Phosphatidylserine-specific phospholipase A₁ (PS-PLA₁), which acts specifically on phosphatidylserine (PS) and 1-acyl-2-lyso phosphatidylserine (lyso-PS) to hydrolyze fatty acids at the sn-1 position of these phospholipids, was first identified in rat platelets (Sato, T., Aoki, J., Nagai, Y., Dohmae, N., Takio, K., Doi, T., Arai, H., and Inoue, K. (1997) J. Biol. Chem. 272, 2192–2198). In this study we isolated and sequenced cDNA clones encoding human PS-PLA₁, which showed 80% homology with rat PS-PLA₁, at the amino acid level. In addition to an mRNA encoding a 456-amino acid product (PS-PLA₁), an mRNA with four extra bases inserted at the boundary of the exon-intron junction was detected in human tissues and various human cell lines. This mRNA is most probably produced via an alternative use of the 5’-splicing site (two consensus sequences for RNA splicing occur at the boundary of the exon-intron junction) and encodes a 376-amino acid product (PS-PLA₁ΔC) that lacks two-thirds of the C-terminal domain of PS-PLA₁. Unlike PS-PLA₁, PS-PLA₁ΔC hydrolyzed exclusively lyso-PS but not PS appreciably. Any other phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphaticid acid (PA), and their lyso derivatives were not hydrolyzed at all. These data demonstrated that PS-PLA₁ΔC exhibits lyso-PS-specific lysophospholipase activity and that the C-terminal domain of PS-PLA₁ is responsible for recognizing diacylphospholipids. In addition, human PS-PLA₁ gene was mapped to chromosome 3q13.13–13.2 and was unexpectedly identical to the nmd gene, which is highly expressed in nonmetastatic melanoma cell lines but poorly expressed in metastatic cell lines (van Groningen, J. J., Bloemers, H. P., and Swart, G. W. (1995) Cancer Res. 55, 6237–6243).

Phosphatidylserine (PS)¹ in cell membranes is known to be an essential cofactor for the activation of protein kinase C (1) and for blood coagulation (2). More recently, PS has been shown to regulate the activity of various enzymes, such as c-Raf-1 protein kinase (3), nitric oxide synthase (4), Na⁺/K⁺-ATPase (5), dynamin GTPase (6), and diacylglycerol kinase (7). PS is predominantly located on the inner leaflet of plasma membranes in various types of cells (8) but appears on the outer leaflet after stimulation by various factors such as cytokines (9, 10), inflammatory reactions, and platelet activation (8, 11–13). Surface-exposed PS has also been shown to act as a signal for the removal of damaged or aged cells by the reticuloendothelial system and is observed in cells undergoing apoptosis (14, 15). Thus, the exposure of PS on the cell surface must be tightly regulated.

Another serine-containing phospholipid, lyso-PS, is implicated to act as a lipid mediator under pathophysiological conditions (16). For example, lyso-PS is demonstrated to interact with local mast cells (17), producing specific and stereoselective activation (18). It also induces transient increases in cytosolic free Ca²⁺ ([Ca²⁺]i) in ovarian and breast cancer cells (19) and lyso-PS; 2-acyl-1-lyso-PS with unsaturated fatty acids especially inhibits mitogen-induced T cell activation (20). Lyso-PS is present in human serum, the aqueous humor and the lacrimal gland fluid of the eye (21). It is likely to be produced from PS by phospholipase A₂ or A₁, but the precise mechanisms of lyso-PS production and elimination in vivo remain to be clarified.

We previously demonstrated that a serine phospholipid-specific phospholipase A₁/lyso phospholipase is secreted from rat platelets when they are activated (22, 23). Very recently we purified this enzyme from rat platelets and cloned its cDNA (24). Although this novel phospholipase, named PS-PLA₁ΔC, has a similar structure to members of the lipase family such as hepatic, pancreatic, and lipoprotein lipases, PS-PLA₁ΔC does not hydrolyze triacylglycerol but specifically acts on PS or 1-acyl-2-lyso-PS to hydrolyze fatty acids at the sn-1 position of these phospholipids (24). Thus, PS-PLA₁ΔC mediates two types of reactions, producing 2-acyl-1-lyso-PS from PS and eliminating 1-acyl-2-lyso-PS. It is secreted from cells and has an affinity for heparin, like other members of the lipase family (24). It is likely that PS-PLA₁ΔC is involved in regulating PS/lyso-PS-dependent reactions under physiological conditions. Indeed, PS-PLA₁ΔC can hydrolyze PS in rat platelets when the cell is activated (23). The physiological function of PS-PLA₁ΔC, however, is still unknown and requires further investigation. As a part of our continuing study of the physiological role of PS-PLA₁ΔC, we have isolated the cDNA for human PS-PLA₁ΔC. In the course of the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank¹⁷/EBI Data Bank with accession number(s) AF035268 (human PS-PLA₁) and AF035269 (human PS-PLA₁ΔC).

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study, we detected an alternative splicing form of human PS-PLA₁ and identified it as a lysosomotropic lysophospholipase.

MATERIALS AND METHODS
cDNA Cloning of Human PS-PLA₁—With the polymerase chain reaction (PCR), we amplified cDNA using a human liver Agt11 cDNA library (CLONTECH, Palo Alto, CA) as a template, and the following primers, based on the EST clone: 5′-CACGAGGATTCTCCAGGTCG-3′ and 5′-CCCTGGACATGGTTCTATTG-3′.
The resulting recombinant baculovirus was used to infect Sf9 cells. The resulting recombinant baculovirus was used to infect Sf9 cells. System (Life Technologies, Inc.) according to the manufacturer’s protocol to generate donor plasmids. Recombinant viruses were amplified, and PS-PLA₁ activity was determined as described below.

Southern Blot Analysis—Human and rat genomic DNAs (12 μg each) were digested with the appropriate restriction enzymes (50 units each) for 12 h then run on a 0.8% SeaKem Gold agarose gel (FMC BioProducts, Rockland, ME). The DNA was then transferred under capillary pressure to a Hybond-N nylon hybridization transfer membrane (Amersham Pharmacia Biotech). The DNA sequence was determined by the dideoxynucleotide chain termination method using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) and an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

Expression of Human PS-PLA₁ in Sf9 Cells—DNA fragments encoding the normal and truncated forms of human PS-PLA₁ were Amplification and Restriction Enzyme Digestion of Human PS-PLA₁ cDNA—Total RNA (1 μg) obtained from various human tissues and cell lines was used as a template in a reverse transcriptase (RT)-PCR. A Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech) was used according to the manufacturer’s protocol to synthesize the first-strand cDNA. PCR DNA fragments were isolated from an agarose gel and amplified using primers based on the sequence of the first-strand reaction product as a template and the following set of primers: 5′-ACAAGGACACCAACTGAGGTTACCTCC-3′ (nucleotide positions 1055–1084 of the cDNA encoding human PS-PLA₁) and 5′-GCTGCAATTCTGTGATATCCG-3′ (nucleotide positions 1312–1339). The RT-PCR products (285 or 289 bp) were digested with BstXI (+4 h at 60 °C) and subjected to 4% NuPage 3:1 agarose (FMC BioProducts) gel electrophoresis.

Amplification of Human PS-PLA₁—The amplified cDNA fragments encoding the normal and truncated forms of human PS-PLA₁ were Amplified using PCR using two synthetic oligonucleotides. The 5′ oligonucleotide, 5′-CAAAGGAGCACTGGATACGTCTCCG-3′ (nucleotide positions 1055–1084 of the cDNA encoding human PS-PLA₁), and 5′-GCTGCAATTCTGTGATATCCG-3′, contained the HindIII site and a complementary human PS-PLA₁ sequence. The amplified DNA fragments were subcloned into the BamHI site in the human PS-PLA₁ vector. Recombinant viruses were then prepared using the BAC-TO-BAC baculovirus expression system (Life Technologies, Inc.) to generate donor plasmids. Recombinant baculovirus was used to infect Sf9 cells. Four days after infection, the culture supernatant of the infected cells was collected, and PS-PLA₁ activity was determined as described below.

RESULTS

Molecular Cloning of Human PS-PLA₁—Southern blot analysis showed the existence of a single gene copy in humans and rats (data not shown). We used the BLASTN program to search the nucleotide databases for sequences similar to that of rat PS-PLA₁, and found two human EST sequences with homology to rat PS-PLA₁ on the GenBank (accession numbers T96213 (5′ region) and T96131 (3′ region)). We prepared one set of PCR primers corresponding to the 5′-untranslated region and the 3′-untranslated region based on the DNA sequences of these EST clones. Because the EST clones were derived from human fetal lung and spleen cDNA libraries, we performed PCR using human liver cDNA as a template.

We then used the resulting 1.7-kb DNA fragment to screen a human liver cDNA library, and one positive clone was isolated. DNA sequence analysis revealed that this clone has a sequence highly homologous with that of rat PS-PLA₁, covering the whole region corresponding to the open reading frame (Fig. 1a). This cDNA clone contained a 1368-bp open reading frame that encoded 456 amino acids, starting with an initiation codon.
FIG. 1. a, nucleotide and amino acid sequences of human PS-PLA₁. The first and second lines indicate the nucleotide and the deduced amino acid sequence, respectively. The nucleotide and amino acid positions are shown at both sides. The putative lid residues are underlined, and the polyadenylation signal is double underlined.

b, nucleotide and amino acid sequences of human PS-PLA₁. The sequences of PS-PLA₁ΔC that differ from those of PS-PLA₁ are shown. The four extra bases, GTAC, are shaded.

c, schematic model of PS-PLA₁ and PS-PLA₁ΔC. The positions of the active serine, aspartic acid, and histidine residues that compose the catalytic triad of PS-PLA₁ are shown by hatched bars.
(ATG) at nucleotide 32 (numbered as 1) and ending with a stop codon (TAG) at position 1369–1371. This open reading frame was flanked by 5′- and 3′-untranslated sequences of 31 and 359 bp, respectively, with a polyadenylation signal 19 bp before the poly(A) tail. Comparison of this open reading frame with rat PS-PLA1 revealed extensive homology at both the nucleotide (82.1%) and amino acid (80.0%) levels. Ser-142, Asp-166, and His-236, which may make up a catalytic triad in rat PS-PLA1 (24), are conserved between the rat and human enzymes. The amino acid sequences around these three amino acids were also highly conserved between the two species. A putative lid, which (24), are conserved between the rat and human enzymes. The insertion points of the four extra bases, GTAC, inserted in the PS-PLA1 mRNA is produced when the first consensus site (gtacgt) is used, and mRNA for the PS-PLA1ΔC is produced when the second site (gtaagt) is used. The nucleotide numbers in Fig. 1 were shown for PS-PLA1 and PS-PLA1ΔC, respectively.

![Diagram](https://via.placeholder.com/150)

**Fig. 2. Alternative splicing of PS-PLA1.** a, the nucleotide sequence of the human and rat PS-PLA1 genes around the insertion point of the four extra bases. The capital letters represent the exon, and the small letters represent the intron for PS-PLA1. The four bases, gate (in italics), were found at the 5′-end of the exon-intron boundary. The nucleotide numbers in Fig. 1 were shown for PS-PLA1 and PS-PLA1ΔC, respectively. b, possible exon-intron structure of the human PS-PLA1 gene. The 5′-splicing donor sites are underlined. Consensus sequences for RNA splicing are shown in the lower panel. The nucleotide numbers in Fig. 1 were shown for PS-PLA1 and PS-PLA1ΔC, respectively. c, model for alternative splicing of PS-PLA1. PS-PLA1 mRNA is produced when the first 5′-splicing donor site (gtacgt) is used, and mRNA for the PS-PLA1ΔC is produced when the second site (gtaagt) is used. The nucleotide numbers in Fig. 1 were shown for PS-PLA1 and PS-PLA1ΔC, respectively.
Expression of PS-PLA₁ and PS-PLA₁ΔC in Human Tissues and Cells—First we used Northern blot analysis to examine the expression of PS-PLA₁ mRNA (PS-PLA₁ and PS-PLA₁ΔC) using the cDNA corresponding to the coding region as a probe (Fig. 4). Transcripts of 1.9-kb messages were seen in most of the tissues examined, with the highest expression in the liver and prostate gland. PS-PLA₁ mRNAs were not detected in leukocytes or platelets (Fig. 4). Because PS-PLA₁ was first identified in rat platelets, we analyzed the expression of PS-PLA₁ in human platelets by RT-PCR analysis and an assay for PS-PLA₁ activity. No appreciable PS-PLA₁ activity or transcripts were detected in human platelets (data not shown).

The PS-PLA₁ transcript present in human tissues was detected as a single band on Northern blot analysis (Fig. 4). To determine the relative amounts of PS-PLA₁ and PS-PLA₁ΔC mRNAs in human tissues, RT-PCR analysis was performed using total RNAs derived from various human tissues. Because the insertion of the four extra bases generates a new restriction enzyme site, BsrGI (TGTACA) (Figs. 1 and 2), we quantified the frequency of the two mRNAs by digesting the RT-PCR products with BsrGI, as described under “Materials and Methods.” As shown in Fig. 5, both PS-PLA₁ and PS-PLA₁ΔC transcripts were detected in various human tissues, including skeletal muscle, kidney, small intestine, spleen, and testis. The amount of PS-PLA₁ΔC in these tissues was about 10 to 20% that of the PS-PLA₁ level (Fig. 5). We also examined the expression of PS-PLA₁ and PS-PLA₁ΔC in several human cell lines. mRNAs for both PS-PLA₁ and PS-PLA₁ΔC were detected in human fibroblast, keratinocyte, melanoma, HepG2, and HeLa cells. Thus, human tissues and cell lines express both PS-PLA₁ and PS-PLA₁ΔC, although the relative amount of PS-PLA₁ΔC is lower than that of PS-PLA₁.

Chromosome Mapping of PS-PLA₁—The location of the PS-baculovirus were applied onto a HiTrap heparin fast protein liquid chromatography column, and PS-PLA₁ and PS-PLA₁ΔC were eluted using a linear gradient of NaCl. The lysophospholipase activity of each fraction was measured using lyso-PS as a substrate.
the first example of determining a protein structure required

reported that the PLA1 and PLA2 activities of

ing. We showed in this study that PS-PLA1

pholipids (PS and lyso-PS) and is responsible for heparin bind-

a structure that recognizes the serine residues of serine phos-

function(s).

whereas the PS-PLA1

of PS-PLA1 in human tissues and cells determined by RT-PCR.

hepatic lipase (28), guinea pig pancreatic phospholipase (29),

human chromosome 3q13.13–13.2 (Fig. 6).

digestion, the PS-PLA1 plasmid gave rise to a single 285 bp band,

only occurs in PS-PLA1

addition to a band of 285 bp (derived from PS-PLA1) in various tissues

cell lines.

PLA1 gene on the human chromosome was assigned by direct

R-bandind fluorescence in situ hybridization using a human
cDNA fragment as a probe. The PS-PLA1 gene was localized to

human chromosome 3q13.13–13.2 (Fig. 6).

DISCUSSION

PS-PLA1ΔC as a Lyso-PS-specific Lysophospholipase—So far it has been reported that the various phospholipases for which the cDNA structure is already known, cytosolic PLA2 (26, 27), hepatic lipase (28), guinea pig pancreatic phospholipase (29), phospholipase B (30), Campylobacter coli PL A2 (31), and PS-PLA1 (24), show both PLA and lysophospholipase activity. Among these phospholipases, hepatic lipase (32), guinea pig phospholipase (33), phospholipase B, and PS-PLA1 exhibited both PLA and lysophospholipase activity. Saito et al. (30) reported that the PLA1 and PLA2 activities of Penicillium notatum phospholipase B are lost completely by limited proteolysis, whereas its lysophospholipase activity remains unchanged (30), although the protein structures required for each activity have not been characterized yet. Separating lyso-PS-specific lysophospholipase activity from PS-phospholipase A1 is the first example of determining a protein structure required for lysophospholipase and PLA activity.

The lipases (lipoprotein and pancreatic lipase) are reported to be composed of two domains (the N-terminal and C-terminal domains) (34, 35). PS-PLA1 is also predicted to possess the similar two domains (24), but in PS-PLA1ΔC, two-thirds of the C-terminal domain is lost (Fig. 1). From our results (Fig. 3, b and c), we conclude that the N terminus of PS-PLA1, which is conserved between PS-PLA1 and PS-PLA1ΔC (Fig. 1c), carries a structure that recognizes the serine residues of serine phospholipids (PS and lyso-PS) and is responsible for heparin binding. We showed in this study that PS-PLA1ΔC fails to hydrolyze PS. In addition, our preliminary experiment shows that when we incorporated lyso-PS as a substrate of PS-PLA1ΔC into PC liposomes, its enzymatic activity was effectively inhibited (data not shown). This indicates that PS-PLA1ΔC may not be able to recognize the lipid surface of PS micelles and that the C terminus of PS-PLA1, which is missing in PS-PLA1ΔC, plays an important role in recognizing diacyl-PS in the lipid bilayers. Several studies have in fact suggested an important role of the C terminus of lipases in recognizing lipid surfaces (36, 37); however, as PS-PLA1 does not show any amino acid homology with other lipases in the C-terminal domain (30), it is also possible that this domain of PS-PLA1 has another specialized function(s).

It is generally known that a lid is involved in the interfacial activation (38) and/or substrate recognition (39). The lid of PS-PLA1 is composed of 12 amino acid residues (Fig. 1), whereas those of many lipases are composed of 22 or 23 residues. This short lid may not play a role in the interfacial activation, because it was demonstrated that pancreatic lipase loses the ability to surface activation when its lid, composed of 23 amino acids, was shortened by site-directed mutagenesis (38). Rather this short lid may be involved in substrate recognition. In fact, both PS-PLA1 and PS-PLA1ΔC possess the same lid structure.

Expression of PS-PLA1 and PS-PLA1ΔC in Human Tissues—We first purified PS-PLA1 from rat platelets. In this study, we showed that PS-PLA1 is expressed in various human tissues. However, human platelets, leukocytes (Fig. 4), and red blood cells do not express PS-PLA1 appreciably. We could not detect PS-PLA1 activity in platelets from rabbits, cattle, or pigs. Thus, PS-PLA1 is expressed in platelets in a species-specific manner. It is not yet known whether the alternative form (PS-PLA1ΔC) exists in species other than humans, although the nucleotide sequence of the rat PS-PLA1 gene around the exon-intron boundary is identical to that of the human PS-PLA1 gene (Fig. 2a). Thus, we expect that the alternative form will be present in the rat and in other species.

Possible Roles of PS-PLA1 and PS-PLA1ΔC—The physiological significance of PS-PLA1 is not yet known. The chromosomal location of the human PS-PLA1 gene does not suggest a possible link to human disease. It is noteworthy, however, that the nucleotide sequence of human PS-PLA1 is identical to that of the nnd gene product (40). This gene is predominantly expressed in nonmetastatic human melanoma cell lines, with a lower expression level in metastatic cell lines (40). Any molecular link between PS and the metastasis of tumor cells is totally unknown at present, but identifying PS-PLA1 as the nnd gene product suggests that serine phospholipids (PS or lyso-PS) are involved in the metastatic process of tumor cells. Calderon et al. (4) reported that some cancer cells secrete PS, and it impairs macrophage cytotoxicity by inhibiting the production of nitric oxide from macrophages. Thus they speculated that cancer cells escape from macrophage recognition by secreting PS. PS-PLA1 may hydrolyze such PS and increase the activity of macrophages in self-defense against tumor cells.

PS-PLA1 mediates three types of reaction to eliminate PS and 1-acyl-2-lyso-PS and to produce 2-acyl-1-lyso-PS. PS-
PLA$_3$C, by contrast, has only an ability to eliminate 1-acyl-2-lyso-PS. Once 2-acyl-1-lyso-PS are produced by the PLA$_1$ reaction, the fatty acid at the sn-2 position readily migrates to the sn-1 position, which results in production of 1-acyl-2-lyso-PS (41). PS-PLA$_1$/C might eliminate such 1-acyl-2-lyso-PS in vivo, or it might eliminate 1-acyl-2-lyso-PS, which is produced by the PLA$_2$ reaction. Because serine phospholipids are suggested to be involved in various pathophysiological conditions (16), both PS-PLA$_1$/C and PS-PLA$_3$C might be key enzymes that regulate the production and elimination of serine phospholipids. Further study is required to clarify the physiological functions of PS-PLA$_1$/C and PS-PLA$_3$C.

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