Surface loops of extracellular phospholipase A_1 determine both substrate specificity and preference for lysophospholipids[S]

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Abstract Members of the pancreatic lipase family exhibit both lipase activity toward triacylglycerol and/or phospholipase A_1 (PLA_1) activity toward certain phospholipids. Some members of the pancreatic lipase family exhibit lysophospholipase activity in addition to their lipase and PLA_1 activities. Two such enzymes, phosphatidylserine (PS)-specific PLA_1 (PS-PLA_1) and phosphatidic acid (PA)-selective $PLA_1\alpha$ (PA-$PLA_1\alpha$, also known as LIPH) specifically hydrolyze PS and PA, respectively. However, little is known about the mechanisms that determine their substrate specificities. Crystal structures of lipases and mutagenesis studies have suggested that three surface loops, namely, β5, β9, and lid, have roles in determining substrate specificity. To determine roles of these loop structures in the substrate recognition of these PLA_1 enzymes, we constructed a number of PS-PLA_1 mutants in which the three surface loops are replaced with those of PA-$PLA_1\alpha$. The results indicate that the surface loops, especially the β5 loop, of PA-$PLA_1\alpha$ play important roles in the recognition of PA, whereas other structure(s) in PS-PLA_1 is responsible for PS preference. In addition, β5 loop of PS-PLA_1 has a crucial role in lysophospholipase activity toward lysophosphatidylserine.[JLR] The present study revealed the critical role of lipase surface loops, especially the β5 loop, in determining substrate specificities of PLA_1 enzymes.—Arima, N., A. Inoue, K. Makide, M. Nonaka, and J. Aoki. **Surface loops of extracellular phospholipase A_1 determine both substrate specificity and preference for lysophospholipids. *J. Lipid Res.* 2012.** 53: **513–521.**

Supplementary key words lysophospholipid • lysophospholipase • lipase • surface loop • lid • phospholipases • phospholipids • phospholipids/phosphatidic acid • phospholipids/phosphatidylserine

Phospholipase A_1 (PLA_1) is an enzyme that hydrolyzes fatty acid bound at the *sn*-1 position of phospholipids. There are several classes of PLA_1 isozymes that differ in their structure and cellular localization. In mammals intracellular PLA_1 consists of three members, $iPLA_1\alpha$ [also known as phosphatidic acid (PA)-preferential PLA_1, PA-PLA_1] (1–3), $iPLA_1\beta$ (also known as p125) (4) and $iPLA_1\gamma$ (also known as KIAA0725) (5), whereas extracellular PLA_1 consists of at least six members [phosphatidylserine (PS)-specific PLA_1 (PS-PLA_1), phosphatidic acid (PA)-selective $PLA_1\alpha$ (PA-$PLA_1\alpha$, also known as LIPH), PA-$PLA_1\beta$ (also known as LIPI), hepatic lipase (HL), endothelial lipase (EL) and pancreatic lipase-related protein 2] (6–8). The latter six belong to the pancreatic lipase family, which hydrolyzes triglyceride (TG), phospholipids or both. Based on amino acid sequences and their substrate specificities, PS-PLA_1, PA-$PLA_1\alpha$, and PA-$PLA_1\beta$ form a subfamily within the pancreatic lipase family (8). Interestingly, these members show unique substrate preference toward specific phospholipids such as PS and PA, but not TG (9–11). PS-PLA_1 is specific to PS, whereas PA-$PLA_1\alpha$ and β are specific to PA. The enzymes produce lysophospholipid mediators such as lysophosphatidylserine (LPS) and lysophosphatidic acid (LPA) from PS and PA, respectively, and are considered to be responsible for production of these lysophospholipid mediators (12–14). However, little is known about the mechanism underlying the strict substrate specificity. In addition to its PLA_1 activity, PS-PLA_1 shows lysophospholipase activity, which cleaves the fatty acid bound at the *sn*-1 position of LPS (9, 15). Among the members of the pancreatic lipase family, HL and EL were also reported to have lysophospholipase activity (16, 17), whereas other members have not been tested.

A crystallographic study of human pancreatic lipase (PL) (18) showed that it possesses three surface loops called the β5, β9, and lid loops that cover the active site. Because the

Abbreviations: EL, endothelial lipase; LPA, lysophosphatidic acid; LPS, lysophosphatidylserine; PA, phosphatidic acid; PA-$PLA_1\alpha$, phosphatidic acid-selective $PLA_1\alpha$; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA_1, phospholipase A_1; PS, phosphatidylserine; PS-PLA_1, phosphatidylserine-specific PLA_1;TG, triglyceride.

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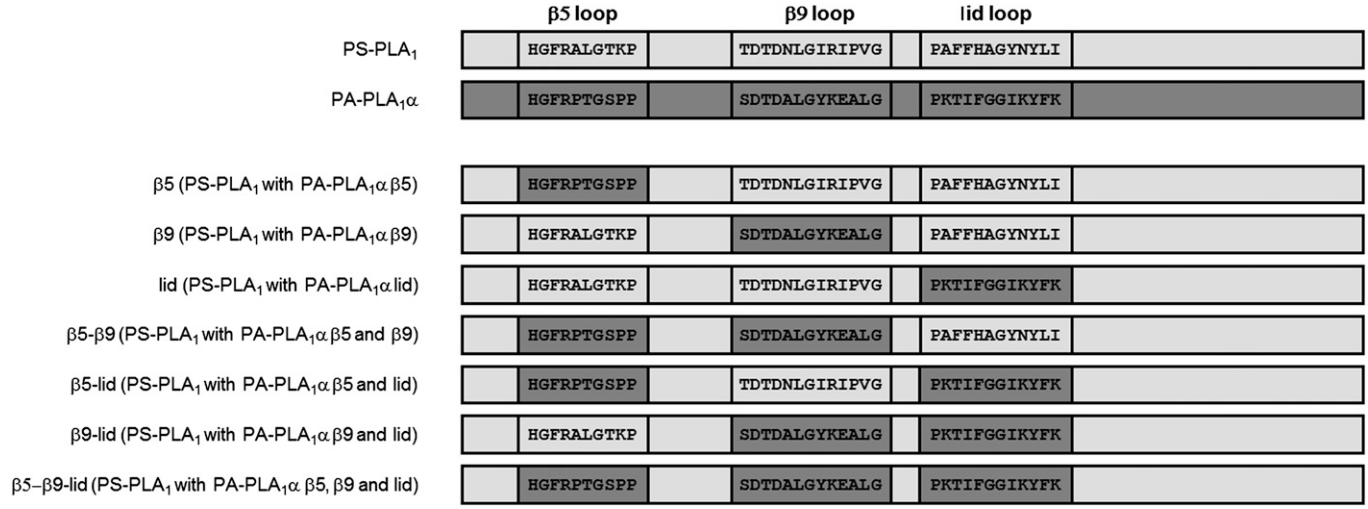

Fig. 1. Schematic diagrams of wild-type PS-PLA$_1$, wild-type PA-PLA$_1$α and PS-PLA$_1$ mutants.

lid loop of PL was found to undergo a conformational change upon contact with its substrate to allow the substrate to access the active site, it has been postulated that the lid loop is involved in substrate specificity (6, 19). In fact, the substrate specificities of lipoprotein lipase (LPL) and EL can be switched by exchanging their lid loops (20). The crystallographic studies of PL also suggested that the β5 and β9 loops need conformational changes to allow full substrate entry (18). Furthermore, the importance of these three loops in substrate recognition was supported by the following evidence: the β9 and lid loops of PLA$_1$s (PS-PLA$_1$, PA-PLA$_1$α and β) are much shorter (composed of 12 amino acids) than those of TG lipases such as PL, HL, and LPL (composed of 22 or 23 amino acids) (8, 21). These notions raise the possibility that the surface loops are involved in the substrate recognition of lipases.

In this study, to test this hypothesis, we constructed a number of chimeric molecules between PS-PLA$_1$ and PA-PLA$_1$α in which the three loop structures, β5, β9, and lid, were interexchanged and examined the substrate specificity. The results indicated that the surface loops of PA-PLA$_1$α, especially β5 and lid, participate in the recognition of PA, whereas other domain(s) are responsible for PS recognition in PS-PLA$_1$. In addition, we found that the β5 loop of PS-PLA$_1$ is crucial for the lysophospholipase activity of the enzyme toward LPS.

MATERIALS AND METHODS

Construction of PS-PLA$_1$ mutants

A series of cDNAs encoding PS-PLA$_1$ mutants were constructed by overlap extension PCR method (22). The strategy is illustrated in supplementary Fig. I. In the first step, two independent PCR reactions were carried out using mouse PS-PLA$_1$ cDNA in pCAGGS as a template. One reaction was performed using Primer 1 and an overlap reverse primer, corresponding to the β5, β9, or lid domain of PA-PLA$_1$α, to amplify the 5′-half of mutant PS-PLA$_1$ cDNA. The other reaction was performed using Primer 2 and an overlap forward primer, corresponding to the β5, β9, or lid domain of PA-PLA$_1$α, to amplify the 3′-half of mutant PS-PLA$_1$ cDNA. To produce the full-length mutant PS-PLA$_1$ cDNA, the resulting two fragments were gel purified and used as templates for the second PCR reaction using Primer 1 and Primer 2. The resulting DNA fragments were subcloned into the *KpnI/XhoI* site of a plasmid expression vector pCAGGS-c-*myc*, which harbors an in-frame myc tag sequence followed by a stop codon at 3′ position of the *XhoI* site. Nucleotide sequences were confirmed by a standard dideoxy method (Fasmac, Japan). The oligonucleotide DNA primers used for PCR are as follows: Primer 1; gactccggtaccaccatgcgtcctggcctc, Primer 2; aggctcctcgagcacgcaggctatttt, β5 overlap fwd; ccaacaggctcccctccttcttggatcgac, β5 overlap rev; aggggagcctgttggcctgaatccatgaat, β9 overlap fwd; gacgcactaggctacaaggaagccctaggacatgtggactac, β9 overlap rev; cttgtagcctagtgcgtcagtgtcagagtgtggatggcttctac, lid overlap fwd; tttggaggtataaagtacttcaagtgtgatcacatgagg, lid overlap rev; ctttatacctccaaatattgtcttagggcatccaggctg.

PS-PLA$_1$ single amino acid mutants of β5 loop

By the overlap extension PCR method (see supplementary Fig. I), we constructed four PS-PLA$_1$ β5 loop mutants in which an amino acid residue of the β5 loop of PS-PLA$_1$ was replaced with that of PA-PLA$_1$α (namely, A93P, L94T, T96S, and K97P). The first PCR was carried out using mouse PS-PLA$_1$ cDNA in pCAGGS as a template. In the first step, one reaction was performed with Primer 1 and a single-mutation reverse primer, A93P, L94T, T96S, or K96P rev primer, to amplify the 5′-end of PS-PLA$_1$. The other reaction was performed with Primer 2 and a single-mutation forward primer, A93P, L94T, T96S, or K96P fwd primer, to amplify the 3′-end of PS-PLA$_1$. The second PCR was carried out using the products of the first PCR reactions with Primer 1 and Primer 2. These DNA fragments were subcloned into the *KpnI/XhoI* site of a plasmid expression vector pCAGGS-c-*myc*. Nucleotide sequences were confirmed by a standard dideoxy method (Fasmac, Japan). The oligonucleotide DNA primers used for PCR are as follows: Primer 1; gactccggtaccaccatgcgtcctggcctc, Primer 2; aggctcctcgagcacgcaggctatttt, A93P fwd; attattcatggattcaggccactcgga, A93P rev; agaaggctttgttccgagtggcctgaa, L94T fwd; agggcgacaggaacaaagccttcttgg, L94T rev; tgttcctgtcgccctgaatccatgaat, T96S fwd; ctcggatccaagccttcttggatcgac, T96S rev; aggcttggatccgagcgccctgaatcc, K97P fwd; ggaacacctcctcttggatcgacaag, K97P rev; agaaggaggtgttccgagcgccctgaa.

Cell culture and transfection

HEK293 cells were maintained in DMEM (Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin

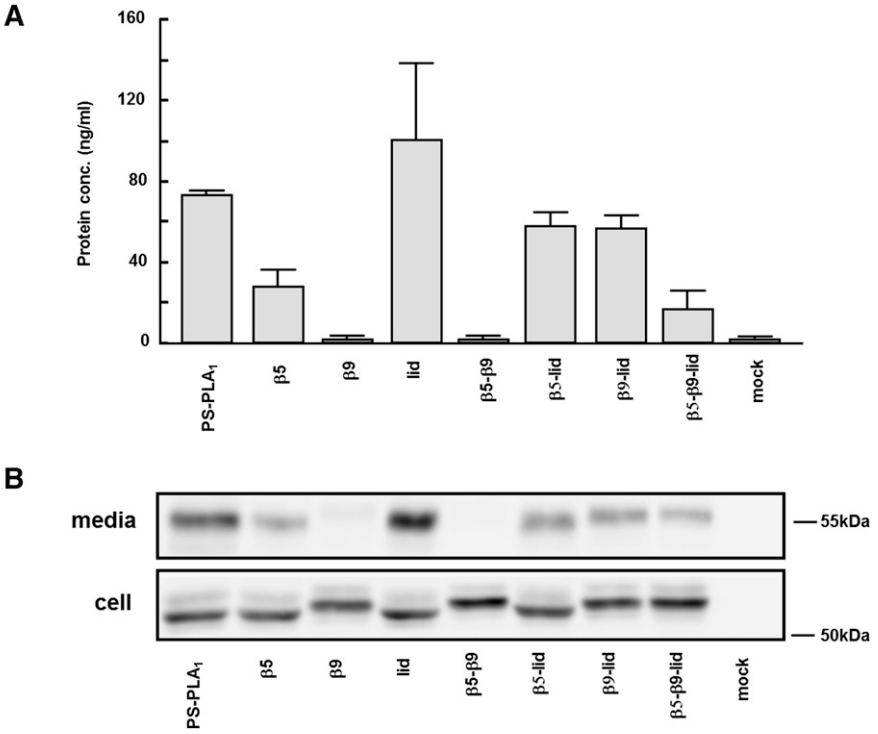

Fig. 2. The expression of PS-PLA$_1$ mutants. HEK 293 cells were transiently transfected with plasmids encoding PS-PLA$_1$ mutants (see Fig. 1). Forty-eight hours after transfection, conditioned media were collected and the protein levels in each conditioned medium were examined by ELISA (A) and by Western blotting (B). In (A), bars indicate mean values and error bars indicate SD.

(GIBCO) in a 37°C incubator with 5% CO_2. For transfections, HEK293 cells were seeded at 2.0×10^5 per well in 12-well plate and cultured for 24 h. The cells were transfected using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturers. Twenty-four hours after transfection, the medium was replaced with 600 μl of serum-free ExCell 302 medium (JRT) and incubated for another 24 h. Conditioned media were collected, clarified by low-speed centrifugation and used as an enzyme source.

Western blotting

Protein in conditioned media was TCA-precipitated. Briefly, 100 μl of conditioned media was mixed with 10 μl of 100% (w/v) TCA. After a brief vortex, the samples were incubated at 4°C for 1 h and centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was aspirated and the pellet was washed with 100 μl of cold acetone. The samples were centrifuged, as above, and the pellet was again washed with cold acetone. After centrifugation, the pellet was air-dried at room temperature and resuspended in 15 μl of SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS and 10% Glycerol). The cells were extracted in 75 μl of lysis buffer (10 mM HEPES (pH 7.3), 10% Glycerol, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml PMSF, 20 μg/ml leupeptin and 2.5 mM p-NPP). Cell lysates were centrifuged at 20,000 *g* and the resulting supernatants were collected and added with 4 x SDS-PAGE sample buffer.

Before loading the samples on SDS-PAGE, they were heated to 100°C. Fifteen microliters of the samples were applied and separated by SDS-PAGE. Protein samples were transferred to nitrocellulose membranes using the Bio-Rad protein transfer system. The membranes were blocked with Tris-buffered saline containing 5% (w/v) skimmed milk and 0.05% (v/v) Tween-20, incubated with 1:50 anti-myc antibody (9E10) and treated with 1:2000 anti-mouse IgG-horseradish peroxidase. Proteins bound to the antibodies were visualized with an enhanced chemiluminescence system (GE Health Science).

PLA$_1$ assays

Substrates [phosphatidylethanolamine (PE), PA, PS, phosphatidylinositol (PI), phosphatidylcholine (PC), LPA and LPS)] were purchased from Avanti Polar Lipids. These substrates were dried under nitrogen gas and dissolved at 400 μM in 100 mM Tris-HCl (pH 7.5) using water bath sonication and stocked in −20°C. We confirmed that phospholipid substrates stored at −20°C gave similar results to those obtained using freshly prepared phospholipid substrates. Substrates (final 80 μM) were added to 10 μl of conditioned media in a total volume of 100 μl in a 96-well plate and incubated at 37°C for several hours (PS, LPA, and LPS: 1 h; PE and PA: 1.5 h; PI: 2 h; PC: 4 h). Then, the resulting fatty acid liberated from phospholipids was measured using NEFA C-test Wako (WAKO) according to the manufacturers. In all experiments we confirmed that reaction was linear with time and amount of protein at 80 μM substrate concentration.

PS-PLA$_1$ sandwich ELISA assay

Monoclonal antibodies against mouse PS-PLA$_1$ were established as described previously (23). The amount of PS-PLA$_1$ in the conditioned media was determined by PS-PLA$_1$ sandwich ELISA assay using two anti-mouse PS-PLA$_1$ monoclonal antibodies. A 96-well plate (Nunc) was coated with purified anti-mouse PS-PLA$_1$ monoclonal antibody (clone 4D2). After soaking with PBS containing 3% (w/v) BSA, conditioned media containing recombinant PS-PLA$_1$ was applied, incubated with biotinylated anti-PS-PLA$_1$ monoclonal antibody (clone 4C10) and treated with HRP-conjugated streptavidin. Bound HRP-conjugated streptavidin was visualized with 3,3',5,5'-tetramethylbenzidine (TMB) as the peroxidase substrate. Standard PS-PLA$_1$ was recombinant PS-PLA$_1$

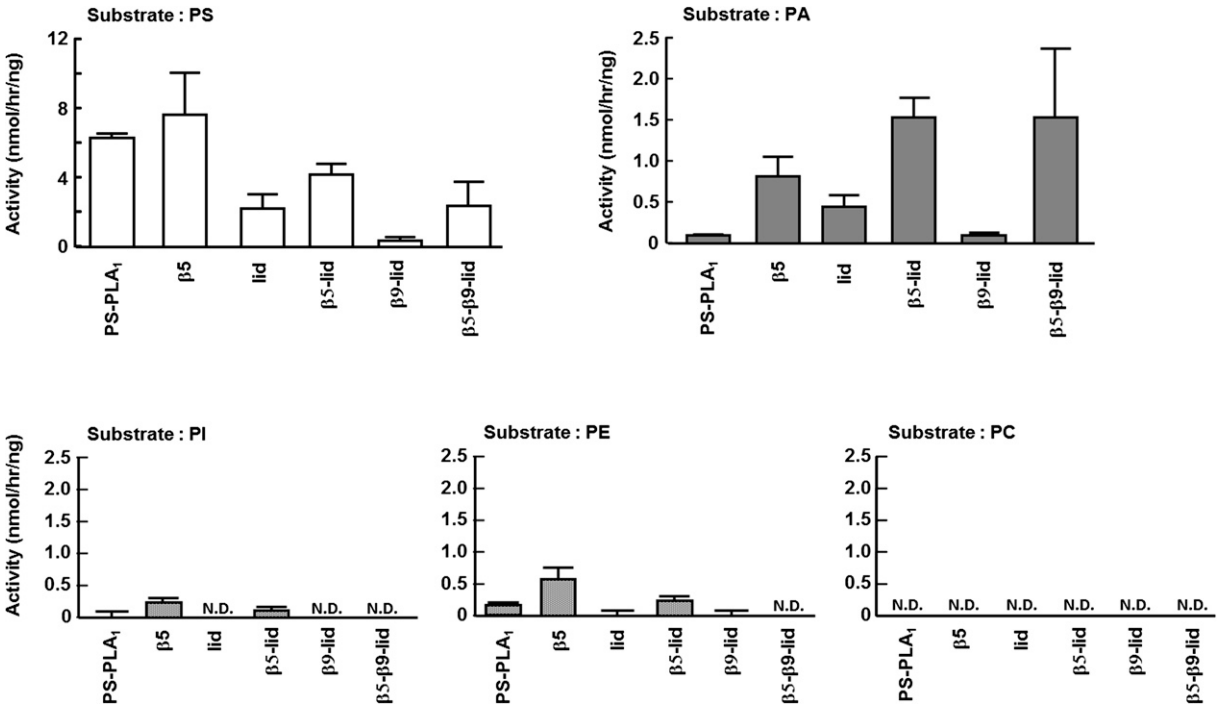

Fig. 3. Substrate specificity of PS-PLA$_1$ mutants. Phospholipid substrates (PS, PA, PI, PE, and PC) were mixed with each PS-PLA$_1$ mutant in 100 mM Tris-HCl (pH 7.5) and incubated at 37°C for several hours. Enzyme activity was determined by quantifying the fatty acids liberated and expressed as calculated enzyme activity per ng protein of PS-PLA$_1$ mutants. Bars indicate mean values and error bars indicate SD. N.D., not detected.

protein expressed by baculovirus system. The standard curve is shown in supplementary Fig. II.

RESULTS

Expression of PS-PLA$_1$ mutants

We previously found that PS-PLA$_1$ was exclusively secreted into the culture media when expressed in cultured cells (9, 24), whereas PA-PLA$_1\alpha$ was secreted but localized exclusively to the plasma membrane (10, 11). Based on these observations, we introduced the PA-PLA$_1\alpha$ loop structures, β5, β9, and/or lid, into the PS-PLA$_1$ backbone. Accordingly, we constructed seven cDNAs encoding PS-PLA$_1$ mutants with PA-PLA$_1\alpha$ loop structures (**Fig. 1**) and transfected HEK293 cells with the resulting plasmids. As is the case for wild-type PS-PLA$_1$, most of the recombinant PS-PLA$_1$ mutant proteins were detected in both cells and conditioned

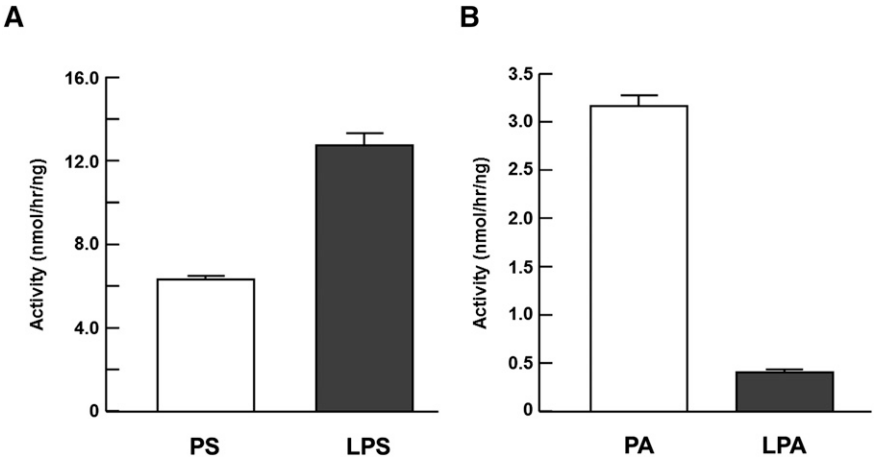

Fig. 4. Lysophospholipase activities of PS-PLA$_1$ (A) and PA-PLA$_1\alpha$ (B). Enzyme activities of PS-PLA$_1$ and PA-PLA$_1\alpha$ were determined by quantifying fatty acid liberated and exhibited as calculated enzyme activity per ng protein of PS-PLA$_1$ (A) or PA-PLA$_1\alpha$ (B). The culture media of HEK293 cells transfected with PS-PLA$_1$ or PA-PLA$_1\alpha$ plasmid were used as an enzyme source. Although PA-PLA$_1\alpha$ is localized exclusively to the plasma membrane, it is slightly secreted into conditioned medium. Substrates are PS and LPS for PS-PLA$_1$ and PA and LPA for PA-PLA$_1\alpha$. Bars indicate mean values and error bars indicate SD.

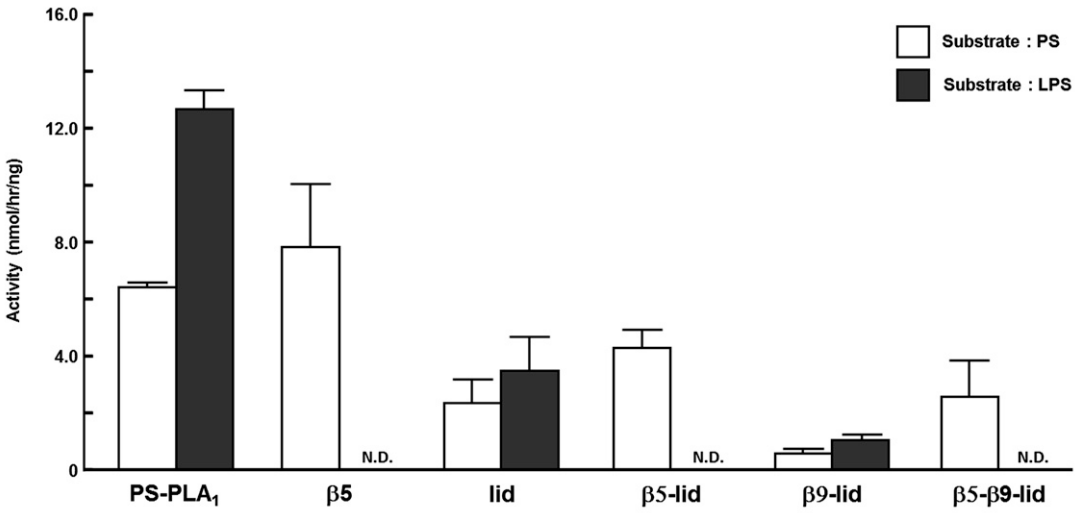

Fig. 5. The β5 mutants lost lysophospholipase activity toward LPS. Phosphatidylserine (PS, white columns) and lysophosphatidylserine (LPS, black columns) were incubated at 37°C for 1 h with each PS-PLA$_1$ mutant in 100 mM Tris-HCl (pH 7.5). Then, enzyme activity was determined by quantifying fatty acids liberated and exhibited as calculated enzyme activity per ng protein of PS-PLA$_1$ mutants. Bars indicate mean values and error bars indicate SD. N.D., not detected.

media as judged by sandwich ELISA (**Fig. 2A**) and Western blotting (Fig. 2B). However, the β9 and β5-β9 mutant proteins were almost exclusively detected in the cells. We therefore examined the enzymatic activity of other five PS-PLA$_1$ mutant proteins.

Substrate preference of PS-PLA$_1$ mutants

To examine the role of surface loops in determining the substrate specificity of PS-PLA$_1$ and PA-PLA$_1$α, we examined the substrate specificity of the above five PS-PLA$_1$ mutant proteins and wild-type PS-PLA$_1$ using various phospholipid substrates including PS, PA, PC, PE, and PI. PLA$_1$ activities were determined by quantifying the fatty acid liberated as described in Materials and Methods (**Fig. 3**). All five mutants (β5, lid, β5-lid, β9-lid, and β5-β9-lid) retained catalytic activity toward PS. Especially, four mutants (β5, lid, β5-lid, and β5-β9-lid) hydrolyzed PA efficiently and hydrolyzed PS to a similar extent as in wild-type PS-PLA$_1$. Like wild-type PS-PLA$_1$, most mutants did not hydrolyze PC, PE, and PI. However, two mutants (β5 and β5-lid) slightly hydrolyzed PI and PE in addition to PS and PA (Fig.3). These results indicate that at least the β5 and lid loops of PA-PLA$_1$α play a role in the recognition of PA but not in the recognition of PS. Because all the mutants still showed a preference for PS, structures of PS-PLA$_1$ other than the three loops must be responsible for the recognition of PS.

The β5 loop of PS-PLA$_1$ is required for lysophospholipase activity

PS-PLA$_1$ liberates fatty acid at the *sn*-1 position of PS and LPS (**Fig. 4A**). By contrast we found that PA-PLA$_1$α did not hydrolyze LPA efficiently (Fig. 4B). Accordingly, we tested whether the three surface loops are involved in the discrimination between di-acylphospholipids and lysophospholipids by examining lysophospholipase activity of PS-PLA$_1$ mutants toward LPS because all the five mutants were found to retain activity toward PS (Fig. 3). Like wild-type PS-PLA$_1$, the lid mutants (lid) hydrolyzed LPS. β9-lid mutants showed a very weak lysophospholipase activity (**Fig. 5**). We found that PS-PLA$_1$ mutants with different combinations of the β5 loop PA-PLA$_1$α (β5, β5-lid and β5-β9-lid) completely lost hydrolysis activity toward LPS, although they hydrolyzed PS efficiently (Fig. 5). Hydrolysis of phospholipids in this assay followed Michaelis-Menten kinetics (supplementary Fig. III). Therefore, we calculated the Michaelis-Menten kinetic parameters of each enzyme from the Michaelis-Menten curve of the phospholipase and lysophospholipase activity and confirmed that the $V_{max,\ LPS/PS}$ ratios of β5 mutants (β5, β5-lid, and β5-β9-lid)

TABLE 1. Kinetic characteristics of PS-PLA$_1$ and PS-PLA$_1$ mutants

Enzyme	Substrate: LPS		Substrate: PS		V_{max} ratio
	V_{max} (pmol/min/ng protein)	apparent K_m (μM)	V_{max} (pmol/min/ng protein)	apparent K_m (μM)	(LPS/PS)
PS-PLA$_1$	245	38	113	26	2.2
β5	N.D.	N.D.	126	19	–
Lid	64.6	30	40.8	13	1.6
β5-Lid	N.D.	N.D.	74.5	22	–
β9-Lid	22.9	28	10.9	11	2.1
β5-β9-Lid	N.D.	N.D.	46.6	22	–

LPS, lysophosphatidylserine; PS, phosphatidylserine; N.D., not detected.

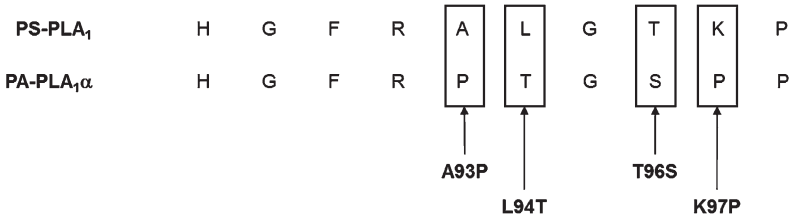

Fig. 6. Alignment of amino acid sequence of the β5 loops of PS-PLA$_1$ and PA-PLA$_1$α. Four amino acids out of nine differ between PS-PLA$_1$ and PA-PLA$_1$α. The positions of single amino acid mutants are also shown.

were markedly decreased (**Table 1**). These data show that the β5 loop of PS-PLA$_1$ is essential for lysophospholipase activity and that it is not exchangeable with the β5 loop of PA-PLA$_1$α.

The amino acid sequences of the β5 loops in PS-PLA$_1$ and PA-PLA$_1$α differ by four amino acids (**Fig. 6**). To identify the amino acid residues responsible for lysophospholipase activity, we generated single amino acid mutants in which each of the four different amino acids of the β5 loop in PS-PLA$_1$ was replaced with that of PA-PLA$_1$α (A93P, L94T, T96S, and K97P; each number represents the amino acid position of PS-PLA$_1$) and examined the lysophospholipase activity of these mutants toward LPS. All the single mutants were detected in conditioned media (**Fig. 7A**). Interestingly, A93P mutant showed a drastic change in the substrate preference (i.e., little lysophospholipase activity toward LPS) (Fig. 7B, C). The substrate preferences of the other three mutants (L94T, T96S and K97P), which hydrolyzed both PS and LPS, did not differ significantly from that of wild-type PS-PLA$_1$. We also calculated the Michaelis-Menten kinetic parameters of each single amino acid mutant from the Michaelis-Menten curve (supplementary Fig. III) and confirmed that the $V_{max,\ LPS/PS}$ ratio of only A93P mutant was markedly decreased (**Table 2**). In addition, we constructed the single amino acid mutant of PA-PLA$_1$α in which the proline in the β5 loop of PA-PLA$_1$α was replaced with alanine (as in PS-PLA$_1$) (**Fig. 8A**) and examined the lysophospholipase activity toward LPS and LPA. We found that this mutant did not show lysophospholipase activity toward LPA, although it was active toward PA (Fig. 8B). From these observations, we concluded that the presence of the proline alone in the β5 loop of PA-PLA$_1$α was not enough to explain why PA-PLA$_1$α lacks lysophospholipase activity. Thus, other factor(s) on PS-PLA$_1$ should be involved in exhibiting lysophospholipase activity. A hydrophobic interaction between some amino acids and acyl chains can be such a factor.

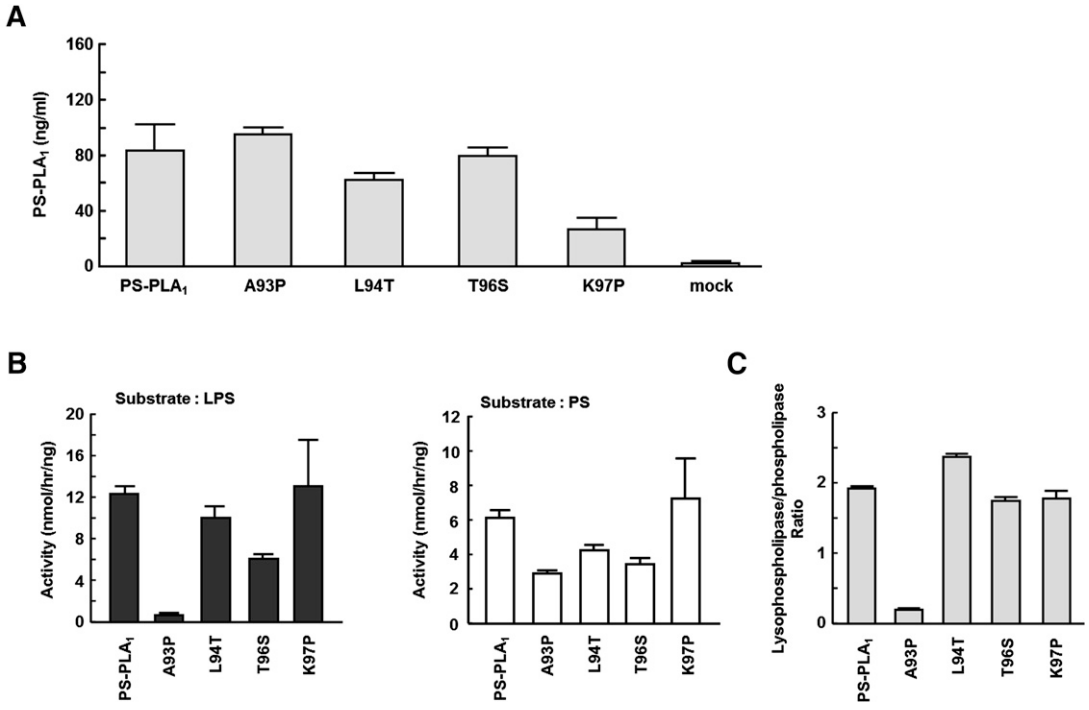

Fig. 7. Lysophospholipase activity of single amino acid mutants. A: HEK 293 cells were transiently transfected with plasmids encoding single amino acid PS-PLA$_1$ mutants. Expression of each mutant was examined by sandwich ELISA. B: Phosphatidylserine (PS, left panel) and lysophosphatidylserine (LPS, right panel) were incubated at 37°C for several h with conditioned media in 100 mM Tris-HCl (pH 7.5). Then, enzyme activity was determined by quantifying fatty acids liberated and exhibited as calculated enzyme activity per ng protein of the single amino acid mutants. Results are presented as bar charts of mean values ± SD depicted by error bars. C: The ratio of lysophospholipase to phospholipase was expressed as calculated enzyme activity to LPS per to PS. Bars indicate mean values and error bars indicate SD.

TABLE 2. Kinetic characteristics of PS-PLA$_1$ and single amino acid mutants

Enzyme	Substrate: LPS		Substrate: PS		V_{max} ratio
	V_{max} (pmol/min/ng protein)	apparent K_m (μM)	V_{max} (pmol/min/ng protein)	apparent K_m (μM)	(LPS/PS)
PS-PLA$_1$	245	38	113	26	2.2
A93P	11.6	20	49.6	24	0.23
L94T	192	25	73.6	21	2.6
T96S	118	31	62.8	16	1.9
K97P	259	28	129.6	11	2.0

LPS, lysophosphatidylserine; PS, phosphatidylserine.

DISCUSSION

PS-PLA$_1$ and PA-PLA$_1\alpha$ specifically recognize PS and PA, respectively (9, 10), although the recognition mechanism is unclear. In this study, we constructed a number of PS-PLA$_1$ mutants in which corresponding the three loop structures of PA-PLA$_1\alpha$ were introduced and tested their substrate specificities (Figs. 1, 3). PS-PLA$_1$ mutant with the triple substitutions (β5-β9-lid) hydrolyzed PA in addition to PS. Hydrolysis of PA was also observed in the PS-PLA$_1$ mutant with the PA-PLA$_1\alpha$ lid loop (lid) or β5 and lid loops (β5-lid). Therefore, at least the β5 and lid loops of PA-PLA$_1\alpha$ appear to act in concert to recognize and hydrolyze PA. Meanwhile, the β9 loop of PA-PLA$_1\alpha$ may be involved in regulating membrane association because the β9 and β5-β9 mutant proteins were almost exclusively detected in the cells (Fig. 2). In addition, two mutants, β5 and β5-lid, slightly hydrolyzed PI and PE in addition to PS and PA (Fig. 3), suggesting that the β5 loop plays a critical role in substrate selectivity. We assume that the exchange of the β5 loop changed the structure of catalytic pocket of PS-PLA$_1$ and resulted in temporary substrate promiscuity.

On the other hand, the finding that the preference for PS was not affected by the introduction of the three loops of PA-PLA$_1\alpha$ clearly shows that the surface loops of PS-PLA$_1$ are not involved in the recognition of PS. We speculate that amino and carboxyl groups of the serine residue in PS enter into the catalytic pocket of PS-PLA$_1$ and, thus, the amino acid residues in the internal surface of the pocket are involved in the recognition of serine in PS. Given that PS-PLA$_1$ doesn't act on PA, the three surface loops of PS-PLA$_1$ probably play a role in accepting PS and excluding PA into the catalytic pocket.

PS-PLA$_1$ hydrolyzes lysophospholipid more effectively than PA-PLA$_1\alpha$ (Fig. 4). We found that lysophospholipase activity of PS-PLA$_1$ was dramatically reduced when the β5 loop was replaced with the β5 loop of PA-PLA$_1\alpha$ (Fig. 5 and Table 1). Furthermore, among the single amino acid mutants of PS-PLA$_1$ (A93P, L94T, T96S and K97P), only A93P mutant markedly lost lysophospholipase activity against LPS (Fig. 7B and Table 2). Interestingly, Pro 93 in

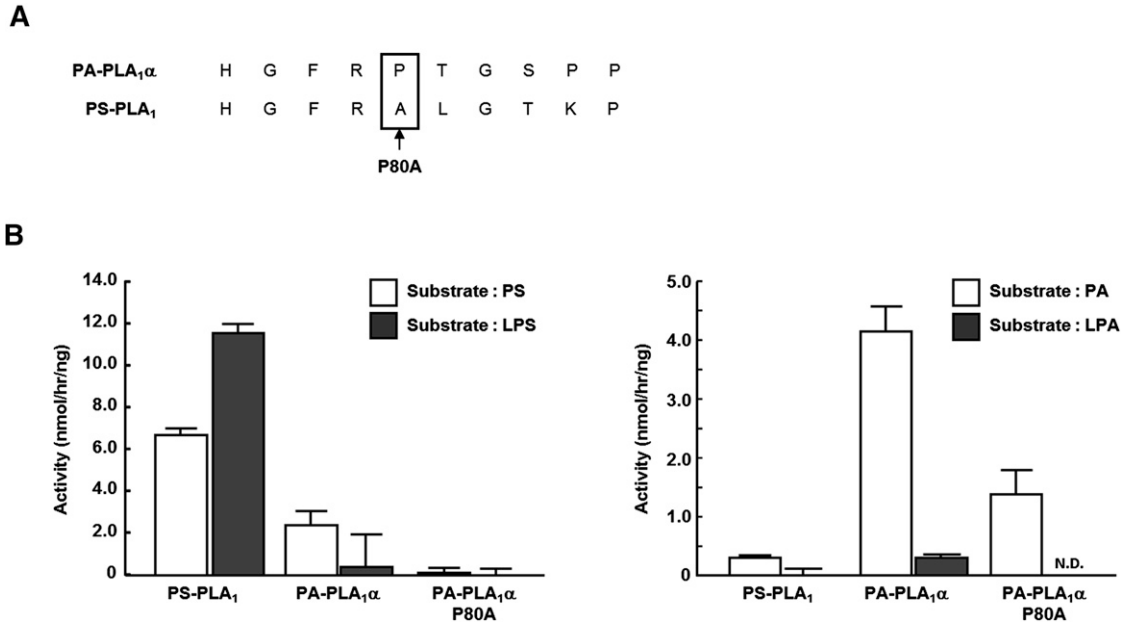

Fig. 8. Lysophospholipase activity of single amino acid PA-PLA$_1\alpha$ mutant. A: The position of single amino acid mutant is shown. B: Phosphatidylserine (PS), lysophosphatidylserine (LPS), phosphatidic acid (PA) and lysophosphatidic acid (LPA) were incubated at 37°C for several hours with conditioned media in 100 mM Tris-HCl (pH 7.5). Then, enzyme activity was determined by quantifying fatty acids liberated and exhibited as calculated enzyme activity per ng protein of PS-PLA$_1$, PA-PLA$_1\alpha$, or PA-PLA$_1\alpha$ P80A mutant. Bars indicate mean values and error bars indicate SD.

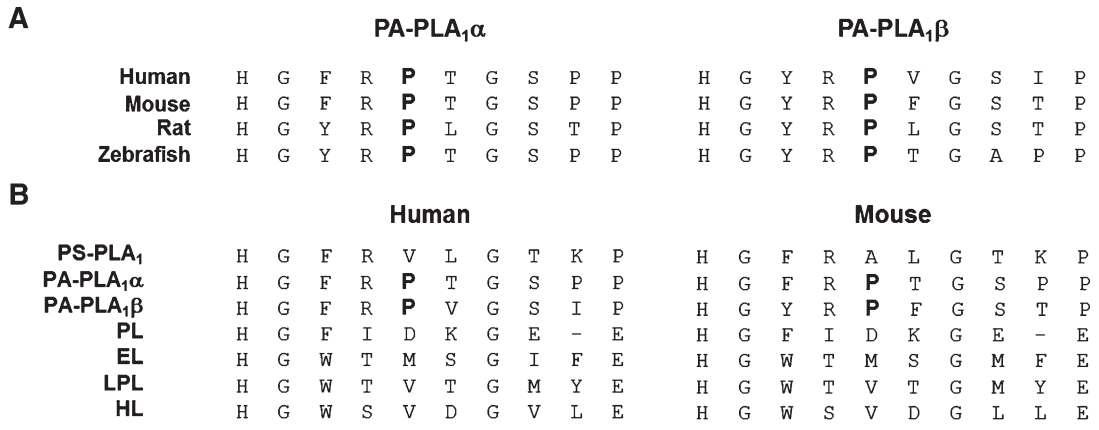

Fig. 9. Comparison of the amino acid sequences of PS-PLA$_1$, PA-PLA$_1$α, β and other lipases. A: Comparison of the amino acid sequences of β5 loop of PA-PLA$_1$α and PA-PLA$_1$β from human to zebrafish. The amino acid, Pro93 of mouse PA-PLA$_1$α, is completely conserved in PA-PLA$_1$α and β from several species. B: Comparison of the amino acid sequences of the β5 loop of PS-PLA$_1$, PA-PLA$_1$α, PA-PLA$_1$β, EL, LPL, and HL. The corresponding residue in other members of the pancreatic lipase family (PS-PLA$_1$, EL, LPL and HL) is not Pro.

the β5 loop of mouse PA-PLA$_1$α and β is completely conserved among PA-PLA$_1$α and β in other vertebrate species (**Fig. 9A**). In addition, the corresponding residue in other members of the pancreatic lipase family is not proline (Fig. 9B). Due to its ring structure, Pro 93 may affect the entire structure of the protein molecule. Taken together, this suggests that the conformation of the PA-PLA$_1$α β5 loop is altered by the presence of a proline residue, which as a result, does not allow the enzyme to hydrolyze lysophospholipid in PA-PLA$_1$α. Another possibility is that hydrophobic interaction has a role in exhibiting lysophospholipase activity. This idea is supported by the fact that the corresponding amino acid is valine and methionine in HL and EL, respectively (Fig. 9B), which also show lysophospholipase activity (16, 17).

PS-PLA$_1$ stimulates degranulation of mast cells and participates in the progression of allergy by producing LPS with fatty acid at the *sn*-2 position of the glycerol backbone (2-acyl-LPS) (25). 2-Acyl-LPS is known to be unstable and is readily converted to 1-acyl-LPS by the quick spontaneous acyl chain migration within a molecule, known as intra molecular acyl migration (26). Thus, it is reasonable to assume that PS-PLA$_1$ has a dual role: production and elimination of LPS. Namely, LPS (2-acyl-LPS) produced by PS-PLA$_1$ is degraded by the same enzyme after the acyl migration reaction occurs. Recent study has identified GPR34 as a cellular receptor for LPS (27). Our preliminary data suggested that 2-acyl-LPS was by far a better ligand for GPR34 than 1-acyl-LPS. This suggests that PS-PLA$_1$ is an activator of GPR34. Hydrolysis of both PS and 1-acyl-LPS by PS-PLA$_1$ may result in an increase in the relative amount of 2-acyl-LPS, which contributes the local action of LPS signaling. Recent studies have shown that PA-PLA$_1$α has a critical role in the formation of hair follicle by producing LPA and activating LPA receptor P2Y5/LPA$_6$ (14). Unlike the LPS system, LPA can be degraded by its dephosphorylation reaction catalyzed by lipid phosphate phosphatases (28). Because of the presence of ipid phosphate phosphatases, PA-PLA$_1$α may not need to have lysophospholipase activity while PS-PLA$_1$ has.

We are now in a position to evaluate the biological importance of the lysophospholipase activity of PS-PLA$_1$ because we have a way to separate its PLA$_1$ and lysophospholipase activities. A93P PS-PLA$_1$ mutant will be a useful tool because it shows only PLA$_1$ activity without any detectable lysophospholipase activity. We suppose that A93P PS-PLA$_1$ mutant is not capable of degrading LPS, thus leading to enhanced LPS-induced effects. These possibilities are now being tested in our laboratory.

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