C-terminal loop of *Streptomyces* phospholipase D has multiple functional roles

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(RECEIVED September 5, 2006; FINAL REVISION October 31, 2006; ACCEPTED November 1, 2006)

Abstract

We have recently shown that two flexible loops of *Streptomyces* phospholipase D (PLD) affect the catalytic reaction of the enzyme by a comparative study of chimeric PLDs. Gly188 and Asp191 of PLD from *Streptomyces septatus* TH-2 (TH-2PLD) were identified as the key amino acid residues involved in the recognition of phospholipids. In the present study, we further investigated the relationship between a C-terminal loop of TH-2PLD and PLD activities to elucidate the reaction mechanism and the recognition of the substrate. By analyzing chimeras and mutants in terms of hydrolytic and transphosphatidyl transfer activities, Ala426 and Lys438 of TH-2PLD were identified as the residues associated with the activities. We found that Gly188 and Asp191 recognized substrate forms, whereas residues Ala426 and Lys438 enhanced transphosphatidyl transfer and hydrolysis activities regardless of the substrate form. By substituting Ala426 and Lys438 with Phe and His, respectively, the mutant showed not only higher activities but also higher thermostability and tolerance against organic solvents. Furthermore, the mutant also improved the selectivity of the transphosphatidyl transfer activity. The residues Ala426 and Lys438 were located in the C-terminal flexible loop of *Streptomyces* PLD separate from the highly conserved catalytic HxKxxxxD motifs. We demonstrated that this C-terminal loop, which formed the entrance of the active well, has multiple functional roles in *Streptomyces* PLD.

Keywords: phospholipase D; *Streptomyces*; phospholipids; transphosphatidyl transfer activity

Phospholipase D (PLD, EC 3.1.4.4), which is present in mammals, plants, and bacteria (Exton 2002), catalyzes the hydrolysis of phospholipids to produce phosphatidic acid (PA) and alcohol moieties of phospholipids. Mammalian PLDs are activated in many cell types in response

to growth factors, hormones, and neurotransmitters (Liscovitch et al. 2000). Recently, it has been reported that PLD1 is associated with the amyloid precursor protein in Alzheimer's disease (Jin et al. 2006). PLD also catalyzes the transphosphatidyl transfer of phosphatidyl groups that produce various phosphatidyl alcohols; thus, this is a very useful reaction synthesizing rare natural phospholipids, such as phosphatidylserine (PS) and phosphatidylglycerol (PG), and novel artificial phospholipids (Juneja et al. 1987, 1989; Takami et al. 1994). Therefore, the elucidation of the catalytic reaction and the recognition of the substrate mechanism of PLD are important for understanding the biological properties of PLD.

Almost all PLDs contain two separate copies of the highly conserved HxKxxxxD (HKD) catalytic motif (Hammond et al. 1995; Koonin 1996; Ponting and Kerr 1996). Previous studies showed that the histidine residue of one HKD motif acts as a nucleophile that attacks the phosphate of the phosphodiester bond to form a

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Abbreviations: PLD, phospholipase D; RIBS, repeat-length independent and broad spectrum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PpNP, phosphatidyl-p-nitrophenol; TLC, thin-layer chromatography; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]; PEtOH, phosphatidylethanol; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; CMC, critical micelle concentration.

Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.062537907>.

phosphor-enzyme intermediate, and the histidine residue of another motif acts as a general acid that induces the protonation of the leaving group (Stanacev and Stuhne-Sekalec 1970; Bruzik and Tsai 1984; Raetz et al. 1987; Gottlin et al. 1998). In particular, *Streptomyces* PLDs show a higher transphosphatidylase activity than those from many other sources (Juneja et al. 1988; Hagishita et al. 2000). *Streptomyces* PLDs conserve three regions, in which two HKD motifs are involved, within the most compact structures among many sources (Iwasaki et al. 1994; Hammond et al. 1995; Waksman et al. 1996; Exton 2002). Therefore, *Streptomyces* PLDs are thought to be the most suitable model for studying the reaction mechanism among PLDs from other sources. Xie et al. (2000) and Iwasaki et al. (1999) showed that the two HKD motifs possessed different roles in the catalytic reaction using the N- and C-terminal halves of rat brain PLD1 and *Streptomyces* PLD. The crystal structure of PLD from *Streptomyces* sp. PMF (PMFPLD) indicates that a histidine residue in the N-terminal HKD motif acts as a nucleophile that attacks the phosphorus atom of the phospholipid substrate (Leiros et al. 2004). As mentioned above, interest in the relationship between the catalytic activity of *Streptomyces* PLDs and their primary structures has focused on HKD motifs from an experimental point of view.

Previously, using RIBS (repeat-length independent and broad spectrum) in vivo DNA shuffling (Mori et al. 2005), we found that the N-terminal HKD motif contained the nucleophile as determined by surface plasmon resonance analysis, and two flexible loop regions, residues 188–203 and 425–442, of *Streptomyces septatus* TH-2 (TH-2PLD) are related to catalytic reactions and substrate recognition (Uesugi et al. 2005). For the N-terminal loop region, it was shown that Gly188 and Asp191 are the key amino acid residues involved in reactions other than those in the two HKD motifs.

In this study, we further identified the key residues related to reactions for the C-terminal loop region (residues 425–442). To investigate the role of these identified residues in phospholipid recognition, we clarified the relationship between these residues and the catalytic reaction using several phospholipids. Furthermore, the effects of these residues on thermostability, tolerance against organic solvents, and selectivity in transphosphatidylase activity were evaluated.

Results

Comparison of activities of chimeras G to I

In a previous study (Uesugi et al. 2005), chimeras G and I were obtained using the *pldp* and *th-2pld* genes and RIBS in vivo DNA shuffling. This is a novel method of random

chimeragenesis based on the combined use of high-frequency deletion formation in the *E. coli ssb-3* strain with an *rpsL*-based chimera selection system using streptomycin (Mori et al. 2005). In our previous study, we found that two flexible loop regions, i.e., residues 188–203 and 425–442, of TH-2PLD are related to catalytic reactions and substrate recognition using chimeric PLDs from two homologous *Streptomyces* PLDs, TH-2PLD, and PLD from *Streptomyces* sp. (PLDP). In this study, we investigated the key residues that are related to reactions for the C-terminal loop region (residues 425–442: corresponding to chimeras G to I) (Fig. 1A). For the C-terminal loop region, chimera G differed from chimera I in 10 residues of the primary structure. This suggests that some of these residues are related to the difference in PLD activities. Therefore, we constructed six chimera G mutants (G-1 to G-6, the primary sequence of which is shown in Fig. 1A), expressed their genes and purified the protein products. The purities of the obtained chimeric PLDs are determined by SDS-PAGE (Fig. 1B). The purified chimeric PLDs were compared in terms of their transphosphatidylase and hydrolysis activities (Fig. 1C). All chimeric PLDs have hydrolysis activities similar to or higher than those of wild-type TH-2PLD and PLDP. However, chimera G and some chimera G mutants exhibited lower transphosphatidylase activities than two of the wild-type PLDs. By comparing the specific activities of the purified chimeric PLDs, the differences between the transphosphatidylase activities were larger than those between the hydrolysis activities. Interestingly, the transphosphatidylase activities of chimeras G-3 and G-4 were similar to that of chimera G, although those of chimeras G-1, G-2, G-5, and G-6 were higher than that of chimera G. We focused on the differences in the transphosphatidylase activities between chimeras G and G-1, chimeras G-4 and G-5, and chimeras G-6 and I. Thus, we assumed that three residues, Ala426, Ala432, and Lys438 of TH-2PLD, are related to transphosphatidylase activity.

Identification of amino acid residues related to PLD activities

To determine the contribution of each residue, we constructed seven chimera G mutants that had a substitution of one, two, or three candidate residues from the chimera G-type to the chimera I-type. Their primary sequences are shown in Figure 2A. These mutants were expressed, purified, and confirmed by SDS-PAGE as mentioned above. The purified chimera G mutants were evaluated in terms of their transphosphatidylase and hydrolysis activities (Fig. 2B). By comparison of specific activities, mutant G-F has the highest transphosphatidylase activity

Figure 1. Primary structures of chimeras G, I, chimera G mutants, and parental PLD, TH-2PLD, and PLDP, and their transphosphatidylase and hydrolytic activities. (A) The schematic primary structures of PLDs (*upper*) and amino acid sequence of the regions related to the catalytic reaction (*lower*) are shown. The regions derived from TH-2PLD and PLDP are indicated in gray and black, respectively. The numbers correspond to TH-2PLD in the amino acid sequence. (B) SDS-PAGE of purified chimera G mutants. Lane M indicates low-molecular-weight marker proteins (molecular weights, 94,000, 67,000, 43,000, 30,000, 20,100 and 14,400). Each sample was applied at 1.5 μ g per lane on a 15% acrylamide gel. The arrow indicates the position of purified PLDs. (C) Specific activities of PLDs in transphosphatidylase (*upper*) and hydrolytic (*lower*). Transphosphatidylase activity was measured at pH 5.5 with 13 mM PpNP. Hydrolytic activity was determined at pH 5.5 with 2 mM PpNP. Data are expressed as mean $\pm$ SD of three independent experiments.

among the single mutants. Among the double mutants examined, each showed a significantly higher activity than chimera G (*t*-test; $P < 0.0001$). In particular, mutant G-FH had 2.5-fold and twofold higher transphosphatidylase and hydrolysis activities than chimera G, respectively. The transphosphatidylase and hydrolysis activities of a triple mutant, G-FDH, were similar to those of mutant G-FH. Chimera G and mutant G-FH differed in two amino acid residues at 426 and 438. From these results, we assumed

that Ala426 and Lys438 of TH-2PLD are the key amino acid residues related to PLD activities.

Kinetic analysis of PLD activities

The effects of PpNP concentration on the transphosphatidylase and hydrolytic activities of PLDs were determined (Table 1). In both reactions, the k_{cat} values of mutant G-FH were higher than those of chimera G. However, the K_m

Figure 2. Identification of amino acid residues related to transphosphatidylase and hydrolytic activities. (A) Primary structures of chimera G, chimera G mutants, and chimera I. (B) Specific activities of chimera G, chimera G mutants, and chimera I in transphosphatidylase (upper) and hydrolytic (lower). Transphosphatidylase activity was measured at pH 5.5 with 13 mM PpNP. Hydrolytic activity was determined at pH 5.5 with 2 mM PpNP. Data are expressed as mean $\pm$ SD of three independent experiments.

values of mutant G-FH differed between the two reactions. In transphosphatidylase, the K_m of chimera G was 6.8-fold lower than that of mutant G-FH. On the other hand, in hydrolysis, these enzymes exhibited similar K_m values. Hence, the k_{cat}/K_m value of mutant G-FH was twofold lower than that of chimera G in transphosphatidylase; however, the k_{cat}/K_m value of mutant G-FH was twofold higher than that of chimera G in hydrolysis. These results suggest that mutant G-FH has different affinities toward emulsions and mixed-micelle with a low substrate concentration, and that at a high substrate concentration, mutant G-FH shows a high k_{cat} in both transphosphatidylase and hydrolysis.

PLD activities toward PC in different physical states

From the above results, chimera G and mutant G-FH show a large difference in PLD activities. Previously, we

assumed that the difference between the PLD activities of chimeras C and C-2 is attributed to the difference between the sensitivities of the chimeras to the substrate form (Uesugi et al. 2005). To evaluate the sensitivities of chimera G and mutant G-FH toward PC in different physical states, their activities were analyzed.

In water, synthetic short-chain phospholipids exist as monomers at lower concentrations below the critical micelle concentration (CMC), whereas they form micelles above the CMC. Long-chain phospholipids form vesicles or are soluble in mixed micelles with detergents (Lichtenberg et al. 1983). Bian and Roberts (1992) reported that pure diC₄PC has a CMC of about 278 mM, and diC₇PC has a CMC of about 1.46 mM. Here, we carried out an assay using 5 mM diC₄PC and diC₇PC; diC₄PC and diC₇PC existed as monomer and micelle forms, respectively.

As shown in Figure 3, mutant G-FH showed much higher activities than chimera G toward all substrate forms (*t*-test; $P < 0.0001$ to $P < 0.01$). These results suggest that N-terminal residues 188 and 191 affect the sensitivity to the substrate form, whereas C-terminal residues 426 and 438 affect catalytic activity regardless of the substrate form when the substrate concentration is sufficient. Each PLD showed the highest activity toward a micelle-forming substrate, and an extremely decreased activity toward vesicles (SUVs and LUVs). This phenomenon is in agreement with the results; the activity of PMFPLD was markedly reduced toward phospholipid vesicles compared with toward micelle-forming and monomer substrates (Yang and Roberts 2003).

Transphosphatidylase activity of PLDs toward different chain-length PCs

Next, we evaluated the transphosphatidylase activities of chimeras G and mutant G-FH toward different chain-length PCs using diC₇PC and POPC (Fig. 4). Mutant G-FH converted PC to PEtOH at an amount approximately twofold higher than that for chimera G toward both short-chain and long-chain PCs (*t*-test; $P < 0.01$ and $P < 0.001$, respectively). These results suggest that the difference in transphosphatidylase activity between chimera G and mutant G-FH is independent of the length of the phospholipid fatty acid chain.

Effects of temperature on PLD activities

The optimum temperatures and thermostabilities of chimera G and mutant G-FH are shown in Figure 5. Mutant G-FH had a higher optimum temperature than chimera G (Fig. 5A). Moreover, the thermostability of mutant G-FH increased above 5°C compared with that of chimera G (Fig. 5B). These results suggest that the substitutions of

Table 1. Kinetic parameters of transphosphatidylation and hydrolysis activities of PLDs toward PpNP and EtOH

	K_m (PpNP) (mM)	k_{cat} (PpNP) (sec^{-1})	k_{cat}/K_m (PpNP) ($\text{mM}^{-1} \text{sec}^{-1}$)	K_m (EtOH) (mM)
Transphosphatidylation ^a				
G	0.84 ± 0.06	113.7 ± 9.4	135.8 ± 17.6	79 ± 14
G-FH	5.69 ± 0.18	350.9 ± 10.7	61.7 ± 1.7	97 ± 15
Hydrolysis ^b				
G	0.104 ± 0.023	65.1 ± 2.3	647.8 ± 134.9	
G-FH	0.106 ± 0.009	133.8 ± 6.4	1260.2 ± 27.2	

^aThe transphosphatidylation assays of chimera G and mutant G-FH toward PpNP were performed with 1.8–20 mM PpNP at pH 5.5, and toward EtOH were performed with 0.11–1.20 M EtOH at pH 5.5.

^bThe hydrolysis assays of chimera G and mutant G-FH were carried out with 0.13–2 mM PpNP at pH 5.5. Data are expressed as mean $\pm$ SD.

Ala426 and Lys438 of chimera G with Phe and His, respectively, enhance both optimum temperature and thermostability. Interestingly, mutant G-FH showed an optimum temperature higher than its thermostable temperature, although chimera G showed the same optimum and thermostable temperatures.

Tolerance of PLDs against organic solvents

We determined the tolerance of PLDs against benzene and ethyl acetate (Fig. 6). This is because benzene is used in our transphosphatidylation assay and ethyl acetate is a common solvent in transphosphatidylation (Juneja et al. 1989; Takami et al. 1994; Hagishita et al. 1999). Moreover, thermostability is frequently associated with tolerance against organic solvents (Hatanaka et al. 2004). Both chimera G and mutant G-FH maintained their activities after incubation with benzene for 1 h (Fig. 6A). In the case of ethyl acetate, mutant G-FH mostly maintained its activity after 1 h of incubation (85%), whereas chimera G markedly lost its activity after 10 min of incubation (Fig. 6B). These results show that C-terminal residues 426 and 438 strongly affect tolerance against organic solvents.

Selectivity in transphosphatidylation

PLD-catalyzed transphosphatidylation is usually carried out in an emulsion system composed of a water–organic solvent (Hirche et al. 1997; Hagishita et al. 1999). Thus, this reaction is accompanied by the production of a PA by-product. Reaction selectivity is an important factor in industrial applications. We analyzed the amounts of PG and PA produced during transphosphatidylation from PC to PG by TLC. The relative amounts of the reaction products determined with chimera G and mutant G-FH are shown in Figure 7. After 10 min of reaction, mutant G-FH converted PC to PG at an amount approximately threefold higher than that for chimera G. In the case of mutant G-FH, the relative PG content reached 35% after 10 min, and increased with reaction time; most of the PC was transformed into PG after 3 h of reaction (Fig. 7B).

On the other hand, the PG content of chimera G was lower than that of mutant G-FH over a reaction time period from 10 min to 6 h (Fig. 7A). Moreover, the PA content of chimera G was approximately fourfold higher than that of mutant G-FH after 6 h of reaction. The PA content of chimera G was 16%, and was similar to that of TH-2PLD (21%, data not shown). These results suggest that mutant G-FH shows not only a higher activity but also a higher selectivity in terms of transphosphatidylation activity than chimera G.

Discussion

Previously, we found that the two regions corresponding to amino acid residues 188–203 and 425–442 of TH-2PLD are related to transphosphatidylation activity. We first focused on the N-terminal region of TH-2PLD (residues 188–203), and found that Gly188 and Asp191 of TH-2PLD are the key residues related to PLD activities (Uesugi et al. 2005).

Figure 3. Hydrolytic activities of PLDs toward phosphatidylcholine in monomers, micelle, and vesicles. The specific activities of chimera G and mutant G-FH were measured with 5 mM diC₄PC, diC₇PC, or 10 mM POPC in acetate buffer (pH 5.5). Data are expressed as mean $\pm$ SD of three independent experiments. The activities of mutant G-FH were significantly different from those of chimera G toward all substrate forms (* P < 0.01, ** P < 0.001, *** P < 0.0001, Student's t -test).

Figure 4. Transphosphatidylase activity of PLDs toward different chain-length phosphatidylcholines. The reaction was performed for 10 min using 10 mM diC₇PC or POPC in acetate buffer (pH 5.5) containing 4 mM CaCl₂, and then the resultant products were analyzed by TLC. Relative PLD activity was determined by measuring the intensity of the spot corresponding to phosphatidylethanol using *Scion Image* software, and indicated as chimera G activity toward diC₇PC or POPC. The relative activities of mutant G-FH were significantly different from those of chimera G toward diC₇PC and POPC (* $P < 0.01$, ** $P < 0.001$, Student's *t*-test).

In this study, we further investigated the residues of the C-terminal region of TH-2PLD (residues 425–442) related to PLD activities. The results suggest that Ala426 and Lys438 of TH-2PLD are the key residues related to both transphosphatidylase and hydrolytic activities. Although, chimera G and mutant G-FH only differed in these two residues (Fig. 2A), they exhibited considerably different activities (Fig. 2B; Table 1). We found that these C-terminal residues are important for the enhancement of the transphosphatidylase and hydrolytic activities. The mutant G-FH showed much higher activities than chimera G toward monomer-, micelle-, and vesicle-form substrates (Fig. 3). By the substitution of the two residues of chimera G with Phe and His, the mutant shows not only thermostability and tolerance against organic solvents but also a higher selectivity of transphosphatidylase than the original chimera G (Figs. 5–7). In hydrolysis, phospholipids exist in many different aggregated forms (Reynolds et al. 1991). Although the geometry of the aggregated substrates plays an important role in the reactions, the recognition mechanism of PLD toward the substrate form is still unclear. Regarding the difference in the selectivity of the transphosphatidylase between chimera G and mutant G-FH, two contributing factors are speculated. The first factor is the stability of emulsion in the reaction system. Pongcharoenkiat et al. (2002) reported that the variation in the surface charge of an emulsion by adding a small amount of PG stabilizes the emulsion. That is, it is considered that the stabilization of an emulsion by the addition of synthesized PG in transphosphatidylase leads to a more reactive reaction system; therefore, the differences between the properties

of these two PLDs tend to be amplified. The second factor is the recognition of particular phospholipids. Recently, the amount of PA formed by the side reaction of *Streptomyces* PLDs has been shown to differ significantly during transphosphatidylase from PC to PG (Sato et al. 2004). In this phenomenon, it is considered that the synthesized PG is hydrolyzed to PA during transphosphatidylase, suggesting that reaction selectivity differs among *Streptomyces* PLDs. Chimera G might hydrolyze PG more than PC similarly to TH-2PLD. Moreover, a Phe residue corresponding to Ala426 of TH-2PLD is highly conserved in all the determined *Streptomyces* PLDs except TH-2PLD, although at Lys438 of TH-2PLD, four of them have a Lys residue and the rest have a His residue. Thus, the main cause of the low selectivity of TH-2PLD seems to be Ala426 in transphosphatidylase.

Leiros et al. (2000, 2004) elucidated the crystal structure of PLD from *Streptomyces* sp. PMF. Based on

Figure 5. Effects of temperature on hydrolytic activities of PLDs. (A) The optimum temperature for PLD hydrolytic activity was determined for 10 min at pH 5.5. Relative PLD activity was calculated with respect to the maximum PLD activity. (B) The thermostability of PLDs was measured for 10 min at pH 5.5. Residual PLD activity was calculated as a ratio to a sample at 15°C. Data are expressed as mean $\pm$ SD of three independent experiments.

Figure 6. Tolerance of PLD against organic solvents. The time course of PLD activity was measured using 50% benzene (A) or ethyl acetate (B). Residual enzyme activity was calculated as a ratio to a sample without an organic solvent. The residual activity of mutant G-FH was significantly different from that of chimera G with ethyl acetate (* $P < 0.0001$, Student's t -test).

this crystal structure, the local environment around key residues related to the catalytic reactions (Gly188, Asp191, Ala426, and Lys438 of TH-2PLD) is shown in Figure 8. These key residues are located in two flexible loops apart from two catalytic HKD motifs. Two flexible loops, in coordination with each loop, form the entrance of the active well consisting of two HKD motifs. It is reasonable to consider that these residues play a role in the recognition of phospholipids from a geometrical view point. Residues 191 and 438 of TH-2PLD were located at the entrance of the predicted pocket for the recognition of phospholipids, and were exposed to a solvent. On the other hand, residues 188 and 426 were located inside the enzyme. These residues were reported to be thermostability related residues (Mori et al. 2005; Negishi et al. 2005). This result suggests that residues 188 and 426 participate in conformational stability. It agrees well with the results that mutant G-FH has a higher thermostability and tolerance against organic sol-

vents than chimera G (Figs. 5,6). Considering the above-mentioned results, we speculate that residues 426 and 438 of TH-2PLD have different roles from residues 188 and 191. Thus, we consider that residues 188 and 191 are involved in the sensitivity of *Streptomyces* PLD to the physical state of the substrate, and residues 426 and 438 participate in the enhancing enzyme activities, enzyme stability, and selectivity of transphosphatidylolation. Therefore, we consider that residues 426 and 438 play more prominent roles than residues 188 and 191 in the catalytic reaction and recognition of phospholipids. It might be possible to change the property of *Streptomyces* PLD by substituting the two residues. In fact, we previously showed that the substitution of residue Ala 426 of TH-2PLD with other residues, such as Gly, Phe, and His, led to this PLD having a much higher thermostability than the wild-type TH-2PLD (K. Mori, T. Mukaiharu, Y. Uesugi, M. Iwabuchi,

Figure 7. Selectivities of chimera G (A) and mutant G-FH (B) in transphosphatidylolation from POPC to POPG. The reaction was performed using 10 mM POPC with 0.5 M glycerol in acetate buffer (pH 5.5) containing 4 mM CaCl_2 for 10 min, 1 h, 3 h, and 6 h, followed by TLC. Relative contents were determined by measuring the intensities of the spots corresponding to phosphatidic acid, phosphatidylglycerol, and phosphatidylcholine using Scion Image software.

Figure 8. (A) Overall structure of TH-2PLD using Swiss-PDB viewer based on crystal structure of PMFPLD. The two regions related to enzyme activities are indicated in green (residues 188–203 of TH-2PLD) and orange (residues 425–442 of TH-2PLD). The identified key residues are indicated in red. The N- and C-terminal HKD motifs are shown in light blue and purple, respectively. (B) The local environment around the identified key residues (188, 191, 426, and 438 of TH-2PLD) is represented. The identified key residues are indicated in red. The light-green circle indicates the predicted pocket for the recognition of phospholipids.

and T. Hatanaka, unpubl.). However, we do not examine the effects of the substitution of Lys 438 with other residues on enzymatic properties. We will investigate the effects of the substitution of these residues with other amino acid residues on phospholipid recognition in the future.

Chimera G and mutant G-FH have C-2-type amino acid residues at 188 and 191. However, they show transphosphatidyltransferase and hydrolysis activities regardless of the substrate form differently from chimera C-2. Unfortunately, we still cannot explain this difference, but we are certain that the two flexible loops affect each other regarding their multiple functions because of their opposite locations.

In summary, we demonstrated that the C-terminal flexible loop of *Streptomyces* PLD (residues 425–442), which are separate from two highly conserved catalytic HKD motifs and formed at the entrance of the active well,

has multiple functional roles. In the future, we will further analyze the relationship between the identified residues and the recognition of several phospholipids, such as phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, and phosphatidylethanol.

Materials and methods

Materials

pETKmS2 (Mishima et al. 1997) was kindly provided by Dr. Tsuneo Yamane (Nagoya University, Japan). Phosphatidyl-*p*-nitrophenol (PpNP) was prepared from soybean phosphatidic acid and *p*-nitrophenol according to the procedure of D'Arrigo et al. (1995). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti. 1,2-Dibutyroyl-*sn*-glycero-3-phosphocholine (diC₄PC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (diC₇PC) were purchased from Sigma. All the other chemicals used were of the highest grade.

Comparison of primary structures between chimeras G and I

As described previously (Uesugi et al. 2005), chimera G was obtained from the random chimeragenesis of the *pldp* and *th-2pld* genes using RIBS in vivo DNA shuffling. Chimera I (recombination site: corresponding to the *th-2pld* gene, nucleotides 1326–1340) was constructed using the Van91I site (corresponding to the *th-2pld* gene, nucleotide 1329–1339), which is adjacent to the C-terminal HKD motif. The plasmid pETKmS2(*pldp*) was digested with PstI and Van91I. The plasmid pATIS4b(G) was digested with Van91I and BamHI. The fragments were ligated into the PstI–BamHI gap of the vector pATIS4b(G) to construct the expression vector. The obtained plasmid was confirmed by DNA sequencing. The amino acid-based primary structures of these chimeras are shown in Figure 1A.

Construction of chimera G and I mutants

To identify the amino acid residues related to PLD activities, we constructed chimera G and I mutants by PCR amplification. To prepare the mutants (G-1, G-2, G-3, and G-F), the following four mutagenic sense primers, in which the NheI site (underlined) was substituted with a silent mutation, were synthesized; 5'-CCTCCAGCT(C→A)GCC(A→G, Thr→Ala)CC(GC→TT, Ala→Phe)CCG-3' (corresponding to the chimera G gene, nucleotides (nt) 1299–1319), 5'-CAGCT(C→A)GCC(A→G, Thr→Ala)CC(GC→TT, Ala→Phe)CCG(T→C)(AGC→GCG, Ser→Ala)(T→G, Ser→Ala)CCG-3' (corresponding to the chimera G gene, nt 1303–1327), 5'-CAGCT(C→A)GCC(A→G, Thr→Ala)CC(GC→TT, Ala→Phe)CCG(T→C)(AGC→GCG, Ser→Ala)(T→G, Ser→Ala)CC(GA→CC, Asp→Pro)CAGCGCC-3' (corresponding to the chimera G gene, nt 1303–1335), and 5'-CCTCCA GCT(C→A)GCCACC(GC→TT, Ala→phe)CCG-3' (corresponding to the chimera G gene, nt 1299–1319), respectively. The target mutation was introduced with the primer sets of 5'-TGGT GGTGGTGTCTCGAGTGC GGC-3' (an antisense primer, corresponding to part of the *His6* tag sequence) and each of the mutagenic primers using the GC-RICH PCR system. The partial

pldp gene was amplified by PCR with a combination of a sense primer 5'-ATGACCACCGCCAAGACCTCC-3' (corresponding to the chimera G gene, nt 499–519) and an antisense primer (5'-GAAGCGGCTAGCTGGAGGTTTC-3' for the silent mutation of the NheI site (underlined), corresponding to the nucleotide sequence from the chimera G gene, nt 1295–1317). The amplified DNA fragments were cloned into the T-vector pGEM-TEasy (Promega), and the resultant plasmids were confirmed by DNA sequencing. The plasmids representing G-1, G-2, G-3, and G-F were digested with NheI and BamHI. The plasmid containing the NheI site was digested with AgeI and NheI. The fragments were ligated into the AgeI–BamHI gap of the vector pETKmS2(*pldp*) to construct the expression vector.

To construct mutant G-FH, the mutagenic gene was amplified by PCR with a combination of the sense primer 5'-CCTCCAGCT(C→A)GCCACC(GC→TT, Ala→phe)CCG-3' (corresponding to the chimera G gene, nt 1299–1319) and the antisense primer 5'-TGCGGTACGG(CTT→GTG, Lys→His)GCCGTCG-3' (corresponding to the chimera G gene, nt 1344–1364). The amplified DNA fragment was cloned into the T-vector pGEM-TEasy (Promega), and the resultant plasmids were confirmed by DNA sequencing. The plasmid representing G-FH was digested with NheI and BsiWI. The plasmid containing the NheI site mentioned above was digested with AgeI and NheI. The fragments were ligated into the AgeI–BsiWI gap of the vector pETKmS2(*G-F*) to construct the expression vector.

To prepare the mutants (G-4, G-5, G-6, and G-H), the following four mutagenic antisense primers were synthesized: 5'-CGCGTACGG(GTG→CTT, His→Lys)GCCGTCGGCCCA(GG→CT, Thr→Lys)TG(TC→GC, Asp→Ala)G-3' (corresponding to the chimera I gene, nt 1332–1362), 5'-CGCGTACGG(GTG→CTT, His→Lys)GCCGTCGGCCCA(GG→CT, Thr→Lys)TGTCGCC-3' (corresponding to the chimera I gene, nt 1330–1362), 5'-TGCGGTACGG(GTG→CTT, His→Lys)GCCGTGCG-3' (corresponding to the chimera I gene, nt 1344–1364), and 5'-TGCGGTACGG(CTT→GTG, Lys→His)GCCGTCG-3' (corresponding to the chimera G gene, nt 1344–1364), respectively. The target mutation was introduced with the sets of the sense primer 5'-ATGACCACCGCCAAGACCTCC-3' (corresponding to the chimera G gene, nt 499–519) and each of the mutagenic primers described above using the GC-RICH PCR system. The amplified DNA fragments were cloned into the T-vector pGEM-TEasy (Promega), and the resultant plasmids were confirmed by DNA sequencing. The plasmids representing G-4, G-5, G-6, and G-H were digested with AgeI and BsiWI. The plasmid pUC19(*th-2pld*) was digested with BsiWI and BamHI. The fragments were ligated into the AgeI–BamHI gap of the vector pETKmS2(*pldp*) to construct the expression vector.

To prepare the other mutants (G-D, G-FD, G-DH, and G-FDH), the following two mutagenic antisense primers were synthesized: 5'-CGCGTACGGCTTGCCGTCGGCCCACTTG(G→T, Ala→Asp)CGC-3' (corresponding to the chimera G gene, nt 1331–1362) for G-D and G-FD, and 5'-CGCGTACGG(CTT→GTG, Lys→His)GCCGTCGGCCCACTTG(G→T, Ala→Asp)CGC-3' (corresponding to the chimera G gene, nt 1331–1362) for G-DH and G-FDH. By using the GC-RICH PCR system, the target mutation was introduced with the sets of the sense primer 5'-CGTCGGCATCCAGAGCGTCGA-3' (corresponding to the chimera G gene, nt 846–866) and each of the mutagenic primers described above with pATIS4b(*G*), pETKmS2(*G-F*), pETKmS2(*G-H*), and pETKmS2(*G-FH*) as templates for G-D, G-FD, G-DH, and G-FDH, respectively. The amplified DNA fragments were cloned into the T-vector pGEM-TEasy (Promega), and the resultant plasmids were

confirmed by DNA sequencing. The plasmids representing G-D, G-FD, G-DH, and G-FDH were digested with EcoO109I and BsiWI. The plasmid pUC19(*pldp*) was digested with PstI and EcoO109I. The fragments were ligated into the PstI–BsiWI gap of the vector pETKmS2(*G-F*) to construct the expression vector.

Expression and purification of PLDs

Escherichia coli BL21-Gold(DE3) (Invitrogen) was transformed with PLD expression plasmids. The expression of PLDs was carried out according to a method described previously (Hatanaka et al. 2002a) in the presence of appropriate antibiotics (chloramphenicol [50 µg/mL] and kanamycin [50 µg/mL]) for strains harboring plasmids obtained from the random chimeragenesis and plasmids derived from pETKmS2, respectively) except for the addition of the premixed protease inhibitor tablet Complete Mini, EDTA-free (Roche) at the induction period (one tablet per culture). Cultures were centrifuged for 60 min at 3500 × *g*. The culture broth was concentrated with an ultrafiltration device using Amicon Ultra (Millipore, 30-kDa cut), and dialyzed against 20 mM Tris–HCl (pH 8.0). His-tagged PLDs were purified using Magextractor-His Tag (TOYOBO). The purified PLDs were dialyzed against 10 mM acetate buffer (pH 5.5) and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). The amounts of purified PLDs were ~50% of the total activities.

Assay of PLD activities using PpNP

Hydrolytic activity was determined on the basis of the hydrolytic activity of PpNP. The procedure was similar to the method described previously (Hatanaka et al. 2002b). PLD-catalyzed transphosphatidylolation activity was determined by measuring the production of *p*-nitrophenol from PpNP and ethanol according to the method that used a biphasic system consisting of benzene and water as described previously (Hatanaka et al. 2002b). One unit of PLD was defined as the amount of the enzyme that releases 1 µmol of *p*-nitrophenol per minute under the assay conditions. The PLDs used in this study showed both hydrolysis and transphosphatidylolation activities in the absence of Ca²⁺. The kinetic assays of both activities were carried out as described previously (Hatanaka et al. 2002b). In the assay of hydrolytic activity, the reactions were carried out in 1.5-mL cuvettes; the 1-mL reaction mixture consisted of PpNP at final concentrations ranging from 0.11 to 2 mM in 0.1 M acetate buffer (pH 5.5) and the purified PLDs. In the assay of transphosphatidylolation activity, the reactions were performed for 10 min in 1.5-mL sample tubes; the 400-µL reaction mixture consisted of PpNP at final concentrations ranging from 1.8 mM to 20 mM, and ethanol at a final concentration of 800 mM, in an emulsion containing benzene and acetate buffer (pH 5.5). The purified PLDs (200 ng) were used in the assay.

Preparation of phospholipids

Appropriate aliquots of diC₄PC, diC₇PC, and POPC dissolved in chloroform were evaporated and further dried under vacuum for at least 3 h. The lipids were hydrated at 10 mM for diC₄PC and diC₇PC, or at 20 mM for PC in 0.1 M acetate buffer (pH 5.5). Then, POPC vesicles were prepared by the extrusion method (MacDonald et al. 1991) using a Lipofast extruder (Avestin).

POPC suspensions were vortexed vigorously and frozen, and thawed 10 times in liquid nitrogen. The multilamellar vesicles were passed 30 times through polycarbonate filters (50-nm or 100-nm pore diameter) using an extruder, to obtain small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs).

Hydrolytic activities toward PC in different physical states

Specific activities toward PC in monomers, micelles, and vesicles were measured by the method using choline oxidase and peroxidase (Nakajima et al. 1994) with several modifications. Purified PLD concentration was adjusted to 0.004 mg/mL with 0.1 M acetate buffer (pH 5.5). Thirty-four microliters of the enzyme solution was added to 136 μ L of 5 mM diC₄PC, diC₇PC, or 10 mM POPC (SUVs, LUVs) in 0.1 M acetate buffer (pH 5.5) containing 4 mM CaCl₂. The reaction mixture was incubated at 37°C for 10 min, and then the reaction was stopped by heat treatment (98°C, 1 min). After centrifugation, 50 μ L of the supernatant was added to 750 μ L of a mixture containing 0.1 M Tris-HCl (pH 8.0), 1.7 U/mL choline oxidase, 6 U/mL peroxidase, 0.5 mM 4-aminoantipyrine, and 21 mM phenol, followed by incubation at 37°C for 10 min. The amount of choline liberated was estimated by measuring the absorbance of the solution at 505 nm.

Transphosphatidylolation activity analyzed by thin-layer chromatography (TLC)

PLD-catalyzed transphosphatidylolation using diC₇PC or POPC as the substrate was analyzed by TLC. The reaction conditions were similar to those mentioned above except for the use of 0.1 M acetate buffer (pH 5.5) containing 4 mM CaCl₂. After centrifugation, the organic layer of the reaction mixture was applied to a TLC plate (Silica gel 60 F₂₅₄, Merck) and developed with a mixture containing chloroform: methanol: water (30:10:1 [v/v]). Lipids on the TLC plate were detected by spraying with the Dittmer-Lester reagent (Dittmer and Lester 1964) or 5% sodium phosphomolybdate/ethanol solution for diC₇PC or POPC, respectively. Then, the plate was dried and scanned. The intensity of the spot corresponding to phosphatidylethanol (PEtOH) was analyzed using *Scion Image* software. Relative PLD activity was calculated as chimera G activity toward diC₇PC or POPC.

Optimum temperature test

The optimum temperature of PLD was determined by a method reported previously on the basis of the hydrolytic activity for PpNP with appropriate heat treatment (Hatanaka et al. 2004). Purified PLD concentration was adjusted to 0.001–0.002 mg/mL with 0.1 M acetate buffer (pH 5.5). Ten microliters of the enzyme solution was added to 90 μ L of 1 mM PpNP in 0.1 M acetate buffer (pH 5.5) and incubated at an appropriate temperature for 10 min. After incubation, the reaction mixture was heated at 98°C for 1 min to stop the enzyme reaction. Then, 30 μ L of the reaction mixture was added to 170 μ L of 0.1 M Tris-HCl (pH 8.0) in a well of a 96-well microtiter plate. Hydrolytic activity was determined by measuring the absorbance at 405 nm using a microplate reader. Relative hydrolytic

activity was calculated with respect to the maximum hydrolytic activity.

Thermostability test

Purified PLD concentration was adjusted to 0.001–0.002 mg/mL with 0.1 M acetate buffer (pH 5.5). The enzyme solution was incubated at an appropriate temperature for 10 min. After incubation, 10 μ L of the enzyme solution was added to 90 μ L of 1 mM PpNP in 0.1 M acetate buffer (pH 5.5). Then, the reaction mixture was incubated at 37°C for 10 min and heated at 98°C for 1 min to stop the enzyme reaction. Thirty microliters of the reaction mixture was added to 170 μ L of 0.1 M Tris-HCl (pH 8.0) in a well of a 96-well microtiter plate. Residual PLD activity was determined by measuring the absorbance at 405 nm using a microplate reader. Residual PLD activity was calculated as a ratio to a sample at 15°C.

Tolerance of PLD against organic solvent

PLD concentration was adjusted to 0.001–0.002 mg/mL with 0.1 M acetate buffer (pH 5.5). The enzyme solution was incubated with an equal volume of benzene or ethyl acetate at 37°C for 0–60 min. After incubation, the mixed solution was centrifuged, and 10 μ L of the resultant aqueous layer was used for the enzyme reaction under the same conditions used in the thermostability test. Residual enzyme activity was calculated as a ratio to a sample without an organic solvent.

Selectivity in transphosphatidylolation

The progress of PLD-catalyzed transphosphatidylolation from PC to PG was monitored by TLC. The reaction conditions were similar to those mentioned above using 10 mM POPC except for the use of acetate buffer (pH 5.5) containing 0.5 M glycerol and 4 mM CaCl₂. After centrifugation, the organic layer of the reaction mixture was applied to a TLC plate (Silica gel 60 F₂₅₄, Merck) and developed using a mixture containing chloroform: methanol: acetic acid (70:30:8 [v/v]). Lipids on the TLC plate were detected by spraying with 5% sodium phosphomolybdate/ethanol solution. Then, the plate was dried and scanned. The intensities of the spots corresponding to POPG, POPA and POPC were analyzed using *Scion Image* software.

Statistical analysis

All statistical analyses were performed using unpaired Student's *t*-test. All data are presented as mean $\pm$ standard deviation (SD) of at least three determinations.

Acknowledgments

This research was financially supported by the Sasakawa Scientific Research Grant from The Japan Science Society.

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