Mapping the Region of the α-type Phospholipase A$_2$ Inhibitor PLI$_\alpha$ Responsible for Its Inhibitory Activity

Kohji Okumura, Ai Ohno, Masanori Nishida, Kyozo Hayashi, Kiyoshi Ikeda, and Seiji Inoue

From the Department of Biochemistry, Osaka University of Pharmaceutical Sciences, Nasahara, Takatsuki, Osaka 569-1094, Japan

Running Title: PLA$_2$ Binding Site on PLI$_\alpha$

Address correspondence to: Seiji Inoue, Department of Biochemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan, Tel. +81-72-690-1075; Fax. +81-72-690-1075; E-mail: inoue@glyoups.ac.jp

α-Type phospholipase A$_2$ inhibitory protein (PLI$_\alpha$) from the serum of the venomous snake Gloydius brevicaudus, GbPLI$_\alpha$, is one of the protective endogenous proteins that neutralizes its own venom phospholipase A$_2$ (PLA$_2\alpha$); and it is a homotrimer of subunits having a C-type lectin-like domain (CTLD). The non-venomous snake Elaphe quadrivirgata has a homologous serum protein, EqPLI$_\alpha$-LP, that does not show any inhibitory activity against various snake venom PLA$_2$s (Okumura, K., Inoue, S., Ikeda, K., and Hayashi, K. (2003) IUBMB Life 55, 539-545). By constructing GbPLI$_\alpha$ – EqPLI$_\alpha$-LP chimeric proteins, we have mapped the residues important in conferring GbPLI$_\alpha$ inhibitory activity on the region 13-36 in the primary structure of GbPLI$_\alpha$. Non-inhibitory EqPLI$_\alpha$-LP showed comparable inhibitory activity only when this region was replaced with that of GbPLI$_\alpha$. Further, mutational analysis of the candidate residues revealed that the individual GbPLI$_\alpha$ to EqPLI$_\alpha$-LP residue substitutions N26K, K28E, D29N, Y144S each produced a mutant GbPLI$_\alpha$ protein with reduced inhibitory activity, with the single N26K substitution having the most significant effect. Residues 13-36 were suspected to be located in the helical neck region of the GbPLI$_\alpha$ trimer. Therefore, the region of GbPLI$_\alpha$ responsible for PLA$_2$ inhibition was distinct from the carbohydrate-binding site of the homologous C-type lectin.

Phospholipases A$_2$ (PLA$_2$s, EC 3.1.1.4)$^1$ catalyze the hydrolysis of the acyl-ester bond at the sn-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. Secretory PLA$_2$s are a growing family of low-molecular-weight, highly disulfide-linked, Ca$^{2+}$-requiring secretory enzymes with a His-Asp catalytic dyad and are classified into six main groups (I, II, III, V, X, and XII) according to their primary structures (1). Snake venom is one of the most abundant sources of secretory PLA$_2$s, which exhibit a wide variety of pharmacological effects including neurotoxicity and myotoxicity (2). Elapidae venom contains group-I PLA$_2$s; and Viperidae venom, group-II ones (3). Venomous snakes have three distinct types of PLA$_2$ inhibitory proteins (PLI$_\alpha$, PLI$_\beta$, and PLI$_\gamma$) in their blood to protect themselves from the leakage of their own venom PLA$_2$s into the circulatory system (4-6). PLI$_\alpha$ has only been identified in the blood of Viperidae snakes, such as Protobothrops flavoviridis (renamed from Trimeresurus flavoviridis according to the present taxonomy) (7), Gloydius brevicaudus (renamed from Agkistrodon blomhoffii sininctus) (8), Bothrops asper (9), and Cerrophidion godmani (10). It is a 75-kDa trimeric glycoprotein of 20-kDa subunits having a C-type lectin-like domain (CTLD) which is homologous to that of collectins, such as serum mannose-binding protein (MBP) and lung surfactant-apoproteins (11). G. brevicaudus, B. asper, and C. godmani PLI$_\alpha$s are composed of three identical subunits, whereas P. flavoviridis PLI$_\alpha$ is a trimer of two homologous subunits. P. flavoviridis and G. brevicaudus PLI$_\alpha$s inhibit specifically the group-II acidic PLA$_2$s from their own venom (12). On the contrary, B. asper PLI$_\alpha$ (BaMIP) and C. godmani PLI$_\alpha$ (CgMIP-II) selectively inhibit the group-II basic myotoxic PLA$_2$s from their own venom (9,10). Recently, we identified a PLI$_\alpha$ homolog (PLI$_\alpha$-LP) from the serum of non-venomous snake Elaphe quadrivirgata (13). This protein had 70% sequence identity with G. brevicaudus PLI$_\alpha$, but did not show any inhibitory activity against various snake venom PLA$_2$s.

CTLDs were first identified as carbohydrate recognition domains (CRDs) of C-
Materials and Methods

Materials – G. breviceaus acidic PLA2 was purified from the venom as described previously (12). A cloned cDNA of E. quadririgada PLIα-LP (pEQ105) was described previously (13).

Construction of PLIα and PLIα-LP Expression Plasmid – The construction of the G. breviceaus PLIα (GbPLIα) expression plasmid, αBB105/16b, was described previously (20). For expression of E. quadririgada PLIα-LP (EqPLIα-LP), two synthetic oligonucleotides were designed and used for the PCR amplification with a cloned PLIα-LP cDNA used as a template. The sense primer, 5’-GGGAATTCCATATGGATCAGCAACAGCAGACACAA CAGTGAACCGACCCCGCTCGAAGGG-3’, contained a NdeI site (underlined) and 15 bases encoding an in-frame enterojenase cleavage site, which was followed by 20 bases encoding the N-terminal residues 1-7 of the mature PLIα-LP. The antisense primer (BamHI-α-3/EQ), 5’-GGGGGAATTCCATATGGAGGATCCTCCAGCTCCTCC C-3’, contained a BamHI site (underlined), followed by 19 bases that were complementary to the nucleotide sequence of 546-564 of the cDNA. After the PCR amplification, the amplified product was cut with NdeI and BamHI, and then ligated between NdeI and BamHI sites of the bacterial expression vector pET-16b (Novagen). The ligated products were used to transform E. coli XL1-Blue competent cells. Plasmid DNAs were recovered, and the nucleotide sequence of the insert was verified by both restriction analysis and DNA sequencing. The resulting plasmid, EqαNB/16b, was then used to transform E. coli strain BL21(DE3) pLysS.

Chimeric and Deletion Mutation – Chimeric PLIαs were constructed by fusing appropriate expression plasmid segments. In order to construct a pair of expression plasmids for the N-terminal hybrid proteins, we digested αBB105/16b and EqαNB/16b with EcoRI, and replaced the purified small EcoRI fragment of one with the other to yield Eq13Gb and Gb13Eq. For construction of a pair of expression plasmids for the C-terminal hybrid proteins, αBB105/16b and EqαNB/16b were double-digested with AarI and HindIII, respectively, and the resultant small AarI-HindIII fragment of one was replaced with that of the other to yield Eq84Gb and Gb84Eq.

In order to prepare a pair of plasmid DNAs for CTLD hybrid proteins, we introduced SnaBI sites into αBB105/16b and EqαNB/16b by using a USE mutagenesis kit (Amersham Biosciences) with mutagenetic primers 5’-CGACCTCTCTTTGGTTAGTCACAAACTTCTCACTGCC-3’ and 5’-CCTACTTGCTTGTTGGTTACGTATAACTT TTCACTGCC-3’ (replaced nucleotides are underlined). The obtained plasmid DNAs were double-digested with ApaI and SnaBI, and the resultant small ApaI-SnaBI fragment of one was replaced with that of the other to yield Eq37Gb and Gb37Eq.

In order to prepare deletion mutants, GbCTLD, having only the CTLD of GbPLIα, we designed the following sense primer: 5’-GGGAATTCCATATGGACGACGACGACACAA GCATGAAAACCGACCCCGCTCGAAGGG-3’, containing a NdeI site (underlined) and 15 bases encoding an in-frame enterojenase cleavage site, which was followed by 20 bases encoding the N-terminal residues 1-7 of the mature PLIα-LP. The antisense primer (BamHI-α-3/EQ), 5’-GGGGGGAATTCCATATGGAGGATCCTCCAGCTCCTCC C-3’, contained a BamHI site (underlined), followed by 19 bases that were complementary to the nucleotide sequence of 546-564 of the cDNA. After the PCR amplification, the amplified product was cut with NdeI and BamHI, and then ligated between NdeI and BamHI sites of the bacterial expression vector pET-16b (Novagen). The ligated products were used to
nucleotide sequences of these chimeric and deletion mutants were confirmed by automated sequencing.

Site-directed Mutagenesis – A USE mutagenesis kit (Amersham Biosciences) was used to introduce point mutations into the GbPLIα coding region of αBB105/16b expression plasmid (20) according to the manufacturer’s instructions. The complementary mutagenetic oligonucleotides used are shown in Table I. DNA sequences were confirmed by automated sequencing of the entire coding sequences.

Expression and Purification of the Recombinant Proteins – The expression plasmid DNAs were used to transform competent E. coli strain BL21(DE3)pLysS. Since all of the recombinant proteins accumulated as insoluble inclusion bodies upon the addition of isopropyl β-D-thiogalactopyranoside (IPTG), the inclusion bodies were solubilized in 50 mM Tris-HCl buffer (pH 8.0) containing 8 M urea and 0.1 M NaCl, and purified by using TALON metal affinity resin (BD Biosciences) as described previously (20). The purified recombinant proteins were denatured in 50 mM Tris-HCl buffer (pH 8.0) containing 6 M guanidine-HCl and 1 mM EDTA, and then gradually refolded by dialysis for 4 days against 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. After the insoluble unfolded protein had been removed by centrifugation, the supernatant was further purified by gel filtration chromatography on a HiLoad 10/30 Superdex 200pg column (Amersham Biosciences).

DNA Sequencing and Analysis – Both strands of DNAs were sequenced with a Thermo Sequenase core sequencing kit with 7-deaza-dGTP (Amersham Biosciences) on an SQ-3000 DNA sequencer (Hitachi) or an ABI Prism 310 genetic analyzer (Applied Biosystems). Analysis of DNA sequencing data was performed by use of the DNASIS software package (Hitachi Software Engineering).

Inhibition of PLA2 Enzymatic Activity – Enzymatic activities of G. breviceaudus acidic PLA2 were measured fluorometrically using 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine (Cayman Chemical) as a substrate, according to the method of Radvanyi et al. (21) in the presence of various concentrations of the recombinant PLIαs. Values of apparent inhibition constant (Kᵢ) were determined by non-linear least-squares analysis of the relative PLA2 activities by using the equation described previously (20).

Binding Analysis by Surface Plasmon Resonance – Bindings of PLIαs to PLA2 were analyzed with a BIAcore X system (Biacore, Uppsala, Sweden). G. breviceaudus acidic PLA2 was directly linked to the carboxymethylated dextran matrix of a CM5 sensor chip (Biacore) surface. For protection of the active site of PLA2 from the modification, 11.8 μg of PLA2 was dissolved in 100 μl of 9 mM sodium acetate buffer (pH 4.0) containing 6.66 mM CaCl₂ and 10 mM n-dodecyl phosphorylcholine, and was then covalently bound to the CM5 chip surface at a flow rate of 5 μl/min by using an amine coupling kit (Biacore) according to the manufacturer’s instruction. Various concentrations of the recombinant proteins were injected at a flow rate of 10 μl/min at 25 °C with running buffer (50 mM Hepes buffer (pH 7.5) containing 0.05% Tween20 and 1 mM EDTA). Sensor chips were regenerated at the end of each run by the injection of 10 mM HCl. Analysis of the association and dissociation curves was performed with the BIAevaluation 3.0 software (Biacore) using the 1:1 Langmuir binding model with drifting baseline (global fitting), and the mean values of the apparent dissociation constants (Kₐ) were calculated from the association rate constant (kₐ) and the dissociation rate constant (kₐₐ) (Kₐ = kₐₐ / kₐ).

Homology Modeling of GbPLIα – Protein homology modeling was performed by using MOE (The Molecular Operating Environment, Version 2004.03, Chemical Computing Group Inc., Montreal, Canada). The amino acid sequence of GbPLIα (NCBI accession code BAA86972) was aligned to the template sequence (rat SP-A, Protein Data Bank code 1R13) by using the protein alignment tools in MOE. Figs. 6A and 6B were prepared by using ViewerLite Version 4.2 (Accelrys Inc.).

RESULTS

Preparation and Characterization of Mutated PLIαs – We previously established the system for bacterial expression of the recombinant Gloydius breviceaudus PLIα (GbPLIα) as a (His)₄⁶-tagged fusion protein, and showed the recombinant protein to have strong inhibitory activity against acidic PLA₂ comparable to that of the natural PLIα (20). Using this expression system, we produced recombinant Elaphe quadrivirgata PLIα homolog (EqPLIα-LP)
having 70% sequence identity to GbPLIα (Fig. 1). Furthermore, we produced various recombinant chimeric proteins between GbPLIα and EqPLIα-LP, as shown in Fig. 2. The far-ultraviolet CD spectra of the purified recombinant proteins were very similar to that spectrum of the native PLIα (data not shown), indicating that they had correctly refolded. The trimeric structure of the recombinant GbPLIα was already confirmed by a chemical cross-linking experiment (20). Similarly, the recombinant EqPLIα-LP was found to form the trimeric structure (data not shown). However, the truncated recombinant proteins, GbCTLD and EqCTLD, lacking the respective N-terminal 48 residues of GbPLIα and EqPLIα-LP, were found to be monomers since they were eluted with a 25-kDa peak corresponding to the monomeric form by Superdex 200 column chromatography. Therefore, it is conceivable that the N-terminal regions preceding the CTLD of GbPLIα and EqPLIα-LP were required for the formation of the trimeric structure. Figure 3A shows the PLA2 inhibitory activities of four recombinant proteins, GbPLIα, EqPLIα-LP, GbCTLD, and EqCTLD. As expected, the recombinant EqPLIα-LP did not inhibit acidic PLA2 just like the natural EqPLIα-LP, whereas the recombinant GbPLIα inhibited it with an apparent inhibition constant (K_{app}) value of 3.05 nM. The truncated recombinant proteins, GbCTLD and EqCTLD, did not show any inhibition against acidic PLA2. The lack of inhibitory activity of monomeric GbCTLD against acidic PLA2 raises two possibilities, that the PLA2 binding site is not in the CTLD or the trimeric structure of CTLD is essential for the complex formation with PLA2, because one trimeric PLIα molecule was previously shown to bind stoichiometrically to one PLA2 molecule (7). Therefore, the loss-of-function experiments should be designed under conditions where the trimeric structure is retained, otherwise, the obtained results would be complicated. All the chimeric proteins and point-mutated PLIαs used in the present study proved to form a trimeric structure, like the native PLIα, as judged from the elution profiles of gel filtration on a Superdex 200 column, which showed peaks corresponding to the apparent molecular mass of 70-75 kDa.

**Chimeric Mutagenesis between GbPLIα and EqPLIα-LP** — In order to specify the regions responsible for the PLA2 inhibition in the GbPLIα, we created various sets of chimeric proteins between GbPLIα and EqPLIα-LP (Fig. 2). Figure 3B shows the inhibitory activity of these chimeric proteins against G. brevicaudus acidic PLA2, and their estimated inhibition constants (K_{app}) are summarized in Table II. The chimeric proteins Eq13Gb, Gb37Eq, Gb84Eq, and Eq13Gb37Eq were inhibitory against acidic PLA2; whereas the others, Gb13Eq, Eq37Gb, Eq84Gb, and Gb13Eq37Gb, showed little or no significant inhibition. These results indicate that the residues 13-36 of GbPLIα were the critical region for the PLA2 inhibition. Most strikingly, Eq13Gb37Eq showed significant inhibition toward the acidic PLA2, with a K_{app} value of 9.38 nM, which was comparable to that of GbPLIα (3.05 nM); that is, non-inhibitory EqPLIα-LP gained inhibitory activity only when its residues 13-36 were replaced with those of GbPLIα. In contrast, the inhibitory activity of GbPLIα was lost when these residues were replaced with those of EqPLIα-LP (Gb13Eq37Gb). Since all these chimeric proteins retained their trimeric structure and showed backbone CD spectra similar to that of the native PLIα, the major conformation would not be affected by the replacement of these residues.

Detailed comparisons of apparent inhibition constants between GbPLIα and Eq13Gb, and between Gb37Eq and Eq13Gb37Eq, suggested that the residues 1-12 of EqPLIα-LP were preferred for the inhibition toward the acidic PLA2. Between the amino acid sequences of GbPLIα and EqPLIα-LP shown in Fig. 1, there are only three amino acid replacements in the residues 1-12. This indicates that the replacements, Asp to Glu at residue 6, His to Gln at residue 8, and Val to Ile at residue 9, can affect the inhibitory activity. Furthermore, the comparison of K_{app} values of GbPLIα and Gb37Eq, and of Eq13Gb and Eq13Gb37Eq, suggested that the residues 37-147 of GbPLIα were preferred for the PLA2 inhibition rather than those of EqPLIα-LP. These preferable residues could be further restricted to the residues 84-147 from the comparison of the K_{app} value between GbPLIα and Gb84Eq.

In order to study the effect of these chimeric mutations on the binding kinetics of the PLA2-PLIα interaction, we immobilized G. brevicaudus acidic PLA2 on a biosensor chip in the presence of the micellar substrate analog (n-
dodecyl phosphorylcholine) and measured the interaction with various chimeric proteins in real-time by monitoring the changes in surface plasmon resonance after the injection of various concentrations of the chimeric PLIαs. Since Ca$^{2+}$ had no apparent effect on the binding curves (data not shown), the formation of PLIα-PLA2 complex was considered to be independent of Ca$^{2+}$. Previously, no significant effect of Ca$^{2+}$ was observed in the gel filtration of PLIα and its venom PLA2 (7). The Ca$^{2+}$-independent binding of PLIα to PLA2 is in contrast with the Ca$^{2+}$-dependent binding of C-type lectins to carbohydrates. Typical binding and dissociation curves at various concentrations of GbPLIα and those obtained at about 20 mM concentrations of the chimeric proteins in the presence of 1 mM EDTA are shown in Fig. 4. GbPLIα formed a stable complex with acidic PLA2, showing fast association and slow dissociation. The dissociation constants ($K_d$) and the association and dissociation rate constants ($k_{ass}$ and $k_{diss}$) were calculated from the curve-fitting analysis of the sensorgrams (Table II). The $K_d$ value of GbPLIα was calculated to be 4.53 nM, which was comparable to the $K_{app}$ value (3.05 nM) obtained from the measurement of its inhibitory activity. The chimeric proteins Gb37Eq and Eq13Gb37Eq formed a complex with the immobilized PLA2 with $K_d$ values of 24.5 and 6.5 nM, respectively. However, the chimeric proteins Eq37Gb and Gb13Eq37Gb did not bind to the immobilized acidic PLA2. This result is consistent with that obtained on the inhibitory activity described above, confirming that the residues 13-36 of GbPLIα were the critical region for the PLA2 binding and PLA2 inhibition. The replacements of the residues 37-147 of GbPLIα with those of EqPLIα-LP caused a 3-fold increase in the dissociation rate constant and a decrease in the association rate constant. Further replacements of the residues 1-12 of Gb37Eq with those of EqPLIα-LP caused a 2-fold increase in the association rate constant. This increase in the association rate constant might explain the preference of the residues 1-12 of EqPLIα-LP in PLA2 inhibition described above.

**Single Amino Acid Substitution of GbPLIα** – When the amino acid sequence of EqPLIα-LP was compared with those sequences of PLIαs from G. brevicaudus, P. flavoviridis, and C. godmani (Fig. 1), there were some unique residues only found in the EqPLIα-LP sequence, including residues 21, 23, 25-29, and 59-63, the insertion of two residues at position 118, and the deletion of the C-terminal residue. Therefore, we performed site-directed mutagenesis to substitute the corresponding amino acid residues of GbPLIα with these unique residues of EqPLIα-LP to specify the residues responsible for the PLA2 inhibition. The inhibitory activities of these mutant proteins are shown in Fig. 5, and the estimated apparent inhibition constants are summarized in Table III. Since all of these mutants showed significant inhibitory activity, no inhibitory activity of EqPLIα-LP was not ascribable to the replacement of a single critical residue with one of these unique residues. Therefore, multiple mutations might be required for the complete loss of the inhibition. Among the mutant proteins substituted at residues within 13-36, N26K showed the weakest inhibition toward the acidic PLA2 with a $K_{app}$ value of 80 nM, which was a value 26-fold higher than that of GbPLIα. From the chimeric mutagenesis experiments described above, the residues 13-36 of GbPLIα were suggested to be essential for both the inhibition and binding of the acidic PLA2. Since other mutant proteins, Q21E, E23K, S25D, K28E, and D29N, showed decreased inhibitory activities, multiple residues including Asn-26 were probably responsible for PLA2 binding and inhibition. Further, binding kinetics of E23K, S25D, N26K, K28E, and D29K with respect to the immobilized acidic PLA2 were investigated by surface plasmon resonance analysis, and the values obtained are also shown in Table III. The calculated $K_d$ value of N26K was 4-fold higher than that of GbPLIα. The disagreement between $K_d$ and $K_{app}$ values might reflect the difference between free and immobilized forms of PLA2, or the difference in the experimental conditions between the presence and absence of phospholipid substrate. N26K caused a 3.3-fold increase in the dissociation rate constants, resulting in a decreased inhibitory activity. The introduction of a positive charge at position 26 is likely to have affected the interaction with PLA2. In spite of the drastic change of the residues to the oppositely charged residues, neither E23K nor K28E caused any significant reduction in the inhibitory and binding activities, suggesting that Glu-23 and Lys-28 were unimportant to the interaction with the acidic PLA2. But there was a remarkable feature found only in the
sensogram of K28E; i.e., the $K_d$ value for K28E increased with a decrease in its concentration, whereas for other mutant proteins was independent of their concentrations (data not shown). The K28E protein might be structurally unstable or might dissociate into monomer at its low concentrations. Although D29N had an inhibitory activity weaker than the intact protein against acidic PLα$_2$, it showed the association and dissociation rate constants of 2.5 and 2.1 fold, respectively, indicating that the binding of PLα$_2$ to D29N was slightly stronger than that for GbPLα$_2$. The negative charge at position 29 might be important in the inhibitory activity but not in the binding activity.

Figure 5B shows the inhibitory activities of GbPLα$_2$ with point mutation in its CTLD. The $K_{\text{app}}^\alpha$ values of Q79E, Q111E, T118K, T118NKL N136D, and L148A toward acidic PLα$_2$ were 10.5, 9.09, 11.8, 3.87, 4.72, and 10.8 nM, respectively, which were comparable to that value of GbPLα$_2$ (3.05 nM). However, the $K_{\text{app}}^\alpha$ values of Y144S was 43.0 nM, which was 14-times higher than that of GbPLα$_2$. Therefore, Tyr-144 might be important in the PLα$_2$ inhibition. This substitution at residue 144 may account for the weak inhibitory activity of Gb84Eq.

Furthermore, the results obtained for the point mutants H129N and S130D, in which the unique residues in PLα$_2$ sequences were substituted by the conserved residues among the extracellular collectins, showed that these unique residues were not involved in PLα$_2$ inhibition.

**Discussion**

Earlier, Nobuhisa et al. (22) investigated the binding of various fragments of Protobothrops (Trimeresurus) flavoviridis PLα$_2$ to the venom PLα$_2$ isozymes and reported that the hydrophobic residues 136-147 of P. flavoviridis PLα$_2$ were critical for binding to the PLα$_2$ isozymes. In the present study, Tyr-144 of GbPLα$_2$ was found to contribute to the PLα$_2$ inhibition to some extent. However, the loss of PLα$_2$ inhibitory activity in EqPLα$_2$-LP could not be rationalized by the replacement of the residues 136-147, since EqPLα$_2$-LP was found to exhibit the inhibitory activity solely by replacing the residues 13-36 with those of GbPLα$_2$. Moreover, they reported that the N-terminal fragment (the residues 1-37) and the CTLD fragment (the residues 38-147) of P. flavoviridis PLα$_2$ also bound to the PLα$_2$ isozymes, although their affinities and stoichiometry were not quantified (22). Since Eq37Gb and GbCTLD lost the binding and inhibitory activities toward PLα$_2$, the role of CTLD in the PLα$_2$ binding appears to be inconsistent with their results for the P. flavoviridis CTLD fragment.

We constructed a 3D structural model of GbPLα$_2$ by homology modeling on the basis of the crystal structure of rat SP-A (23). Figure 6 compares the overall fold of the homology-built model of GbPLα$_2$ with that of rat SP-A. The surface loops and calcium binding sites of SP-A CTLD, which are responsible for the carbohydrate binding, were drastically changed in the GbPLα$_2$ CTLD, presumably due to the deletion of 13 amino acid residues starting at residue 97 of GbPLα$_2$ (Fig. 1). This finding may agree with the previous result that GbPLα$_2$ does not have any Ca$^{2+}$-dependent carbohydrate binding activity (8). The residues 13-36 of GbPLα$_2$, which were found to be critical for PLα$_2$ binding and inhibition in the present study, are located at the helical neck region in this model. This $\alpha$-helical coiled-coil neck region is known to mediate the trimerization of CTLD in many C-type lectins, including human MBL (24), rat MBP-A (25), human SP-D (26), rat SP-A (23), and tetranectin (27). This is likely to apply to GbPLα$_2$ as well since the truncated recombinant protein GbCTLD lacking the N-terminal 48 residues of GbPLα$_2$ was in monomeric form. Therefore, the residues 13-36 of GbPLα$_2$ are important both in binding of acidic PLα$_2$ and in trimerization of PLα$_2$ subunits. It should be noted that the N-terminal 37-amino acid fragment of P. flavoviridis PLα$_2$ was reported to bind directly to its venom PLα$_2$ isoenzymes (22). As was depicted in Fig. 6C, we suppose that the central pore formed by the trimerization of GbPLα$_2$ would be the PLα$_2$ binding site, since one trimeric PLα$_2$ stoichiometrically binds one PLα$_2$ molecule. In this model, the region of residues 13-36 may not function as a direct binding site of PLα$_2$, but serve as a dominant determinant of the central pore structure formed by the trimerization through the interactions of these $\alpha$-helical neck regions. The central pore formed by the residues 13-36 of EqPLα$_2$-LP might be too small to interact with G. brevicaudus acidic PLα$_2$, and their replacement with GbPLα$_2$ residues would enlarge the pore to permit the interaction.
Since the region of residues 13-36 are the most variable region among various PLIαs, the amino acid substitutions in this region might contribute to the high specificity of PLIα toward various PLA2s. This possible PLA2 binding site in the central pore is distinct from the carbohydrate-binding sites of the homologous SP-A trimer (Fig. 6D). Since Tyr-144 is expected to be located in the central pore, this residue might be one of the residues responsible for the direct interaction with to the PLA2 molecule. Further studies using site-directed mutagenesis targeting the residues in the central pore will be required to confirm this model.

Recently SP-A was reported to be an endogenous inhibitor of groups IIA and X PLA2s (19). The inhibition of P. flavoviridis PLA2 by SP-A was not affected by the presence of various monosaccharides (28), suggesting that the carbohydrate-binding domain of SP-A is not involved in the PLA2 inhibition. Therefore, the PLA2 binding site of SP-A is likely to reside apart from the carbohydrate-binding sites, as similarly suggested for PLIα. But there are some differences between SP-A and PLIα in the interactions with the PLA2 molecule; i.e., SP-A interacts directly with group IIA PLA2 in a Ca2+-dependent manner (29), whereas PLIα in a Ca2+-independent manner. This difference might be due to the loss of the calcium-binding site in PLIα. Another endogenous inhibitor of group IB and X PLA2s is a soluble form of the PLA2 receptor, which contains eight tandem CTLDs (18). Since three CTLDs (domains 3-5) of the PLA2 receptor were shown to be PLA2-binding region (30), these three CTLDs might bind one PLA2 molecule in a way similar to that of trimeric PLIα. Further information about the role of CTLDs in PLA2 inhibition will be obtained by examining the crystal structure of the PLIα-PLA2 complex.

**REFERENCES**

1. Murakami, M., and Kudo, I. (2002) *J. Biochem.* **131**, 285-292
2. Kini, R. M. (2003) *Toxicon* **42**, 827-840
3. Heinrikson, R. L., Krueger, E. T., and Keim, P. S. (1977) *J. Biol. Chem.* **252**, 4913-4921
4. Ohkura, N., Okuhara, H., Inoue, S., Ikeda, K., and Hayashi, K. (1997) *Biochem. J.* **325**, 527-531
5. Lambeau, G., and Lazdunski, M. (1999) *Trends Pharmacol. Sci.* **20**, 162-170
6. Dunn, R. D., and Broady, K. W. (2001) *Biochim. Biophys. Acta* **1533**, 29-37
7. Kogaki, H., Inoue, S., Ikeda, K., Samejima, Y., Omori-Satoh, T., and Hamaguchi, K. (1989) *J. Biochem.* **106**, 966-971
8. Ohkura, N., Inoue, S., Ikeda, K., and Hayashi, K. (1993) *J. Biochem.* **113**, 413-419
9. Lizano, S., Lomonte, B., Fox, J. W., and Gutierrez, J. M. (1997) *Biochem. J.* **326**, 853-859
10. Lizano, S., Angulo, Y., Lomonte, B., Fox, J. W., Lambeau, G., Lazdunski, M., and Gutierrez, J. M. (2000) *Biochem. J.* **346**, 631-639
11. Inoue, S., Kogaki, H., Ikeda, K., Samejima, Y., and Omori-Satoh, T. (1991) *J. Biol. Chem.* **266**, 1001-1007
12. Inoue, S., Shimada, A., Ohkura, N., Ikeda, K., Samejima, Y., Omori-Satoh, T., and Hayashi, K. (1997) *Biochem. Mol. Biol. Int.* **41**, 529-537
13. Okumura, K., Inoue, S., Ikeda, K., and Hayashi, K. (2003) *IUBMB Life* **55**, 539-545
14. Drickamer, K. (1988) *J. Biol. Chem.* **263**, 9557-9560
15. Drickamer, K. (1999) *Curr. Opin. Struct. Biol.* **9**, 585-590
16. Lambeau, G., Ancian, P., Barhanin, J., and Lazdunski, M. (1994) *J. Biol. Chem.* **269**, 1575-1578
17. Ishizaki, J., Hanasaki, K., Higashino, K., Kishino, J., Kikuchi, N., Ohara, O., and Arita, H. (1994) *J. Biol. Chem.* **269**, 5897-5904
18. Higashino, K., Yokota, Y., Ono, T., Kamitani, S., Arita, H., and Hanasaki, K. (2002) *J. Biol. Chem.* **277**, 13583-13588
19. Chabot, S., Koumanov, K., Lambeau, G., Gelb, M. H., Balloy, V., Chignard, M., Whitsett, J. A., and Touqui, L. (2003) *J. Immunol.* **171**, 995-1000
20. Okumura, K., Inoue, S., Ikeda, K., and Hayashi, K. (1999) *Biochim. Biophys. Acta* **1441**, 51-60
21. Radvanyi, F., Jordan, L., Russo-Marie, F., and Bon, C. (1989) *Anal. Biochem.* **177**(1), 103-109
22. Nobuhisa, I., Chiwata, T., Fukumaki, Y., Hattori, S., Shimohigashi, Y., and Ohno, M. (1998) *FEBS Lett.* **429**, 385-389
23. Head, J. F., Mealy, T. R., McCormack, F. X., and Seaton, B. A. (2003) *J. Biol. Chem.* **278**, 7
24. Sheriff, S., Chang, C. Y., and Ezekowitz, R. A. (1994) *Nature Struct. Biol.* **1**, 789-794
25. Weis, W. I., and Drickamer, K. (1994) *Structure* **2**, 1227-1240
26. Hakansson, K., Lim, N., Hoppe, H., and Reid, K. (1999) *Structure* **7**, 255-264
27. Nielsen, B. B., Kastrup, J. S., Rasmussen, H., Holtet, T. L., Graversen, J. H., Etzerodt, M., Thogersen, H. C., and Larsen, I. K. (1997) *FEBS Lett.* **412**, 388-396
28. Fisher, A., Dodia, C., Chander, A., Beers, M., and Bates, S. (1994) *Biochim. Biophys. Acta* **1211**, 256-262
29. Arbibe, L., Koumanov, K., Vial, D., Rougeot, C., Faure, G., Havet, N., Longacre, S., Vargaftig, B. B., Bereziat, G., Voelker, D. R., Wolf, C., and Touqui, L. (1998) *J. Clin. Invest.* **102**, 1152-1160
30. Higashino, K., Ishizaki, J., Kishino, J., Ohara, O., and Arita, H. (1994) *Eur. J. Biochem.* **225**, 375-382
31. Hakansson, K., and Reid, K. B. M. (2000) *Protein Sci.* **9**, 1607-1617
FOOTNOTES

* This work was supported in part by a grant (No. 12672137, to S. I.) from the program Grants-in-Aid for Scientific Research (C) of the Ministry of Education, Science, Sports, and Culture of Japan.

1 The abbreviations used are: PLA₂, phospholipase A₂; PLI, phospholipase A₂ inhibitor; PLIα, α-type phospholipase A₂ inhibitor; GbPLIα, Gloydius brevicaudus PLIα; EqPLIα-LP, Elaphe quadrivirgata PLIα-like protein; CTLD, C-type lectin-like domain; CRD, carbohydrate recognition domain; SP-A, surfactant protein A.

FIGURE LEGENDS

Fig. 1. Sequence alignment of GbPLIα, EqPLIα-LP, rat SP-A, and rat MBP-A. The variable amino acid residues found in other PLIαs (P. flavoviridis PLIα, B. asper BaMIP, and C. godmani CgMIP-II) are shown above the GbPLIα sequence. The dotted residues of EqPLIα-LP are different from those of the other PLIα sequences. The target residues for the site-directed mutagenesis in the present study are boxed. Twenty-three amino acids conserved among 22 different extracellular collectins (31) are shown in reversed type. NCBI Accession codes of these proteins are as follows: GbPLIα, BAA86972; EqPLIα-LP, BAC53925; rat SP-A, 1R13_A; rat MBP-A, 1MSB_A.

Fig. 2. GbPLIα – EqPLIα-LP chimera and associated mutants. A schematic representation of GbPLIα – EqPLIα-LP constructs. The C-type lectin-like domain (CTLD) of PLIα and its disulfide bonding are shown (top). All recombinant proteins were expressed as fusion proteins with an additional N-terminal 31 amino acid residues including ten histidine residues (His-tag).

Fig. 3. Inhibition of the enzymatic activities of G. brevicaudus acidic PLA₂ by recombinant PLIαs. The PLA₂ activity was measured fluorometrically with 1-palmitoyl-2- pyrenecanoylphosphatidylcholine used as a substrate in the presence of various concentrations of the recombinant proteins. The solid curves are theoretical ones constructed by using the $K_{app}$ values determined by non-linear least-squares analysis. A. Effects of GbPLIα (○), EqPLIα-LP(●), GbCTLD (□), and EqCTLD (■). B. Effects of Gb13Eq37Gb (○), Gb84Eq (□), Gb37Eq (△), Gb13Eq (▽), Eq13Gb (▼), Eq37Gb (▲), Eq84Gb (■), and Eq13Gb37Eq (●). The dotted curve in panel B shows the theoretical curve of the GbPLIα in panel A.

Fig. 4. Binding curves for the interactions of GbPLIα and the chimeric proteins with the immobilized G. brevicaudus acidic PLA₂, measured in real-time by surface plasmon resonance. A. Sensorgrams for GbPLIα at various concentrations. Various concentrations of the recombinant GbPLIα (40 μl) were introduced at a flow rate of 10 μl/min in 50 mM Hepes buffer (pH 7.5) containing 1 mM EDTA and 0.05% Tween 20 onto a CM5 sensor chip bearing immobilized G. brevicaudus acidic PLA₂. After the injection pulse, buffer flow was continued for 4 min in order to measure the dissociation. The concentrations of GbPLIα used are as follow: a, 15.6 nM; b, 7.8 nM; c, 3.9 nM; d, 2.0 nM; e, 1.0 nM. B. Comparison of the sensorgrams for chimeric proteins at about 20 nM concentrations. The recombinant proteins used are as follow: a, 15.6 nM GbPLIα; b, 18.0 nM Eq13Gb37Eq; c, 21.5 nM Gb37Eq; d, 22.0 nM Eq37Gb; e, 20.0 nM Gb13Eq37Gb.

Fig. 5. Inhibition of the enzymatic activities of G. brevicaudus acidic PLA₂ by point-mutated GbPLIαs. A. Effects of Q21E (○), E23K (□), S25D (△), N26K (●), K28E (■), and D29N (▲). B. Effects of Q79E (○), Q111E (□), T118K (◇), T118NKL (△), H129N (▽), S130D (●), N136D (■), Y144S (◇), and L148Δ (▲).

Fig. 6. Comparison of the structural model of GbPLIα with the structure of rat SP-A. A. Schematic representation of the GbPLIα monomer built by homology modeling. α-helices and β-strands are colored red and blue, respectively. B. Schematic representation of rat SP-A monomer...
C. A possible model in which the GbPLI\(\alpha\) trimer binds one molecule of PLA\(_2\) (shaded). D. Trimeric form of rat SP-A with carbohydrate binding sites (shown by arrows).
### TABLE I.

**Primers used for the site-directed mutagenesis of GbPL1α.**

Sequences of the synthetic oligonucleotide primers with the mutated codons in bold type are shown.

| Mutant | Mutagenetic primer |
|--------|-------------------|
| Q21E   | 5'-GAACCTCTCTTTCAAGTCTCATTAC-3’ |
| E23K   | 5'-CAGGTGGAAGACTTTCTTGGAAGTCTC-3’ |
| S25D   | 5'-GCCATCTTTCCAGGTGGTCGAACTCTCCTTGAGTC-3’ |
| N26K   | 5'-GCCATCTTTCCAGTTGGGAAC-3’ |
| K28E   | 5'-GTCAAGGAAGCTCTCCAGGTGGGAACCTC-3’ |
| D29N   | 5'-CTGTCAGGAAGCCATTTTTCAGGTGGGAAC-3’ |
| Q79E   | 5'-GGCCTTGTCTCATTTTTGAAGTGAAG-3’ |
| Q111E  | 5'-CTTCTCTGTTCTCCTCCAG-3’ |
| T118K  | 5'-GCTTTACACACTTTCCATCAG-3’ |
| T118NKL| 5'-GCTTATCTCCACACACAACCTGGTTCATCCTCAGCTTCC-3’ |
| H129N  | 5'-GACGTAGAGTCCAGAATCCG-3’ |
| S130D  | 5'-CATCACAGGACGTATCGTGCCAGAAATC-3’ |
| N136D  | 5'-CGACTAAGAGTTCGTCATCAGG-3’ |
| Y144S  | 5'-CATAAATGAAAGAAACTCACACACG-3’ |
| Y148Δ  | 5'-GGTCTTTCTCTCATCAAATGAAATAAACCTC-3’ |
TABLE II.

Inhibition constants ($K_{i,app}$) of recombinant proteins toward *G. brevicaudus* acidic PLA$_2$, and dissociation constants ($K_d$) and association and dissociation rate constants ($k_{ass}$ and $k_{diss}$) for the interaction of the recombinant proteins with immobilized *G. brevicaudus* acidic PLA$_2$.

| Recombinant Protein | PLA$_2$ Inhibition |                     | Binding to immobilized PLA$_2$ |                     |
|---------------------|---------------------|----------------------|----------------------|----------------------|
|                     | $K_{i,app}$ (nM)    | $K_d$ (nM)           | $k_{ass}$ (M$^{-1}$s$^{-1}$) $\times 10^{-5}$ | $k_{diss}$ (s$^{-1}$) $\times 10^3$ |
| $GbPLI\alpha$       | 3.05 ± 0.12         | 4.53 ± 0.08          | 8.76 ± 0.04          | 3.97 ± 0.05          |
| $EqPLI\alpha-LP$    | > 1000              | NB$^a$               | NB                   | NB                   |
| $GbCTLD$            | > 1000              |                      |                      |                      |
| $EqCTLD$            | > 1000              |                      |                      |                      |
| $Gb13Eq$            | > 1000              |                      |                      |                      |
| $Eq13Gb$            | 1.71 ± 0.33         |                      |                      |                      |
| $Gb13Eq37Gb$        | > 1000              | NB                   | NB                   | NB                   |
| $Eq13Gb37Eq$        | 9.38 ± 1.03         | 6.54 ± 0.13          | 11.6 ± 0.1           | 7.62 ± 0.08          |
| $Gb37Eq$            | 97.7 ± 12.8         | 24.5 ± 0.6           | 4.68 ± 0.05          | 11.5 ± 0.1           |
| $Eq37Gb$            | > 1000              | NB                   | NB                   | NB                   |
| $Gb84Eq$            | 60.7 ± 2.3          |                      |                      |                      |
| $Eq84Gb$            | 882 ± 91            |                      |                      |                      |

$^a$ NB, no binding
TABLE III.
Inhibition constants ($K_{i, app}$) of recombinant proteins toward *G. brevicaudus* acidic PLA$_2$, and dissociation constants ($K_d$) and association and dissociation rate constants ($k_{ass}$ and $k_{diss}$) for the interaction of the recombinant proteins with immobilized *G. brevicaudus* acidic PLA$_2$.

| Recombinant Proteins | PLA$_2$ Inhibition | Binding to immobilized PLA$_2$ |
|----------------------|---------------------|-------------------------------|
|                      | $K_{i, app}$ (nM)    | $K_d$ (nM)                    | $k_{ass}$ (M$^{-1}$s$^{-1}$) $\times 10^{-5}$ | $k_{diss}$ (s$^{-1}$) $\times 10^{3}$ |
| *GbPLI*α             | 3.05 ± 0.12         | 4.53 ± 0.08                   | 8.76 ± 0.04                                   | 3.97 ± 0.05                           |
| Q21E                 | 11.8 ± 0.6          |                               |                                              |                                     |
| E23K                 | 13.6 ± 1.1          | 6.89 ± 0.14                   | 9.21 ± 0.06                                   | 6.35 ± 0.09                           |
| S25D                 | 6.24 ± 0.95         | 8.21 ± 0.21                   | 11.3 ± 0.1                                    | 9.28 ± 0.14                           |
| N26K                 | 80.0 ± 3.5          | 18.1 ± 0.7                    | 7.28 ± 0.14                                   | 13.2 ± 0.3                            |
| K28E                 | 23.5 ± 2.0          | 11.3 ± 0.2 $^a$              | 4.72 ± 0.03 $^a$                              | 5.34 ± 0.07 $^a$                     |
| D29N                 | 23.1 ± 2.9          | 3.75 ± 0.07                   | 21.8 ± 0.2                                    | 8.17 ± 0.11                           |
| Q79E                 | 10.5 ± 0.5          |                               |                                              |                                     |
| Q111E                | 9.09 ± 0.19         |                               |                                              |                                     |
| T118K                | 11.8 ± 0.5          |                               |                                              |                                     |
| T118NKL              | 3.87 ± 0.22         |                               |                                              |                                     |
| H129N                | 8.02 ± 0.41         |                               |                                              |                                     |
| S130D                | 12.1 ± 0.6          |                               |                                              |                                     |
| N136D                | 4.72 ± 0.56         |                               |                                              |                                     |
| Y144S                | 43.1 ± 1.9          |                               |                                              |                                     |
| L147Δ                | 10.8 ± 0.7          |                               |                                              |                                     |

$^a$ Values were calculated from the data obtained by using 46 nM K28E.
Variable residues found in other PLIαs

GbPLIα

EqPLIα-LP

Conserved residues among collectins
Rat SP-A (81-228)
Rat MBP-A (107-221)

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Mapping the region of the α-type phospholipase A2 inhibitor PLIα responsible for its inhibitory activity
Kohji Okumura, Ai Ohno, Masanori Nishida, Kyozo Hayashi, Kiyoshi Ikeda and Seiji Inoue

J. Biol. Chem. published online September 2, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M507250200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts