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Cloning, Expression, and Catalytic Mechanism of Murine Lysophospholipase I*

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A lysophospholipase (LysoPLA I) has been purified and characterized from the mouse macrophage-like P388D$_1$ cell line (Zhang, Y. Y, and Dennis, E. A. (1988) *J. Biol. Chem.* 263, 9965–9972). This enzyme has now been sequenced, cloned, and expressed in *Escherichia coli* cells. The enzyme contains 230 amino acid residues with a calculated molecular mass of 24.7 kDa. It has a high helical content in its predicated secondary structure, which is also indicated in its CD spectrum. The cloned LysoPLA I was purified to homogeneity from the transformed *E. coli* cells by a gel filtration column and an ion exchange column. The specific activity of the purified protein is 1.47 μmol/min·mg toward 1-palmitoyl-*sn*-glycero-3-phosphorylcholine at pH 8.0 and 40 °C, corresponding to the reported value of 1.3–1.7 μmol/min·mg for the protein purified from the P388D$_1$ cells. In addition, the cloned protein cross-reacted with an antibody raised against LysoPLA I also purified from the P388D$_1$ cells. The deduced LysoPLA I sequence contains a well conserved G*X*S*X*G motif found in the active site of many serine enzymes, and the activity of the LysoPLA I was irreversibly inhibited by the classical serine protease inhibitor diisopropyl fluorophosphate. Furthermore, site-directed mutagenesis was employed to change Ser-119 in the G*X*S*X*G motif to an Ala. The resulting mutant protein lost all of its lysophospholipase activity, even though it had the same overall protein conformation as that of the wild-type LysoPLA I. Therefore, LysoPLA I has been demonstrated to be a serine enzyme with Ser-119 at the active site.

Lysophospholipases (LysoPLAs)[1] are widely distributed enzymes that hydrolyze lysophospholipids, the detergent-like intermediates in phospholipid metabolism. The *in vivo* levels of lysophospholipids are critical for cell survival and function, since the accumulation of lysophospholipids can perturb the activities of many membrane-bound signal-transducing enzymes (1–4), distort cell membrane integrity, and even cause cell lysis (5, 6). Several enzymes are involved in regulating lysophospholipid levels. However, LysoPLAs are considered to be the major route by which lysophospholipids are removed because of their relatively high activities (7–11).

LysoPLA activities have been identified in many mammalian tissues and cells, including human brain (10), pancreas (12, 13), eosinophil (14–16), spermatozoa (17), amnionic membranes (9), and myelocytic leukemia cell line HL-60 (18) as well as rabbit heart (11, 19), rat liver (20, 21), beef pancreas and liver (22–25), pig gastric mucosa (26), mouse macrophage cell lines P388D$_1$ and WEHI 265.1 (7, 8, 27). However, most studies on LysoPLAs have been limited to the purification and preliminary characterization of the proteins. Research on LysoPLA is further complicated by the fact that more than one isoform of LysoPLA can exist in a single cell and that the high molecular mass enzymes (>50 kDa) generally have other enzymatic activities as well as lysophospholipase activity (12, 14, 19, 28–30). The low molecular mass enzymes (<30 kDa), on the other hand, often exhibit only lysophospholipase activity. Among the small mammalian LysoPLAs, only two have been sequenced and cloned, namely, a human Charcot-Leyden crystal protein (16.5 kDa) and a rat liver (24.7 kDa) protein (15, 20). These two LysoPLAs seem to be very different from one another in terms of their primary sequence and enzymatic properties. Despite its importance, the catalytic mechanism of LysoPLA action and the relative roles these enzymes play in regulating lysophospholipid levels in cells are largely unknown.

As part of our continuing effort to study phospholipid metabolism and its regulation in the mouse macrophage-like P388D$_1$ cells (31), we previously reported the purification and kinetic characterization of two small lysophospholipases, LysoPLA I (27 kDa) and LysoPLA II (28 kDa) (7, 8, 32). The macrophage-like P388D$_1$ cells express at least four enzymes that have lysophospholipase activity, providing a model system for studying the relative contribution of each enzyme to lysophospholipid metabolism in intact cells. The two large enzymes (the Group IV cytosolic PLA$_2$ and the Group VI Ca^{2+}-independent PLA$_2$, both of 80–85 kDa) have PLA$_2$ and transacylase activities as well as lysophospholipase activity (30, 31, 33, 34),[2] while the small LysoPLA I and LysoPLA II are specific LysoPLAs (7, 8). In the present work, we report the sequencing, cloning, and expression of the LysoPLA I. We have also carried out inhibition and mutation studies to determine the catalytic requirements of the enzyme.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse macrophage-like P388D$_1$ cells were obtained from the American Type Culture Collection and were maintained at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/ml), and streptomycin sulfate (100 mg/ml). The cell cultures were started with 10^5 cells/ml in 150-cm^2 culture flasks. After 2 days, the cultures were inoculated into 1-liter roller bottles containing 450 ml of culture medium and incubated at 0.3 rpm on a bottle roller at 37 °C without CO_2. After

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U89352.

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[1] The abbreviations used are: LysoPLA, lysophospholipase; PLA$_2$, phospholipase A$_2$; RT-PCR, reverse transcriptase-mediated polymerase chain reaction; DFP, diisopropyl fluorophosphate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

[2] Y.-C. Lio and E. A. Dennis, unpublished results.

growing for 5 days, all of the adherent cells were suspended into the medium by agitation and then harvested by centrifugation at 700 × *g* for 15 min at 4 °C.

LysoPLA I Purification from P388D$_1$ Cells, Activity Assay, and Inhibition Study—LysoPLA I was purified from the mouse macrophage-like P388D$_1$ cells using the procedure of Zhang *et al*. (8). LysoPLA I activity was measured at 40 °C in 0.1 M Tris buffer (pH 8.0), 125 μM 1-[^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcoline (1.6 μCi/μmol) (obtained from Avanti and DuPont NEN) in a total volume of 0.5 ml. The assay was initiated by adding an aliquot of enzyme solution to the substrate mixture and incubating for the desired time. The ^{14}C-labeled palmitic acid formed was extracted by the Dole method and then quantified by scintillation counting (7). Protein concentration was quantified by the Bio-Rad protein assay using bovine serum albumin as standard. For inhibition studies, various amounts of DFP (CalBiochem) were included in the assay mixture, and the LysoPLA I activities in the presence and absence of inhibitor were measured. To examine whether the inhibition was reversible, LysoPLA I was preincubated in the presence or absence of 63.7 mM DFP for 10 min at 40 °C and then diluted 100-fold into the enzyme assay mixture equilibrated at 40 °C. At the indicated time intervals, aliquots of the reaction were removed and quenched, and the fatty acid released was measured as described above.

Protein and DNA Sequencing—LysoPLA I purified from the mouse macrophage-like P388D$_1$ cells was subjected to SDS-PAGE, and then stained by Coomassie Blue. After destaining the gel, the LysoPLA I band at about 27 kDa was cut and digested by trypsin. The resulting peptide fragments were separated by HPLC (Pharmacia Biotech Inc. Smart System), and selected peptide peaks were sequenced using a Perkin-Elmer Sequencer Precise model 492 at the Scripps' Sequencing Facility. For DNA sequencing, both strands were sequenced by the dideoxy chain termination method using a DNA sequencing kit (Amersham Corp.) and/or the automated DNA sequencer (Applied Biosystems 373 from Perkin-Elmer) at the University of California at San Diego Center for AIDS Research Molecular Biology Core.

Messenger RNA Isolation and RT-PCR—Total RNA from the mouse macrophage-like P388D$_1$ cells was isolated using a guanidinium thiocyanate phenol/chloroform extraction method. Then, the mRNA was selected from the total RNA by oligo(dT) columns (Stratagene), and the eluted mRNA was ethanol-precipitated and washed. Finally, the mRNA was dissolved in diethylpyrocarbonate-treated water at about 1 mg/ml and stored at −70 °C. For RT-PCR, the first-strand cDNA was synthesized at 37 °C for 1 h using Moloney murine leukemia virus reverse transcriptase (Stratagene) and random primers (Promega). Aliquots of the synthesized first-strand cDNA were used to amplify the LysoPLA I gene by PCR (Microcycler, Eppendorf) using *Pfu* DNA polymerase (Stratagene). The sequences of the primers used in the PCR cycles were: primer set A (internal primers), 5′-TTTGAAGGTTACATTGGCTGGATT-3′ and 5′-GCCTTGATAGATCAAGAAGTGAAG-3′; primer set B (external primers), 5′-CGCTGTCCGCCAGCCGGTGG-3′ and 5′-CGTCTACTCAAGGCCTCTTAGTGACA-3′; primer set C (cloning primers), 5′-GCGCGAATTCTCAATCAATTGGAGGTAGGAAGCTTAT-3′ and 5′-CCGGCATATGTGCGGCAACAACATGTC-3′. All of these primers were custom-synthesized by Life Technology, Inc., and their positions are indicated in Fig. 1.

Cloning and Expression of LysoPLA I—To clone the cDNA encoding the enzyme, the open reading frame of LysoPLA I was amplified by RT-PCR using primer set C (sequences shown above), which has the restriction sites (*Eco*RI and *Nde*I) near the 5′-ends. The amplified product was purified and then digested by *Eco*RI and *Nde*I restriction enzymes (Pharmacia). The digested insert was purified and ligated to the pLEX vector (pL expression system from Invitrogen) linearized by the same two restriction enzymes. The ligated products were transformed into chemically competent *Escherichia coli* GI724 cells. To confirm the cloning procedures, plasmids were isolated and the insert was verified by both restriction enzyme analysis and DNA sequencing. To express the cloned enzyme, a glycerol stock of GI724 cells harboring pLEX/LysoPLA I vector was streaked on a RMG-Amp plate (Invitrogen) and grown overnight at 30 °C. Then, a liquid culture in RM medium (Invitrogen) was started from a single colony on the RMG-Amp plate, and it was grown overnight in a 30 °C shaker. The overnight liquid culture was diluted to $A_{550\ nm}$ of 0.1 with the induction medium, and incubated at 30 °C until the $A_{550\ nm}$ reached 0.5. A tryptophan stock solution (10 mg/ml, from Sigma) was added to a final concentration of 100 μg/ml, and the cells were put into a 37 °C shaker. After growing to the desired time intervals, the cell density was measured, and the cells were collected by centrifugation and stored at −20 °C until needed.

Site-directed Mutagenesis—A pair of mutagenic primers with the Ser to Ala mutation at position 119 in the protein sequence was synthesized (primer set D: 5′-ATTTTGGGAGGATTTGCTCAGGGAGGCGCC-3′ and 5′-GGCGCCTCCCTGAGCAAATCCTCCCAAAAT-3′) and used to generate the mutated LysoPLA I (S119A) by PCR, according to the method of QuickChange site-directed mutagenesis from Stratagene. Here, the pLEX/LysoPLA I plasmid isolated from the GI724 cells was used as the template for the *Pfu* DNA polymerase (Stratagene). After PCR, the wild-type parent plasmids remaining in the PCR product were selectively digested by the *Dpn*I restriction enzyme (Stratagene), and the resultant mixture was used to transform the chemically competent *E. coli* GI724 cells. The Ser to Ala mutation at position 119 was confirmed by DNA sequencing, and it was found to be the only change introduced in the S119A mutant when the entire coding region of the mutated cDNA was sequenced.

Purification of the Cloned Wild-type and S119A Mutant Proteins from E. coli Cells—After protein expression had been induced by tryptophan for about 4 h, the *E. coli* cells (about 500 ml) were collected by centrifugation at 4 °C. The collected cells were resuspended in cold lysis buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 5% glycerol). Lysozyme (10 mg/ml, Sigma) was then added to a final concentration of 300 μg/ml, and the cells/lysozyme were incubated at 4 °C for about 2 h. The cell debris was pelleted by centrifugation at 20,000 × *g* for 30 min (4 °C) and discarded. To the cleared supernatant, 10% streptomycin sulfate (w/v, Sigma) was added dropwise to a final concentration of 1%. After stirring for 20 min at 0 °C, the mixture was centrifuged at 15,000 × *g* for 10 min (4 °C), and the pellet was discarded. Ammonium sulfate (Fisher) was added slowly to the cleared supernatant to 70% saturation, and the mixture was stirred for an additional 30 min at 0 °C to precipitate proteins. The precipitated proteins were collected by centrifugation at 15,000 × *g* for 15 min, resuspended in 20 ml of cold buffer A (10 mM Tris (pH 8.0), 10 mM β-mercaptoethanol, and 2 mM EDTA), and applied to a Sephadex G-75 column (2.5 × 80 cm, Pharmacia) already equilibrated in buffer A at 4 °C. The proteins were eluted from the column with buffer A at a flow rate about 1.4 ml/min. The LysoPLA I fractions (as judged by SDS-PAGE and/or enzyme activity) were pooled and loaded onto a DEAE-Sephacel column (2.5 × 34 cm, Pharmacia) equilibrated in buffer A. After washing the column with more than 200 ml of buffer A, the proteins were eluted with a NaCl linear gradient (0–0.24 M) at a flow rate of 1.5 ml/min, and the LysoPLA I fractions were saved.

Analysis of the Enzyme Conformation by CD Spectroscopy—CD spectra were measured using a Cary 61 spectropolarimeter that was modified by replacing the original Pockel cell with a 50-KHz photoelastic modulator (Hinds International FS-5/PEM-80), used in conjunction with a lock in amplifier (EG and G Princeton Applied Research No. 128) to detect and integrate the modulation. System automation, multiple scan signal averaging, and base-line subtraction were accomplished by a DEC 11/02 computer interfaced directly to both the Cary 61 and the amplifier. The system software and custom hardware interfaces were designed by Allen MicroComputer Services, Inc. CD spectra were collected at 7 °C using a cylindrical quartz cuvette with path length of 0.5 mm. The proteins used in CD measurements were purified from the DEAE columns (in 10 mM Tris (pH 8.0), 10 mM β-mercaptoethanol, 2 mM EDTA, and 0.2 M NaCl) and were concentrated to 0.26 and 0.34 mg/ml for the wild-type and S119A mutant, respectively. For each sample and blank solution, 10 separate spectra were collected and averaged. The final protein spectra were obtained by subtracting the blank spectra from the sample spectra and converting the difference to mean residue ellipticity.

SDS-PAGE and Western Analysis—Proteins were separated by 12% SDS-PAGE along with prestained protein molecular weight markers (Bio-Rad) using the method of Laemmli (35). For Western analysis, the proteins in the gel were transferred to polyvinylidene difluoride membrane (Millipore). After blocking the nonspecific binding by 5% non-fat milk, the membrane was probed first with anti-mouse LysoPLA I antibody (8) and then with the horseradish peroxidase-linked protein A (Amersham). Finally, the protein bands were detected by the ECL system (Amersham).

RESULTS

Cloning and Primary Sequence of Mouse LysoPLA I—Since the N terminus of the LysoPLA I purified from mouse P388D$_1$ cells was found to be blocked for direct sequencing, the protein was subjected to trypsin digestion, and the resulting peptide fragments were separated by HPLC and then sequenced. Three peptide sequences were obtained, as indicated in Fig. 1. These peptides showed very high homology to a recently sequenced

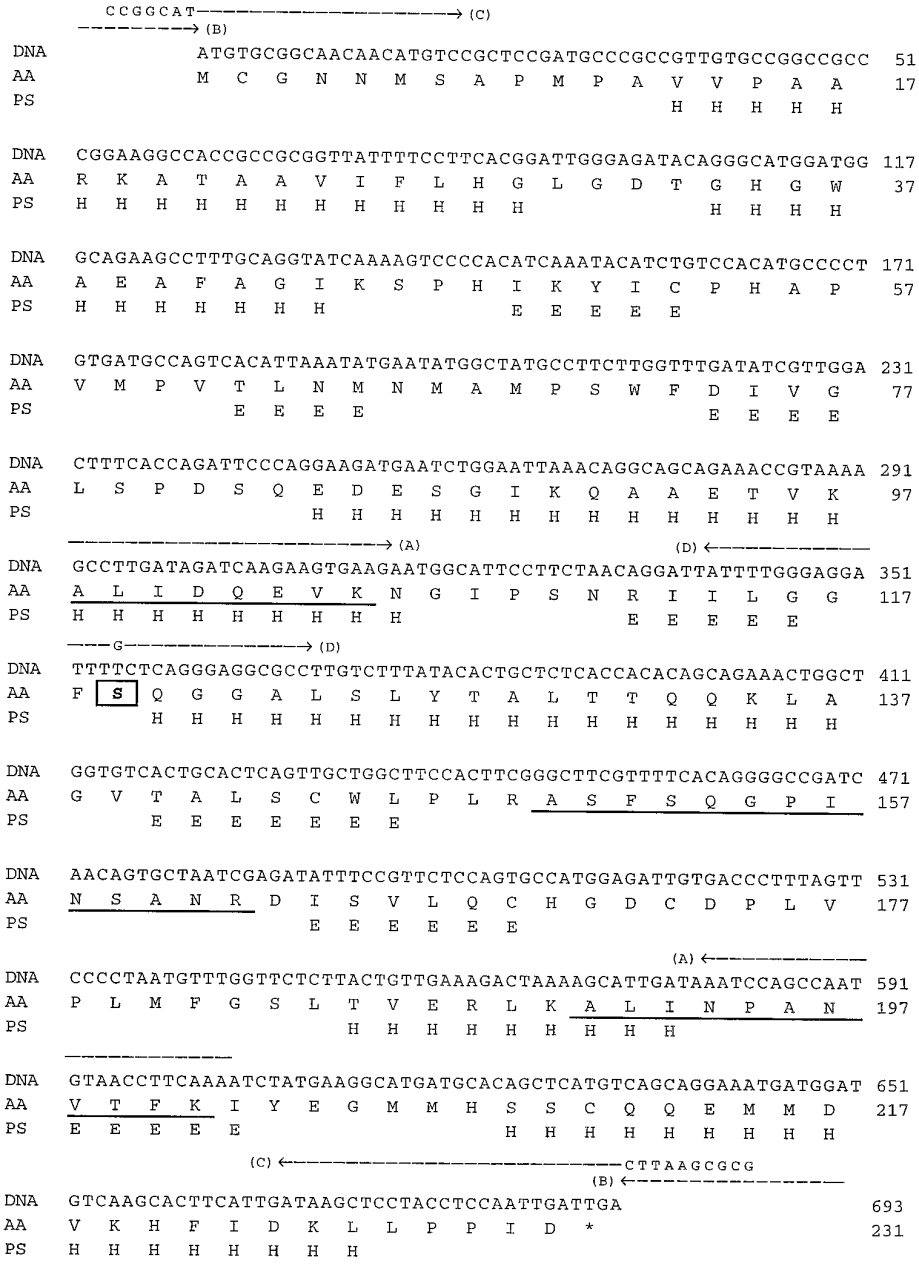

FIG. 1. **The cDNA sequence, deduced amino acid (*AA*) sequence, and the predicted secondary structure (*PS*) of the mouse LysoPLA I.** In the predicted secondary structure, *H* and *E* represent helix and sheet, respectively. The three peptide sequences determined for the trypsin-digested enzyme are *underlined*. The serine residue in the G*X*S*X*G motif is *boxed*. The positions of the primer sets (*A*, *B*, and *C*) used for RT-PCR are indicated by the *arrows* above the cDNA with 5′ to 3′ direction. The mutagenic primers used to obtain the S119A mutant (primer set D) are also indicated by the *arrows*. The actual sequences of all these primers are given under "Experimental Procedures."

rat liver lysophospholipase (20). To obtain the sequence for the mouse LysoPLA I, mRNA from P388D$_1$ cells was isolated, and PCR primers (shown as primer set A in Fig. 1) were designed according to the mouse peptide sequences and the codon usage of the rat lysophospholipase. RT-PCR using cDNA synthesized from the mouse mRNA gave a dominant product of about 310 base pairs, a size expected if the mouse and rat proteins have similar sequences. Furthermore, RT-PCR with primer set B, which was designed according to the sequences adjacent to the coding region of the rat protein (Fig. 1), resulted in a dominant DNA band of about 700 base pairs. For genes that are highly conserved among different species, the noncoding regions are often less conserved; however, it appears that in this case, the noncoding regions of the rat and mouse sequences are sufficiently conserved to allow primer set B to hybridize to the mouse cDNA under our RT-PCR conditions. The sequence of this 700-base pair DNA band is given in Fig. 1, along with the translated protein sequence. The deduced amino acid sequence contained all three peptide sequences that had been determined for the mouse LysoPLA I. The calculated molecular mass for the 230-residue mouse protein is 24.7 kDa, with an isoelectric point of 6.1. The mouse protein seems to have many secondary structural elements such as helix and sheet (Fig. 1), as predicted by the method of Rost (36).

The mouse LysoPLA I and the rat lysophospholipase share 95.5% homology on the DNA level, and 96.5% on the protein level, indicating that these two proteins are of the same origin. In addition, several other proteins with less homology were identified using the BLAST (Basic Local Alignment Search Tool) program (Fig. 2). This included a *Pseudomonas fluorescens* protein reported as carboxylesterase (37) and two putative esterases obtained by chromosome sequencing of *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. As shown in Fig. 2, all of these proteins share more than 30% homology to each other, with certain residues conserved in all five proteins. Interestingly, the most conserved regions include the G*X*S*X*G motif found in the active site of serine proteases, esterases, and lipases.

To clone the cDNA encoding the mouse protein, the LysoPLA I coding region with restriction sites (*Eco*RI and *Nde*I) at the

```
MOUSE      MCGNNMSAPMPAVVPAARKATAAVIFLHGLGDTGHGW------AEAF-AGIKSPHI--K
RAT        MCGNNMSAPMPAVVPAARKATAAVIFLHGLGDTGHGW------AEAF-AGIKSSHI--K
PF               MTEPLILQPAKPADACVIWLHGLGADRYDF------MPVA-EALQESLLTTR
SC               MNGLRVAAKIQPARQTIIFLHGLGDTGSGWGFLAQYLQQR-DPAAFQHT--N
CE     110 TRMSAVSNGNPSIVSPRGEHKGTLIFLHGLGDQGHGW------ADAFKTEAKHDNI--K

MOUSE      YICPHAPVMPVTLNMNMAMPSWFDIVGLSPD-SQEDESGIKQAAETVKALIDQEVKNGI
RAT        YICPHAPVMPVTLNMSMMMPSWFDIIGLSPD-SQEDESGIKQAAETVKALIDQEVKNGI
PF         FVLPQAPTRPVTINGGYEMPSWYDIKAMSPARSISLEE-LEVSAKMVTDLIEAQKRTGI
SC         FVFPNAPELHVTANGGALMPAWFDILEWDPSFSKVDSDGFMNSLNSIEKTVKQEIDKGI
CE         FICPHSSERPVTLNMGMRMPAWFDLFGLDPN-AQEDEQGINRATQYVHQLIDAEVAAGI

MOUSE      PSNRIILGGFSQGGALSLYTALTT-QQKLAGVTALSCWLPLRASFSQG-PINSANRDIS
RAT        PSNRIILGGFSQGGALSLYTALTT-QQKLAGVTALSCWLPLRASFSQG-PINSANRDIS
PF         DASRIFLAGFSQGGAVVFHTAFINWQGPLGGVIALSTYAP---TFGDELELSASQQRIP
SC         KPEQIIIGGFSQGAALALATSVTL-PWKIGGIVALSGFCSIPGILKQ--HKNGINVKTP
CE         PASRIAVGGFSMGGALAIYAGLTY-PQKLGGIVGLSSFFLQRTKFP-G-SF-TANNATP

MOUSE      VLQCHGDCDPLVPLMFGSLTVERLKALINPANVTFKIYEGMMHSSCQQEMMDVKHFIDK
RAT        VLQCHGDCDPLVPLMFGSLTVERLKGLVNPANVTFKVYEGMMHSSCQQEMMDVKYFIDK
PF         ALCLHGQYDDVVQNAMGRSAFEHLKS--RGVTVTWQEY-PMGHEVLPQEIHDIGAWLAA
SC         IFHGHGDMDPVVPIGLGIKAKQFYQDSCEIQNYEFKVYKGMAHSTVPDELEDLASFIKK
CE         IFLGHGTDDFLVPLQFGQMSEQYIKKF-NP-KVELHTYRGMQHSSCGEEMRDVKTFLSA

MOUSE      LLPPID
RAT        LLPPID
PF         RLG
SC         SLSS
CE         HIAH
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FIG. 2. **Amino acid sequence comparison.** The mouse LysoPLA I sequence (*MOUSE*, accession number U89352) is compared with those of a rat lysophospholipase (*RAT*, accession number D63885), a *P. fluorescens* carboxylesterase (*PF*, accession number 77710), and two putative esterases obtained by chromosome sequencing of *S. cerevisiae* (*SC*, accession number U53877) and *C. elegans* (*CE*, accession number Z75712). Spaces (-) are inserted for the optimum alignment of the proteins. The amino acids that are conserved in at least three proteins are *highlighted*, and the conserved G*X*S*X*G motif is *underlined*.

ends was amplified by RT-PCR using the mouse cDNA and primer set C. The amplified product was digested by the restriction enzymes *Eco*RI and *Nde*I and then ligated to the pLEX vector linearized by the same two restriction enzymes. The resultant pLEX/LysoPLA I was used to transform chemically competent *E. coli* cells. The LysoPLA I gene in the pLEX/LysoPLA I vector was confirmed by both restriction enzyme analysis and DNA sequencing.

Expression and Purification of Wild-type LysoPLA I—To verify that pLEX/LysoPLA I indeed encoded a lysophospholipase, the lysophospholipase activities in *E. coli* cells transformed either with pLEX/LysoPLA I vector, or with a control vector pLEX/LacZ (encoding β-galactosidase), were examined. This expression system allows the regulated expression of foreign proteins by a tryptophan induction mechanism and is under the strong P_L promoter from bacteriophage λ. After protein expression was induced by tryptophan for different times, cells were harvested and then lysed with lysozyme. The resultant cell homogenate was subjected to both the lysophospholipase activity assay and Western blot analysis using the antibody raised against the LysoPLA I from the $P388D_1$ cells. As shown in Fig. 3*A*, no protein band was recognized by the LysoPLA I antibody at the beginning of tryptophan induction, indicating that *E. coli* itself does not have LysoPLA I. However, after 90 min of induction, a protein band at about 27 kDa was recognized by the LysoPLA I antibody, and this protein band became more intense as the induction time became longer. It should be noted that the apparent molecular mass of this induced protein band was the same as that of LysoPLA I purified from the mouse $P388D_1$ cells (Fig. 3*A*). Furthermore, lysophospholipase activity in *E. coli* cells harboring the pLEX/LysoPLA I vector also became higher as induction time went longer. After about 4 h of induction, it reached over 20-fold higher activity than the control, demonstrating that the induced protein is an active lysophospholipase (Fig. 3*B*).

To purify the cloned LysoPLA I, *E. coli* cells with the pLEX/LysoPLA I vector were induced by tryptophan for 4 h and then harvested. After cell lysis by lysozyme, the homogenate was centrifuged, and the supernatant was subjected first to 1% streptomycin sulfate and then to 70% ammonium sulfate precipitation. The resultant pellet was resuspended in Buffer A (10 mM Tris (pH 8.0), 2 mM EDTA, and 10 mM β-mercaptoethanol) and loaded onto a Sephadex G-75 column. The LysoPLA I-containing fractions, which were determined by both the activity assay and SDS-PAGE, were applied to a DEAE-Sephacel column. More than half of the active fractions from the DEAE column were essentially free of contamination and were used for CD measurements after being concentrated. The specific activity of the purified LysoPLA I was 1.47 μmol/min·mg, agreeing well with the reported value of 1.3–1.7 μmol/min·mg for LysoPLA I purified from mouse $P388D_1$ cells (8).

Inhibition Studies on LysoPLA I—As the mouse LysoPLA I contains the conserved G*X*S*X*G motif (Fig. 2) characteristic of serine proteases, esterases, and lipases, we examined whether the classical serine protease inhibitor DFP would inhibit the LysoPLA I activity. It was found that DFP inhibited LysoPLA I activity with an IC_{50} of 5 mM under the experimental conditions employed (Fig. 4*A*). Furthermore, the inhibition was found to be irreversible (Fig. 4*B*). As DFP is known to inactivate serine proteins by covalent attachment to the serine residue in the active site, the irreversible inhibition of LysoPLA I by DFP suggested that LysoPLA I has an essential serine residue for its function.

Site-directed Mutagenesis—To identify the serine residue

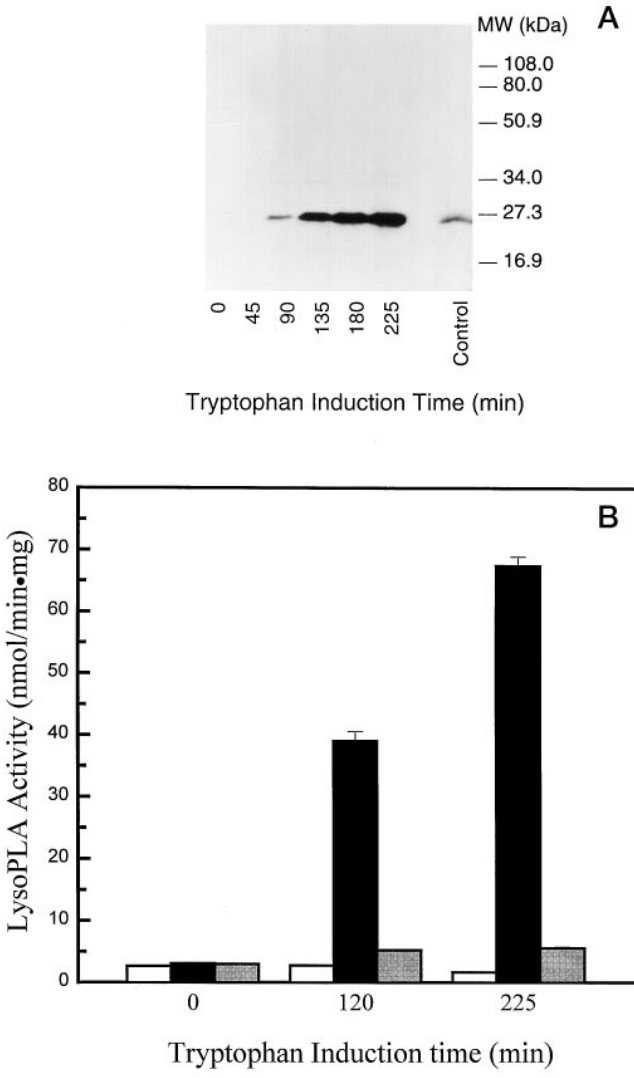

FIG. 3. **Expression of LysoPLA I in *E. coli* cells.** *A*, Western analysis showing the increased expression levels of LysoPLA I as a function of tryptophan induction time. At indicated induction times, *E. coli* cells were lysed, and 25 μg of the protein from the *E. coli* homogenate was loaded in each lane. In the control, 25 ng of LysoPLA I purified from mouse P388D1 cells was also included. *B*, LysoPLA activity in *E. coli* cells harboring pLEX/LacZ control vector (*white*), pLEX/LysoPLA I vector (*black*), or pLEX/S119A vector (*gray*). The activities are the average of two experiments, each with a duplicate determination.

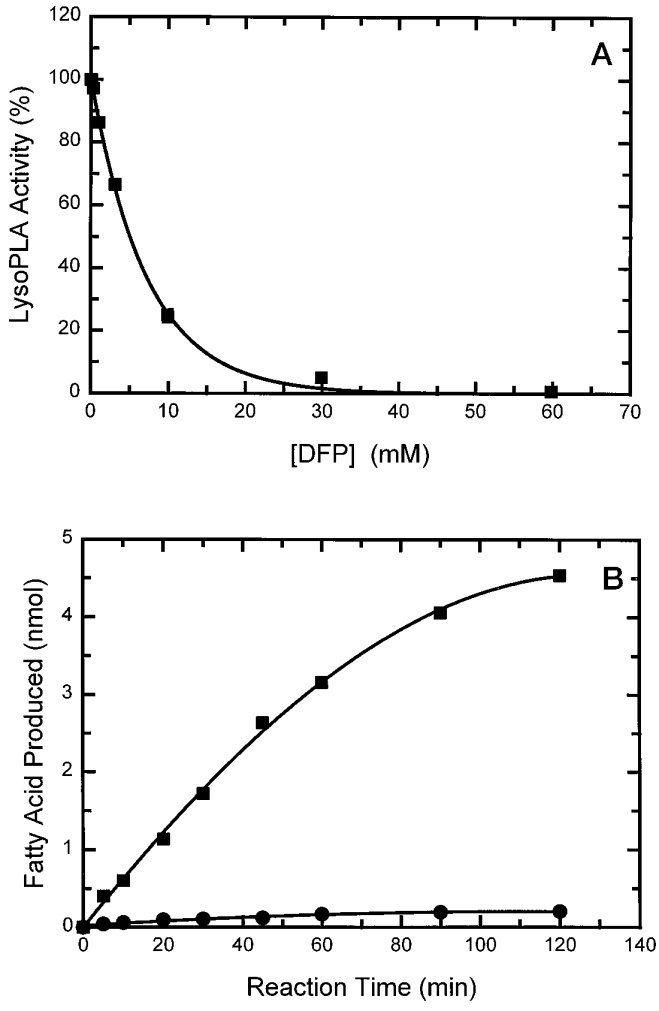

FIG. 4. **Inhibition of LysoPLA I by DFP.** *A*, LysoPLA I activity as a function of DFP concentration. *B*, reversibility test of DFP inhibition. LysoPLA I was preincubated in the presence (●) or absence (■) of 63.7 mM DFP for 10 min at 40 °C and then diluted 100-fold into the enzyme assay mixture. After incubation for the indicated time periods, the reactions were quenched, and fatty acid release was measured.

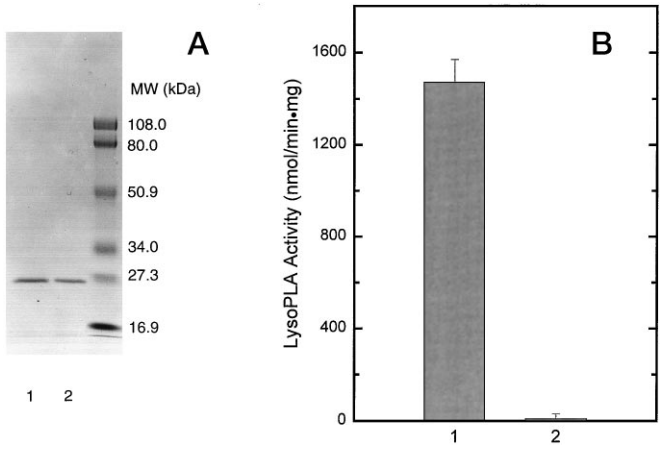

FIG. 5. **Comparison of the purified wild-type LysoPLA I and S119A mutant.** *A*, SDS-PAGE analysis of wild-type (*lane 1*) and S119A (*lane 2*) purified from *E. coli* cells. About 600 ng of protein was applied to each lane, and then silver-stained. Panel B. LysoPLA activity of purified wild-type (*column 1*) and S119A mutant (*column 2*).

that is essential for LysoPLA I function, the Ser-119 residue in the conserved G*X*S*X*G motif was changed to Ala by site-directed mutagenesis. *E. coli* cells transformed with the pLEX/S119A vector expressed the S119A mutant protein at about the same efficiency as that of the wild-type protein. However, the lysophospholipase activity in the *E. coli* homogenate expressing the mutant protein was more than 10-fold lower than that of the wild-type, just a little above the control level (Fig. 3*B*). When the S119A mutant protein was purified by the procedures developed for the wild-type protein (where the mutant protein fractions were followed by SDS-PAGE), it was found that the activity of the purified mutant was reduced to 0.5 nmol/min·mg, significantly less than the 1470 nmol/min·mg of the wild-type enzyme (Fig. 5).

CD Spectra of Wild-type LysoPLA I and S119A Mutant—To examine whether the loss of the enzyme activity in the S119A mutant was due to a conformational change in the mutant, CD spectra were measured for both the purified wild-type protein and the S119A mutant. As shown in Fig. 6, the CD spectra of the two proteins were essentially identical, demonstrating that the significant loss of enzyme activity in the S119A mutant is not the result of misfolding or a conformational change of the

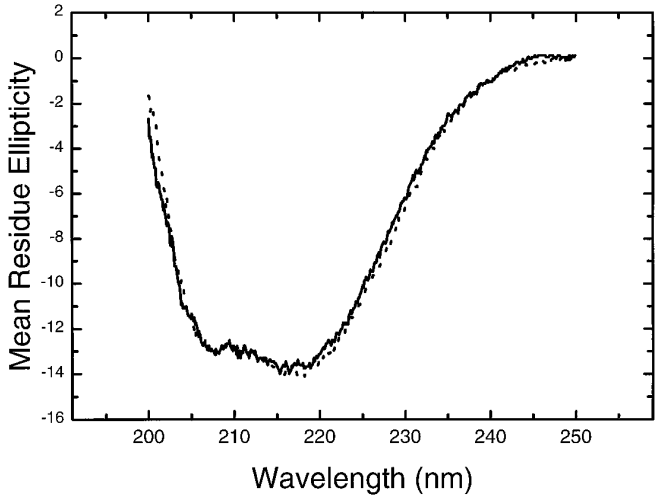

FIG. 6. **CD spectra of the wild-type LysoPLA I (—) and its mutant S119A (– – – –).** The mean residue ellipticity was reported in units of millidegree × cm^2 $dmol^{-1}$.

S119A. In addition, the CD spectra indicate that LysoPLA I has a high helical content, agreeing well with its secondary structure predication (Fig. 1).

DISCUSSION

Lysophospholipids are important components of cell membranes and are involved in a variety of physiological and pathological processes. To understand the roles of LysoPLAs in lysophospholipid metabolism and cell function, we have further characterized a mouse lysophospholipase previously reported as LysoPLA I (7, 8, 32). By RT-PCR, we were able to clone the cDNA encoding an active mouse lysophospholipase, which is composed of 230 amino acid residues with a calculated molecular mass of 24.7 kDa and an isoelectric point of 6.1. Because we used primers to the noncoding regions of the rat sequence that immediately proceed and follow the coding region, it is possible that the mouse cDNA contains an additional initiator codon further upstream that is not present in the rat sequence. If such a N-terminal extension does exist, it should not be very long as the recombinant enzyme appears to have the same molecular mass as that of the native enzyme purified from the $P388D_1$ cells (Fig. 3), and it should have little significance, since the recombinant and the native enzymes have identical characteristics and specific activity. LysoPLA I appears to have a high helical content in its secondary structure, as indicated by both its CD spectrum and the theoretical structure predication of its primary sequence. The enzyme contains a conserved G*X*S*X*G motif characteristic of many serine enzymes, and the serine residue in the center of the motif has been implicated as being part of the active site of LysoPLA I by the following evidence: 1) mutation of the central serine residue in the motif abolished all of the lysophospholipase activity (Figs. 3 and 5); 2) the global conformation of the mutant was the same as that of the wild-type protein (Fig. 6). The identification of LysoPLA I as a serine enzyme was also supported by the inhibition studies with the classical serine esterase inhibitor DFP, which inactivates serine esterases by covalent attachment to the serine in the active site. It was found that DFP inhibited the LysoPLA I activity with IC_{50} at 5 mM. The inhibition was essentially irreversible, presumably due to covalent modification of LysoPLA I at the active site Ser119 (Fig. 4).

Many LysoPLAs have been purified from a variety of mammalian cells. However, no systematic groupings have been made for these LysoPLAs, apparently due to the lack of sequence information as well as the different conditions used to characterize the enzymes. As the sequence of the mouse LysoPLA I is now known, we have compared it with the human eosinophil LysoPLA (Charcot-Leyden crystal protein, 16.5 kDa) (15, 16) and concluded that the two enzymes should be grouped differently based on the following reasons: 1) no sequence homology was found between them; 2) the crystal structure of the eosinophil lysophospholipase shows that it is mainly composed of β sheets, whereas LysoPLA I seems to have a high helical content; 3) the specific activity of the eosinophil lysophospholipase is only 0.39 nmol/min·mg, significantly lower than that of LysoPLA I (1,300–1,700 nmol/min·mg); 4) a putative catalytic site composed of a water, a tyrosine, and a histidine has been identified in the crystal structure of the eosinophil lysophospholipase, whereas LysoPLA I has been demonstrated herein to be a serine enzyme.

On the other hand, the mouse LysoPLA I should be grouped together with the rat lysophospholipase, since the two enzymes share very high sequence homology (96% match) as well as similar properties (7, 8, 20). Other LysoPLAs that may belong to this group include (i) the major 22-kDa LysoPLA from pig gastric mucosa (26); (ii) the 24-kDa LysoPLA from HL60 (18, 20, 38); (iii) the 27-kDa LysoPLA from mouse macrophage WEHI 265.1 cells (27); (iv) the 23-kDa LysoPLA from rabbit heart (11); (v) the 25-kDa LysoPLA from beef liver (24, 25). All of these LysoPLAs exist as monomers with molecular masses around 25 kDa and are most active around pH 8. Generally, these enzymes have broad substrate specificity toward lysophospholipids, but lack other activities such as phospholipase or carboxylesterase activity. Also, the activity of all these enzymes are not affected by Ca^{2+}, Mg^{2+}, or EDTA. In addition, the first two enzymes in the list cross-reacted with the antibody raised against the rat liver lysophospholipase (20).

Several other enzymes that shared more than 30% homology to the mouse LysoPLA I were identified in the protein data base maintained at the National Institutes of Health, namely, a *P. fluorescens* carboxylesterase (37) and two putative enzymes from *S. cerevisiae* and *C. elegans* (Fig. 2). Since the sequences in the G*X*S*X*G motif region are well conserved in all these proteins, it is likely that they are serine hydrolases as well. Besides the G*X*S*X*G motif, several His and Asp residues were also conserved in these proteins, suggesting that they may form the catalytic triad (Ser-His-Asp), the catalytic mechanism found in many hydrolytic enzymes. Currently, site-directed mutagenesis experiments on LysoPLA I are in progress to identify other residues that may contribute to the catalytic triad. It is interesting to note that LysoPLA I shared sequence homology to the esterases even though it has no esterase activity.

In summary, we have sequenced, cloned, and expressed a mouse LysoPLA I, and the Ser119 in the G*X*S*X*G motif was identified to be part of the active site of the enzyme. It will be of interest to identify the other residues in the catalytic triad, but it appears that the LysoPLA is a new member of the serine enzyme superfamily.

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