrin is reminiscent of that of genes involved in lipogenesis, whereas the regulation of Desnutrin/ATGL is consistent with a role in lipolysis.

Similar to previous reports (11, 13), we observed lipase activity for both Adiponutrin and Desnutrin/ATGL in a cell-free system. Although Desnutrin has been shown to modulate lipolysis in cells upon either overexpression or knockdown (11, 19), the activity of Adiponutrin in cells was not examined. We found that, although overexpression of Desnutrin/ATGL reduces intracellular triglycerides, as reported previously, overexpression of Adiponutrin had no effect on intracellular triglycerides in most experiments (and showed a trend toward an increase in some studies). Interestingly, both Adiponutrin and Desnutrin/ATGL have transacylase activity in vitro (13); therefore, it is possible that additional determinants present in cells, such as substrate presentation, subcellular localization, and accessory proteins, may determine whether a particular Adiponutrin family member acts primarily as a lipase or a transacylase.

GS2-Like protein had been mentioned as a possible Adiponutrin family member in previous publications (15, 19) but has not yet been characterized. Here, we report that GS2-Like is indeed a member of the Adiponutrin family. Similar to Adiponutrin and Desnutrin/ATGL, GS2-Like has an N-terminal patatin motif followed by a C-terminal variable domain. Human, mouse, and rat orthologs of GS2-Like are clearly identifiable in the respective genomes, demonstrating evolutionary conservation of this gene. Interestingly, GS2-Like is located adjacent to Adiponutrin in the human, rat, and mouse genomes (our unpublished observations) and therefore may be the result of an Adiponutrin gene duplication event. Although GS2-Like is qualitatively expressed and regulated in a manner similar to Adiponutrin (Figs. 2, 3), the absolute expression levels of GS2-Like are dramatically lower, at least at the RNA level in the mouse (Fig. 2). We have not yet quantitated the relative expression of GS2-Like in human adipose tissue; however, our inability to detect expression by Northern blot analysis is consistent with low levels of expression of GS2-Like in the human adipose tissue as well. Although the regulation of GS2-Like is reminiscent of that of Adiponutrin, its ability to decrease triglyceride incorporation when overexpressed in cells suggests that it can function as a lipase similar to Desnutrin/ATGL. In mouse adipose, GS2-Like mRNA is present at very low levels. For this reason, we consider it unlikely that GS2-Like contributes significantly to lipolysis in this tissue. It is possible, however, that GS2-Like becomes functionally more important under particular pathophysiological conditions, especially in tissues that do not express large amounts of Desnutrin/ATGL. For example, we show that GS2-Like, but not Desnutrin/ATGL, expression is upregulated significantly in the liver of *ob/ob* mice.

GS2 is an Adiponutrin family member that was originally identified as part of the genome-sequencing effort (24). Recently, GS2 was shown to be expressed in the human liposarcoma cell line SW872 and to have triglyceride lipase and transacylase activity (13). Interestingly, GS2 lacks the C-terminal variable region present in all other Adiponutrin family members. Here, we demonstrate for the first time that GS2 is expressed in human adipose tissue and that adipose tissue, as well as other tissues with significant lipid metabolism, such as heart, skeletal muscle, liver, kidney, and sections of the gastrointestinal tract, are the major sites of GS2 expression in humans. Given the relatively broad pattern and high levels of expression of GS2 in human tissues, we were surprised by the complete absence of sequences corresponding to GS2 transcripts in mouse databases. Because the genomic region predicted to contain GS2 is lacking in the mouse genomic sequence, we currently do not have any evidence for a mouse GS2 gene. One possible explanation is that mouse GS2 is expressed at much lower levels compared with human GS2, making the identification of transcripts more difficult; alternatively, GS2 may be lacking in the mouse genome. Our data confirm that, similar to Desnutrin/ATGL, GS2 has lipase activity in vitro and that it can function as a lipase in cells upon overexpression. This raises the possibility that GS2 may contribute significantly to adipocyte lipolysis in humans. Furthermore, given our inability to detect GS2 in mice, it is possible that human and mouse adipose tissue use a different subset of Adiponutrin family members for lipolysis. Further investigation of the function of

GS2 in human adipose tissue and of the relative contributions of Desnutrin/ATGL and GS2 to lipolysis in human adipocytes is clearly warranted.

In summary, we have conducted a detailed investigation of the expression, regulation, and function for multiple Adiponutrin family members. Our data support previously published reports on the function of Desnutrin/ATGL as a major triglyceride lipase in mouse adipose tissue, extend our knowledge of the regulation of Adiponutrin and Desnutrin/ATGL gene expression, and demonstrate a distinct behavior for Adiponutrin and Desnutrin/ATGL when overexpressed in cells. Furthermore, we show that two additional Adiponutrin family members, GS2 and GS2-Like, are expressed and regulated in a manner consistent with a role in metabolic processes and can function as lipases both in vitro and in cells. Thus, our data provide a basis for additional work on this important gene family.

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