

# Expression and Characterization of a PNPLA3 Protein Isoform (I148M) Associated with Nonalcoholic Fatty Liver Disease\*<sup>§</sup>

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**Background:** A genetic variant of PNPLA3 (I148M), an enzyme of unknown function, confers susceptibility to fatty liver disease.

**Results:** Purified human PNPLA3 catalyzed hydrolysis of glycerolipids with a preference for oleoyl esters; the I148M substitution substantially decreased hydrolytic activity.

**Conclusion:** PNPLA3 catalyzes hydrolysis rather than synthesis of triacylglycerol.

**Significance:** PNPLA3-I148M impairs hydrolytic activity against multiple glycerolipids.

A genetic variant of PNPLA3 (patatin-like phospholipase domain-containing 3; PNPLA3-I148M), a serine protease of unknown function, is associated with accumulation of triacylglycerol (TAG) in the liver. To determine the biological substrates of PNPLA3 and the effect of the I148M substitution on enzymatic activity and substrate specificity, we purified and characterized recombinant human PNPLA3 and PNPLA3-I148M. Maximal hydrolytic activity of PNPLA3 was observed against the three major glycerolipids, TAG, diacylglycerol, and monoacylglycerol, with a strong preference for oleic acid as the acyl moiety. Substitution of methionine for isoleucine at position 148 markedly decreased the  $V_{max}$  of the enzyme for glycerolipids but had only a modest effect on the  $K_m$ . Purified PNPLA3 also catalyzed the hydrolysis of oleoyl-CoA, but the  $V_{max}$  was 100-fold lower for oleoyl-CoA than for triolein. The thioesterase activity required the catalytic serine but was only modestly decreased by the I148M substitution. The enzyme had little or no hydrolytic activity against the other lipid substrates tested, including phospholipids, cholesteryl ester, and retinyl esters. Neither the wild-type nor mutant enzyme catalyzed transfer of oleic acid from oleoyl-CoA to glycerophosphate, lysophosphatidic acid, or diacylglycerol, suggesting that the enzyme does not promote *de novo* TAG synthesis. Taken together, our results are consistent with the notion that PNPLA3 plays a role in the hydrolysis of glycerolipids and that the I148M substitution causes a loss of function, although we cannot exclude the possibility that the enzyme has additional substrates or activities.

Triacylglycerols (TAGs)<sup>3</sup> are the predominant energy storage molecules in higher organisms and accumulate to high levels in adipocytes. In other cells, TAGs are maintained at much lower concentrations and comprise only a small fraction of tissue mass. Under conditions of sustained caloric excess, TAG may be deposited at higher levels in non-adipose tissues, particularly in the liver. In humans, the levels of hepatic TAG vary markedly among individuals, ranging from <1% to >50% of liver weight (1). Several factors are associated with an increase in liver TAG content, including obesity, insulin resistance, and alcohol ingestion, but the factors responsible for the wide individual differences in hepatic TAG levels have not been fully elucidated (2).

We showed previously that a missense variant of PNPLA3 (patatin-like phospholipase domain-containing 3) is strongly associated with hepatic TAG content (3). Substitution of isoleucine with methionine at position 148 (PNPLA3-I148M) is associated with increased liver TAG and the full spectrum of nonalcoholic fatty liver disease, including steatohepatitis, cirrhosis, and hepatocellular carcinoma (4–7). A second variant (S453I) is associated with lower levels of liver fat in African-Americans (3). These findings are consistent with PNPLA3 playing a role in the metabolism of hepatic TAG, although its specific function is not known.

Elucidation of PNPLA3 function will be essential to understand the mechanism by which sequence variation in the gene confers susceptibility to liver disease. PNPLA3, alternatively referred to as adiponutrin, is expressed predominantly in the liver and adipose tissue of humans (8), where it is strongly associated with membranes and lipid droplets (9). The protein is highly regulated in response to nutritional stimuli at both the transcriptional and post-translational levels (8). During fasting, low transcription of PNPLA3 mRNA and efficient degradation of PNPLA3 protein ensure very low levels of PNPLA3 in the liver. With carbohydrate refeeding, the transcription factor SREBP-1c stimulates PNPLA3 transcription and concomi-

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<sup>3</sup> The abbreviations used are: TAG, triacylglycerol; ATGL, adipose TAG lipase; DAG, diacylglycerol; MAG, monoacylglycerol; LPA, lysophosphatidic acid; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; DGAT, diacylglycerol acyltransferase.

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tantly up-regulates fatty acid biosynthesis. Fatty acids retard the degradation of PNPLA3 protein, leading to a marked postprandial rebound in PNPLA3 levels.

PNPLA3 belongs to a family of proteins that share an evolutionarily ancient domain first identified in patatin, the major protein of potato tubers (10). The domain comprises a  $\beta$ -sheet sandwiched between two  $\alpha$ -helices and includes a consensus serine lipase motif (Gly-X-Ser-X-Gly) but differs from classical lipases by employing a catalytic dyad (Ser-Asp), rather than a catalytic triad, to effect hydrolysis (11). Whereas patatin shows nonspecific lipid hydrolase activity against a range of lipid substrates, including phospholipids (11), subsequently discovered patatin domain family members have different substrate specificities. The human genome encodes nine patatin domain-containing proteins, of which PNPLA3 is most closely related to PNPLA2 (adipose TAG lipase (ATGL)), the major TAG hydrolase of adipose tissue (12, 13). Partially purified preparations of human PNPLA3 were shown to hydrolyze TAGs *in vitro*, but overexpression of PNPLA3 in mouse liver was not associated with changes in cellular TAG levels (9, 14). Furthermore, siRNA-mediated knockdown of PNPLA3 does not affect the TAG content of 3T3-L1 cells (15), and mice lacking PNPLA3 do not have increased liver TAG levels (16, 17). Thus, it remains unclear if PNPLA3 functions as a TAG hydrolase *in vivo*.

To examine the role of PNPLA3 in TAG metabolism and to elucidate the mechanism by which the I148M substitution affects liver fat content, we tested the activity of purified recombinant wild-type and mutant forms of the protein against a range of substrates from the major classes of lipids. Here, we show that PNPLA3 is active against glycerolipids (TAG, diacylglycerol (DAG), and monoacylglycerol (MAG)) with a strong preference for C18:1 fatty acids. The I148M mutation markedly reduced hydrolase activity against all three glycerolipids. PNPLA3 also has thioesterase activity against oleoyl-CoA, but the I148M mutation had little effect on this activity. In contrast, the enzyme showed little activity against phospholipids, cholesterol esters, NBD C<sub>12</sub>-ceramide, or retinyl esters. Finally, neither WT nor mutant PNPLA3 catalyzed transfer of oleic acid from oleoyl-CoA to glycerophosphate, lysophosphatidic acid (LPA), or DAG, suggesting that the enzyme does not promote *de novo* TAG synthesis.

### EXPERIMENTAL PROCEDURES

**Materials**—FLAG peptide and mouse anti-FLAG monoclonal antibody M2 were purchased from Sigma. [<sup>14</sup>C]Oleoyl-CoA was obtained from PerkinElmer Life Sciences, and [<sup>3</sup>H]triolein [<sup>14</sup>C]triolein, [<sup>14</sup>C]diolein, [<sup>14</sup>C]monoolein, [<sup>14</sup>C]phosphatidylcholine, [<sup>14</sup>C]stearoyl-CoA, and [<sup>14</sup>C]palmitoyl-CoA were from American Radiolabeled Chemicals (St. Louis, MO). Whatman Silica Gel 60 TLC plates were purchased from VWR International, and tripalmitin, dipalmitin, monopalmitin, triolein, diolein, monoolein, trilinolein, dilinolein, and monolinolein were from Nu-Chek Prep (Elysian, MN). Phosphatidic acid, LPA, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were obtained from Avanti Polar Lipids (Alabaster, AL). The protease inhibitor mixture was obtained from Roche Applied Science, and all other chemicals were from Sigma.

**Purification of Human PNPLA3**—Recombinant human wild-type and mutant (I148M and S47A) PNPLA3-FLAG fusion proteins were produced in insect (Sf9) cells as described (9). 48 h after the cells were infected with recombinant baculoviruses, the cells were collected, washed twice with PBS, frozen in liquid N<sub>2</sub>, and stored at  $-80^{\circ}\text{C}$ . Cell pellets were thawed on ice before homogenization using a Wheaton Potter-Elvehjem tissue grinder (10 strokes, setting of 4) in 15 ml of homogenization buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 20% glycerol, and protease inhibitors). Homogenates were centrifuged at  $2000 \times g$  for 20 min to remove cellular debris, and the supernatants were subjected to high speed centrifugation ( $100,000 \times g$ ) for 1 h. The membrane pellets were resuspended in 15 ml of lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium deoxycholate, 1% FOS-CHOLINE®-12, 20% glycerol, and protease inhibitors) and homogenized using a handheld electric homogenizer. After incubation for 3 h at  $4^{\circ}\text{C}$ , the cell lysates were centrifuged at  $100,000 \times g$  for 1 h to remove insoluble material. Supernatants were mixed with 0.5 ml of anti-FLAG affinity gel for 4 h at  $4^{\circ}\text{C}$  and then applied to an Econo-Column chromatography column (Bio-Rad, 737-1512). Beads were washed with 25 ml of wash buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% FOS-CHOLINE®-12, 20% glycerol and protease inhibitors) and then resuspended in 5 ml of wash buffer containing no detergent. Bound proteins were eluted in elution buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 20% glycerol, and 100  $\mu\text{g}/\text{ml}$  FLAG peptide) and stored at  $-80^{\circ}\text{C}$ .

**Purification of GST-CGI-58**—CGI-58 was amplified from a cDNA library prepared from Huh-7 cells using primers 5'-ATATGGATCCATGGCGGCGGAGGAGGAGGAG-3' and 5'-GATCTGCGACACTGTGGACTGAGCGGCCGCATTA-3' and inserted into the pGEX-4T3 vector after digestion with BamHI and NotI. The plasmid encoding the GST-CGI-58 fusion protein was expressed in BL21(DE3) competent *E. coli* cells after induction with 0.25 mM isopropyl  $\beta$ -D-thiogalactopyranoside at  $30^{\circ}\text{C}$ . The cell pellet was lysed in cold PBS containing 1 mg/ml lysozyme, 4 mM PMSF, and protease inhibitors. The cells were disrupted by sonication, and the lysates were centrifuged at  $100,000 \times g$  for 45 min. The supernatant was transferred to new tubes containing glutathione-Sepharose beads (Amersham Biosciences) prewashed with cold PBS. After a 2-h incubation at  $4^{\circ}\text{C}$ , the column was washed with 40 volumes of PBS prior to eluting the bound proteins with buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 20% glycerol, protease inhibitors, and 10 mM reduced glutathione.

**Immunoblot Analysis**—Purified PNPLA3 was size-fractionated on an 8% SDS-polyacrylamide gel, and the gel was stained with Coomassie Blue. Protein concentrations were estimated by comparing stain intensity with known concentrations of bovine serum albumin that was fractionated at different concentrations on the same gel. A total of 25  $\mu\text{g}$  of protein (or 30  $\mu\text{l}$  of each FPLC fraction) was mixed with  $5\times$  sample loading buffer (0.313 M Tris-HCl (pH 6.8), 10% SDS, 0.05% bromophenol blue, 50% glycerol, and 0.4 M dithiothreitol) to a final concentration of  $1\times$ . After heating to  $95^{\circ}\text{C}$  for 5 min, the proteins were size-fractionated by 8% SDS-PAGE at 200 V. The proteins were transferred from the gel to a nitrocellulose membrane (Amer-

sham Biosciences) at 100 V for 1 h. The membrane was incubated in TBST buffer (0.05 mM Tris, 0.138 mM NaCl, 2.7  $\mu$ M KCl, and 0.1% Tween 20 (pH 8.0)) containing 5% dry nonfat milk (Nestlé) at room temperature for 30 min, and then anti-FLAG monoclonal antibody (1:5000) was added. After an overnight incubation at 4 °C, the membranes were washed three times for 10 min each in TBST buffer and incubated with horseradish peroxidase-conjugated donkey anti-mouse IgG (1:5000 dilution in TBST buffer plus 2% dry milk) for 60 min at room temperature. The membrane was washed three times for 10 min each with TBST buffer, and the protein signal was visualized using SuperSignal-enhanced chemiluminescence (Pierce).

**FPLC Analysis of Purified Protein**—A total of 500  $\mu$ l of purified human PNPLA3 (0.024  $\mu$ g/ $\mu$ l) was loaded onto a Superdex 200 column mounted on an ÄKTA FPLC system (GE Healthcare). Proteins were eluted in 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, and 20% glycerol. Fractions (0.5 ml) were collected, and fractions 16–32 were subjected to immunoblotting to detect PNPLA3 as described above.

**Lipase Assay**—Substrates were prepared by combining 25  $\mu$ l of radiolabeled [<sup>3</sup>H]triolein, [<sup>14</sup>C]diolein, or [<sup>14</sup>C]monoolein with 50  $\mu$ M phosphatidylcholine and 200  $\mu$ M triolein, diolein, or monoolein, respectively. The lipids were dried using N<sub>2</sub> prior to adding 1 ml of sonication buffer (170 mM K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) and 200  $\mu$ M sodium taurocholate), and the mixture was sonicated using a Branson Digital Sonifier® 450 sonicator (1-s bursts, 30% power, 4 min). A total of 2  $\mu$ g of PNPLA3 was mixed with 100  $\mu$ l of substrate (final volume of 200  $\mu$ l), warmed to 37 °C for 20 min, and returned to ice. Folch solution (1 ml) was added, and the mixture was vortexed for 30 s prior to centrifugation at 2000  $\times$  g for 10 min. The aqueous phase was removed, and the solvent was evaporated using N<sub>2</sub>. The lipids were suspended in 80  $\mu$ l of chloroform and separated by TLC using chloroform/methanol/ammonium hydroxide (65:25:5, v/v/v) as the mobile phase. The lipids were stained with iodine, and the bands corresponding to oleate were scraped and subjected to scintillation counting.

**Thioesterase Assay**—[<sup>14</sup>C]Acyl-CoA substrates were dissolved in sodium acetate and ethanol (50:50, v/v) and then dried using N<sub>2</sub>. Radiolabeled (20  $\mu$ M) and unlabeled (80  $\mu$ M) substrates were mixed in 100 mM Tris-Cl (pH 7.4). Substrate (50  $\mu$ l) and purified PNPLA3 (1  $\mu$ g) were combined in 1.5-ml Eppendorf tubes on ice. The reaction mixture was transferred to a 37 °C water bath and incubated for 20 min prior to the addition of 0.5 ml of Folch solution. Lipids were extracted and resolved as described above. Fatty acids were scraped from the plates and counted.

**Glycerol-3-phosphate Acyltransferase (GPAT), Lysophosphatidic Acid Acyltransferase (LPAAT), and Diacylglycerol Acyltransferase (DGAT) Assays**—To measure GPAT activity, substrate containing 200  $\mu$ M glycerol 3-phosphate and 20  $\mu$ M [<sup>14</sup>C]oleoyl-CoA or [<sup>14</sup>C]palmitoyl-CoA was prepared in buffer containing 75 mM Tris-HCl (pH 7.5), 4 mM MgCl<sub>2</sub>, 1 mg/ml BSA (essentially fatty acid-free), 1 mM DTT, and 8 mM NaF and vortexed gently. Purified PNPLA3 (2  $\mu$ g) was added to the substrate (50  $\mu$ l) on ice and then incubated at 37 °C for 15 min. Crude liver lysates (100  $\mu$ g) functioned as a positive control for the assay. Folch solution (1.2 ml) and 2% orthophosphoric acid

(300  $\mu$ l) were added to stop the reaction. The mixture was vortexed for 15 s twice and centrifuged at 2000  $\times$  g for 10 min at room temperature. After discarding the top phase, the lipids were dried, reconstituted in 80  $\mu$ l of chloroform, and spotted on a TLC plate. The lipids were resolved in solvent containing chloroform/methanol/acetone/glacial acetic acid/water (50:10:20:12:5) and exposed overnight to a phosphorimaging screen. The plate was stained with iodine vapor to locate the position of LPA.

To measure LPAAT activity, a stock solution of LPA (3 mM) was prepared in 50 mM Tris-Cl (pH 7.5) and sonicated (25% output) twice for 10 s each on ice. Substrate containing 200  $\mu$ M LPA, 80  $\mu$ M unlabeled oleoyl-CoA, and 20  $\mu$ M [<sup>14</sup>C]oleoyl-CoA was prepared and vortexed gently. Purified PNPLA3 (2  $\mu$ g) was added to the substrate (50  $\mu$ l) on ice and then incubated at 37 °C for 15 min. GST-CGI-58 prepared as described above was used as a positive control. Lipids were extracted and resolved on a TLC plate as described for the GPAT assay. The plate was stained with iodine vapor to locate the position of phosphatidic acid.

The LPAAT activity of PNPLA3 and CGI-58 was also measured using recombinant proteins immunoprecipitated from COS-7 cells. Cells were transfected using FuGENE 6 (Roche Applied Science) with expression vectors encoding PNPLA3-WT, PNPLA3-I148M, and PNPLA3-S47A as well as CGI-58. Each cDNA encoded a fusion protein that contained a V5 tag followed by a His tag at the C terminus. 48 h after transfection, cells were collected and disrupted by sonication on ice in buffer containing 50 mM Tris-Cl, 200 mM NaCl, 10% glycerol, 1 mM benzamide, and protease inhibitor mixture. Cell lysates were centrifuged at 12,000 rpm for 15 min, and the supernatants were collected. Nickel-nitrilotriacetic acid-agarose beads (Qiagen) were added to the supernatants and rotated at 4 °C for 2 h. Beads were washed four times with the sonication buffer, and bound proteins were eluted with 200 mM imidazole solubilized in the same buffer. Protein eluates were subjected to 4 h of dialysis at 4 °C in buffer containing 50 mM Tris-Cl, 200 mM NaCl, 10% glycerol, and protease inhibitor mixture (>100 volumes). Dialyzed samples were concentrated using Millipore filters, and the protein concentration was determined using BCA reagents. 10  $\mu$ g of protein from each sample was used in the LPAAT assay as described above.

Liposome substrate containing 200  $\mu$ M DAG and 20  $\mu$ M [<sup>14</sup>C]oleoyl-CoA was prepared as described for the lipase assay. Purified PNPLA3 (4  $\mu$ g) was added to the substrate (100  $\mu$ l) on ice and then incubated at 37 °C for 15 min. Crude liver lysates (100  $\mu$ g) were used as a positive control of DGAT activity. 1 ml of Folch solution was added to stop the reaction. The total lipids were extracted and resolved on a TLC plate as described for the lipase assay and exposed overnight to a phosphorimaging screen. The plate was stained with iodine vapor to locate the position of TAG.

**Quantification of Free Fatty Acids Released from Acylglycerols Using GC-MS**—Unlabeled acylglycerol substrate (0.5 ml) was mixed with 25  $\mu$ g of PNPLA3 and incubated at 37 °C for 4 h. Total lipids were extracted with Folch solution, and the individual lipids were isolated as follows. Aminopropyl SPE cartridges were washed with hexane before addition of lipid extract (<15



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mg of lipids in 200  $\mu$ l of chloroform). The cartridges were then washed with hexane/ethyl acetate (85:15, 2 ml) to elute neutral lipids. To elute free ceramides, monoglycerides, and some sphingolipid bases, 4 ml of chloroform/methanol (23:1) was applied to the cartridges. Free fatty acids were eluted using diisopropyl ether/acetic acid (98:5, 3 ml). The fatty acids were converted to fatty acid methyl esters using anhydrous methanol (18) and analyzed via GC-MS.

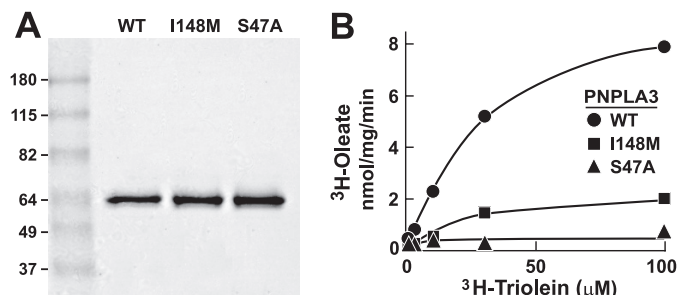
**Enzyme Kinetics**—GraphPad Prism 5 software was used for Michaelis-Menten curve fitting and concurrent calculation of  $V_{\max}$  and  $K_m$  values.

## RESULTS

Previously, we showed that PNPLA3 is strongly associated with membranes, although the enzyme is not predicted to have any membrane-spanning domains (9). To identify a detergent that efficiently solubilizes the enzyme, we expressed recombinant human PNPLA3 in Sf9 cells and solubilized membrane pellets from the cells in different detergents at a concentration of 1% (v/v). The lysates were subjected to high speed centrifugation, and the relative amounts of PNPLA3 in the membrane fraction (pellet) and supernatant were determined (supplemental Fig. S1A). As reported previously (9), 1% Triton failed to solubilize the protein, whereas PNPLA3 was efficiently solubilized in 1% SDS. No detergents tested other than those in the FOS-CHOLINE® class solubilized the enzyme (supplemental Fig. S1A). We then tested the relative ability of FOS-CHOLINE® detergents with chain lengths ranging from 8 to 14 to solubilize the enzyme. PNPLA3 was well solubilized when the chain length of the detergent was >11 (supplemental Fig. S1B). FOS-CHOLINE®-12 was selected as the detergent for protein purification.

Recombinant PNPLA3-WT, PNPLA3-I148M, and PNPLA3-S47A proteins were isolated by affinity chromatography (supplemental Fig. S2). Size fractionation of PNPLA3-WT by FPLC indicated that the protein was present in a complex with a molecular mass of >670 kDa (supplemental Fig. S3). The recombinant proteins were estimated to be >99% pure based on PAGE and staining with Coomassie Blue (Fig. 1A). To determine the effect of the I148M variant on TAG hydrolase activity, the wild-type or mutant enzymes were incubated with [<sup>3</sup>H]triolein liposomes, and the release of free radiolabeled oleate was measured (Fig. 1B). The substitution of methionine for isoleucine at position 148 caused a modest reduction in the  $K_m$  (from 32.2 to 21.91  $\mu$ M) and a sharp decrease in the  $V_{\max}$  (from 10.48 to 2.40 nmol/mg/min) of the enzyme (Table 1). Substitution of alanine for the catalytic serine at codon 47 almost completely abolished TAG hydrolase activity. Thus, the I148M substitution markedly reduced but did not completely inactivate TAG hydrolase activity.

To determine which steps of TAG hydrolysis are catalyzed by PNPLA3, we incubated the recombinant enzyme with an emulsion of triolein in which the glycerol backbone was labeled with <sup>14</sup>C. Separation of the reaction products of the WT enzyme by TLC revealed spots corresponding to 1,3-DAG, 1,2-DAG, and MAG, with greater accumulation of 1,2-DAG than 1,3-DAG (Fig. 2). The intensities of all three spots were much lower for



**FIGURE 1. Triglyceride (TAG) lipase activity of PNPLA3.** A, recombinant human PNPLA3-WT, PNPLA3-I148M, and PNPLA3-S47A were purified from insect (Sf9) cells. A total of 1.8  $\mu$ g of protein was size-fractionated on an 8% SDS-polyacrylamide gel, and the gel was stained with Coomassie Blue. B, wild-type or mutant PNPLA3 (2  $\mu$ g) was incubated with [<sup>3</sup>H]triolein liposome substrate for 20 min at 37 °C as described under "Experimental Procedures." Lipids were extracted and then fractionated by TLC using chloroform/methanol/ammonium hydroxide (65:25:5, v/v/v). Free oleate was isolated, and the radioactivity quantitated by scintillation counting. Values were normalized to the levels in the buffer-only control sample and fitted to Michaelis-Menten kinetic curves. The experiments were performed four times, and the results from a representative experiment are shown.

the I148M isoform, whereas the S47A isoform generated no detectable reaction intermediates.

These data indicate that PNPLA3 catalyzes the sequential cleavage of TAG to DAG and of the resulting DAG to MAG. The accumulation of 1,2-DAG suggests that the enzyme preferentially hydrolyzes fatty acids in the *sn*-1 and *sn*-3 positions of TAG. Both TAG and DAG hydrolase activities are markedly reduced by substitution of methionine for isoleucine at position 148, although the relative preference for fatty acids at the *sn*-1 and *sn*-3 positions was preserved.

Incubation of PNPLA3 with labeled DAG and MAG confirmed that the WT enzyme hydrolyzed both substrates (Fig. 3). The  $K_m$  for both substrates was similar to that observed for TAG, whereas the  $V_{\max}$  increased in inverse proportion to the number of fatty acids in the molecule. These data indicate that PNPLA3 binds TAG, DAG, and MAG with similar affinities and hydrolyzes the ester bonds in the three molecules with comparable efficiency. The I148M substitution markedly reduced the  $V_{\max}$  for DAG and MAG but had little effect on the apparent  $K_m$  for DAG. The substitution decreased the apparent  $K_m$  for MAG.

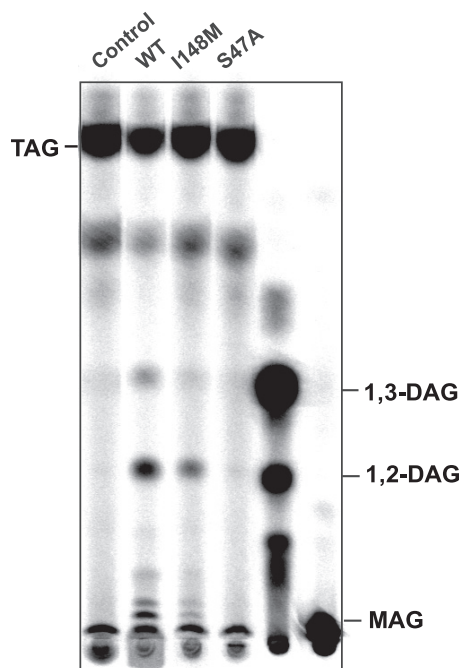
To assess the effects of fatty acid chain length and saturation on PNPLA3 activity, we incubated the purified enzyme with emulsions containing one of five simple TAGs: trimyristin, tripalmitin, triolein, trilinolein, and triarachidonin. PNPLA3 showed a distinct preference for oleic acid (Table 2). Release of free fatty acids was consistently >15% for triolein and <6% for the other four triglycerides tested. Similar results were obtained for DAG and MAG, for which three different fatty acids (C16:0, C18:1, and C18:2) were examined. PNPLA3 consistently hydrolyzed diolein better than dipalmitin or dilinolein and monoolein better than monopalmitin or monolinolein. Thus, PNPLA3 shows a strong preference for glycerolipids in which the acyl group is oleic acid.

Finally, we tested whether the TAG activity of PNPLA3 is activated by CGI-58, the cofactor that activates ATGL (19). For this experiment, we expressed PNPLA3 in COS-7 cells and isolated the protein by affinity chromatography. The TAG hydro-

**TABLE 1** **$K_m$  and  $V_{max}$  of wild-type and mutant PNPLA3 for different lipid substrates**

Acylglycerol substrates TAG, DAG, and MAG and acyl-CoA substrates oleoyl-CoA, stearoyl-CoA, and palmitoyl-CoA were incubated with the buffer control, purified PNPLA3-WT, PNPLA3-I148M, or PNPLA3-S47A as described under "Experimental Procedures." Lipids were extracted and resolved on TLC plates. Released free fatty acids were scraped and counted. Values were normalized to the levels in the buffer-only control and fitted to Michaelis-Menten kinetics curves to determine  $V_{max}$  and  $K_m$  values in the same model. NA, not applicable.

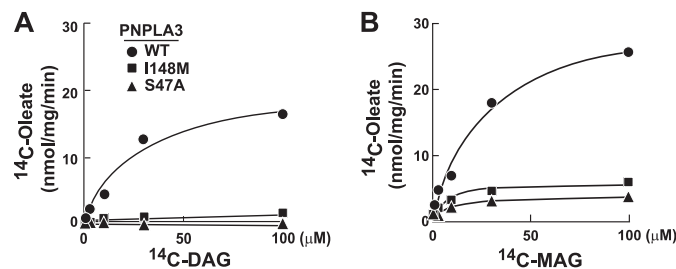
Substrate	WT		I148M		S47A	
	$V_{max}$	$K_m$	$V_{max}$	$K_m$	$V_{max}$	$K_m$
	nmol/mg/min	$\mu\text{M}$	nmol/mg/min	$\mu\text{M}$	nmol/mg/min	$\mu\text{M}$
TAG	10.5 $\pm$ 0.4	32.2 $\pm$ 3.5	2.4 $\pm$ 0.3	21.9 $\pm$ 8.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1
DAG	21.8 $\pm$ 2.2	29.1 $\pm$ 7.6	1.0 $\pm$ 1.2	32.2 $\pm$ 23.6	~0	NA
MAG	32.9 $\pm$ 3.2	27.4 $\pm$ 7.0	5.9 $\pm$ 0.8	5.4 $\pm$ 2.7	3.9 $\pm$ 0.7	7.6 $\pm$ 4.6
Oleoyl-CoA	2.8 $\pm$ 0.2	6.2 $\pm$ 1.1	1.9 $\pm$ 0.1	4.3 $\pm$ 0.8	~0	NA
Stearoyl-CoA	0.3 $\pm$ 0.01	3.9 $\pm$ 0.64	0.3 $\pm$ 0.02	2.7 $\pm$ 0.81	~0	NA
Palmitoyl-CoA	0.1 $\pm$ 0.03	2.7 $\pm$ 2.1	0.3 $\pm$ 0.04	2.4 $\pm$ 1.6	~0	NA



**FIGURE 2. PNPLA3 hydrolysis of TAG.** A total of 100  $\mu\text{M}$  [ $^{14}\text{C}$ ]triolein that was uniformly labeled on glycerol was prepared in liposomes. Substrate (100  $\mu\text{l}$ ) was incubated with 5  $\mu\text{g}$  of wild-type or mutant PNPLA3 in a 200- $\mu\text{l}$  reaction volume for 60 min at 37  $^{\circ}\text{C}$ . The extracted lipids were spotted onto a TLC plate that was flushed by 2.3% boric acid in ethanol and dried at 100  $^{\circ}\text{C}$ . The plate was developed in chloroform/acetone (96:4, v/v) and subjected to phosphorimaging analysis. Positions of TAG, 1,3-DAG, 1,2-DAG, and MAG standards were determined. The experiment was performed twice, and the results were similar.

lase activity of ATGL was 60% higher than that of PNPLA3 in this experiment. The addition of CGI-58 increased the activity of ATGL by 3-fold but had no effect on the TAG lipase activity of PNPLA3-WT or on either of the two mutant proteins (Fig. 4). Thus, CGI-58 does not serve as a cofactor for PNPLA3 under conditions in which it enhances the activity of PNPLA2. Substitution of the isoleucine in ATGL that corresponds to residue 148 in PNPLA3 with methionine inactivated ATGL in the absence of CGI-58 and greatly reduced activity in the presence of the cofactor. As expected, substitution of alanine for serine in the active site of both PNPLA family members destroyed TAG activity.

Inactivation of PNPLA3 in mice does not result in accumulation of TAG in the liver (16, 17). Moreover, expression of high levels of PNPLA3-I148M causes a dramatic increase in hepatic



**FIGURE 3. PNPLA3 hydrolyzes DAG and MAG.** A, PNPLA3-WT, PNPLA3-I148M, or PNPLA3-S47A (2  $\mu\text{g}$ ) was incubated for 20 min at 37  $^{\circ}\text{C}$  with [ $^{14}\text{C}$ ]diolein (A) or [ $^{14}\text{C}$ ]monoolein (B) prepared in liposomes as described under "Experimental Procedures." Total lipids were extracted, and free oleate was measured as described in the legend to Fig. 1. The values were normalized to the levels obtained when buffer alone was added to the reaction. The experiments were performed three times for either substrate, and the results from one representative experiment are shown.

**TABLE 2****Effect of fatty acid chain length and saturation on percentage of substrate hydrolyzed by PNPLA3**

TAGs and DAGs containing a single species of fatty acid (myristate, palmitate, oleate, linoleate, and arachidonate) were prepared as substrates at a concentration of 200  $\mu\text{M}$ . Substrates were incubated with purified PNPLA3 at 37  $^{\circ}\text{C}$ . The acylglycerol substrates (TAG or DAG) and released free fatty acids were isolated and quantified by GC-MS. The percentages of hydrolyzed substrates were calculated. Each reaction was repeated at least three times. NA, not applicable.

Fatty acid	TAG	DAG
Myristate (14:0)	5.4 $\pm$ 1.6	NA
Palmitate (16:0)	5.7 $\pm$ 0.4	6.0 $\pm$ 2.6
Oleate (18:1)	15.4 $\pm$ 1.1	15.2 $\pm$ 3.5
Linoleate (18:2)	5.4 $\pm$ 0.2	7.2 $\pm$ 0.7
Arachidonate (20:4)	5.6 $\pm$ 1.6	NA

TAG content (9). These observations suggest that the elevated hepatic TAG levels associated with the I148M allele may be caused by a gain of function rather than a loss of function. To examine the possibility that PNPLA3 promotes the synthesis of TAG, we measured the GPAT, LPAAT, and DGAT activities of the enzyme.

The conversion of glycerol 3-phosphate to LPA is the first step in hepatic TAG synthesis. Incubation of [ $^{14}\text{C}$ ]oleoyl-CoA and glycerol 3-phosphate with purified PNPLA3-WT or PNPLA3-I148M did not result in formation of LPA (Fig. 5A). A similar assay was performed to assess LPAAT activity. Neither wild-type nor mutant PNPLA3 catalyzed the conversion of LPA to phosphatidic acid (Fig. 5B). CGI-58, a protein with LPAAT activity, served as a positive control for this experiment. Both GPAT and LPAAT activities were readily detectable in crude liver lysates assayed under identical conditions.

## Purification and Biochemical Characterization of PNPLA3

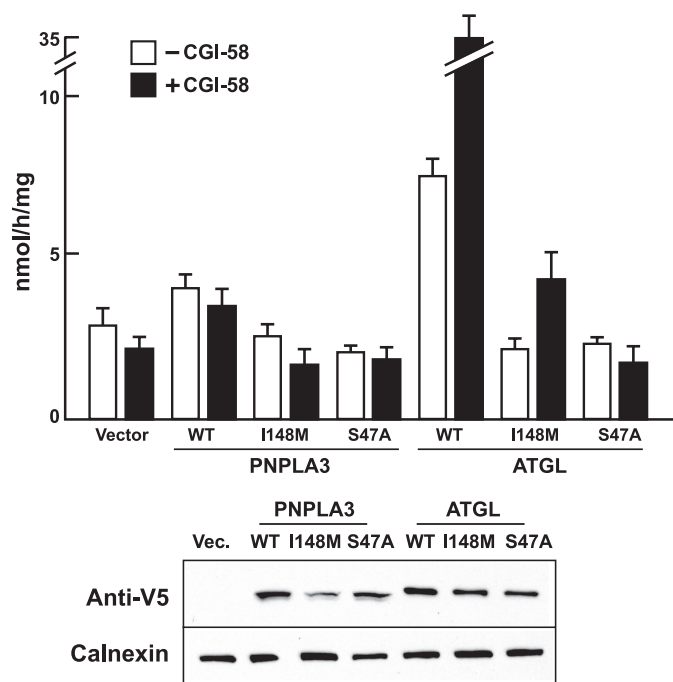


FIGURE 4. **CGI-58 does not activate PNPLA3.** COS-7 cells were transfected with the vector control (Vec.), PNPLA3-WT, PNPLA3-I148M, PNPLA3-S47A, ATGL-WT, ATGL-I148M, or ATGL-S47A. Cells were disrupted by sonication, and 100 mg of cell lysates was used for TAG hydrolysis assay in the presence or absence of 200 ng of bacterially purified GST-CGI-58. The released free oleate was extracted and measured by scintillation counting. Expression of PNPLA3, PNPLA3 mutants, ATGL, ATGL mutants, and calnexin was determined by Western blotting using antibodies against the V5 tag and calnexin. This experiment was performed twice, and the results were similar.

To rule out the possibility that the detergents used to isolate PNPLA3 inhibited LPAAT activity, we repeated the experiment using recombinant PNPLA3 and CGI-58 isolated in an identical manner from cells expressing the recombinant proteins. CGI-58 had robust LPAAT activity under these conditions, whereas both wild-type and mutant PNPLA3 failed to generate any increase in phosphatidic acid (supplemental Fig. S4).

Finally, we tested whether PNPLA3 has DGAT activity, which is the final step in the TAG biosynthetic pathway. No DGAT activity was detected using the purified enzymes.

Jenkins *et al.* (14) reported that PNPLA3 could catalyze the acyl-CoA-independent synthesis of TAG by sequential transacylation of MAG. Incubation of purified PNPLA3 with labeled DAG or with unlabeled DAG and labeled oleate yielded trace amounts of labeled TAG (Fig. 6), but the amount of TAG formed was a linear function of substrate concentrations from 1 to 300  $\mu\text{M}$  (data not shown). Transacylation activity was decreased by the I148M substitution and abolished by the S47A substitution.

To identify other substrates of the enzyme, we tested purified PNPLA3-WT for hydrolytic activity against various lipids. The enzyme hydrolyzed oleoyl-CoA with first-order saturation kinetics (Fig. 7A). Other acyl-CoAs were hydrolyzed much less efficiently (Fig. 7, B and C). Whereas substitution of the catalytic serine abolished acyl-CoA thioesterase activity, the I148M substitution caused only a modest decrease in the  $V_{\text{max}}$  for this substrate. PNPLA3 showed modest but detectable activity against

phosphatidylcholine (~100-fold lower than that observed against triolein) but not against any of the other lipids tested (Table 3).

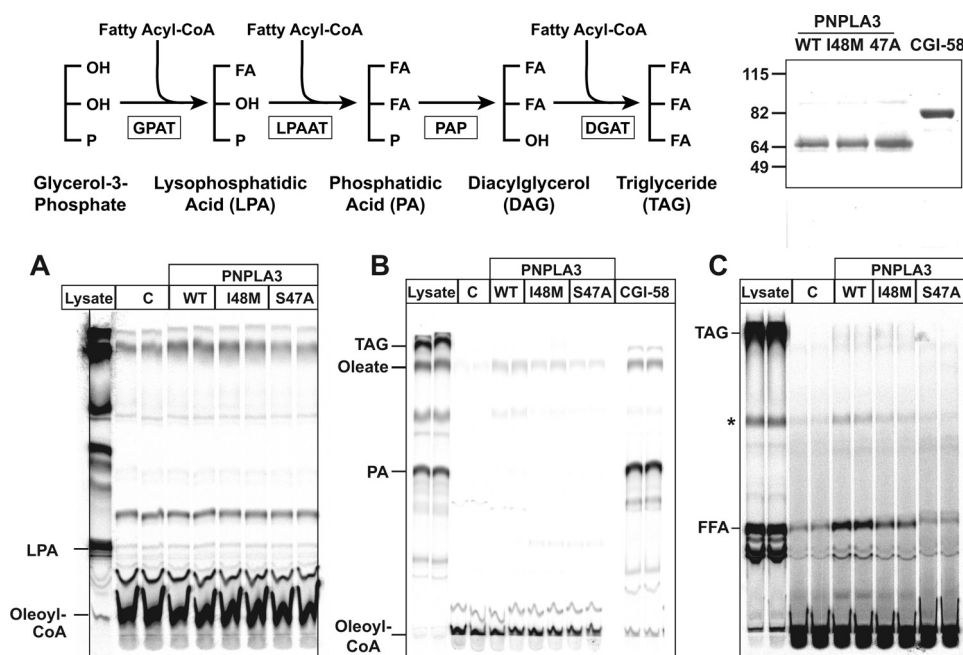
## DISCUSSION

The major finding of this study is that PNPLA3 catalyzes the hydrolysis of the three major glycerolipids (TAG, DAG, and MAG) with similar first-order Michaelis-Menten kinetics. For all three substrates, the enzyme exhibited a strong preference for oleic acid as the fatty acid moiety. The substitution of methionine for isoleucine at position 148 markedly decreased the  $V_{\text{max}}$  of the enzyme for the glycerolipid substrates, with little effect on the  $K_m$ . These findings are consistent with a model in which residue 148 forms part of a hydrophobic substrate-binding groove in the active site of PNPLA3. In this model, the substitution of methionine for isoleucine at position 148 does not alter the orientation of the serine (Ser-47) or aspartate (Asp-166) residues that comprise the catalytic dyad. Rather, the longer side chain of methionine restricts access of substrate to the catalytic serine, thereby inactivating the enzyme (9). Purified PNPLA3 also catalyzed the hydrolysis of oleoyl-CoA. The thioesterase activity required the catalytic serine but was only modestly decreased by the I148M substitution. The enzyme had little or no hydrolytic activity against the other lipid substrates tested and did not exhibit GPAT, LPAAT, or DGAT activity. Taken together, our results are consistent with the notion that PNPLA3 plays a role in the hydrolysis of glycerolipids and that the I148M substitution causes a loss of function. We cannot rule out the possibility that the enzyme has other substrates or activities *in vivo*.

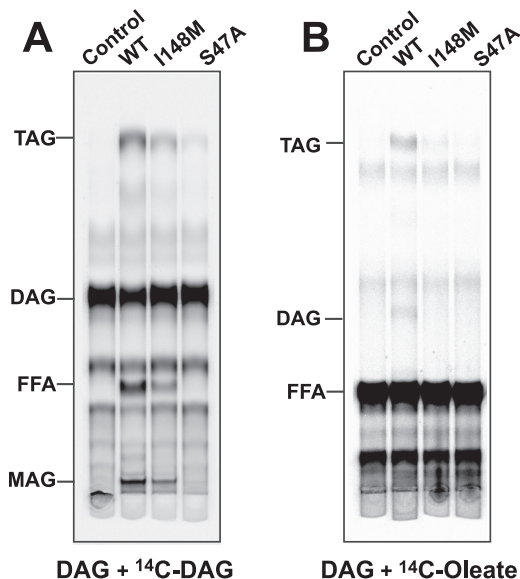
The TAG hydrolysis activity of purified recombinant PNPLA3 observed in this study (10.5 nmol/mg/min) is comparable with that reported previously by Jenkins *et al.* (14) for the partially purified enzyme (6.7 nmol/mg/min). In our assay and in that of Jenkins *et al.*, the triolein lipase activity of PNPLA3 was similar to that of PNPLA2, the major TAG lipase of adipose tissue (13). Subsequently, Zechner and co-workers (19) showed that PNPLA2 is activated by the abhydrolase domain protein ABHD5 (also called CGI-58). ABHD5 is essential for ATGL function; in mice and humans, the phenotype of ABHD5 deficiency is similar in scope and severity to that of PNPLA2 deficiency (20). Because PNPLA2 is the closest family member to PNPLA3, this finding raises the possibility that PNPLA3 may also require a cofactor for activity. ABHD5 failed to stimulate PNPLA3 activity in our assay, suggesting that it does not function as a cofactor for the enzyme. However, we cannot exclude the possibility that another protein acts as a cofactor for PNPLA3. Therefore, although our data suggest that PNPLA3 appears to be a glycerolipid hydrolase with little activity against several other lipids, our assay system may fail to reflect true biological activity of the enzyme if an essential as yet unidentified cofactor is missing from the reaction.

Although PNPLA3 hydrolyzes TAG, DAG, and MAG with similar efficiency and can fully hydrolyze TAG to free fatty acids and glycerol *in vitro*, the enzyme is not required for efficient TAG mobilization in adipose tissue or liver. In contrast to PNPLA2 knock-out mice, which accumulate excess TAG in liver and fat, mice lacking PNPLA3 show no abnormal TAG accumulation in either tissue (13). Together with the observa-





**FIGURE 5. Purified PNPLA3 has no detectable GPAT, LPAAT, or DGAT activity.** A schematic of the Kennedy pathway of TAG synthesis (upper left) and Coomassie Blue staining of PNPLA3-WT, PNPLA3-I148M, PNPLA3-S47A, and GST-CGI-58 used in the experiments (upper right) are shown. A total of 4  $\mu\text{g}$  of PNPLA3-WT, PNPLA3-I148M, or PNPLA3-S47A in a volume of 100  $\mu\text{l}$  was mixed with 100  $\mu\text{l}$  of (A) 200  $\mu\text{M}$  glycerol 3-phosphate and 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]oleoyl-CoA (B), 200  $\mu\text{M}$  LPA and 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]oleoyl-CoA (C), or 200  $\mu\text{M}$  DAG and 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]oleoyl-CoA and incubated at 37  $^{\circ}\text{C}$  for 15 min. Liver lysate (100  $\mu\text{g}$ ) was used as a positive control. For the LPAAT assay, recombinant GST-CGI-58 (4  $\mu\text{g}$ ) was also used as a positive control. Total lipids were extracted and resolved as described under "Experimental Procedures." The positions of oleoyl-CoA and of the reaction end products are indicated. Each of these experiments was repeated at least three times, and the results were similar. PAP, phosphatidic acid phosphohydrolase; FA, fatty acid; C, control. \*, unidentified lipid.



**FIGURE 6. Transacylation activity of PNPLA3.** WT and mutant PNPLA3 (5  $\mu\text{g}$ ) were incubated with 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]diolein (A) or [ $^{14}\text{C}$ ]oleate (B) for 20 min at 37  $^{\circ}\text{C}$ . Total lipids were extracted and loaded onto a TLC plate pretreated with 2.3% boric acid in ethanol. The plate was developed in chloroform/acetone (96:4, v/v) and subjected to phosphorimaging analysis. The positions of TAG, DAG, MAG, and free fatty acid (FFA) are indicated. The experiment was repeated twice, and the results were similar.

tion that PNPLA3 expression is strongly suppressed by fasting and stimulated by refeeding (8), this finding suggests a more specific role for the enzyme. The strong preference of the enzyme for C18:1 is also consistent with a more specific metabolic role, perhaps in the hydrolysis of a particular subset of oleoyl-containing glycerolipids.

Hepatic overexpression of PNPLA3-I148M recapitulates the fatty liver phenotype associated with the allele in humans (9). This finding suggests that the I148M substitution may confer a gain of function, possibly by promoting the synthesis rather than decreasing the hydrolysis of TAG, although overexpression of a catalytically dead enzyme (PNPLA3-S47A) also recapitulates the fatty liver phenotype. In *in vitro* assays, neither the WT nor mutant proteins exhibited GPAT, LPAAT, or DGAT activity. Thus, the enzyme failed to catalyze any of the reactions required for *de novo* TAG synthesis. Jenkins *et al.* (14) reported that PNPLA3 catalyzes formation of TAG by transacylation of MAG and DAG. This activity was confirmed in our study, but product formation increased linearly with substrate concentration and was decreased, rather than enhanced, by the I148M substitution. The finding that the transacylation reaction catalyzed by PNPLA3 requires the catalytic serine but does not exhibit saturation kinetics suggests that the active site of the enzyme does not readily accommodate (or has very low affinity for) two DAG molecules or one DAG and one free fatty acid simultaneously. Thus, our data do not support the hypothesis that the I148M substitution promotes liver fat accumulation by directly increasing TAG synthesis.

The biological significance of the PNPLA3-mediated acyl-CoA hydrolase activity we observed in this study is not known. The low micromolar  $K_m$  of the enzyme is compatible with cellular concentrations of acyl-CoA, which range from 20 to 60  $\mu\text{M}$  in rat liver (21). The localization of PNPLA3 in membranes and lipid droplets is also compatible with the cellular distribution of acyl-CoAs, which partition strongly to membranes and are present only at low concentrations in the cytoplasm. Thus, PNPLA3 may play a role in the regulation of cellular acyl-CoA during the post-prandial period. The strong preference of the enzyme for oleoyl-

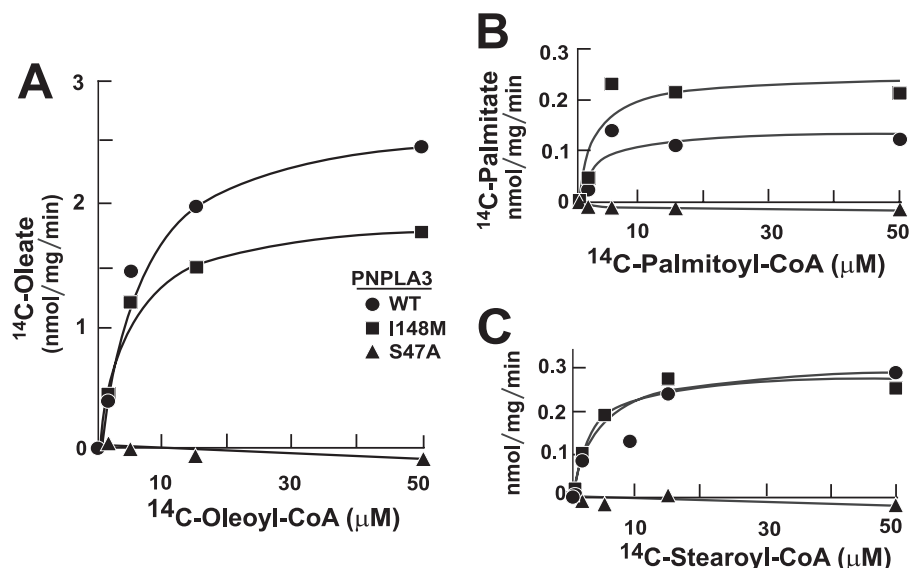


FIGURE 7. **PNPLA3 has thioesterase activity.** PNPLA3-WT, PNPLA3-I148M, or PNPLA3-S47A was incubated for 20 min at 37 °C with [<sup>14</sup>C]oleoyl-CoA (A), [<sup>14</sup>C]palmitoyl-CoA (B), or [<sup>14</sup>C]stearoyl-CoA (C). Lipids were extracted, applied to TLC plates, and resolved in chloroform/methanol/ammonium hydroxide (65:25:5, v/v/v). The activity of the released free oleate was analyzed by scintillation counting and calculated after normalization to the levels obtained using buffer alone. The final values obtained were plotted using GraphPad Prism 5 software. The experiments were performed three times for each substrate, and the results from one representative experiment are shown.

**TABLE 3**

**Lipid substrates for which PNPLA3 did not have detectable enzymatic activity *in vitro***

Lipid substrates were incorporated into liposomes as described under "Experimental Procedures." The substrate was incubated with the buffer control, PNPLA3-WT, PNPLA3-I148M, or PNPLA3-S47A. Lipids were extracted and resolved on TLC plates, and the radiolabeled free fatty acids were scraped and counted. Fluorescently labeled fatty acids were visualized with an Amersham Typhoon 8600 scanner. NBD, [(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-dodecanamide.

Acylcarnitine ( palmitoylcarnitine)
Cholesterol ester (cholesteryl oleate)
NBD-ceramide (NBD C <sub>12</sub> -ceramide)
Lysophosphatidic acid (1-oleoyllysophosphatidic acid)
Phosphatidylethanolamine (1-palmitoyl-2-arachidonylphosphatidylethanolamine)
Phosphatidylinositol (1-stearyl-2-arachidonylphosphatidylinositol 4,5-diphosphate)
Phosphatidylserine (1,2-dipalmitoylphosphatidylserine)
Retinyl ester (retinyl palmitate)

CoA raises the alternative possibility that PNPLA3 may mediate the transfer of oleic acid from oleoyl-CoA to another substrate and may thus catalyze the biosynthesis of a lipid or the post-translational modification of a protein. Finally, acyl-CoA hydrolase activity may be an adventitious property of some serine esterases. Both lipoprotein lipase and hepatic lipase efficiently hydrolyze acyl-CoAs *in vitro* (22) even though both enzymes are active at luminal surfaces of capillaries and are thus exposed to very low concentrations of acyl-CoAs.

Elucidation of the biological function of PNPLA3 has important clinical implications. An increasing number of reports document that the substitution of methionine for isoleucine at position 148 in PNPLA3 contributes to fatty liver disease and possibly other liver disorders (4, 23). However, fundamental questions remain concerning the mechanistic underpinnings of this association. Does the increased risk of disease arise from the loss of normal PNPLA3 function or from a new activity conferred specifically by the I148M substitution? The observation that overexpression of the catalytically inactive enzyme causes hepatic TAG accumulation suggests that the mutant protein may interfere with TAG homeostasis. Previously, we showed that the addition of mutant PNPLA3 to the wild-type protein (1:1) resulted in a 50% reduction in TAG hydrolase activity (9). We have also expressed the mutant protein in the

liver of PNPLA3 knock-out mice, which resulted in an increase in hepatic TAG content (data not shown). Thus, it appears unlikely that the mutant protein promotes hepatic steatosis by interfering with the function of wild-type enzyme. The I148M substitution may cause the sequestration or displacement of another protein required for maintenance of hepatic TAG levels. Alternatively, the protein variant may promote the synthesis (or degradation) of a regulatory molecule that impacts hepatic TG metabolism. The I148M variant increases risk for the full spectrum of disorders that compose fatty liver disease, from simple steatosis (accumulation of TAG in hepatocytes) to steatohepatitis (liver inflammation) and cirrhosis (24). Do these pathological changes result from TAG accumulation *per se* or from an as yet unidentified metabolite that accumulates in the absence of normal PNPLA3 activity (or in the presence of the mutant isoform)? Whereas the results of this study support the hypothesis that PNPLA3 is primarily a triglyceride hydrolase, further studies will be required to fully define the *in vivo* role of this enzyme in hepatic TAG metabolism.

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