Structure/Function Relationships of Adipose Phospholipase A2 Containing a Cys-His-His Catalytic Triad


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#Running title: Analysis of the enzyme activity and structure of AdPLA

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Capsule

Background: AdPLA is the predominant phospholipase in adipose tissue where it suppresses lipolysis.

Results: AdPLA associates with membranes and displays PLA2 and PLA1 activities using a Cys-His-His catalytic triad.

Conclusion: The enzymatic mechanism and structure of AdPLA resembles that of NlpC/P60 cysteine peptidases.

Significance: The structure and enzymatic properties of AdPLA shed light on its physiological function.

ABBREVIATIONS: AdPLA; adipose phospholipase A2; ATGL, adipose triglyceride lipase; BODIPY, boron-dipyrromethene; CAD, charged aerosol detection; cPLA2, cytosolic PLA2; CGI-58, Comparative Gene Identification-58; DXMS, hydrogen deuterium exchange coupled with mass spectrometry; EP3, prostanoglandin E receptor 3; FL-AdPLA, full-length AdPLA; FRET, fluorescence resonance energy transfer; H/D, hydrogen/deuterium; HSL, hormone-sensitive lipase; HRASLS3, H-Ras-like suppressor 3; H-Rev107, Hras-Revertant gene 107; iPLA2, calcium-independent PLA2; L-α-GPC, L-α-glycerolphosphorylcholine; LC, liquid chromatography; LIC, ligation-independent cloning; LPA, lysophosphatidic acid; LpPLA2, lipoprotein-associated PLA2; LRAT, lecithin: retinol acyltransferase; MGC, Mammalian Gene Collection; MBP, maltose binding protein; MGL, monoglyceride lipase; MS, mass spectrometry; PA, phosphatidic acid; PGE2, prostaglandin E2; PLA1, phospholipase A1; PLA2, phospholipase A2; sPLA2, secreted PLA2; SSM, secondary structure matching; PNPLA6, patatin-like phospholipase domain containing 6; PNPLA7, patatin-like phospholipase domain containing 7; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; T-AdPLA, truncated AdPLA; SUV, small unilamellar vesicles; TAG, triacylglycerols; TEV, tobacco etch virus; TIG3, tazarotene-induced gene 3.
SUMMARY
Adipose phospholipase A₂ (AdPLA or Group XVI PLÅ₂) plays an important role in the onset of obesity by suppressing adipose tissue lipolysis. As a consequence, AdPLA-deficient mice are resistant to obesity induced by high-fat diet or by leptin deficiency. It has been proposed that AdPLA mediates its anti-lipolytic effects by catalyzing the release of arachidonic acid. Based on sequence homology, AdPLA is part of a small family of acyltransferases and phospholipases related to lecithin: retinol acyltransferase (LRAT). To better understand the enzymatic mechanism of AdPLA and related LRAT proteins, we solved the crystal structure of AdPLA. Our model indicates that AdPLA is structurally related to the NlpC/P60 family of cysteine proteases, having its secondary structure elements configured in a circular permutation of the classic papain fold. Using both structural and biochemical evidence we demonstrate that the enzymatic activity of AdPLA is mediated by a distinctive Cys-His-His catalytic triad and that the C-terminal transmembrane domain of AdPLA is required for the interfacial catalysis. Analysis of the enzymatic activity of AdPLA toward synthetic and natural substrates indicates that AdPLA displays PLA₁ activity in addition to PLA₂ activity. Thus our results provide insight into the enzymatic mechanism and biochemical properties of AdPLA and LRAT-related proteins, and lead us to propose an alternate mechanism for AdPLA in promoting adipose tissue lipolysis that is not contingent on the release of arachidonic acid and which is compatible with its combined PLA₁/PLA₂ activity.

INTRODUCTION
Adipose tissue plays an important role in the regulation of energy balance. While most other tissues can hydrolyze triacylglycerols (TAG) for their own use, adipose tissue alone can hydrolyze TAG to export glycerol and fatty acids for use by other tissues. The net rate of TAG hydrolysis in adipose tissue, or lipolysis, is a determinant of whole-body insulin sensitivity (1); hence, a potentially important therapeutic target in the treatment of obesity. Adipose tissue lipolysis proceeds through the sequential action of the TAG hydrolases, desnutrin/adipose triglyceride lipase (ATGL) (2-4), hormone-sensitive lipase (HSL) (5,6) and monoglyceride lipase (MGL) (7). The rate of adipose tissue lipolysis is carefully controlled by endocrine mediators such as insulin and catecholamines and locally through the action of autocrine /paracrine signaling mediators (8).

In addition to defining the structure and permeability of cells and cellular organelles, phospholipids are a source of local-acting signaling mediators. Hydrolysis of fatty acids linked to the sn-1 or sn-2 positions of phospholipids by phospholipase A₁ (PLÅ₁) or A₂ (PLÅ₂) enzymes, respectively, leads to formation of lipid signaling mediators such as free fatty acids and lysophospholipids (reviewed in (9)). Adipose-specific phospholipase A₂ (AdPLA) classified as Group XVI PLÅ₂ (PLÅ₂G16) (9), is the most abundant PLÅ₂ in adipose tissue (10,11) and its expression increases during adipose differentiation (12). Expression of AdPLA is also upregulated by feeding and by insulin in vivo (11). Importantly, AdPLA was shown to control adipose tissue lipolysis via the production of eicosanoid mediators (11). The level of the prostaglandin PGE₂ was shown to be markedly reduced in the adipose tissue of AdPLA⁻/⁻ mice (11). PGE₂ activates the prostaglandin E receptor 3 (EP3), which suppresses lipolysis in a cAMP-dependent manner (13). As a result, AdPLA⁻/⁻ mice have an increased rate of lipolysis and fatty acid oxidation and higher energy expenditure than wild type mice (11). AdPLA⁻/⁻ mice are lean and resistant to diet-induced obesity (11). Moreover, crossing AdPLA⁻/⁻ with the leptin-deficient (ob/ob) generates AdPLA⁻/⁻;ob/ob double-knockout mice which gain considerably less weight than single knockout ob/ob leptin-deficient mice (11). These data suggest that AdPLA is an important regulator of the rate of adipose tissue lipolysis via production of prostanoid mediators.

The enzymatic activity of AdPLA was initially described to be a calcium-dependent PLA₂ employing a His-Cys catalytic dyad (10). Later
Analysis of the enzyme activity and structure of AdPLA

reports described AdPLA to be a calcium-independent phospholipase with combined PLA_1, PLA_2 and transacylase activities; its PLA_1 activity was reported to be higher than its PLA_2 activity (14). The sequence and enzymatic activity of AdPLA resembles that of a small family of proteins related to lecithin: retinol acyltransferase (LRAT). All members of the LRAT family have been shown to catalyze PLA_1, PLA_2 or acyltransferase reactions (15-18). Thus the LRAT family could be appropriately described as a phospholipase A/acyltransferase (PLA/AT) family based on a recent proposal by by Uyama et al. (19). In this case, AdPLA would be referred to as PLA/AT-3.

The inhibitory effect of AdPLA expression on adipose tissue lipolysis was proposed to be a result of the production of PGE2 through the release of arachidonic acid from the sn-2 position of phospholipids (11). However, the significant PLA_1 activity of AdPLA led to suggestions that AdPLA might have other functions in controlling cellular metabolism and lipid accumulation in addition to production of free arachidonic acid (14,20). In one such example, overexpression of AdPLA in human embryonic kidney cells 293 (HEK293) cells (21) leads to peroxisome dysfunction and a decrease in ether-linked linked triglycerides and plasmalogens (22). Importantly, these effects were not accompanied by a significant release of arachidonic acid in such cells.

AdPLA was originally identified as being specifically expressed in phenotypic revertants of H-Ras-transformed cells, thus it was characterized as a class II tumor suppressor (23) and referred to as Hras-Revertant gene 107 (H-Rev107) or H-Ras-like suppressor 3 (HRASLS3). Expression of AdPLA is downregulated in several tumor cell lines (24-27); meanwhile overexpression of AdPLA induces growth inhibition of transformed cells (28) and apoptosis of ovarian carcinoma cells (29). The relationship between the enzymatic activity and the tumor suppressive effect of AdPLA is not well understood.

To gain a better understanding of the physiological role of AdPLA, we propose to investigate its structure and enzymatic properties. Based on sequence analysis, members of the LRAT family were predicted to have a circular permutation of the catalytic domain found in members of the superfamily of proteins related to Escherichia coli NlpC and Listeria monocytogenes P60 proteins (NlpC/P60) (30,31). Several of the NlpC/P60 enzymes were suggested to employ a conserved Cys-His pair or a Cys-His-His triad in their catalytic mechanism (32,33). In the case of LRAT and other LRAT-family members, the Cys residue was shown to act as a nucleophile and forms a covalent thiol-acyl intermediate in the catalytic process (20,34). A truncated fragment of AdPLA lacking the transmembrane domain was recently characterized by a solution NMR structure (35) and X-ray crystallography (20). Both structures show that AdPLA conforms to the permuted-papain domain fold seen in a subset of NlpC/P60 proteins and predict the location of residues potentially involved in the catalytic triad of AdPLA.

Herein we further describe the enzymatic mechanism of AdPLA by presenting a novel crystal structure of AdPLA which provides additional support for its enzymatic mechanism. We describe the expression and purification of a soluble full-length form of AdPLA that displays robust enzymatic activity and we examine the PLA_1/PLA_2 specificity of AdPLA for various natural phospholipid substrates. We provide experimental support for the role of the residues proposed to be involved in the catalytic mechanism and we probe the structural dynamic difference between full length and truncated AdPLA based on hydrogen/deuterium (H/D) exchange experiments coupled to mass spectrometry (DXMS). Finally, we evaluate the possible role of AdPLA in the generation of arachidonic acid in adipose tissue in light of its activity and substrate preference.

EXPERIMENTAL PROCEDURES

Cloning mutagenesis and purification of full-length and truncated AdPLA – We cloned human AdPLA cDNA from a full-length expressed sequence tag (EST) clone available
Analysis of the enzyme activity and structure of AdPLA

from the Open Biosystems-Mammalian Gene Collection (MGC) MGC:118754

IMAGE:40000132. A truncation of human AdPLA (T-AdPLA) retaining amino acid residues 1-134 was amplified and cloned through ligation-independent cloning (LIC) in the vector pTB-MalE (36) digested with Ssp I. The ligation places T-AdPLA downstream of an N-terminal fusion partner consisting of a hexahistidine-tagged maltose binding protein (MBP) and a tobacco etch virus (TEV) protease cleavage site. Alternatively, the full-length AdPLA (FL-AdPLA) cDNA was cloned in frame with MBP and separated by a TEV cleavage site to generate a construct coding for an MBP-FL-AdPLA fusion protein.

Mutagenesis of the MBP-FL-AdPLA protein was performed using Quickchange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The expression constructs were sequenced and transformed in the Bl21(DE3) E. coli and grown in Terrific Broth under the selection of 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. The primers used for cloning T-AdPLA and FL-AdPLA and for site-directed mutagenesis reactions of MBP-FL-AdPLA are shown in supplementary Fig. S1. The sequence of the expressed MBP-T-AdPLA and MBP-FL-AdPLA proteins are shown in Fig. S2.

Protein expression was induced using the Overnight Express Autoinduction System (EMD Millipore-Merck KGaA, Billerica, MA) based on a method described previously (37). The bacteria were harvested by centrifugation at 4,000 g for 10 minutes at 4 °C. The pellet was resuspended in 50 mM Tris pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 25 U/ml Benzonase (EMD) and 50 mM arginine and lysed either by two passages in a French press or through sonication at 50% amplitude for 6×20 s. The lysate was spun by centrifugation at 19,000 rpm for 30 min to pellet membranes and insoluble complexes, then filtered through a 0.2 µm filter. Purification of MBP-FL-AdPLA or MBP-T-AdPLA fusion proteins was carried out by metal affinity chromatography. The bacterial supernatant was loaded onto tandem 5 ml His Trap HP columns (GE HealthCare Biosciences, Pittsburgh, PA) in conjunction with an AKTA Xpress purification system at flow rate of 1 ml/min. The columns were washed with Buffer A (0.2% Triton X-100, 50mM Tris pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol) over 4 column volumes. Another wash with 5% Buffer B (500 mM imidazole, 50mM Tris pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol) was used to remove non-specifically bound proteins. Elution was carried out with Buffer B in a linear gradient from 5 to 100%. The resulting fractions were examined by SDS-PAGE and the protein-containing fractions were pooled and filtered through a 0.2µm filter. The protein solution was concentrated and desalted using a Spin-X UF centrifugal concentrator 20 mL, 5,000 MWCO (Corning Inc. Life Sciences, Tewksbury, MA) to a final concentration of 10 mg/ml in 50 mM Tris pH 8.0, 50 mM NaCl and 5 mM β-mercaptoethanol. MBP-FL-AdPLA or MBP-T-AdPLA fusion proteins were next purified by anion-exchange chromatography using tandem 5 ml HiTrap Q HP columns (GE HealthCare). The column was washed using Buffer A (50 mM Tris pH 8.0, 50 mM NaCl) over 4 column volumes followed by a wash with 10% Buffer B (50 mM Tris pH 8.0, 1.0 M NaCl, 5 mM β-mercaptoethanol) to remove non-specifically bound proteins. Elution was carried out with Buffer B in a linear gradient from 10 to 100% at a flow rate of 1 ml/min and the protein containing fractions were pooled and filtered through a 0.2 µm filter. Cleavage with TEV for removing the MBP fusion was performed using 30 mg of MBP-T-AdPLA protein in 30 ml of 150 mM NaCl, 25 mM Tris pH 8.0, 5 mM β-mercaptoethanol supplemented with 600 µg of hexahistidine-tagged-TurboTEV (2 mg/ml stock 10,000 U/mg (Nacalai USA, San Diego, CA) for 48 hr at 20°C with gentle shaking. The hexahistidine-tagged MBP and TurboTEV proteins were removed by metal affinity chromatography using 2 ml of His-Pur Ni-NTA resin (Fisher Scientific, Waltham, MA) in sodium phosphate pH 7.4, 10 mM imidazole, 5 mM β-mercaptoethanol. The eluted untagged T-AdPLA protein was filtered through a 0.2µm filter and concentrated to 4 mL using a Spin-X UF centrifugal concentrator 20 mL, 5,000 MWCO (Corning Inc. Life Sciences, Tewksbury, MA) and purified by using size exclusion chromatography on HiLoad 16/600
Superdex 75 pg column equilibrated with a buffer of 50 mM Tris pH 8.0, 300 mM NaCl and 5 mM dithiothreitol (DTT) and eluted at 0.8 ml/min over 2.5 column volumes. The T-AdPLA containing fractions were pooled and filtered through a 0.2µm filter and concentrated to 10 mg/ml. The resulting untagged T-AdPLA protein is stable at 4 °C for more than four weeks. To verify the identity of the purified protein, the recombinant T-AdPLA protein was examined by SDS-PAGE. The purified protein migrated as a single band as identified by Coomassie Blue staining. The protein band was cut from the gel and treated with iodoacetamide to amidate the Cys residues and digested in gel with TPCK-trypsin. Analysis of the tryptic fragments by tandem MS-MS identified the purified protein as AdPLA showing 90% peptide coverage of the T-AdPLA protein Fig. S3. The T-AdPLA protein was set up for crystallization screens or for enzyme assays.

In the case of FL-AdPLA, cleavage with TEV of the MBP-FL-AdPLA fusion protein results in precipitation of the FL-AdPLA protein, therefore MBP-FL-AdPLA was purified sequentially by affinity chromatography, ion exchange and size exclusion chromatography as described for MBP-T-AdPLA and then used as an intact MBP-FL-AdPLA fusion protein in enzyme assays. The uncleaved MBP-FL-AdPLA protein is stable at 4 °C for several months. We also expressed and purified a C-terminal truncated fragment of AdPLA representing the residues 1-132 of human AdPLA fused to an N-terminal hexahistidine tag denoted as (His)_6-(1-132)AdPLA which has been previously described (20). The plasmid coding for (His)_6-(1-132)AdPLA was obtained as a kind gift from Marcin Golczak and Kris Palczewski. The plasmid was used to express and purify the (His)_6-(1-132)AdPLA protein following the same procedure as MBP-FL-AdPLA.

Crystallization, X-ray Diffraction and Data Collection – A purified sample of T-AdPLA was screened for crystallization in Compact Jr. (Emerald Biosystems, Bainbridge Island, WA) sitting drop vapor diffusion plates at 20 °C using 0.5 µL of protein (10 mg/ml in 50 mM Tris pH 8.0, 300 mM NaCl and 5 mM DTT) and 0.5 µL of crystallization solution equilibrated against 100 µL of the latter. Small prismatic crystals were obtained in 1-2 days from the screen Wizard 4 screen (Emerald Biosystems) condition 21 (25% polyethylene glycol (PEG) 1500, 100 mM malonate / imidazole / borate 2:3:3 (MIB) buffer pH 5.0) and in approximately 1 week from the Wizard 3 screen condition #4 (Emerald Biosystems, 20% PEG 3350, 200 mM ammonium formate). Samples were transferred to a fresh drop composed of 80% crystallization solution and 20% PEG 200 before flash freezing in liquid nitrogen for data collection. Data were collected at the Advanced Photon Source beamline 17-ID using a Dectris Pilatus 6M pixel array detector.

Structure Solution and Refinement – Intensities were integrated using XDS (38) and the Laue class check and data scaling were performed with Aimless (39). The highest probability Laue class was -3m1 and space groups P3121 or P3221. The Matthew’s coefficient (40) (V_m=2.8 / 55.6 %) indicated that there was a single AdPLA molecule in the asymmetric unit. Structure solution was conducted by molecular replacement with Phaser (41) via the Phenix (42) interface. All space groups in the Laue class -3m1 were tested using a single molecule from a previously determined AdPLA NMR structure (PDB: 2KYT) as the search model. The top solution was obtained in the space group P3121 which was used from this point forward. Due to the anisotropy of the diffraction data, the model was refined with a resolution cut-off of 2.65Å. Structure refinement and manual model building were conducted with Buster (43) and Coot (44), respectively. Structure validation was conducted with Molprobity (45). Figures were prepared using the CCP4MG package (46). The coordinates and structure factors were deposited in the Protein Data Bank under accession code 4FA0.

DXMS experiments – Proteins were incubated in D2O buffer consisting of 12 mM Tris, 50 mM
NaCl in 99% D$_2$O, pD$_{read}$ 7.5, as previously described (47). Exchange experiments were initiated by mixing 2µg of T-AdPLA, MBP-T-AdPLA or MBP-FL-AdPLA in protein buffer, or with 6 µL of D$_2$O buffer to a final concentration of 75% D$_2$O. The mixtures were incubated at 23°C for 10, 30, 100, 300, 1000, 3000 or 10,000 s and then the exchange reaction was quenched by adding 12 µl of ice-cold quench solution (0.96% formic acid, 0.8 M guanidine hydrochloride (GdHCl)) resulting in samples with final concentrations of 0.58% formic acid and 0.5 M GdHCl, pH 2.5. The samples were then immediately frozen on dry ice and stored at -80°C.

Proteolysis- Liquid Chromatography-Mass Spectroscopic Analysis of Samples – All steps were performed at 0°C as described previously (47). The digested peptides were separated by C18 column and detected by Thermo Orbitrap Elite mass spectrometer. Data processing of DXMS experiments utilized specialized software as previously described (DXMS Explorer, Sierra Analytics Inc) (47).

In Vitro Phospholipase Activity Assays – Liposomes were created using mixtures of phosphatidylcholine (PC) and phosphatidylglycerol (PG) phospholipids (Avanti Polar Lipids, Alabaster, AL). The PC species examined were 18:0-16:0 PC, 16:0-18:0 PC, 16:0-20:4 PC, 16:0-18:2 PC, 18:1-18:1 PC, 16:0 Lyso PC, 18:0 diether PC. 100 µl aliquots from 10 mg/ml stock solutions of any of the PC species examined were mixed with an equal amount of 10 mg/ml 18:1-18:1 PG. The phospholipid mixture was dried down in a borosilicate glass test tube under vacuum to remove the solvent (chloroform). The lipid cake was hydrated in the presence of 50 mm Tris, pH 8, and 100 mM NaCl, 1mM CaCl$_2$ for 1 hr at a temperature above the solid–fluid transition temperature (T$_c$) of the phospholipid mixture, followed by sonication of the hydrated lipid for 10-30 min in a bath sonicator to produce a suspension of small unilamellar vesicles (SUV) representing a concentration of 1 mM for the combined phospholipids in a ratio of 1:1 PC/PG. The assay was conducted by incubating purified MBP-FL-AdPLA, T-AdPLA, MBP-T-AdPLA, bee venom PLA$_2$ (Invitrogen, Carlsbad, CA) or Thermomyces lanuginosus lipase (TLL) with sn-1,3 specificity (Lecitase Ultra-Novozymes A/S, Denmark, obtained from Invitrogen), for 15 min with various liposomes in the presence of 5 mM tris(2-carboxyethyl)phosphine (TCEP) in a total volume of 220 µl. The reaction was stopped by adding 1 ml Dole’s reagent (48) (40 ml isopropanol, 10 ml heptane and 1 ml 1M H$_2$SO$_4$) and vortexed for 5 mins. The organic phase was extracted with 600 µl heptane and 400 µl water. Phase separation was induced by centrifugation at 3,000 rpm for 5 mins; the organic phase was collected and dried down under vacuum. The residue was resuspended in 200 µl of a mixture of chloroform and methanol (1:1), 10 µl of which were injected into the HPLC system for analysis. Free fatty acids were analyzed by reverse-phase HPLC coupled to a charged aerosol detector using an HPLC1200 system (Agilent Technologies, Santa Clara, CA) equipped with a Halo C8 column 150 x 4.6 mm, 2.7 μm (MAC-MOD Analytical, Inc, Chadds Ford, PA) and a mobile phase A composed of methanol / water / acetic acid (750:250:4) and a mobile phase B composed of acetonitrile / methanol / tetrahydrofuran / acetic acid (500:375:125:4) and a flow rate of 0.8 ml/min with a column temperature of 40°C. The samples were injected in methanol / chloroform (1:1). The gradient followed from 100% A to 30% A over 40 min, 30% A to 10 % A over 20 min, holding 10% A for 5 min followed by re-equilibration in 100% A. The eluted lipids were detected using a Corona charged aerosol detector (CAD, ESA Biosciences) operated at 200 pA and 35 psi nitrogen pressure as described (49). Experimental samples consisting of various enzyme preparations or buffer control were set up in triplicate. Authentic fatty acid standards were used to establish the identity of the eluted peaks.

We also investigated the phospholipase activity of AdPLA using a boron-dipyrromethene (BODIPY)-labeled fluorogenic phospholipid substrate 1-O-(6-BODIPY 558/568-Aminohexyl)-2-BODIPY FL C5-Sn-Glycero-3-
Phosphocholine (BODIPY PC-A2; A10072, Invitrogen). Liposomes were prepared by the ethanolic injection method (50). Equal volumes of 10 mM 18:1-18:1 PC, 10 mM 18:1-18:1 PG and 1 mM BODIPY PC-A2 were combined to prepare a lipid mix containing 10:10:1 molar ratios of 18:1-18:1 PC/18:1-18:1 PG/BODIPY PC-A2. 10µl of the lipid mix was injected while vigorously vortexing in glass tube containing 1 mL of buffer composed of 50 mm Tris, pH 8, and 100 mM NaCl, 1mM CaCl₂. 100 µl liposome substrate was aliquoted in 96-well plate and examined using a Synergy H1 multi-mode microplate reader equipped with dispensers. The liposome suspension was allowed to equilibrate at 37 °C while the fluorescence signal 488 nm / 515 nm was recorded for 1 min to monitor the background emission. No significant increase in background fluorescence emission was observed. Purified MBP-T-AdPLA, T-AdPLA, MBP-T-AdPLA, (His)₆-(1-132)AdPLA and wild type and mutants of MBP-FL-AdPLA were diluted in 50 mm Tris, pH 8, and 100 mM NaCl, 1mM CaCl₂ and 100 µl of protein was dispensed in the well containing the liposome suspension and the fluorescence signal 488 nm / 515 nm signal was recorded for the next 4 minutes. In calculating the rate of reaction as a function of the amount of enzyme, we used a final concentration of BODIPY PC-A2 of 1.67 µM while the protein concentration ranged from 7.8 to 125 µg/ml (0.12 to 1.9 µM). A standard curve of fluorescence signal 488 nm / 515 nm was recorded for 1 min to monitor the background emission. No significant increase in background fluorescence emission was observed. Purified MBP-T-AdPLA, T-AdPLA, MBP-T-AdPLA, (His)₆-(1-132)AdPLA and wild type and mutants of MBP-FL-AdPLA were diluted in 50 mm Tris, pH 8, and 100 mM NaCl, 1mM CaCl₂ and 100 µl of protein was dispensed in the well containing the liposome suspension and the fluorescence signal 488 nm / 515 nm signal was recorded for the next 4 minutes. In calculating the rate of reaction as a function of the amount of enzyme, we used a final concentration of BODIPY PC-A2 of 1.67 µM while the protein concentration ranged from 7.8 to 125 µg/ml (0.12 to 1.9 µM). A standard curve of fluorescence signal 488 nm / 515 nm of 3a,4a-Diaza-s-Indacene-3-Pentanoic Acid (BODIPY FL C5, D3834, Invitrogen), the cleavage product of BODIPY PC-A2, was used for evaluating the percentage of substrate conversion. To measure enzyme velocities as a function of substrate concentration we used a titration of substrate concentrations in molar excess of enzyme concentrations. Initial rates of reaction for various substrate concentrations were derived from steady-state kinetic measurements based on rates measured in the first 20 s of reaction. The assay was carried out in triplicate and the data was evaluated using the SigmaPlot and GraphPad software packages.

RESULTS

The Crystal Structure of AdPLA Reveals the Geometry of the Active Site

We tested several strategies to express human AdPLA as a recombinant protein in E. coli for structural studies. It was shown that the C-terminal domain of AdPLA is embedded in the membrane while the N-terminus of the AdPLA protein is found in the cytoplasm (10,51). Analysis of the amino acid sequence of AdPLA reveals the absence of a cleavable signal sequence and the presence of a single hydrophobic domain at its C-terminus. In accordance with this model, the C-terminal domain is required for targeting AdPLA to the membrane of the endoplasmic reticulum (ER) (10) by acting as both a targeting signal and a membrane anchor domain (51). To increase the yield of soluble protein, we tested the effect of removing the C-terminal hydrophobic domain and also expressed both the full-length and the truncated version of AdPLA fusion proteins with a hexahistidine-tagged MBP protein to increase their solubility (52). The linker between the MBP and AdPLA contains a TEV cleavage site (ENLIFQ/S), allowing the MBP fusion partner to be cleaved with TEV protease and removed in the final stages of purification. Cleavage with TEV protease produces a protein that retains the sequence of either full-length AdPLA (FL-AdPLA) or truncated AdPLA (T-AdPLA) and a stretch of three additional amino acids (SNA-) at the N-terminus of the recombinant proteins (Fig. S2).

Our results indicate that the presence of the C-terminal hydrophobic domain adversely affects the solubility of the bacterially-expressed AdPLA protein. Though MBP-FL-AdPLA is a soluble protein, upon cleavage with TEV protease, the FL-AdPLA protein precipitated from solution. Inclusion of chaotropic agents and detergents led to a modest improvement in the solubility of cleaved FL-AdPLA and attempts to crystallize the intact MBP-FL-AdPLA protein were not successful. On the other hand, cleavage of purified MBP-T-AdPLA with TEV protease produced a soluble protein. The protein was confirmed as T-AdPLA by...
MS/MS analysis (Fig. S3). The purified T-AdPLA protein is monomeric in solution based on its elution profile on a size-exclusion chromatography column calibrated with protein standards (Fig. S3). Crystallization screens of T-AdPLA produced small crystals in several conditions. The crystals were indexed as trigonal/hexagonal with \(a = 60.05\, \text{Å}, c = 74.06\, \text{Å}\) unit cell parameters. Analysis of the axial reflections indicated the presence of a 3-fold screw axis as systematic absences 00l; l=3n were observed.

The structure solution of T-AdPLA was carried out by molecular replacement with Phaser (41) using the NMR model (PDB: 2KYT) of AdPLA as the search model (35,53). The terminal ends and residues 39-55 were truncated in the search model as they were shown to be disordered in PDB: 2KYT. The top solution was obtained for a single monomer in the asymmetric unit in the space group \(P\overline{3}121\). Intensities were integrated and scaled using XDS and Scala (54) respectively (Table 1). The Wilson \(B\)-factor was unusually high, \(\sim 65\, \text{Å}^2\), for data processed to this resolution (2.3 Å). Due to the anisotropy of the diffraction data, the observed electron density maps were not clear in certain regions of the polypeptide chain making it difficult to discern certain side chains. Therefore, a second data set was collected at a longer wavelength (\(\lambda=1.7463\) Å) and phased anomalous difference electron density maps were calculated in an effort to confirm the position of the Cys sulfur atoms and ensure that the sequence register was correctly assigned. Indeed, anomalous difference map peaks were observed greater than 4\(\sigma\) for the two Cys residues C89 and C113, respectively (Fig. 1) The structure reported here was deposited as PDB: 4FA0.

Overall, the structure is similar to the solution NMR structure of AdPLA (PDB: 2KYT) with a RMSD of 1.43 Å between \(\text{C}_\alpha\) atoms (P6 to V124) as determined using secondary structure matching (SSM) (55) via the CCP4 interface (56) (Fig. 2). Residues between P39 and K57 were disordered and could not be fit to the electron density maps. The largest deviations between the AdPLA crystal structure and the solution NMR structure are observed in regions containing loops 27-33, 71-74, 79-87 and 106-112 as shown in Fig. 3A and 3C. The crystal structure we describe here also closely resembles the one recently reported by Golczak et al. (20) of an AdPLA protein shorter by two amino acids at its C-terminus (PDB: 4DOT) with a RMSD of 0.54 Å between \(\text{C}_\alpha\) atoms (P6 to V124). The main difference between the crystal structure reported by Golczak et al. and the one reported here occurs in a loop region between V104-S110 as shown in Fig. 3B and 3C. This difference could be attributed to the relatively high conformational flexibility of the V104-S110 loop as observed in the values of the average \(B\)-factors for the residues encompassing this domain (Fig. 3D).

The crystal structure of AdPLA reported here (PDB: 4FA0) reveals an \(\alpha+\beta\) fold composed of three \(\alpha\)-helices and five anti-parallel \(\beta\)-sheets in a circular permutation of the topology of papain and members of the NlpC/P60 superfamily of cysteine peptidases (31) (Fig. 4). A Cys residue (C113), which is highly conserved in both LRAT-family proteins and the NlpC/P60 superfamily of proteins, is found on helix \(\alpha\)3. In AdPLA and all LRAT-like proteins studied, thus far, the conserved C113 residue was shown to be required for their catalytic activity (10,14,22) by acting as nucleophile in forming the covalent acyl-enzyme intermediate (20,34). Our model indicates the presence of a well-conserved His residue (H23) on a neighboring \(\beta\)-strand which is 3.6 Å from C113. A second His (H35), which resides on an adjacent \(\beta\)-strand, is positioned 3 Å from H23. The three C113-H23-H35 residues comprising the catalytic triad of AdPLA are found in a conserved arrangement in the active site of NlpC/P60 proteins such as \(E.\, coli\) Spr (32).

**The Residues Comprising the Catalytic Triad Are Required for the Activity of AdPLA.**

We assayed the contribution of the C-terminal transmembrane domain to the phospholipase activity of AdPLA. For this, we examined the activity of MBP-FL-AdPLA and MBP-T-AdPLA proteins using a continuous assay based
on monitoring the release of a fluorescently-labeled acyl group from the PLA2-specific substrate BODIPY PC-A2 (structure depicted in top panel of Fig. 5). Release of the BODIPY FL pentanoic acid acyl chain from sn-2 of BODIPY PC-A2 results in an increase in the fluorescence signal (excitation 488 nm /emission 515) nm due to decreased intramolecular quenching by fluorescence resonance energy transfer (FRET) of the BODIPY 558/568 dye attached at the sn-1 position. The assay allowed us to conduct a sensitive kinetic assay of AdPLA and to calculate its $V_{\text{max}}$ (2.5 ± 0.2 μmol/min/mg) and $K_m$ (0.7 ±0.15 μM) using BODIPY PC-A2 as a substrate. These values are within the range of reported values for AdPLA in previous studies (10,14). To account for the interfacial mechanism of the PLA2-catalyzed reaction, other kinetic models, such as the “surface dilution model” (57,58) have been developed, but we were not successful in adapting the assay based on detergent-phospholipid mixed micelles to the analysis of the MBP-FL-AdPLA reaction. In conclusion, our findings establish that MBP-FL-AdPLA exhibits characteristics consistent with a physiological role of AdPLA in the hydrolysis of phospholipids.

Next, we used site-directed mutagenesis to examine the effect of mutating the active site residues C113, H23, H35 on the phospholipase activity of AdPLA. Substituting either one of the C113, H23 and H35 residues with alanine or for more sterically conserved substitutions C113S, H23Q and H35Q results in a significantly reduced AdPLA activity of the resulting proteins (Fig. 6). Both C113 and H23 (H21 in mouse AdPLA) had been previously implicated to be important for the activity of AdPLA (10,14). Here, we also demonstrate that H35 is also required for AdPLA activity. We propose that the function of H23 is to form an ion pair with C113, acting as a base and subsequently as an acid in the reaction cycle. Meanwhile H35 maintains H23 in its proper orientation as shown for the NlpC/P60 protein Spr (32). Thus our studies provide experimental support for the requirement of the C113, H23 and H35 residues in the enzymatic activity of AdPLA.

**Lack of the C-terminal Domain Impairs the Association of AdPLA with Membranes.**

Our results indicate that deletion of the C-terminal transmembrane domain of AdPLA severely affects the activity of MBP-T-AdPLA against liposome substrates. Using a liposome-based assay of AdPLA activity we were not able to detect any hydrolytic activity associated with MBP-T-AdPLA (Fig. 6) or T-AdPLA (not shown). In comparison, MBP-FL-AdPLA had a robust activity (specific activity of 720 pmol/min/mg) (Fig. 6). These results are in agreement with the requirement of an intact C-terminal domain for the activity of AdPLA demonstrated by other studies (10,14). Thus the hydrophobic C-terminal domain is necessary for interfacial catalysis by AdPLA.

In recently published findings, Golczak et al. examined the structure and activity of a 1-132 fragment of AdPLA with nearly the same sequence and structure as the T-AdPLA protein described here (Fig. 3B and 3C). While MBP-T-AdPLA, T-AdPLA and (His)$_6$-(1-132)AdPLA do not display significant activity against phospholipids embedded in liposomes (results not shown), it was shown that (His)$_6$-(1-132)AdPLA protein can become acylated in the presence of phospholipids with very short fatty acid chains, such as diheptanoyl PC (20). These findings suggest that the C-terminal domain of AdPLA does not include residues which participate in the catalytic reaction, however, this domain is required for the association of AdPLA with membranes.

**Amide H/D Exchange Dynamics of MBP-FL-AdPLA, MBP-T-AdPLA and T-AdPLA**

To shed light on the structural differences between T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA, we used a DXMS approach to probe the solvent accessibility of backbone amides of T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA. We incubated purified T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA with buffer supplemented with D$_2$O. Aliquots of the H/D exchange reaction were quenched, digested with pepsin and analyzed by LC-MS/MS. Pepsin...
digestion of the denatured T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA proteins resulted in a complete coverage of the AdPLA protein based on the identification of 36 and 30 peptides derived from the FL-AdPLA or the T-AdPLA fusion partner, respectively. The peptides with the best signal to noise ratio and least overlapping regions for representation of the H/D exchange data are shown in Fig. S4. The kinetics of the rates of deuteration of T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA show the same behavior, with the exchange steadily increasing with time, consistent with the premise that the FL-AdPLA and T-AdPLA proteins have similar structures (Fig. 7A). Comparison of the exchange rates of MBP-T-AdPLA and T-AdPLA showed a similar rate of solvent accessibility arguing that the presence of the MBP fusion partner does not affect the folding of T-AdPLA. The rates of deuteration are also consistent with the crystal structure model as shown in Fig. 7A, B. Residues 39-59 were found to be disordered in the crystal structure and could not be modeled. Using DXMS we observed high rates of hydrogen/deuterium exchange for the region 39-59 (greater than 80% in 10 s and reaching a maximum after 1000 s). These fast rates of exchange reflect the inherent flexibility of the region encompassing residues 39-59. The α3 helix (111-121) containing the C113 catalytic residue is packed tightly against the β-sheet β2 and thus displays relatively low rates of exchange in T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA. We noticed very subtle differences between MBP-FL-AdPLA versus MBP-T-AdPLA or T-AdPLA as the rates of deuteration for α3 appear to be slightly lower in the case of MBP-FL-AdPLA than those observed for MBP-T-AdPLA or T-AdPLA, perhaps indicating a more compact structure of this domain.

Two domains of AdPLA encompassing residues I16-Y27 and Y66-E98 display much lower rates of solvent accessibility compared to the rest of the protein Fig. 7A, B (colored green). Both domains Y66-E98 also display low conformational flexibility based on average B-factors (Fig. 3D). These results are consistent with the tight packing around the I16-Y27 domain which includes the β-sheet β2 and the H23 catalytic residue. In addition, the Y66-E98 domain of AdPLA adopts a compact structure that excludes water consistent with a possible role of this domain in additional interactions with the membrane interface. Overall, the T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA proteins appear to have similar solvent accessibility and to adopt similar conformations.

FL-AdPLA Displays Combined PLA1 and PLA2 Activities

AdPLA was initially identified as a PLA2 enzyme (10); however, later reports suggested that the PLA1 activity of AdPLA is in fact higher than its PLA2 activity (14). The positional specificity, sn-1 versus sn-2 of AdPLA, is important for evaluating its physiological role. In physiological settings, unsaturated fatty acids, such as arachidonic acid, are found primarily in the sn-2 position of phospholipids. Arachidonic acid release is the key argument in explaining the physiological role of AdPLA in PGE2 production, therefore, determining the positional specificity of AdPLA is essential for explaining its physiological function.

The process of determining the positional specificity of phospholipases is affected by the rapid intramolecular rearrangement of lysophospholipids. Following cleavage by PLA1, the 2-acyl lyso-PC product rearranges to form the more thermodynamically favored 1-acyl lyso-PC (59). Subsequent action of PLA1 can result in a second round of hydrolysis and the production of L-α-glycerylphosphorylcholine (L-α-GPC) through the release of the migrated acyl group. Thus both sn-1 and sn-2 derived fatty acids can be released by a PLA1 enzyme despite its specificity for the sn-1 position of phospholipids. Since AdPLA was reported to release both sn-1 and sn-2 fatty acids we sought to determine if the combined PLA1/PLA2 activity could be explained by PLA1 activity alone.

Based on a fluorescence-based continuous assay using BODIPY PC-A2, our data argues that AdPLA displays intrinsic PLA2 activity by accessing the sn-2 acyl group directly. As seen...
in Fig. 5, MBP-FL-AdPLA can hydrolyze the BODIPY PC-A2 resulting in the increase of the fluorescence of the BODIPY FL dye. Since the mechanism of detection in this assay depends on the reduced quenching of the BODIPY FL dye by FRET of BODIPY 558/568, this implies that either the sn-1 or sn-2 labeled probes of BODIPY PC-A2 were hydrolyzed and released by MBP-FL-AdPLA. BODIPY PC-A2 contains an alkyl group linked through an ether bond in the sn-1 position. The ether bond of BODIPY PC-A2 confers PLA2 specificity by virtue of the fact that AdPLA, like most other PLA2 enzymes, does not hydrolyze ether bonds, as found in 18:0 18:0 diether PC (results not shown). Together these results demonstrate that the release of BODIPY FL pentanoic acid acyl chain from sn-2 of BODIPY PC-A2 is the result of direct hydrolysis of the sn-2 ester bond by MBP-FL-AdPLA and that MBP-FL-AdPLA has authentic PLA2 activity.

It is important to determine the specificity of AdPLA for physiological substrates incorporated in liposomes that resemble the composition of cellular membranes. Thus we employed a secondary method to assess the activity of MBP-FL-AdPLA using a direct assay of the hydrolysis of natural phospholipid substrates. To determine the PLA1 or PLA2 specificity of MBP-FL-AdPLA, we used PC substrates with mixed acyl chains. We incubated MBP-FL-AdPLA, MBP-T-AdPLA, commercial PLA2 (bee venom) or PLA1 (TLL from Thermomyces lanuginosus) preparations with liposomes composed of an equimolar mix of 18:1-18:1 PG and various species of PC. We then extracted and examined the fatty acids produced by HPLC using charged aerosol detection (CAD).

Our results indicate that MBP-FL-AdPLA displays potent PLA1 and PLA2 activities by catalyzing the hydrolysis of roughly equivalent amounts of stearate (18:0) or palmitate (16:0) whether linked to the sn-1 or the sn-2 positions of 16:0-18:0 PC or 18:0-16:0 PC (Fig. 8A,B). In the case of phospholipids containing unsaturated acyl chains, MBP-FL-AdPLA hydrolyzed the release of slightly more palmitate from the sn-1 position of 16:0-20:4 PC or 16:0-18:2 PC than arachidonate (20:4) or linoleate (18:2) from the sn-2 position, respectively (Fig. 8D, E). To confirm the specificity of the substrates, we examined the hydrolysis of PC species with mixed acyl groups using authentic PLA1 or PLA2 enzyme preparations. By incubating liposomes composed of PC species with mixed acyl groups with the PLA1 enzyme from T. lanuginosus we observed the release of fatty acids linked predominantly to the sn-1 position in 18:0-16:0 PC (Fig. 8C) or 16:0-18:0 PC (not shown). Meanwhile, hydrolysis of PC species with mixed acyl groups by PLA2 resulted in the release of fatty acids linked to the sn-2 position of 18:0-16:0 PC (Fig. 8C) and 16:0-18:0 PC (not shown). Comparison of the rates of hydrolysis indicates that MBP-FL-AdPLA does not appear to show any preference for the hydrolysis of arachidonic acid. Our results also show that MBP-FL-AdPLA can hydrolyze PG to release olate (18:1) (Fig. 8F) indicating that additional phospholipid head groups other aminophospholipids can be recognized by AdPLA. As predicted, based on the fluorescent AdPLA assays, the levels of fatty acids measured in all assays of MBP-T-AdPLA activity are equivalent to the buffer control reaction and, therefore, MBP-T-AdPLA does not exhibit significant hydrolytic activity against substrates embedded in liposomes (Fig. 8). In conclusion, our data presented in Fig. 6 and 8 support a model in which AdPLA has combined PLA1 and PLA2 activities as opposed to exclusively PLA1 or PLA2 activities.

**DISCUSSION**

Our findings describe the structure and enzymatic activity of AdPLA, an enzyme that plays an important role in the regulation of adipose tissue function. To gain a better understanding of the molecular mechanism of AdPLA we solved the crystal structure of a soluble fragment of AdPLA to 2.65 Å resolution (PDB: 4FA0). The model presented here closely resembles the previously reported NMR model (PDB:2KYT) (Fig. 2, Fig. 3A, C) and the crystal structure model of AdPLA (PDB: 4DOT) (Fig. 3B,C). The model indicates that
AdPLA adopts a globular fold consisting of three \( \alpha \)-helices and five anti-parallel \( \beta \)-sheets (Fig. 4) organized in a circular permutation of the classic papain fold. AdPLA utilizes a conserved Cys-His-His catalytic triad to orchestrate the nucleophilic attack by Cys on the \( sn-1 \) and \( sn-2 \) acyl groups of phospholipids. In line with this observation, residues C113, H23, H35 are required for the its activity (Fig. 6). The use of a Cys residue for catalyzing the hydrolysis of phospholipids is unique to the phospholipases and acyltransferases found in the LRAT family. For C113 to act as a nucleophile it would require a \( pK_a \) of less than 7.0, however, the \( pK_a \) of free Cys of roughly 8.3-8.8 cannot account for the nucleophilic character of this residue. Therefore, the active site of AdPLA provides an environment that is critical for catalysis by decreasing the \( pK_a \) of C113. In papain, such function is provided by a His residue positioned 3.01 Å from the active thiol to form a thiolate-imidazolium ion-pair (60,61). Based on the similarity of AdPLA to papain, we propose that H23 assists in the deprotonation of C113. The distance from the N\( \varepsilon \) of H23 to the S\( \gamma \) of C113 is greater than the one generally observed in other proteins employing a Cys-His pair, nevertheless, the role of the H23-C113 ion-pair in the catalytic mechanism of AdPLA is supported by the abolished activity of the H23A and H23Q mutants (Fig. 6). We cannot rule out that additional residues or the dipole of the \( \alpha_3 \) helix (62) could also interact with C113 and contribute to its reduced \( pK_a \) to generate a strong enough nucleophile.

To explore the enzymatic activity of AdPLA, we generated a MBP-fused, full-length recombinant form of AdPLA, MBP-FL-AdPLA, which exhibits robust PLA\(_1\) and PLA\(_2\) activities against natural substrates (Fig. 8). We characterized the activity of MBP-FL-AdPLA using a continuous fluorescent-based assay and we observed enzyme kinetics consistent with a physiologically relevant PLA enzyme (Fig. 5). The use of a fluorescent substrate containing a non-hydrolyzable bond in the \( sn-1 \) position provides additional support for the intrinsic PLA\(_2\) activity of AdPLA (Fig. 5). In addition to the residues involved in catalysis, we determined that the transmembrane domain of AdPLA is also required for interfacial catalysis. As a result, MBP-T-AdPLA or T-AdPLA, which are recombinant fragments of AdPLA that lack the transmembrane domain, have no detectable PLA\(_1\)/PLA\(_2\) activity against liposome substrates (Fig. 6, 8).

We probed the solvent accessibility of backbone amides of MBP-FL-AdPLA, T-AdPLA and MBP-T-AdPLA using a DXMS approach. The Y66-E98 domain shows low rates of H/D-exchange consistent with the tight globular packing around the \( \beta \)-sheet \( \beta_2 \) (Fig. 7A, B). On the other hand, the residues that surround the active site of AdPLA display relatively high rates of H/D-exchange (Fig. 7B). Thus we propose that in the process of catalysis, water can readily access the active site of AdPLA and allow the acyl-enzyme thioester intermediate to decompose to free fatty acid and lysophospholipid. In agreement with this mechanism, AdPLA displays a robust PLA\(_2\) activity rather than an exclusive acyltransferase activity as observed in the case of LRAT. Analysis of MBP-FL-AdPLA, MBP-T-AdPLA and T-AdPLA by DXMS also indicates that there are no large changes in conformation between the different forms of the AdPLA protein; thus it is likely that the structure we report for T-AdPLA resembles the catalytically active MBP-FL-AdPLA protein (Fig. 7) and we attribute the lack of interfacial catalysis by MBP-T-AdPLA and T-AdPLA to the absence of a membrane targeting domain.

**Membrane targeting of AdPLA.**

The association of AdPLA with membranes is an essential part of the interfacial catalysis process. With the exception of several calcium-independent PLA\(_2\)s (iPLA\(_2\)), such as Group VIC PLA\(_2\) (patatin-like phospholipase domain containing 6 (PNPLA6) (63) and patatin-like phospholipase domain containing 7 (PNPLA7) (64,65), most PLA\(_2\) enzymes characterized so far are soluble. Generally, secreted PLA\(_2\) (sPLA\(_2\)) and Group IVA cytosolic PLA\(_2\) (cPLA\(_2\)) target the phospholipid bilayer in response to signals such as binding of calcium. Other PLA\(_2\)s, like group VI A PLA\(_2\) (iPLA\(_2\)β) or...
lipoprotein-associated PLA2 (LpPLA2) target to the membrane via hydrophobic pockets (66,67). In contrast to most PLA2 enzymes, AdPLA is an integral membrane protein. Like the related tazarotene-induced gene 3 (TIG3) (68) and LRAT proteins (69), AdPLA targets to membranes via a C-terminal signal-anchor domain to assume a “tail-anchored” membrane topology (10,51). Truncated AdPLA lacking the membrane-spanning C terminus domain is not active against phospholipids embedded in liposomes (reported here and (10,14)) as it does not associate with membranes (10). By virtue of the targeting signal being the last domain to exit the ribosome, tail-anchored proteins have to target to membranes in a post-translational fashion, i.e., after protein translation has been completed (70). A substantial fraction of AdPLA is found to be localized in the cytosol (51). Thus post-translational translocation of AdPLA could allow AdPLA to target to subcellular compartments that have different lipid pools, such as the ER or peroxisomes (22) and play a role in its mechanism of action.

Relationship of AdPLA to NlpC/P60 enzymes

The structure of AdPLA, the first member of the LRAT family to be structurally characterized (model presented here and (20,35)), appears to be related to the papain-like NlpC/P60 bacterial cysteine peptidases which are involved in remodeling of the bacterial cell wall (31). The evolutionary relationship between the LRAT-family of proteins and the NlpC/P60 protein superfamily is intriguing and could provide clues to the function of these related enzymes. For example, our results indicate that AdPLA shares the conformation of the Cys-His-His catalytic triad first described in case of the classical NlpC/P60 protein, Spr from E. coli (32). AdPLA also resembles the structure of YiiF an NlpC/P60 permuted domain protein from E. coli whose structure indicates the presence of a bound stearate molecule (71). These observations suggest that eukaryotic representatives of the LRAT family of proteins, are related in both structure and enzymatic mechanism with cysteine hydrolases from the NlpC/P60 family which catalyze various acylation / deacylation reactions involving proteins and lipids.

Role of AdPLA in the regulation of lipolysis

One of the strongest arguments in favor of a role for AdPLA in the generation of free arachidonic acid in fat is the dramatic decrease in the phospholipase A activity (18% of wild type levels) and PGE2 levels (12% of wild type levels) in adipose tissues of AdPLA-/- mice compared to wild type mice (11). In addition, treatment of adipocytes isolated from AdPLA-/- mice with PGE2 can restore lipolysis and cAMP to wild type levels (11). These observations are consistent with a role of AdPLA in the formation of PGE2 in fat whereby AdPLA-/- mice have a higher net rate of adipose tissue lipolysis as a result of decreased EP3 signaling (11). However, the robust PLA1 activity of AdPLA brings into question its role in the release of arachidonic acid as this fatty acid is generally bound to the sn-2, not the sn-1 position of phospholipids.

In addition to PLA1 and PLA2 activities, it was reported that AdPLA also displays N-acylation activity (14,20), suggesting it might have a potential role in the formation of N-acyl PE, precursors of endocannabinoids, an important class of endocrine regulators (20). Nevertheless, AdPLA displays very low N-acylation activity (results not shown and (19)) in comparison with the PLA1/PLA2 activities described here and elsewhere (10,14) which suggests that in a cellular environment, fatty acid hydrolysis would occur more often than acyl transfer to PE. However, we cannot exclude the possibility that AdPLA contributes to the synthesis of endocannabinoid precursors in fat or that there might still be other unidentified acyl acceptors which become esterified by AdPLA.

Other products of the AdPLA-mediated hydrolysis beside free fatty acids can have important regulatory roles in fat. Our studies show that AdPLA is a potent combined PLA1/PLA2 enzyme, leading to release of free fatty acids and the formation of 2- or 1-
lysophospholipids, respectively. In our studies, AdPLA displays very low lysophospholipase activity and, thus does not further hydrolyze lysophospholipids to generate L-\(\alpha\)-GPC (results not shown, and (10,14)). Lysophospholipids can become precursors to important bioactive lipid mediators such as lysophosphatidic acid (LPA) through the action of lysophospholipase D / autotaxin (72). LPA can in turn be acylated by either Comparative Gene Identification-58 (CGI-58) or adiponutrin to produce phosphatidic acid (PA) (73-75). Since AdPLA, autotaxin, CGI-58 and adiponutrin are highly expressed in adipose tissue (10,76-79), the contribution of AdPLA to the formation of LPA or PA in adipose tissue is particularly pertinent. PA is a central lipid signaling mediator in fat as seen in both the resistance to obesity of CGI-58 knockdown mice (80) as well as the lipodystrophy seen in patients suffering from Chanarin-Dorfman syndrome due to CGI-58 deficiency (81-83). LPA is, in its own right, a potent inducer of white adipose tissue formation and a negative regulator of thermogenic, brown fat (84). Mice deficient in the LPA receptor LPA1R are resistant to diet induced obesity (85). Similarly, AdPLA\(-/-\) are lean and the white adipose tissue of AdPLA\(-/-\) mice display increased thermogenesis and fatty acid oxidation (11), which is consistent with the possibility that AdPLA\(-/-\) mice have decreased levels of LPA. This is an attractive hypothesis since it offers an explanation for a role of AdPLA in adipose tissue that is independent of the formation of PGE2. This hypothesis also fits with the lack of positional or acyl-chain specificity of AdPLA. The role of AdPLA in the generation of LPA could be explored by examining the levels of LPA and the activation of cognate LPA receptors in the tissues of AdPLA\(-/-\) and wild type mice.

**Conclusion**

We solved the structure of AdPLA leading us to the identification of the Cys-His-His residues involved in the catalytic activity of AdPLA. Our results demonstrate that the Cys-His-His catalytic triad and the signal-anchor domain of AdPLA are both required for its function. We describe the purification of a recombinant form of full-length AdPLA which we characterize as a combined PLA\(_1\)/PLA\(_2\) enzyme. The potent PLA\(_1\) activity exhibited by AdPLA suggests that other products of AdPLA-mediated hydrolysis other than arachidonic acid could also play a role in its anti-lipolytic effects.

Recent developments in the understanding of the physiological role, enzymology and structure of AdPLA pave the way for the development of inhibitors of AdPLA. Such inhibitors are expected to have a positive impact in the treatment of obesity by promoting adipose tissue lipolysis and mimicking the phenotype of the AdPLA\(-/-\) mice. Though this goal makes AdPLA an attractive therapeutic target in obesity settings, an important caveat is that unchecked lipolysis could lead to ectopic lipid accumulation if not compensated by increased fatty acid oxidation. As a consequence, both AdPLA\(-/-\), ob/ob double knockout mice and AdPLA\(-/-\) mice on high-fat diet exhibit hepatic lipid accumulation and resistance to insulin (11). Milder increases in lipolysis, however, as seen in mice overexpressing ATGL, can improve insulin sensitivity (86). It is possible that pharmacological inhibition of AdPLA will not elicit lipolysis and ectopic lipid deposition to the same extent as in AdPLA\(-/-\) mice maintained on high-fat diet, particularly if treatment with AdPLA inhibitors is administered in conjunction with a low-fat diet or with therapies aimed at increasing fatty acid oxidation. To achieve this goal and improve upon these first insights into the AdPLA enzymatic mechanism, the structure of AdPLA in complex with substrates or inhibitors is greatly needed. Such findings would shed light on the residues involved in the binding and substrate specificity of AdPLA. Trapping the enzyme-substrate complex would allow us to observe the formation of the presumed tetrahedral intermediate and to define the position of the oxyanion hole necessary to polarize the carbonyl group of the substrate. In parallel with structural approaches, more studies are also needed to explore the physiological role and the consequences of the disruption of AdPLA in vivo.
Analysis of the enzyme activity and structure of AdPLA

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Figure 1. Phase anomalous difference map (green mesh) contoured at 4σ using diffraction data collected at λ = 1.7463 Å. The largest peaks were observed near Cys residues 89 and 113.

Figure 2. Superposition of AdPLA structures determined by NMR (PDB: 2KYT, colored cyan) and X-ray crystallography (PDB: 4FA0, colored magenta). The N- and C-terminal ends are noted and the disordered loop in the crystal structure is indicated by the asterisks.

Figure 3. Comparison of the NMR (PDB: 2KYT) and X-ray structures (PDB: 4DOT and 4FA0) of AdPLA. (A) Loop regions 27-33, 71-74, 79-87 and 106-112 in AdPLA structures determined by NMR (PDB: 2KYT, colored cyan) and X-ray crystallography (PDB: 4FA0, colored magenta) show the largest difference between the two structures. (B) Loop region 104-110 contains the largest differences between the AdPLA crystal structure reported here PDB: 4FA0 (colored magenta) and the previously reported structure PDB: 4DOT (colored green). (C) Plot of RMSD deviations between Cα atoms of PDB: 2KYT versus PDB: 4FA0 (solid gray line) and PDB: 4DOT versus PDB: 4FA0 (dashed black line) models of the AdPLA. (D) Plot of average B-factors values of PDB: 4DOT (dashed black line) and PDB: 4FA0 (solid gray line).

Figure 4. Crystal structure of AdPLA colored by secondary structure and showing the C113, H23, H35 catalytic triad.

Figure 5. Phospholipase A2 Activity of MBP-FL-AdPLA. BODIPY PC-A2 was used to measure the rate of reaction as a function of substrate concentration. The 488 nm /515 nm fluorescence signal of liposomes containing various concentrations of BODIPY PC-A2 substrate (structure shown in top panel) was recorded for 1 minute to monitor the background emission. No significant increase in background fluorescence emission was observed. Purified MBP-FL-AdPLA protein was injected at 1 min (indicated by arrow) and the fluorescence signal 488 nm /515 nm signal was recorded for the next 4 minutes. A standard curve of BODIPY FL C5, the cleavage product of BODIPY PC-A2, was used for evaluating the amount of product formed by MBP-FL-AdPLA-mediated hydrolysis. Inset shows the rate of reaction as a function of substrate concentration. The enzyme kinetic data were analyzed through sum-of-squares nonlinear regression to derive the $V_{max}$ and $K_m$.

Figure 6. Deletion and mutagenesis analysis of MBP-FL-AdPLA. (Top panel) Rate of reaction of MBP-FL-AdPLA and MBP-T-AdPLA as a function of enzyme concentration. The rate of PLA2 activity of MBP-FL-AdPLA and MBP-T-AdPLA was measured during steady state conditions using the fluorescent substrate BODIPY PC-A2. A standard curve of BODIPY FL C5 was used for evaluating the amount of product formed by MBP-FL-AdPLA-mediated hydrolysis. (Bottom panel) The rate of activity of wild type and active site mutants of MBP-FL-AdPLA. Equal amounts of purified recombinant proteins were examined for hydrolysis of BODIPY PC-A2. The rate of activity is expressed as a percentage of wild type protein. The experiment was conducted in triplicate and repeated.
Figure 7. Amide hydrogen/deuterium exchange analysis of T-AdPLA, MBP-T-AdPLA and MBP-FL-AdPLA. (A) The percent of maximal deuterium incorporation in T-AdPLA (upper ribbon), MBP-T-AdPLA (middle ribbon) and MBP-FL-AdPLA (lower ribbon) peptidic regions is shown. In each ribbon, the percent deuteration at each of the labeling duration is shown from 10s to 10,000s (top to bottom). Differential coloring indicates the percentage of maximal labeling in a given time point. (B) Crystal structure of AdPLA colored based on fast (red) or intermediate (green) H/D exchange rates. The N-terminus and the residues H23, H35 and C113 composing the catalytic triad residues are indicated. Dotted lines represent the C-terminal strands and the 35-59 loop region for which there are no X-ray data.

Figure 8. Analysis of the specificity of the phospholipase activity of MBP-FL-AdPLA and MBP-T-AdPLA. PLA₁/PLA₂ activity was assayed using liposome compositions containing an equimolar mixture of 18:1-18:1 PG and one of five different PC species examined as is indicated at the top of each panel. The liposomes were incubated for 15 min with either MBP-FL-AdPLA, MBP-T-AdPLA, buffer control (labeled no enzyme), bee venom PLA₂ or PLA₁ from T. lanuginosus (panel C). The resulting fatty acids were extracted and analyzed by HPLC-CAD. The fatty acid products of reactions carried out (solid-black line chromatograms) were identified based on the elution profile in comparison with authentic standards (dashed-gray line chromatograms). The oleate (18:1) fatty acid product of the hydrolysis of 18:1-18:1 PG (panels A-F) or 18:1-18:1 PC (panel F) by MBP-FL-AdPLA, bee venom PLA₂ or PLA₁ from T. lanuginosus is indicated by a black star (*). The experiment was conducted in triplicate and repeated.
Table 1. Crystallographic data for AdPLA refined to 2.65Å resolution.

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</tr>
</thead>
<tbody>
<tr>
<td>Unit-cell parameters (Å)</td>
<td>$a=59.99$, $c=74.01$</td>
</tr>
<tr>
<td>Space group</td>
<td>$P3_121$</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>74.01-2.30 (2.39-2.30)</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Observed reflections</td>
<td>30,627</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>7,196</td>
</tr>
<tr>
<td>$&lt;I/\sigma(I)&gt;^1$</td>
<td>14.6 (2.3)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.9)</td>
</tr>
<tr>
<td>Multiplicity $^1$</td>
<td>4.3 (4.4)</td>
</tr>
<tr>
<td>$R_{merge}$ ($^1$)$^{1,2}$</td>
<td>5.2 (43.7)</td>
</tr>
<tr>
<td>$R_{meas}$ ($^1$)$^{1,4}$</td>
<td>6.0 (49.7)</td>
</tr>
<tr>
<td>$R_{pim}$ ($^1$)$^{1,4}$</td>
<td>2.8 (23.2)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30.14-2.65</td>
</tr>
<tr>
<td>Reflections (working/test)</td>
<td>4,743/227</td>
</tr>
<tr>
<td>$R$ factor / $R_{free}$ (%)</td>
<td>20.6/24.7</td>
</tr>
<tr>
<td>No. of atoms</td>
<td>802</td>
</tr>
<tr>
<td>Model Quality</td>
<td></td>
</tr>
<tr>
<td>R.m.s deviations</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.010</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.050</td>
</tr>
<tr>
<td>Average B factor (Å$^2$)</td>
<td></td>
</tr>
<tr>
<td>All Atoms</td>
<td>68.8</td>
</tr>
<tr>
<td>Wilson Plot</td>
<td>65.0</td>
</tr>
<tr>
<td>Coordinate error, Luzatti plot (Å)</td>
<td>0.37</td>
</tr>
<tr>
<td>Ramachandran Plot</td>
<td></td>
</tr>
<tr>
<td>Most favored (%)</td>
<td>97.9</td>
</tr>
<tr>
<td>Additionally allowed (%)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

1) Values in parenthesis are for the highest resolution shell.
2) $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl)| - <I(hkl)> |/ \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity measured for the $i$th reflection and $<I(hkl)>$ is the average intensity of all reflections with indices hkl.
3) $R_{factor} = \sum_{hkl} |F_{obs} (hkl)| - |F_{calc} (hkl)| / \sum_{hkl} |F_{obs} (hkl)|$; Rfree is calculated in an identical manner using 5% of randomly selected reflections that were not included in the refinement.
4) $R_{meas} =$ redundancy-independent (multiplicity-weighted) $R_{merge}$ (39,54). $R_{pim} =$ precision-indicating (multiplicity-weighted) $R_{merge}$ (87,88).
FIGURE 1
FIGURE 2
FIGURE 3

A

27-33
71-74
106-112

B

C-Term
104-110

N-term

C

4FA0 vs. 2K YT
4FA0 vs. 4DOT

D

4FA0
4DOT

RMSD (Å)

Average B-factor (Å²)

Residue Number

Disordered residues

Disordered residues

Residue Number
FIGURE 4
FIGURE 6

PLA$_2$ Activity of MBP-FL-AdPLA Active Site Mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>H23A</th>
<th>H23Q</th>
<th>H35A</th>
<th>H35Q</th>
<th>C113S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (% wild type)</td>
<td>2.8±0.3</td>
<td>2.7±0.7</td>
<td>2.8±1.0</td>
<td>3.0±0.7</td>
<td>1.4±0.7</td>
</tr>
</tbody>
</table>
Structure/Function Relationships of Adipose Phospholipase A<sub>2</sub> Containing a Cys-His-His Catalytic Triad
Xiao-Yan Pang, Jian Cao, Linsee Addington, Scott Lovell, Kevin P. Battaile, Na Zhang, J.L. Uma Maheshwar Rao, Edward A. Dennis and Alexander R. Moise

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