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 P388D1 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 predicted 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 P388D1 cells. In addition, the cloned protein cross-reacted with an antibody raised against LysoPLA I also purified from the P388D1 cells. The deduced LysoPLA I sequence contains a well conserved GXGXS 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 Ser119 in the GXGXS 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 Ser119 at the active site.

LysoPLAs 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), amnion membranes (9), and myeloid 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 P388D1 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 isofrom 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 P388D1 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 P388D1 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 PLA2 and the Group VI Ca2+-independent PLA2, both of 80–85 kDa) have PLA2 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 P388D1 cells were obtained from the American Type Culture Collection and were maintained at 37 °C in a humidified atmosphere of 90% air and 10% CO2 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 105 cells/ml in 150-cm2 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 CO2. After

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

**LysoPLA I Purification from P388D1 Cells, Activity Assay, and Inhibition Study—**LysoPLA I was purified from the mouse macrophage-like P388D1 cells. The procedure of purification of LysoPLA I was described in detail by Morgenstern et al. (Stratagene). The activity assay was measured at 40 °C in 0.1 M Tris buffer (pH 8.0), 125 mM \( \cdot \) (1-Chol)palmitoyl-sn-glycero-3-phosphorylcholine (1.6 \( \mu \)g/\( \mu \)ol) (obtained from Avanti and DuPont NEN) in a 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 Bole method and 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 and absence of inhibitor, and it was grown overnight in a 30 °C shaker. The overnight liquid culture was diluted to 10 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 spectrum 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 with 5% non-fat milk, the membrane was probed first with anti-mouse LysoPLA I antibody (Amersham) 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 P388D1 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 protein, as indicated in Table 1. The results are consistent with the prediction of the amino acid sequence of the cloned 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 PCR DNA polymerase (Stratagene). After PCR, the wild-type parent plasmids remaining in the PCR product were selectively digested by the DpnI 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 sequence 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 \( \mu \)g/ml, and the cells/lysozyme were incubated at 4 °C for about 2 h. The cell debris was pelleted by centrifugation at 20,000 \( \times g \) 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, the mixture was centrifuged at 15,000 \( \times g \) 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 \( \times g \) for 15 min, resuspended in 20 ml of cold buffer A (10 mM Tris (pH 8.0), 10 mM \( \beta \)-mercaptoethanol, and 2 mM EDTA), and applied to a Sephadex G-75 column (2.5 \( \times \) 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-Sephalocolumn (2.5 \( \times \) 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 (Hinda International FS-5/FEM-80). All spectra were measured on the UV/visible or the visible/visible accessory detector (EG and G Princeton Applied Research No. 128) to detect and integrate the modulation. System automatic, 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 cm. The proteins used in CD measurements were purified from the DEAE columns (in 10 mM Tris (pH 8.0), 10 mM \( \beta \)-mercaptoethanol, 2 mM EDTA, and 0.2 mM 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 spectrum from the sample spectra and converting the difference to mean residue ellipticity.
rat liver lysophospholipase (20). To obtain the sequence for the mouse LysoPLA I, mRNA from P388D1 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 GXXG 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 (EcoRI and NdeI) at the

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**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 GXSG 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.”

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To clone the cDNA encoding the mouse protein, the LysoPLA I coding region with restriction sites (EcoRI and NdeI) at the
ends was amplified by RT-PCR using the mouse cDNA and primer set C. The amplified product was digested by the restriction enzymes EcoRI and NdeI 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 PL 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 P388D1 cells. As shown in Fig. 3A, 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 P388D1 cells (Fig. 3A). 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. 3B).

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 P388D1 cells (8).

Inhibition Studies on LysoPLA I—As the mouse LysoPLA I contains the conserved GXXG 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 IC50 of 5 μM under the experimental conditions employed (Fig. 4A). Furthermore, the inhibition was found to be irreversible (Fig. 4B). As DFP is known to inactivate serine proteins by covalent attachment to the serine residue, 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
that is essential for LysoPLA I function, the Ser-119 residue in the conserved GXXG 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. 3B).

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 × cm² dmol⁻¹.

S119A. In addition, the CD spectra indicate that LysoPLA I has a high helical content, agreeing well with its secondary structure prediction (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 precede 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 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 GXSXG 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 IC₅₀ 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 is not affected by Ca²⁺, Mg²⁺, 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 GXSXG motif region are well conserved in all these proteins, it is likely that they are serine hydrolases as well. Besides the GXSXG 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 GXSXG 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 I is a new member of the serine enzyme superfamily.

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