Structural elements of *Trimeresurus flavoviridis* serum inhibitors for recognition of its venom phospholipase A_2 isozymes

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Abstract Five inhibitors (PLI-I–V) against *Trimeresurus flavoviridis* (*Tf*, habu snake, Crotalinae) venom phospholipase A_2 (PLA_2) isozymes have been isolated from its serum. PLI-I, which is composed of two repeated three-finger motifs, and PLI-IV and PLI-V, which contain a sequence similar to the carbohydrate recognition domain (CRD) of C-type lectins, were expressed in the forms fused with glutathione *S*-transferase (GST). The resulting GST-PLIs showed ability to bind to three *Tf* venom PLA_2 isozymes. The binding study with the truncated forms indicated that one of two three-finger motifs of PLI-I was able to bind to PLA_2 isozymes. The N-terminal 37-amino acid fragment and the CRD-like domain of PLI-IV and PLI-V were bound to PLA_2 isozymes. On the other hand, their C-terminal 12-amino acid segment also associated with PLA_2 isozymes. When either of two units of a hydrophobic tripeptide in this sequence was replaced by trialanine, the binding was completely abolished, indicating that the C-terminal hydrophobic cores of PLI-IV and PLI-V were critically responsible for the binding to venom PLA_2 isozymes.

Key words: Snake venom phospholipase A_2 isozyme; Serum phospholipase A_2 inhibitor; Three-finger motif; Carbohydrate recognition domain; Binding study

1. Introduction

Phospholipase A_2 (PLA_2, EC 3.1.1.4), which is one of the major enzymes and often forms an isozyme family in snake venoms, catalyzes the hydrolysis of the 2-acyl ester bond of 3-*sn*-phosphoglycerides to produce fatty acids and lysophospholipids. Snake venom PLA_2s are known to exhibit a variety of physiological functions [1–5]. PLA_2 inhibitors (PLIs), proteins capable of inhibiting snake venom PLA_2s, have been isolated from various snake sera and their primary structures have been determined [6–12]. PLIs can be divided into two groups based on their structural characteristics. One group contains a segment similar to the carbohydrate recognition domain (CRD) of Ca^{2+}-dependent (C-type) lectins [13,14] and the other has two repeats of a unit termed the three-finger motif [11], which has also been found in urokinase-type plasminogen activator receptor [15], ly-6 [16], CD59 [17] and neurotoxins [18].

Five *Trimeresurus flavoviridis* (*Tf*, habu snake, Crotalinae) serum proteins, named PLI-I–V, that bind to its venom PLA_2 isozymes were obtained through affinity chromatography [9]. They are all glycoproteins [9]. PLI-I is a major component of inhibitory proteins against three *Tf* venom basic PLA_2 isozymes, that is, PLA-B (a basic [Asp^{49}]PLA_2) (Y. Yamaguchi, unpublished) and BPI [19] and BPII [20] (both [Lys^{49}]PLA_2s), and is composed of two units of the three-finger motif. The functions of three-finger motifs in different proteins appear to differ from one another [21–23]. PLI-IV and PLI-V [7] are known to bind to a major [Asp^{49}]PLA_2 [24], termed PLA2, which is less basic than PLA-B. They are similar in sequence and have a CRD-like domain [7]. Recently, we cloned cDNAs and genes encoding PLI-I [9], PLI-VI and PLI-V [25] and determined their nucleotide sequences. Exons 3 of genes encoding PLI-IV and PLI-V are exceptionally abundant in nonsynonymous nucleotide substitutions causing amino acid changes.

The mutagenesis study of erabutoxin a, a neurotoxin from *Laticauda semifasciata* venom, with a three-finger motif revealed that $\sim$10 amino acid residues involved in three loops are functionally important for the binding to acetylcholine receptors [26,27]. To date, however, there is no structural information in terms of the interactions between the three-finger motif and PLA_2. The CRD was originally defined as a functional unit in C-type lectins responsible for the binding to carbohydrate [13,14]. PLA_2 receptors were isolated from various mammalian tissues [28–31] and their structural similarity to mannose receptors was noted because they have eight tandem repeats of CRDs which involve the cysteine and tryptophan residues at conserved positions [29]. PLA_2 receptors can bind pancreatic group I PLA_2 and secretory group II PLA_2 which are not glycosylated [28,31]. Although interactions occur between the CRD-like domain of PLA_2 receptors and PLA_2 [30], little is known about the particular region of the CRD-like domain which is responsible for the binding to PLA_2.

The present study was focused on the three-finger motifs of PLI-I and the CRD-like domain of PLI-VI and PLI-V to determine the structural elements required for the binding to *Tf* venom PLA_2 isozymes. For this purpose, these serum inhibitors and their truncated forms were expressed as glutathione *S*-transferase (GST)-fused proteins or peptides in *Escherichia coli*. The interactions between GST-fused proteins or peptides and PLA_2 isozymes immobilized on magnetic beads

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Abbreviations: PLA_2, phospholipase A_2; PLI, PLA_2 inhibitor; *Tf*, *Trimeresurus flavoviridis*; GST, glutathione *S*-transferase; CRD, carbohydrate recognition domain; PCR, polymerase chain reaction

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were assessed by means of in vitro immunoblot analysis using anti-GST antibody.

2. Materials and methods

The DNA encoding full-length PLI-I was amplified by polymerase chain reaction (PCR) of its cloned cDNA and inserted into a GST gene-fused vector, pGEX4T-1 (Pharmacia), at *Eco*RI sites. The PCR-amplified DNA, which encodes the fragment of positions 96–181 of PLI-I, termed I(96–181), was inserted into *Eco*RI or *Bam*HI/*Eco*RI sites of the pGEX4T-1 vector. DNAs encoding full-length PLI-IV and PLI-V were amplified by PCR of their cloned cDNAs and inserted into the pGEX4T-1 vector at *Eco*RI sites. The DNA fragments encoding IV(38–147), IV(38–84), IV(38–126) and IV(127–147) which are common in sequence between PLI-IV and PLI-V were cloned into the pGEX4T-1 vector at *Eco*RI or *Bam*HI/*Eco*RI sites. Synthetic oligonucleotides encoding IV(1–13), IV(127–135) and IV(136–147) which are also identical between PLI-IV and PLI-V were cloned into the pGEX4T-1 vector at *Bam*HI/*Eco*RI sites. The DNA fragments encoding IV(1–37), V(1–37), IV(14–37) and V(14–37) in which amino acid substitutions occur between PLI-IV and PLI-V were prepared by PCR and ligated into the pGEX4T-1 vector at *Eco*RI or *Bam*HI/*Eco*RI sites. A mutant of IV(136–147) carrying trialanine in place of Val-Leu-Leu at positions 137–139, represented as IV(136–147:137AAA139), was produced by introducing the corresponding synthetic oligonucleotide into the pGEX4T-1 vector at *Bam*HI/*Eco*RI sites. IV(136–147:143AAA145) with trialanine instead of Phe-Tyr-Phe was also prepared.

E. coli DH5α harboring expression plasmid was incubated overnight at 37°C in 5 ml L-broth medium containing 100 μg/ml ampicillin. The culture was inoculated into 200 ml L-broth or Terrific broth medium and incubated at 37°C for 3 h. Isopropyl-β-D-thiogalactopyranoside (0.1 mM concentration) was added to the medium and incubation was continued at 25°C for 12 h. The cells were collected by centrifugation, resuspended in 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.1% Nonidet P-40 and sonicated with a Branson Sonifier Model 200 (Danbury). After centrifugation, the supernatant was applied onto a glutathione-Sepharose 4B column with the same buffer. The matrix was collected by centrifugation and washed four times with 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1% Nonidet P-40. The fusion protein bound to the matrix was eluted with 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 10 mM reduced glutathione.

To study the interaction of PLA_2 isozymes with GST-fused PLIs and their fragments, each of the PLA_2 isozymes was conjugated chemically to magnetic beads (MPG Long Chain Alkylamino, CPG) according to the manufacturer's protocol. Three *Tf* venom PLA_2 isozymes, PLA2, BPI and BPII, were fractionated from its lyophilized crude venom via three successive chromatographies [19,20,25]. Magnetic beads were pretreated in 5% glutaraldehyde and 10 mM phosphate buffer (pH 7.5) for 1.5 h at room temperature. After washing the beads (1.5 mg) five times with 10 mM phosphate buffer (pH 7.5), PLA_2 isozyme (200 μg) was added and incubated at 4°C for 1.5 h in 10 mM phosphate buffer (pH 7.5) containing 0.02% $NaCNBH_3$ (total 1.0 ml). After removal of the supernatant, the beads were further incubated in 10 mM phosphate buffer (pH 7.5) containing 0.02% $NaCNBH_3$ and 0.75% glycine for 1 h at 4°C and then washed five times with 10 mM phosphate buffer (pH 7.5) containing 1 M NaCl. The GST-fused proteins and their fragments were incubated with PLA_2-conjugated beads in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 0.5% bovine serum albumin for 1.5 h at room temperature. The beads were then washed four times with the same buffer containing 0.1% Nonidet P-40 to remove unbound proteins. The proteins liberated from the enzyme-conjugated beads with 0.1 M acetic acid were run on 12.5% SDS-PAGE under reducing condition and transferred onto PVDF membrane. The membrane was incubated in 25 mM Tris-HCl (pH 7.6) containing 500 mM NaCl, 0.05% Tween-20, 0.05% NaN_3 and 5% non-fat dry milk for 1.5 h at room temperature. The membrane was then treated with anti-GST monoclonal antibody (a generous gift from Dr. Hideki Sumimoto of Kyushu University) in the same buffer for 1 h at room temperature. After washing three times with the buffer without dry milk, the membrane was stained with alkaline phosphatase-conjugated anti-IgG antibody.

3. Results and discussion

After purification of *Tf* serum PLI-I–V, the nucleotide sequences of three cDNAs encoding PLI-I, PLI-IV and PLI-V were determined [9,25]. For assay of the interactions between *Tf* serum PLIs and its venom PLA_2 isozymes, PLI-I, PLI-IV and PLI-V were expressed in *E. coli* as GST-fused proteins. GST-fused PLIs bound to PLA2, BPI and BPII immobilized on magnetic beads were detected by anti-GST monoclonal antibody as described in Section 2. Magnetic beads alone did not show any affinity to GST-fused PLIs (data not shown). GST-fused PLI-I, PLI-IV and PLI-V were bound to three PLA_2 isozymes, PLA2, BPI and BPII (Figs. 1 and 2). No binding occurred for GST alone. When an excess amount of non-conjugated PLA2, for example, was added, GST-fused PLIs failed to bind to conjugated PLA2 (data not shown), indicating that the binding between immobilized PLA_2 isozymes and GST-fused PLIs is specific. The binding modes to BPII of all the proteins and peptides tested here were identical to those to BPI (or PLA2), so that all the data for BPII are omitted hereafter except in Fig. 1.

Our previous study indicated that each of PLA_2 isozymes forms complex with plural different PLIs [9]. The present results showed that one inhibitor protein alone can interact directly with each of PLA_2 isozymes. Although native PLI-I, PLI-IV and PLI-V are glycoproteins, GST-fused PLIs expressed in *E. coli* have no carbohydrate. However, recombinant PLIs can interact with PLA_2 isozymes, so that the carbohydrate moiety of PLI-I, PLI-IV and PLI-V is not necessarily crucial for recognition of PLA_2 isozymes.

For evaluation of the binding abilities of PLI-I segments, they were expressed as GST-fused forms in *E. coli*. A segment containing one of two three-finger motifs of PLI-I, I(96–145), was effectively bound to PLA2, BPI and BPII (Fig. 1). This

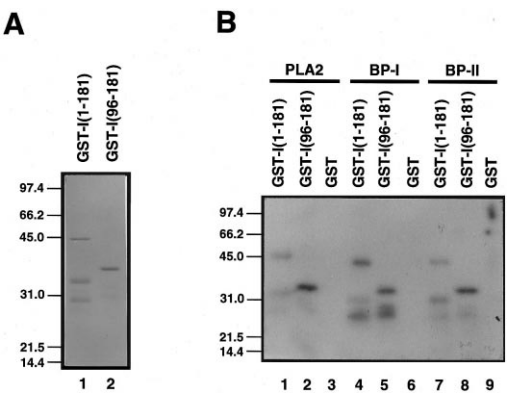

Fig. 1. Binding of PLI-I and its C-terminal half to venom PLA_2 isozymes. A: Electrophoretograms of GST-fused PLI-I (GST-I(1–181)) (lane 1) and GST-I(96–181) (lane 2) on 12.5% SDS-PAGE. Stained with Coomassie brilliant blue. B: In vitro binding of GST-I(1–181) and GST-I(96–181) to PLA_2 isozymes, PLA2 (lanes 1–3), BPI (lanes- 4-6) and BPII (lanes 7–9), which had been immobilized on magnetic beads. The GST-fused proteins bound to each of PLA_2 isozymes were eluted from the affinity column, subjected to SDS-PAGE, transferred onto PVDF membrane, treated with anti-GST monoclonal antibody and stained with alkaline phosphatase-conjugated anti-IgG antibody. GST alone was used as control. Lanes 1, 4 and 6, GST-I(1–181); lanes 2, 5 and 8, GST-I(96–181); lanes 3, 6 and 9, GST. The mobilities of molecular size markers are indicated on the left in kDa.

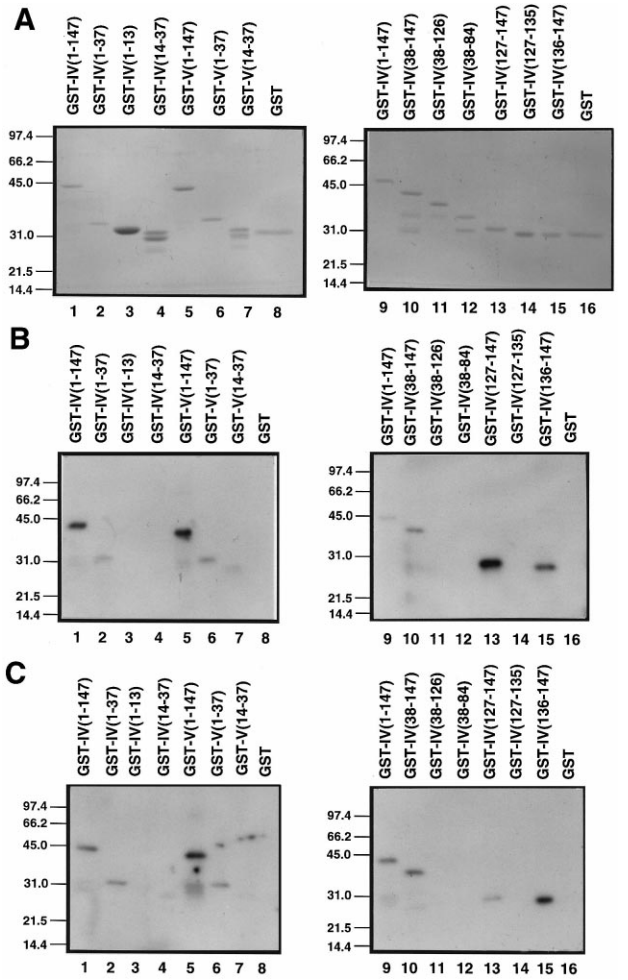

Fig. 2. Assay of the binding regions of PLI-IV and PLI-V to PLA_2 isozymes. A: Electrophoretograms of GST-fused proteins and peptides on 12.5% SDS-PAGE. B: In vitro binding of PLI-I, PLI-V and their fragments to PLA2 which had been immobilized on magnetic beads. The binding was detected in the same manner as described in the legend to Fig. 1. Lanes 1 and 9, GST-IV(1–147); lane 2, GST-IV(1–37); lane 3, GST-IV(1–13); lane 4, GST-IV(14–37); lane 5, GST-V(1–147); lane 6, GST-V(1–37); lane 7, GST-V(14–37); lanes 8 and 16, GST; lane 10, GST-IV(38–147); lane 11, GST-IV(38–126); lane 12, GST-IV(38–84); lane 13, GST-IV(127–135); lane 14, GST-IV(136–147); lane 15, GST-IV(127–147). C: In vitro binding of PLI-I, PLI-V and their fragments to BPI which had been immobilized on magnetic beads. The proteins and peptides employed and the lane numbering are exactly the same as in B. The binding modes to BPII of all the proteins and peptides tested here were identical to those to BPI (or PLA2), so that all the data for BPII are omitted hereafter.

suggests that only one three-finger motif of PLI-I is capable of binding to PLA_2 isozymes.

Various fragments of PLI-IV and PLI-V were prepared in order to identify the region required for the binding to PLA_2 isozymes. The amino acid sequences of PLI-IV and PLI-V are highly identical except for the region encoded by exon 3 [25]. The fragments containing this variable region, IV(1–37) and V(1–37), effectively interacted with PLA_2 isozymes (Fig. 2). When this region was divided into two fragments, IV(1–13) and V(1–13) with identical sequences exhibited considerably reduced binding activity whereas no binding was observed for IV(14–37) and V(14–37) which contain the variable region (Fig. 3). These results clearly indicate that the full portion of N-terminal 37 amino acid residues of PLI-IV and PLI-V is essential for interacting with PLA_2 isozymes.

IV(38–147), which contains a sequence similar to CRDs of some mammalian cellular PLA_2 receptors, was found to bind to PLA_2 isozymes (Fig. 2). This is in accord with the fact that the CRD-like domain of PLA_2 receptors is bound to certain PLA_2s [28,30,31]. To identify the particular region involved in the binding, several fragments of the CRD-like domain of PLI-IV and PLI-V were prepared. A positive interaction was detected for IV(127–147), the C-terminal portion of PLI-IV or PLI-V (Fig. 2). On the other hand, the fragments from the CRD-like domain in which the C-terminal region (positions 127–147) is not involved, that is, IV(38–84) and IV(38–126), failed to interact with any of the PLA_2 isozymes (Fig. 2). When IV(127–147) was divided into IV(127–135) and IV(136–147), strong binding was observed for IV(136–147) (Fig. 3), indicating that the C-terminal segment of positions 136–147 of PLI-IV or PLI-V is critically important for interacting with PLA_2 isozymes. This sequence, $(Asn/Lys)^{136}$-Leu-Leu-Val-Val-Cys-Glu-Phe-Tyr-Phe-Ile-Leu^{147}, is particularly rich in hydrophobic amino acid residues. Recently, pentapeptides with the sequence of Xxx-Val-Leu-Leu-Yyy (Xxx and Yyy are any amino acid residues) were found to bind to PLA_2 isozymes (T. Chiwata, unpublished). A similar sequence, (Asn/Lys)-Leu-Leu-Val-Val, is noted in the above sequence (positions 136–141). The importance of two extremely hydrophobic tripeptide regions, Leu-Leu-Val (positions 137–140) and Phe-Tyr-Phe (positions 143–145), of PLI-IV and PLI-V was then assessed. GST-fused mutants of IV(136–147) in which these tripeptide segments were replaced by trialanine, namely IV(136–147:137AAA139) and V(136–147:143AAA145), were synthesized. The experiment showed that the binding capacity was completely lost by these replacements (Fig. 3). These results emphasize the importance of the

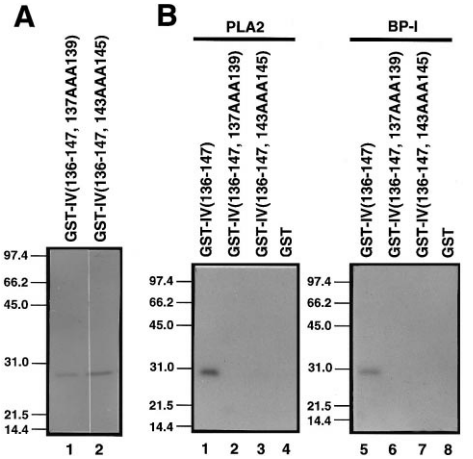

Fig. 3. Binding of the C-terminal peptides of PLI-IV and PLI-V to PLA_2 isozymes. A: Electrophoretograms of GST-IV(136–147:137AAA139) (lane 1) and GST-IV(136–147:143AAA145) (lane 2) on 12.5% SDS-PAGE. B: In vitro binding of GST-IV(135–147), GST-IV(136–147:137AAA139), GST-IV(136–147:143AAA145) and GST to PLA_2 isozymes, PLA2 (lanes 1–4) and BPI (lanes 5–8), which had been immobilized on magnetic beads. The binding was detected in the same manner as described in the legend to Fig. 1. Lanes 1 and 5, GST-IV(136–147); lanes 2 and 6, GST-IV(136–147:137AAA139); lanes 3 and 7, GST-IV(136–147:143AAA145); lanes 4 and 8, GST.

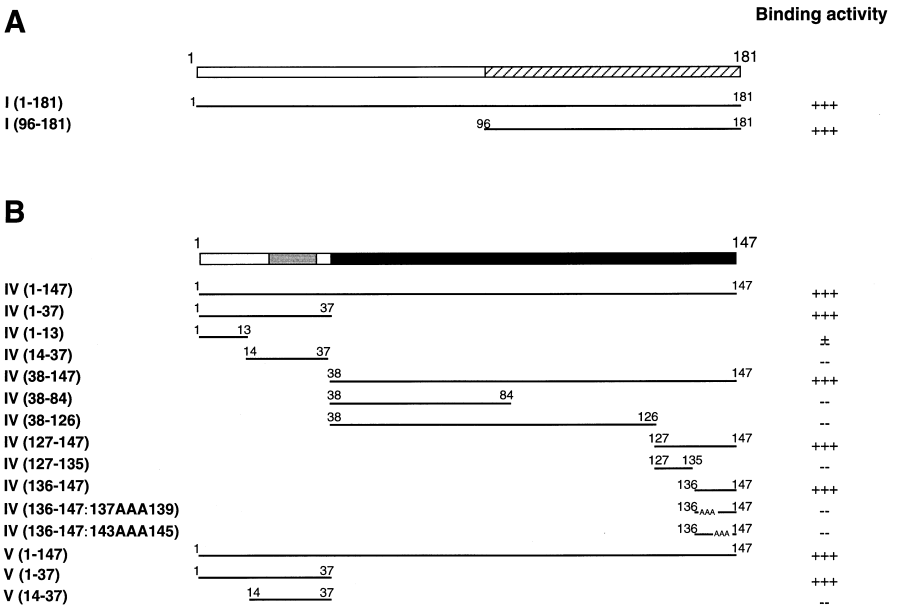

Fig. 4. Schematic representation of the structural units of PLI-I and of PLI-IV and PLI-V and the binding capacities of their segments to PLA_2 isozymes. A: Two three-finger motifs are shown at the top as open and hatched boxes. B: A variable region between PLI-IV and PLI-V and their CRD-like domain are shown at the top as shaded and solid boxes, respectively. The binding capacities of the segments are indicated on the right.

C-terminal segment of PLI-IV and PLI-V for association to PLA_2 isozymes. The structural units of PLI-I and of PLI-IV and PLI-V and the binding capacities of their segments to PLA_2 isozymes are summarized in Fig. 4.

A recent study showed that the pentapeptide from positions 70–74 of three group II PLA_2s inhibits its own PLA_2 activity in mixed micelles [32]. This segment is involved in a β-loop of this molecule which is in contact with its N-terminal region by hydrogen bonding [33]. The properties of the pentapeptide constituting positions 70–74 of *Tf* PLA_2 isozymes and other three group II PLA_2s are different from those of peptides constituting the C-terminal hydrophobic region of PLI-IV and PLI-V, indicating that the mechanism of inhibition of PLA_2s differs between peptides derived from the β-loop segment of PLA_2 itself and peptides from the C-terminal hydrophobic region of PLI-IV and PLI-V.

The present study ignored the roles of the disulfide bonds of the three-finger motif and the CRD-like domain of inhibitors. The data, therefore, show that *E. coli*-expressed PLI-I, PLI-IV and PLI-V and some of their segments have affinities to venom PLA_2 isozymes strong enough to make a stable and isolatable complex. If they have any proper disulfide bonds, it is likely that their affinities must be strengthened.

As mentioned, the hydrophobic core of the C-terminal region of PLI-IV and PLI-V is critical for binding to PLA_2 isozymes. It was noted that the C-terminal 12 amino acid residues of PLI-IV are identical to those of PLI from *Agkistrodon blomhoffii siniticus* serum [10], implying that the CRD-like domain in snake serum PLIs is one of the essential structural elements to recognize PLA_2 isozymes. Since CRDs of PLA_2 receptors do not have such a hydrophobic core in their C-terminal regions [28–31], it is likely that the binding of the C-terminal region of PLI-IV and PLI-V to PLA_2 isozymes is mechanistically different from the binding of PLA_2 receptors to PLA_2. It was found that a pentapeptide Ser-Phe-Tyr-Phe-Trp, which is similar in property to the C-terminal region of PLI-IV and PLI-V, inhibit PLA2 in a dose-dependent manner (T. Chiwata, unpublished). This strongly suggests that the C-terminal region of PLI-IV and PLI-V actually inhibits PLA_2 activity through the binding to the active site cleft or its vicinity of PLA_2 isozymes. It is conceivable that GST-fused PLIs not only bind to PLA_2 isozymes but also inhibit their activity.

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