A Novel Phospholipase A2 Inhibitor with Leucine-rich Repeats from the Blood Plasma of Agkistrodon blomhoffii siniticus

SEQUENCE HOMOLOGIES WITH HUMAN LEUCINE-RICH α2-GLYCOPROTEIN* (Received for publication, February 17, 1998, and in revised form, May 1, 1998)

Kohji Okumura, Naoki Ohkura, Seiji Inoue, Kiyoshi Ikeda, and Kyozo Hayashi
From the Department of Biochemistry, Osaka University of Pharmaceutical Sciences, Nasahara, Takatsuki, Osaka 569-1094, Japan

The phospholipase A2 (PLA2) inhibitor PLIβ, purified from the blood plasma of Chinese mamushi snake (Agkistrodon blomhoffii siniticus), is a 160-kDa trimer with three 50-kDa subunits; and it inhibits specifically the enzymatic activity of the basic PLA2 from its own venom (Ohkura, N., Okuhara, H., Inoue, S., Ikeda, K., and Hayashi, K. (1997) Biochem. J. 325, 527–531). In the present study, the 50-kDa subunit was found to be glycosylated with N-linked carbohydrate, and enzymatic deglycosylation decreased the molecular mass of the 50-kDa protein, which contained the sequences of all the peptides derived by lysyl endopeptidase digestion of the subunit. The molecular mass of the mature protein was calculated to be 34,594 Da, and the deduced amino acid sequence contained four potential N-glycosylation sites. The sequence of PLIβ showed no significant homology with that of the known PLA2 inhibitors. But, interestingly, it exhibited 33% identity with that of human leucine-rich α2-glycoprotein, a serum protein of unknown function. The most striking feature of the sequence is that it contained nine leucine-rich repeats (LRRs), each of 24 amino acid residues and thus encompassing over two-thirds of the molecule. LRRs in PLIβ might be responsible for the specific binding to basic PLA2, since LRRs are considered as the motifs involved in protein-protein interactions.

Phospholipases A2 (PLA2s, EC 3.1.1.4) catalyze the hydrol-

* This work was supported in part by Grant 07672400 (to S. I.) from the program Grants-in-Aid for Scientific Research (C) of the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1704 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1084, Japan. Tel.: 81-726-90-1075; Fax: 81-726-90-1005; E-mail: inoue@vosun11.oups.ac.jp.

‡ The abbreviations used are: PLA2, phospholipase A2; PLI, phospholipase A2 inhibitor; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; LRR, leucine-rich repeat; LRG, leucine-rich α2-glycoprotein.

Materials—The blood plasma, venom, and liver of Chinese mamushi (A. blomhoffii siniticus) were obtained from Ueda Trading Co. (Gifu, Japan). A. blomhoffii siniticus basic PLA2 was purified from the venom as described previously (4). Chinese mamushi PLIβ was purified to homogeneity from blood plasma as described previously (3).

Experimental Procedures

The phospholipase A2 inhibitor PLIβ was isolated from a Chinese mamushi liver cDNA library by use of a probe prepared by a polymerase chain reaction on the basis of the partially determined amino acid sequence of the subunit. The cDNA contained an open reading frame encoding a 23-residue signal sequence followed by a 308-residue protein, which contained the sequences of all the peptides derived by lysyl endopeptidase digestion of the subunit. The molecular mass of the mature protein was calculated to be 34,594 Da, and the deduced amino acid sequence contained four potential N-glycosylation sites. The sequence of PLIβ showed no significant homology with that of the known PLA2 inhibitors. But, interestingly, it exhibited 33% identity with that of human leucine-rich α2-glycoprotein, a serum protein of unknown function. The most striking feature of the sequence is that it contained nine leucine-rich repeats (LRRs), each of 24 amino acid residues and thus encompassing over two-thirds of the molecule. LRRs in PLIβ might be responsible for the specific binding to basic PLA2, since LRRs are considered as the motifs involved in protein-protein interactions.

The phospholipase A2 (PLA2) inhibitor PLIβ, purified from the blood plasma of Chinese mamushi snake (Agkistrodon blomhoffii siniticus), is a 160-kDa trimer with three 50-kDa subunits; and it inhibits specifically the enzymatic activity of the basic PLA2 from its own venom (Ohkura, N., Okuhara, H., Inoue, S., Ikeda, K., and Hayashi, K. (1997) Biochem. J. 325, 527–531). In the present study, the 50-kDa subunit was found to be glycosylated with N-linked carbohydrate, and enzymatic deglycosylation decreased the molecular mass of the 50-kDa protein, which contained the sequences of all the peptides derived by lysyl endopeptidase digestion of the subunit. The molecular mass of the mature protein was calculated to be 34,594 Da, and the deduced amino acid sequence contained four potential N-glycosylation sites. The sequence of PLIβ showed no significant homology with that of the known PLA2 inhibitors. But, interestingly, it exhibited 33% identity with that of human leucine-rich α2-glycoprotein, a serum protein of unknown function. The most striking feature of the sequence is that it contained nine leucine-rich repeats (LRRs), each of 24 amino acid residues and thus encompassing over two-thirds of the molecule. LRRs in PLIβ might be responsible for the specific binding to basic PLA2, since LRRs are considered as the motifs involved in protein-protein interactions.

Phospholipases A2 (PLA2s, EC 3.1.1.4) catalyze the hydrol-

The phospholipase A2 (PLA2) inhibitor PLIβ, purified from the blood plasma of Chinese mamushi snake (Agkistrodon blomhoffii siniticus), is a 160-kDa trimer with three 50-kDa subunits; and it inhibits specifically the enzymatic activity of the basic PLA2 from its own venom (Ohkura, N., Okuhara, H., Inoue, S., Ikeda, K., and Hayashi, K. (1997) Biochem. J. 325, 527–531). In the present study, the 50-kDa subunit was found to be glycosylated with N-linked carbohydrate, and enzymatic deglycosylation decreased the molecular mass of the 50-kDa protein, which contained the sequences of all the peptides derived by lysyl endopeptidase digestion of the subunit. The molecular mass of the mature protein was calculated to be 34,594 Da, and the deduced amino acid sequence contained four potential N-glycosylation sites. The sequence of PLIβ showed no significant homology with that of the known PLA2 inhibitors. But, interestingly, it exhibited 33% identity with that of human leucine-rich α2-glycoprotein, a serum protein of unknown function. The most striking feature of the sequence is that it contained nine leucine-rich repeats (LRRs), each of 24 amino acid residues and thus encompassing over two-thirds of the molecule. LRRs in PLIβ might be responsible for the specific binding to basic PLA2, since LRRs are considered as the motifs involved in protein-protein interactions.

Phospholipases A2 (PLA2s, EC 3.1.1.4) catalyze the hydrol-
Deglycosylation and SDS-PAGE of PLIβ—PLIβ was deglycosylated with an enzymatic deglycosylation kit (Bio-Rad). O-Linked oligosaccharides were cleaved by the combination of O-glycanase and N-acetylgalactosaminidase at 37 °C for 1 h in 50 mM sodium phosphate buffer, pH 6.0. N-Linked oligosaccharides were cleaved by peptide-N-glycosidase F (PNGase F). For the detection of sugars in glycoproteins, proteins on SDS-polyacrylamide gel were transferred electrophoretically to a nitrocellulose membrane. The glycoproteins on the membrane were detected with a DIG glycan detection kit (Boehringer Mannheim). They were oxidized, labeled with digoxigenin, and then detected by an enzyme immunnoassay using a digoxigenin-specific antibody conjugated with alkaline phosphatase.

Mass Spectrometry Analysis of Deglycosylated PLIβ—Mass analysis was performed on a Voyager DE STR (PerSeptive Biosystems) matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometer. The instrument was operated in the linear positive ion mode with an accelerating potential in the source of 20 kV. Ionization was accomplished with the 357-nm beam from a nitrogen laser with the laser energy precisely tuned for the optimal signal to noise ratio. Calibration was performed with a mixture of three standard compounds (bovine serum albumin and horse heart myoglobin).

An aliquot (2 μl) of the HPLC-purified, deglycosylated PLIβ in 40% acetonitrile was mixed with an equal volume of matrix solution (10 mg/ml of sinapinic acid in 30% acetonitrile containing 0.1% TFA). This mixture was deposited on the metal target, allowed to air-dry, and introduced into the MALDI source.

Chemical Cross-linking—Aliquots (5 μl) of the purified PLIβ (140 μg/ml) in 50 mM Hepes buffer (pH 7.5, ionic strength 0.2) were treated with bis(sulfosuccinimidyl) suberate (Pierce) for 3 h at room temperature. The samples were diluted with the double-strength gel sample buffer containing 0.2 M dithiothreitol, heated to 100 °C for 5 min, and analyzed by SDS-PAGE, followed by Coomassie Blue staining. Direct Binding of Basic PLA2 to PLIβ—A 0.25-nmol amount of PLIβ was incubated for 1 h at room temperature with 0.7, and 2 nmol of basic PLA2 in 100 μl of 50 mM Hepes buffer (pH 7.5, ionic strength 0.2) containing 0.05% (w/v) Tween 20. The mixture was then applied to a Superose 12 HR10/30 column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The fractions containing the PLIβ/PLA2 complex were further analyzed by reversed-phase HPLC on a Vydac C4 column (The Separations Group).

Amino Acid Sequencing of Peptides Derived from PLIβ—PLIβ was pyrolythediated in the presence of 6 M guanidine hydrochloride by the method described previously (13). The pyrolythediated PLIβ was digested with lysyl endopeptidase (Wako Chemicals), and the resultant fragments were separated by HPLC on a Vydac C4 column with 0.1% trifluoroacetic acid in the mobile phase. The fragments thus obtained were sequenced with an Applied Biosystems model 477A protein sequencer equipped with an ECL direct nucleic acid detection system (Amersham Pharmacia Biotech) on an O-Sq-3000 DNA sequencer (Hitachi) after subcloning into pBluescript (Stratagene). Analysis of DNA sequencing data and alignments of protein sequences were performed by use of DNASTAR (Hitachi Software Engineering Co. Ltd.) and BLAST (15) software on EMBL (16) and GenBankTM (17) data bases.

RNA and DNA Blotting Analyses—A 0.23-μg amount of poly(A)-RNA prepared from Chinese manushi liver was electrophoresed on a 1.2% agarose gel containing 2 % formaldehyde and transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech). The biotinylated probes for RNA blotting analysis were prepared from the amplified 550-bp product with a BioPrime DNA labeling kit (Life Technologies, Inc.). After prehybridization, the membrane was incubated with the hybridization buffer (Amersham Pharmacia Biotech) containing 5% blocking agent, washed twice for 5 min with 2× SSC containing 0.1% SDS at room temperature, twice for 5 min with 0.2× SSC containing 0.1% SDS at room temperature, and twice for 15 min with 0.2× SSC containing 0.1% SDS at 42 °C. After the final wash, the PLIβ transcript was chemiluminoscenently detected with a Supersignal NA complete blotting kit for detection of biotinylated probes (Pierce) according to the manufacturer’s instructions.

Genomic DNA was prepared from Chinese manushi liver by the standard procedure (18). For DNA blotting, DNA was completely digested with various restriction endonucleases, electrophoresed on a 0.7% agarose gel, and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). The membrane was hybridized with the peroxidase-labeled 550-bp fragment at 42 °C overnight in an ECL gold hybridization buffer (Amersham Pharmacia Biotech) containing 5% blocking agent, washed twice for 20 min with 0.5× SSC containing 0.4% SDS and 8 μg/ml at 42 °C, and then twice for 5 min with 2× SSC at room temperature. The positive phages were located with ECL detection reagents (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Plasmids were obtained from the isolated phages by in vitro excision.

RESULTS

Chemical Characterization of PLIβ—PLIβ was purified from the blood plasma of Chinese manushi, A. blochhoffii sinicus, by sequential chromatography on Sephadex G-200, Q-Sephrose, Hi-Trap Blue, Hi-Trap Phenyl HP, and Mono-Q HR5/5 columns, as described previously (3). SDS-PAGE of the purified PLIβ showed one protein band with an apparent molecular mass of 50 kDa (Fig. 1). When the PLIβ was treated with N-glycosidase F and a combination of N-acetylneuraminidase and O-glycanase, the apparent molecular mass was decreased to 47 kDa, respectively. Simular PLIβ treated with N-glycosidase F after the treatment with the combination of N-acetylneuraminidase and O-glycanase gave the same molecular mass of 39 kDa as that treated only with N-glycosidase F (data not shown).
A Snake PLA₂ Inhibitor with Leucine-rich Repeats

Since the molecular mass of 39 kDa, estimated from the migration of the deglycosylated PLIβ on SDS-PAGE gel, seemed to be different from that of 34,594 Da, which was calculated from the sequence determined in the present study, the molecular mass of the deglycosylated PLIβ was confirmed by means of mass spectrometry. The observed molecular mass was 34,674 ± 61.8 Da, which was comparable to the calculated value, indicating that the leucine-rich structure of PLIβ may affect its migration on SDS-PAGE. The molecular mass of 50 kDa obtained from SDS-PAGE of the intact PLIβ subunit was also determined to be 43,857.2 ± 81.9 Da by the mass spectrometry.

As described previously (3), the molecular mass of PLIβ was determined to be 160 kDa by gel filtration. Thus the native PLIβ was considered to be a trimer of 50-kDa subunits. In order to confirm the trimeric structure of PLIβ, chemical cross-linking experiments were performed. As shown in Fig. 2, the subunits of PLIβ could be cross-linked to form dimer and trimer, but no other higher multimer were formed. Therefore, native PLIβ was found to be composed of three homogeneous 50-kDa subunits.

Table I shows the quantification of free thiol groups of PLIβ. In the presence of denaturating agents, 1.7 mol of free thiol group was detected in one mol of the PLIβ subunit, while only 0.2 mol was detected in the absence of the denaturating agents. Therefore, two half-cystine residues of the PLIβ subunit were linking experiments were performed. As shown in Fig. 2, the respective apparent molecular masses of 160 and 8 kDa. However, when their mixture was applied to the column, they were eluted with a single peak at 28 min and 42 min, corresponding to their respective apparent molecular masses of 160 and 8 kDa. However, when their mixture was applied to the column, the latter peak disappeared, and the height of the former peak increased.

**Direct Interaction of PLIβ with Basic PLA₂—**As shown in Fig. 3, complex formation of PLIβ with A. blomhoffii siniticus basic PLA₂ was investigated by gel filtration on a Superose 12 column. When 0.23 nmol of PLIβ and 0.7 nmol of PLA₂ were separately applied to the column, they were eluted with a single peak at 28 min and 42 min, corresponding to their respective apparent molecular masses of 160 and 8 kDa. However, when their mixture was applied to the column, the latter peak disappeared, and the height of the former peak increased, suggesting the formation of a stable complex between PLIβ and basic PLA₂. The fraction containing the complex of PLIβ and basic PLA₂ was obtained by the gel filtration of the mixture containing an excess amount of the PLA₂ over PLIβ. Then, PLIβ and basic PLA₂ in this fraction were separately quantified by reverse-phase HPLC. The molar ratio of PLA₂ to PLIβ was found to be 3.17, suggesting that each subunit of PLIβ may bind one molecule of basic PLA₂.

**Cloning of PLIβ cDNA—**Previously, we determined the N-terminal amino acid sequence of PLIβ (3). In order to elucidate the internal sequences of PLIβ, we digested reduced and S-pyridylethylated PLIβ with lysyl endopeptidase. Of the resulting peptides, fourteen peptides were isolated by reverse-phase HPLC, and their complete or partial amino acid sequences were determined. On the basis of the determined partial amino acid sequences of PLIβ and its peptides, we synthesized degenerate primers, ABSPR5 and ABSPR6, corresponding to residues 11–17 (ENVTEFV) and 186–192 (NPIQCIV), respectively. Using these degenerate primers for RT-PCR with mamushi liver total RNA as template, we obtained a cDNA fragment of 550 bp. When this fragment was subcloned and sequenced, the deduced amino acid sequence coincided with that of some lysyl endopeptidase peptides, suggesting that the fragment was a part of PLIβ cDNA. Thus this fragment was used as a probe to screen the mamushi liver aMOSExox cDNA library. From the 2 × 10⁶ plaques screened, five positive clones were obtained. Clone pABS205, which contained the largest insert (2.2 kb), was selected and sequenced. The complete nucleotide sequence of PLIβ cDNA and the deduced amino acid sequence are shown in Fig. 4. It contained 2256 bp with a 5′-noncoding region of 12 bp, followed by an open reading frame region of 993 bp, and a 3′-noncoding region of 1241 bp and a poly(A) tail. The open reading frame of this cDNA sequence

**Sulphydryl titration of PLIβ**

The thiol content was determined as described under “Experimental Procedures” in the presence or absence of 6 M guanidine HCl.

| 6 M guanidine HCl | Mol of thiol/mol of PLIβ subunit | No. of determination |
|------------------|-------------------------------|---------------------|
| +                | 1.7 ± 0.5                      | 3                   |
| −                | 0.2                           | 1                   |

**Gel filtration of the mixture of PLA₂ and PLIβ on a Superose 12 column.** The column had been equilibrated with Heps buffer containing 0.05% (w/v) Tween 20 (pH 7.5, μ = 0.2). (a) 0.23 nmol of PLIβ, (b) 0.7 nmol of PLA₂, (c) 0.23 nmol of PLIβ and 0.7 nmol of PLA₂, (d) 0.23 nmol of PLIβ and 2.1 nmol of PLA₂.
encoded a protein of 331 amino acids, and its deduced amino acid sequence included the amino acid sequences determined from the lysyl endopeptidase peptides of the purified PLIβ (Fig. 4, underlined). The first 23 amino acids following the initiator methionine have the features of a signal sequence (19). Valine at the residue 1 was regarded as the N-terminal residue of the mature protein on the basis of the N-terminal sequence determined from the purified PLIβ. Thus the mature PLIβ was predicted to be composed of 308 amino acid residues with a calculated molecular mass of 34,594 Da, which was consistent with the corresponding value of the deglycosylated PLIβ determined by mass spectrometry. There were four potential N-linked glycosylation sites (Asn-X-Ser/Thr) in the deduced sequence (Fig. 4, boldface). One of them, asparagine at residue 12, would actually be glycosylated, since no PTH derivatives of asparagine were detected at the corresponding position on the amino acid sequence study of the purified PLIβ (3). Two potential polyadenylation signals (AATAAA) occurred at 1503 and 2226 (Fig. 4). Since the latter signal was present 17 bp upstream of the poly(A) tail, it was used as the actual signal by the mRNA from which this cDNA clone was derived.

**RNA and Genomic DNA Blotting Analysis for PLIβ**—Northern blot analysis revealed that a single hybridizing band of approximately 2.7 kb was observed on the blot of the poly(A)1 RNA isolated from Chinese mamushi liver, indicating that the size of the obtained cDNA is close to that of the full-length copy of mRNA (Fig. 5). Since no other shorter transcripts could be detected, no alternative polyadenylation would occur.

Genomic Southern analysis by use of the fragment corresponding to the coding region (nucleotide 112–656) as a probe showed about four bands of different intensity in each lane of genomic DNA digested with different restriction endonucleases (Fig. 6A). A nearly identical pattern of hybridizing bands was observed, when the fragment corresponding to the 3′-noncoding region (nucleotide 1132–2256) was used as a hybridization probe (Fig. 6B). The restriction digest with EcoRI revealed a major hybridizing band of 2.5 kb on probing with the coding fragment as well as on probing with the noncoding fragment;
although the recognition sites of EcoRI were present at positions 1102 and 1132 of PLIβ cDNA, suggesting that the PLIβ gene was not a single copy and that PLIβ-related sequences formed a multigene family in the snake genome.

**DISCUSSION**

Three distinct types of PLA2 inhibitors (PLIα, PLIβ, and PLIγ) are present in the blood plasma of the Chinese mamushi, *A. blomhoffii sinicus* (3). These inhibitors inhibit three group-II PLA2s (acidic, neutral, and basic PLA2s) from the venom with different specificity. PLIα specifically inhibits the acidic PLA2 by binding of 1 mol of the PLA2 to 1 mol of the PLIα, which is a trimer of 20-kDa subunits (4). PLIγ shows a broad inhibition spectrum; i.e., it inhibited all three venom PLA2s almost equally and, furthermore, it inhibited other groups of PLA2s, including Elapidae venom PLA2s (group I) and honeybee PLA2 (group III) (3). PLIβ is a specific inhibitor of the venom basic PLA2 of *A. blomhoffii sinicus*, and it does not inhibit the other two PLA2s (3). As shown in Fig. 3, 1 mol of the PLIβ, which was composed of three identical subunits of 50-kDa, was found to bind 3 mol of the basic PLA2, suggesting that each subunit would bind one PLA2 molecule.

In the present study, the complete nucleotide sequence of cDNA encoding PLIβ was determined. The mature PLIβ was composed of 308 amino acid residues with a calculated molecular weight of 34,594. This value is consistent with that obtained by mass spectrometry of the deglycosylated PLIβ. PLIβ was found to be a heavily glycosylated protein having N-linked glycosidic chains. Four potential N-linked glycosylation sites were identified in the deduced sequence. The amino acid sequence of PLIβ showed no significant

![Fig. 5. Northern blot analysis of PLIβ. Poly(A)+ RNA prepared from Chinese mamushi liver was electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a positive-charged nylon membrane. The membrane was hybridized with the biotinylated PCR fragment corresponding to nucleotides 112–656. RNA size markers are indicated on the right. The arrow indicates the position of the PLIβ transcript.](image)

![Fig. 6. Southern blot analysis of PLIβ. Chinese mamushi genomic DNA was digested with various restriction endonucleases, electrophoresed on a 0.7% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with the peroxidase-labeled probe of the portion of the coding region corresponding to nucleotides 112–2256 (Panel A) or with that of the portion of the 3′-noncoding region corresponding to nucleotides 1132–2256 (Panel B). Lanes 1, PstI; 2, KpnI; 3, EcoRV; 4, PstI; 5, EcoRI; 6, HindIII; 7, BamHI. DNA size markers are indicated.](image)

![Fig. 7. Comparison of the amino acid sequences of *A. blomhoffii sinicus* PLIβ and human LRG. The amino acid sequence of *A. blomhoffii sinicus* PLIβ was compared with that of human LRG (22). Identical residues are shown in stippled boxes.](image)
The sequence of A. blomhoffii siniticus PLIβ was divided into signal sequence, N-flanking, leucine-rich tandem repeats, and C-flanking regions. Conserved residues in leucine-rich repeat predicted on the basis of the three-dimensional structure of ribonuclease inhibitor (23) are indicated. Cysteine residues in N-flanking and C-flanking regions are also shown in stippled boxes. Structural similarities to those of PLIα and PLIγ. PLIβ contained neither the CRD sequences, which were found in the sequences of PLIα (6) and PLIγ receptors (20, 21), nor the two tandem patterns of cysteine residues found in the sequences of two homologous subunits of PLIγ (11) or in Ly-6-related proteins. Searching for homologies by use of the BLAST program revealed that PLIβ shared 33% identity over the whole molecule with human leucine-rich α-glycoprotein (LRG) (Fig. 7). LRG is a human serum protein in which leucine-rich repeats (LRRs) were first discovered (22). Like LRG, PLIβ contained 9 tandem LRRs of 24 residues each, which encompassed over two-thirds of the molecule (Fig. 8). An alignment of LRRs of PLIβ showed that they contained the consensus sequence of X-L-X-L-D-L-S-X-N-X-L-X-L-X-X-X-X-F-X-X-X-L (X denotes any amino acid). Similar LRR consensus sequences have been found in the primary structure of a large number of proteins, including proteins that participate in biologically important processes, such as receptors for hormone, enzymes, enzyme inhibitors, proteins for cell adhesion, and ribosome-binding proteins (reviewed in Refs. 23 and 24). Although the functions of these proteins are different, all proteins containing LRRs are thought to be involved in protein-protein interactions. The functions of LRG remain unknown, and its physiologic ligand remains unidentified. But, both LRG and PLIβ are serum proteins, and there is 33% identity between their sequences. This raise the possibility that PLIβ corresponds to the snake LRG and, furthermore, that human LRG functions as a PLA2 inhibitor, although evidence on this hypothesis has yet to be demonstrated.

Many LRR-containing proteins contain homologous regions flanking the LRR domains. These regions are characterized by the patterns of cysteine residues; the consensus sequences can be described as CP{−2X}CX C{−6X}C for the amino-flanking and PXXCXC{−20X}C{−20X}C for the carboxy-flanking regions (23). The carboxy-flanking region of PLIβ was nearly identical to the consensus sequence. The amino-flanking region of PLIβ did not conform to the consensus sequence, but contained a related sequence with two cysteine residues. In addition, there was a proline-rich cluster in the amino-flanking region of PLIβ. This proline-rich cluster is not present in any other LRR-containing proteins, and thus might play an important structural or functional role in PLIβ.

Two cysteine residues, Cys-147 and Cys-190, were found in the LRR domain of PLIβ, while no cysteine residues were found in that of LRG. One of the LRR-containing proteins, ribonuclease inhibitor (RI), contained most of cysteine residues in its LRR domain, and all of the cysteine residues occur as free thiol groups (25). Of the 10 cysteine residues present in the PLIβ subunit, two were found to occur as free thiols as shown in Table I. Therefore, the 2 cysteine residues with free thiol groups might be assign to be Cys-147 and Cys-190 found in the LRR domain, because the intrachain disulfide bonding of these cysteine residues could be expected to be unfavorable geometry for the conformation of LRR.

The crystal structure of porcine liver RI has been determined (26). RI was found to be a nonglobular, horseshoe-shaped molecule with a curved parallel β-sheet lining the inner circumference of the horseshoe and the helices flanking its outer circumference. In this structure, the individual LRR units have essentially the same conformation of β-strand structural units, consisting of a short β-strand and an α-helix approximately parallel to each other. The crystal structure of the complex of RI and bovine ribonuclease A (RI-RNase A) has also been determined (27). RNase A binds to a concave surface of the inhibitor, mainly comprising its parallel β-sheet and the loops C-terminal to the β-strands. Therefore, the structure of RI-RNase A could serve as a model for the interaction between PLIβ and PLA2. If the LRRs in PLIβ constitute β-strand structural units that occur in RI and the whole molecule reveals a horseshoe structure just like the molecule of RI, the PLA2 molecule can be expected to bind to the concave surface of the horseshoe structure of PLIβ. That is, the concave surface would contain a large excess of negatively charged residues, especially aspartate at the sixth residue of the consensus sequence of LRR, and these negative charges could electrostatically interact with the positively charged residues on the basic PLA2 molecule. PLIβ is a selective inhibitor against the group II basic PLA2s from Crotalidae venom, and it does not inhibit other kind of PLA2s. The residues His-1, Arg-6, Glu-17, Trp-70, Lys-111, and Ile-124 are conserved among these group II basic PLA2s, but they are replaced by other residues in all the PLA2s not inhibited by PLIβ. Some of these residues in the PLA2 molecule are therefore thought to be involved in the interaction with the PLIβ molecule.

The very extensive binding surface of the RI-RNase A complex was suggested to be in part responsible for the extremely low inhibitory constant (Ki) of 5.9 × 10^{-12} M (28). The inhibitory constant of PLIβ for A. blomhoffii siniticus basic PLA2 was 7.5 × 10^{-10} M (3). That this interaction is much weaker than that of RI and RNase suggests that the number of amino acid residues of PLIβ closely contacting with the PLA2 molecule is much smaller than that for the RI-RNase complex and that they were located scatteringly and specifically on the extensive concave surface of the horseshoe. The narrow inhibition spectrum of PLIβ may be accounted for by the local distribution of the contact residues despite the extensive contact area.
4. Ohkura, N., Inoue, S., Ikeda, K., and Hayashi, K. (1993) *J. Biochem.* **113**, 413–419
5. Kogaki, H., Inoue, S., Ikeda, K., Samejima, Y., Omori-Satoh, T., and Hayashi, K. (1991) *J. Biochem.* **106**, 966–971
6. Inoue, S., Kogaki, H., Ikeda, K., Samejima, Y., and Omori-Satoh, T. (1991) *J. Biol. Chem.* **266**, 1901–1907
7. Inoue, S., Shimada, A., Ohkura, N., Ikeda, K., Samejima, Y., Omori-Satoh, T., and Hayashi, K. (1997) *Biochem. Mol. Biol. Int.* **41**, 529–537
8. Ohkura, N., Inoue, S., Ikeda, K., and Hayashi, K. (1991) *Biochem. Biophys. Res. Commun.* **204**, 1212–1218
9. Fortes-Dias, C. L., Lin, Y., Ewell, J., Diniz, C. R., and Liu, T.-Y. (1994) *J. Biol. Chem.* **269**, 5897–5904
10. Perales, J., Villete, C., Domont, G. B., Choumet, V., Saligou, B., Moussatché, H., Bon, C., and Faure, G. (1995) *Eur. J. Biochem.* **227**, 19–26
11. Ohkura, N., Inoue, S., Ikeda, K., and Hayashi, K. (1994) *Biochem. Biophys. Res. Commun.* **204**, 1212–1218
12. Laemmli, U. K. (1970) *Nature* **227**, 680–685
13. Cavins, J. F., and Friedman, M. (1970) *Anal. Biochem.* **33**, 489–495
14. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) *Methods Enzymol.* **91**, 49–60
15. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1992) *J. Mol. Biol.* **215**, 403–410
16. Emmert, D. B., Stoehr, P. J., Stuesser, G., and Cameron, G. N. (1994) *Nucleic Acids Res.* **22**, 3445–3449
17. Benson, D. A., Boguski, M., Lipman, D. J., and Ostell, J. (1994) *Nucleic Acids Res.* **22**, 3441–3444
18. Sambrook, K., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Von Heijne, G. (1988) *Biochim. Biophys. Acta* **947**, 307–333
20. Lambeau, G., Ancien, P., Barhanin, J., and Lazdunski, M. (1994) *J. Biol. Chem.* **269**, 1575–1578
21. Ishizaki, J., Hanasaki, K., Higashino, K., Kishino, J., Kikuchi, N., Obara, O., and Arita, H. (1994) *J. Biol. Chem.* **269**, 5897–5904
22. Takahashi, N., Takehashi, Y., and Putnam, F. W. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1906–1910
23. Kobe, B., and Deisenhofer, J. (1994) *Trends Biochem. Sci.* **19**, 415–421
24. Kobe, B., and Deisenhofer, J. (1995) *Cur. Opin. Struct. Biol.* **5**, 409–416
25. Lee, F. S., Fox, E. A., Zhou, H.-M., Strydom, D. J., and Vallee, B. L. (1988) *Biochemistry* **27**, 8545–8553
26. Kobe, B., and Deisenhofer, J. (1993) *Nature* **366**, 751–756
27. Kobe, B., and Deisenhofer, J. (1995) *Nature* **374**, 183–186
28. Vicentini, A. M., Kieffer, B., Mathies, R., Meyhack, B., Hemmings, B. A., Stone, S. R., and Hofsteenge, J. (1990) *Biochemistry* **29**, 8827–8834