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Isolation and Characterization of a Novel Subunit of Phospholipase A_2 Inhibitor in the Serum of *Trimeresurus flavoviridis*

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Abstract

Phospholipase A_2 inhibitor γ (PLIγ) is an oligomeric protein containing two homologous subunits, A- and B-chains (also called α- and β-chains). Three proteins termed PLI-I to PLI-III have already been identified in the serum of Habu snake (*Trimeresurus flavoviridis*) as the constituting subunits of PLIγs. PLI-I was assigned to be the subunit A of *T. flavoviridis* PLIγs and other two were the subunits B. We here describe the isolation and characterization of a novel subunit of PLIγs from the same serum. The complete amino acid sequence of this protein (named PLI-VI) has been determined by peptide analysis. It was a glycoprotein having three sugar chains and consisted of 193 amino acids. The cDNA encoding PLI-VI was cloned from a cDNA library of the *T. flavoviridis* liver using probes prepared by polymerase chain reaction on the basis of the amino acid sequence of PLI-VI. The cDNA clone for PLI-VI encoded a polypeptide of 211 amino acid residues including 21 residues of the signal sequence. Phylogenetic analysis of snake serum PLIs and the related family proteins has revealed that PLI-VI is a novel protein, and it is the first protein belonging a new subtype of PLIγ.

Introduction

Phospholipase A_2 (EC 3.1.1.4) is the major component of most snake venoms[1], and the detailed insight into the structure and mechanism of these enzymes is available[2]. It catalyzes the hydrolysis of phospholipids at its *sn*-2 position. Snake venom PLA_2 induces several pharmacological effects including presynaptic neurotoxicity, myotoxicity and cardiotoxicity as well as anticoagulant, hemolytic, hemorrhagic, oedema-inducing and platelet-aggregation inhibitory effects[3-5]. Habu snake (*T. flavoviridis*) inhabiting in the southwestern islands of Japan contains several PLA_2 and the related proteins such as acidic [Asp^{49}]-PLA_2s (PLA2 and PLA-N), a basic [Asp^{49}]-PLA_2 (PLA-B), and basic proteins I and II (BPI and BPII, both [Lys^{49}]-PLA_2s) [5-8].

Venomous snakes have PLA_2 inhibitors (PLIs), proteins capable of inhibiting snake venom PLA_2s, in their blood sera[9]. The role of these proteins is postulated to protect themselves from the leakage of

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Abbreviations: BPI, basic protein I; BPII, basic protein II; MALDI, matrix-assisted laser desorption ionization; PE, S-pyridylethylated; PLA_2, phospholipase A_2; PLI, phospholipase A_2 inhibitor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TOF, time-of-flight; uPAR, urokinase plasminogen activator surface receptor; UTR, untranslated region.

their own venomous PLA_2s into their circulatory system. Many PLIs have been isolated from various snake sera and their primary structures have been determined[10-14]. PLIs can be divided into three groups (PLIα, PLIβ and PLIγ) based on their structural characteristics. PLIα is a glycoprotein having three 20-kDa subunits[15-16], which show the sequence homology to a carbohydrate-recognition domain of Ca^{2+}-dependent (C-type) lectins[12]. PLIβ is a 160 kDa glycoprotein composed of three identical subunits that have leucine-rich repeats[17]. PLIγ also has an oligomeric structure consisting of 25 and 20 kDa subunits[12-14, 18], the primary structure of which had two tandem patterns of cysteine residues and was structurally related to proteins belonging to the uPAR/ly-6 superfamily[13].

Five proteins (PLI-I–V) relating to PLA_2 inhibitors have already been identified in *T. flavoviridis* serum by affinity chromatography[19]. PLI-I is a main component of γ type PLIs and a repeated three-finger motifs are present in the molecule. PLI-IV and V (also called PLI-A and B, respectively) are the components of α type of PLIs[10]. Recently, we have reported the primary structures of PLI-II and III[20], which are the minor components of γ type PLIs.

In the present paper, we describe the isolation of a novel subunit of γ type PLIs named PLI-VI from *T. flavoviridis* serum. We also determined the primary structure of PLI-VI and the nucleotide sequence of cDNA encoding this protein.

Experimental

Materials

The blood of Habu (*T. flavoviridis*) in the Amami Oshima islands was collected by decapitation. The serum was separated by centrifugation and stored at − 20 ℃. Ethanol precipitation was carried out as described[21]. All the other reagents were purchased from Wako Pure Chem. (Osaka).

Electrophoresis

SDS–PAGE was carried out on 12.5 % gels by Laemmli's method[22], or on 10 % polyacrylamide gels as described by Schagger and von Jagow[23]. Prestained XL-ladder marker kit (Apro science) was used for the estimation of molecular weights. After running the gels under a constant current, they were stained with 0.1 % Coomassie brilliant blue R-250 and destained with 10 % acetic acid.

Mass spectrometric analysis

Mass spectrum was measured on a Voyager DE-STR matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometer (PerSeptive Biosystems). Sample was dissolved in 0.1 % trifluoroacetic acid–50 % acetonitrile containing α-cyano-4-hydroxycinnamic acid (10 mg/ml) as the matrix, and 2-μl aliquots were analyzed. Spectrum was calibrated by the molecular mass of apomyoglobin.

HPLC

Preparative reverse-phase HPLC was performed on a μBondashere 5μ-C8-300Å column (1.9 × 15 cm, Waters), and the proteins were eluted with a linear gradient of acetonitrile in 0.1 % trifluoroacetic acid (TFA) at a flow rate of 5 ml/min. Analytical gel filtration was carried out using a TSKgel G3000SW column (0.75 × 30 cm) equilibrated with 0.2 M NaCl –50 mM phosphate buffer (pH 7.0).

Protein sequencing

Protein was reduced and S-pyridylethylated according to Friedman *et al.*[24]. The pyridylethylated (PE)-protein (500 μg each) was digested at 37 ℃ with Lys-C (E/S = 1 : 100) for 5 h in 2 M urea-20 mM Tris–HCl (pH 9.0), Asp-N (E/S = 1 : 100) for 16 h in 50 mM Tris–HCl (pH 8.0), V8 protease (E/S = 1 : 30) for 16 h, thermolysin (E/S = 1 : 100) for 6 h in 100 mM NH_4HCO_3, or cleaved at room temperature by CNBr (100 equivalents) for 21 h in 70 % HCOOH. The digests were lyophilized, dissolved in 0.1 % TFA, and fractionated by reverse-phase HPLC on a YMC-Pack ODS column in 0.1 % TFA with an appropriate gradient of acetonitrile. The amino acid sequences of proteins were determined by an automatic protein sequencing system PPSQ 21 (Shimadzu).

Analytical gel filtration

A sample was dissolved in 0.2 M NaCl-50 mM phosphate buffer (pH 7.0), and put on a TSKgel G3000SW column (0.75 × 30 cm, Tosoh). Elution was carried out by the same buffer at a flow rate of

1.0 ml/min. Elution was monitored at 280 nm and the peak area was determined by 807-IT integrator (Jasco). Molecular weight was calibrated by the retention times of bovine serum albumin (67,000), ovalbumin (46,000), and soybean trypsin inhibitor (21,500).

Synthesis of partial fragment of PLI-VI cDNA

Total RNA was extracted from 0.5 g of *T. flavoviridis* liver by acid guanidinium-phenol-chloroform (AGPC) method, and reverse transcribed to synthesize cDNA first strands using adaptor-linked Oligo(dT) primer (5'-GGCCACGCGTCGACT AGTAC-$(dT)_{17}$-3'). cDNAs obtained were used as template for 3'-RACE (rapid amplification of cDNA ends) reaction. Synthetic oligonucleotides, *PLI-VI_NS* (5'-RTGYCARAARTGYGTNGCNCCNATHA AYGAR-3') and 3'-Adp (5'-GGCCACGCGTCGACTA GTAC-3'), were used for PCR amplification. *PLI VI_NS* primer was designed upon the N-terminal amino acid sequence of PLI-VI and 3'-Adp corresponded to the adaptor sequence within adaptor-linked oligo(dT) primer. Amplification product was once subcloned into plasmid vector, and its nucleotide sequence was determined. As the result, the sequence was confirmed to be the cDNA partial fragments for PLI-VI. PLI-II(IIa) cDNA fragment obtained previously[20] was radiolabeled with [α-^{32}P]-dCTP (3000 Ci/mmol) using random primer DNA labeling kit (Takara Bio, Japan) and used for hybridization screening of cDNA library.

Construction of *T. flavoviridis* liver cDNA library

T. flavoviridis liver cDNA library was constructed using Creator SMART cDNA Library Construction kit (BD Biosciences) according to manufacturers' instruction. Briefly, cDNA first strand was synthesized using 1 μg of total RNA, followed by five cycles of PCR for non-specific enrichment of full-length cDNAs. The cDNA fragments were then ligated to pDNR-LIB vector. When the plasmid clones were used to transform *E. coli* JM109, resulting library contained 2.0 x 10^6 independent clones.

Cloning and sequence determination of cDNA encoding PLI-VI

Clones (9.6 x 10^4) from unamplified cDNA library were placed on LB agar plates and bacterial colonies were transferred onto Hybond-NX membranes (GE Healthcare Bio-Science, USA) and fixed by UV irradiation. The resulted replica membranes were prehybridized in Church's hybridization solution at 65 ℃ for 30 min and then hybridized with radiolabeled PLI-II(IIa) cDNA overnight at 50 ℃ in Church's hybridization solution. Membranes were finally washed twice for 15 min at 50 ℃ with 1x SSC, 0.1 % SDS and hybridization signals were visualized using BioImage Analyzer (Fuji Film, Japan). Accordingly, 14 bacterial colonies were isolated, cultured and their plasmids were purified by standard Alkali-SDS method. Nucleotide sequences of cDNA inserts were determined using ABI PRISM 377 DNA Sequencing System (Applied Biosystems, CA, USA).

Results

Purification of phospholipase A_2 inhibitors

Ethanol fraction $E_{2.0}$ of the habu serum was prepared as described previously[21], and subjected to reverse-phase HPLC using a C8 column. Among several peaks shown in Fig. 1, peaks 1 – 3 were assigned to be PLI-I, PLI-II, and PLI-III[19, 20], respectively. The protein in peak 6 had the N-terminal sequence of LKCQKCVAPI..., which was never been reported previously. Since the sequence XXCXXC at the N-terminal, where X is any amino acid residue other than Cys, is a characteristic feature of PLIγ subunits, we designated phospholipase inhibitor VI (PLI-VI) for the new protein in peak 6. The apparent molecular mass of PLI-VI was estimated to be 66 kDa from SDS-PAGE (Fig. 1A, inset). The exact mass determined by MALDI–TOF mass spectrometry was 32,718.7.

Oligomer formation of PLI-VI

Analytical gel filtration using a TSKgel G3000SW column showed that PLI-VI was eluted at 7.88 min which corresponded to the molecular mass of 60 kDa (Fig. 1B). This indicates that PLI-VI can easily associate to form a homodimer. As shown previously, PLI-I, II, and III were eluted under the same conditions as its monomer of 24–30 kDa[20]. PLI-I, II, and III also gave additional peaks which corresponded to the dimer or oligomer.

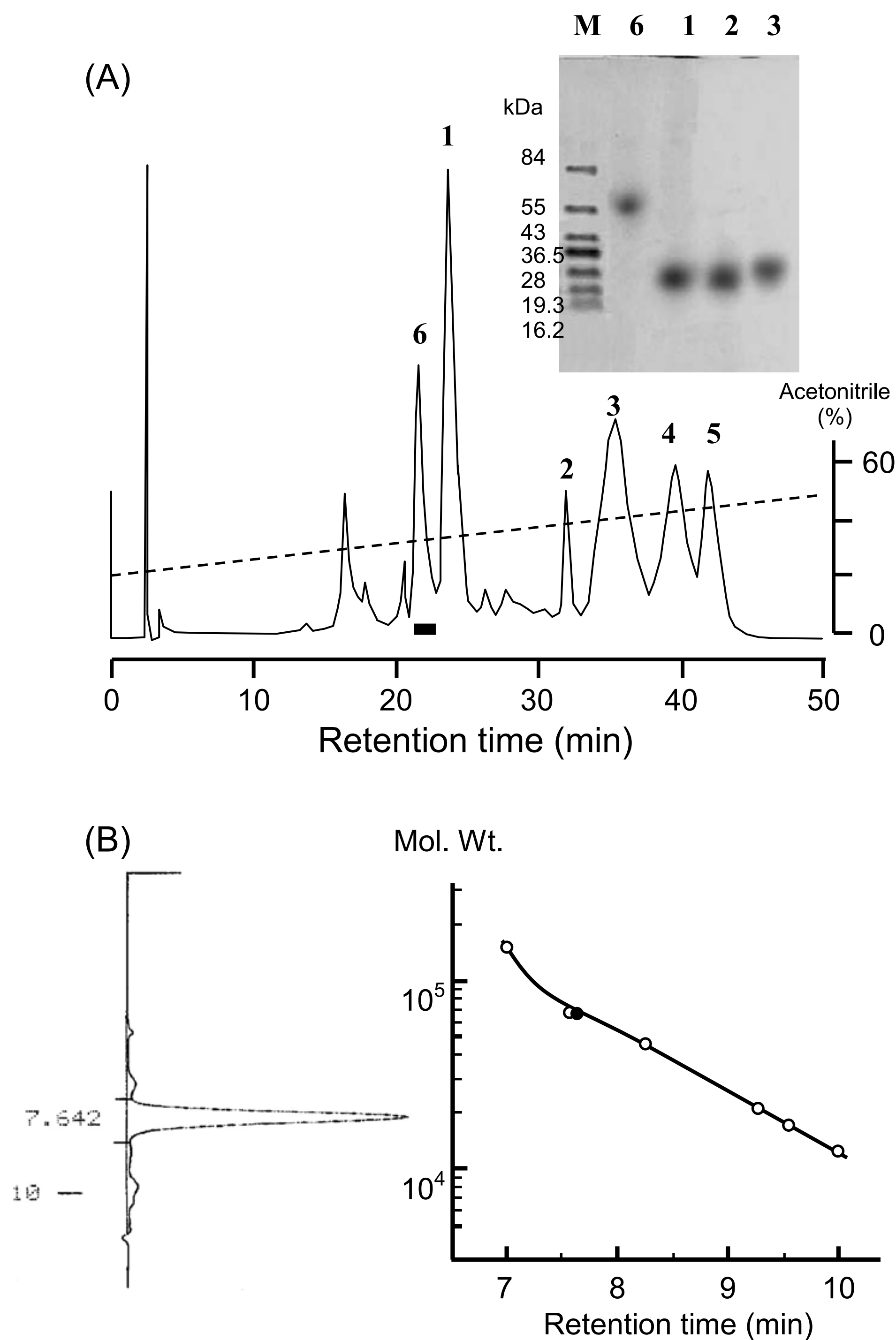

Fig. 1. (A) Purification of PLA_2 inhibitors by reverse-phase HPLC on a μBondashere 5μ-C8-300Å column (1.9 × 15 cm). Inset, SDS-PAGE analysis of purified proteins on a 10 % gel. M, molecular weght marker; 1, PLI-I; 2, PLI-II; 3, PLI-III, 6, PLI-VI, respectively. (B) Analysis of molecular size of PLI-VI. Analytical gel filtration of PLI-VI on a TSKgel G3000SW column (0.75 × 30 cm) in 0.2 M NaCl–50 mM phosphate buffer (pH 7.0). Closed circle represents a position of PLI-VI.

Sequence analysis of PLI-VI

The N-terminal 52 residues of S-pyridylethylated PLI-VI was directly determined as shown in Fig. 2. The complete amino acid sequence was determined on the basis of the results of sequence analysis of peptide fragments generated by several enzymatic digestions. The sequence of PLI-VI consisting of 193 amino acids were determined by the enzymatic cleavage using Lys-C, V8 protease, Asp-N, and thermolysin (Fig. 2). Residues 36, 142, and 175 could not be determined by Edman degradation, indicating that they as Asn residues having sugar chains. This may explain the higher molecular weight than the other PLIs.

Cloning of a cDNA encoding PLI-VI

T. flavoviridis liver cDNA was synthesized using an adaptor-linked oligo(dT) primer. Partial cDNA fragments for PLI-VI was then amplified by PCR using a oligonucleotides *PLI-VI_NS* that was originally designed based on N-terminal amino acid sequences of PLI-VI. As a result, a DNA fragment of the expected size (approx. 0.9 kb) was obtained. The nucleotide sequence was confirmed to be the cDNA partial fragment in accord with the amino acid sequence of PLI-VI. The cDNA encoding PLI-VI was obtained from Habu liver cDNA by 3'-RACE fragment and this sequence was confirmed to be the cDNA fragment for PLI-VI. Fig. 3 shows the nucleotide sequence of full-length cDNA for PLI-VI. It consisted of 947 bp, including a 28-bp 5'-untranslated region (UTR), 633-bp open reading frame encoding 211 amino acids including a potential signal peptide of 21 amino acids, and 274-bp 3'-UTR. The nucleotide sequence in the mature protein-coding region of PLI-VI was 53.3 % and 57.1 % identical to PLI-I[19] and PLI-II[20], respectively. Amino acid sequence deduced from the nucleotide sequence of cDNA was agreed well to the protein sequence (Fig. 2).

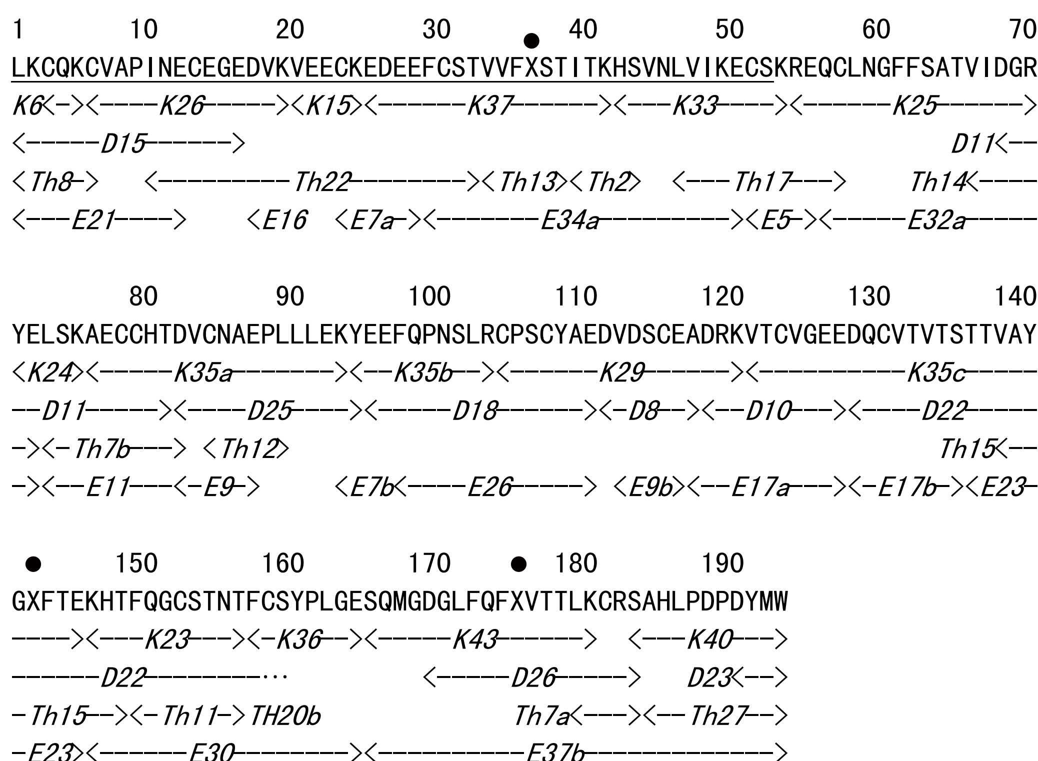

Fig. 2. Sequence determination of *T. flavoviridis* phospholipase A_2 inhibitor VI. Sugar-attached Asn residues are marked by closed circles. Sequences determined directly from the intact proteins are underlined. Peptides derived from the digestion with Lys-C, Asp-N, V8 protease, and thermolysin are designated a K, D, E, and Th, respectively.

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  1 ATAGGTGACTGAGTTGAGTTCTACCAACATGAGGGCTGCTGGAATTCTTGTCTTCTGTCT
                                 M  R  A  A  G  I  L  V  F  C  L
 61 CTTCTCTTCAATCTTATCAACAGTGACTTCTCTGAAGTGTCAGAAATGTGTTGCCCCTAT
      F  S  S  I  L  S  T  V  T  S  L  K  C  Q  K  C  V  A  P  I
121 AAATGAATGTGAAGGAGAAGATGTGAAGGTGGAGGAATGCAAAGAAGATGAAGAATTTTG
      N  E  C  E  G  E  D  V  K  V  E  E  C  K  E  D  E  E  F  C
181 TTCTACTGTAGTTTTTAATAGTACTATAACCAAGCATTCCGTGAATTTGGTTATCAAGGA
      S  T  V  V  F  N  S  T  I  T  K  H  S  V  N  L  V  I  K  E
241 ATGCTCAAAACGTGAGCAGTGTCTCAATGGCTTCTTCAGCGCCACAGTGATAGATGGTAG
      C  S  K  R  E  Q  C  L  N  G  F  F  S  A  T  V  I  D  G  R
301 ATATGAATTGTCAAAAGCTAACTGTTGCCACACTGATGTCTGTAATGCAGAACCGCTTCT
      Y  E  L  S  K  A  N  C  C  H  T  D  V  C  N  A  E  P  L  L
361 GTTGGAAAAGTATGAAGAGTTTCAACCAAATTCGTTAAGGTGCCCAAGCTGCTATGCCGA
      L  E  K  Y  E  E  F  Q  P  N  S  L  R  C  P  S  C  Y  A  E
421 AGATGTGGATTCTTGTGAAGCCGATCGGAAAGTGACGTGTGTCGGTGAAGAGGATCAGTG
      D  V  D  S  C  E  A  D  R  K  V  T  C  V  G  E  E  D  Q  C
481 TGTTACCGTCACTAGTACCACAGTGGCTTATGGAAATTTCACTGAAAAGCACACTTTCCA
      V  T  V  T  S  T  T  V  A  Y  G  N  F  T  E  K  H  T  F  Q
541 AGGATGTTCAACAAACACTTTTTGTTCCTACCCTCTAGGAGAGTCACAGATGGGCGATGG
      G  C  S  T  N  T  F  C  S  Y  P  L  G  E  S  Q  M  G  D  G
601 GCTGTTTCAGTTCAATGTCACCACCCTGAAATGCAGAAGTGCTCATCTTCCTGATCCTGA
      L  F  Q  F  N  V  T  T  L  K  C  R  S  A  H  L  P  D  P  D
661 TTATATGTGGTAGTTTGGGTAGAGTTGAACCTCCTTCACAATTATTGGAAGATTTGTAAA
      Y  M  W  *
721 AAACGAGAAAGGTGGAGATTTAATGCTGAGATAAGTGAAAATTGGGATCAAAGATGCTTG
781 AGTTTCTTCTCTGAGTCAGTGGTGGAAAGTTGGAAATTTGGGGCAAAAAAATTGAAATAT
841 TATGCTTTGAGACCATAGCTCAAGAAAATGTTGTGTGTTTTCAATCTTCGAAGTTCACAG
901 AATCATCGTAAAAATAAATTGATATGACAAAAAAAAAAAAAAAAAAA
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Fig. 3. Nucleotide sequence of cDNA encoding PLI-VI. Deduced amino acid sequence is cited below the nucleotide sequence. Signal peptide is shown in italic. Start and stop codons are boxed. Polyadenylation signal is underlined.

Comparison of amino acid sequences of several PLIs

The primary structure of PLI-VI was compared with other snake PLIs and the related proteins (Fig. 4). Habu PLI-I and *G. blomhoffi siniticus* PLI-Ib (gPLI-Ib)[25] have common features: the presence of 16 cysteine residues and a single N-linked carbohydrate site at Asn[157] (Asn[165] in Fig. 4). On the other hand, other proteins showed the high sequence similarity with the well-conserved 18 cysteine residues, whereas Habu PLI-VI showed a low homology and a different pattern of residue insertion/deletion. Moreover, the high contents of sugar chains are characteristic of PLI-VI. The primary structure of PLI-VI showed the highest homology to that of a bovine protein (Accession No. E1BFZ1). However, the amino acid identity was only 37 % and the function of the bovine protein is not known, but it was supposed the PLA_2 inhibitory activity.

Discussion

Two groups of PLIs have been identified in the serum of *T. flavoviridis*[19]. One group belongs to PLIα that contains a segment similar to the carbohydrate recognition domain of Ca^{2+}-dependent (C-type) lectins[26, 27]. The others are the member of PLIγ having two repeats of a unit termed the three-finger motif[13], which has also been found in uPAR/ly6 family proteins[29, 30]. PLIβ has not yet been reported from the serum of *T. flavoviridis*. Interestingly, PLIβ from the serum of a snake (*G. brevicaudus*) can neutralize a basic PLA_2 from its own venom, and it has 33 % sequence homology with human leucine-rich α_2-glycoprotein, which can bind cytochrome c[31]. *G. brevicaudus* PLIβ can tightly bind snake cytochrome c with a K_d of 2.37×10^{-12} M.

In the present study, we have purified PLI-VI, a novel PLIγ-type protein from the serum of *T. flavoviridis*. PLI-VI has some unique features; it has 193 residues that is the longest sequence compared with other PLIγs and the related proteins, and 3 sugar chains were attached (Fig. 4). Moreover, no similar PLI was yet reported. Recently, we have found a similar protein from *Gloydius blomhoffii* serum having an N-terminal sequence of LECQTCVSSINECEGE. This may be an ortholog of PLI-VI.

As cited in Fig. 4, the protein with the highest similarity to PLI-VI was a bovine protein (Accession No. E1BFZ1). Though the exact nature of this protein is not known, it belongs to the uPAR/ly6 family. Although few was known about the roles of PLI-II and PLI-III (see Fig. 1A), these proteins are

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          1       10        20        30        40        50        60        70        80        90       100
PLI-VI    LKCQKCVAPINECEGEDVKVEECKEDEEFCSTVVFNSTITKHSVNLVIKECSKREQCLNGFFSATVIDGRYELSKAECCHTDVCNAEPLLLEKYEEFQPN
E1BFZ1    LSCEVCVGDGPNC---RGKLQTCAPDEDSCIVVVTETNRKASLAVTSYKGCSKSSNCESGLFGFTVNHENYMGSKRRCCQKDGCNQDPLPAFV-RNHTEN
Q9PTC7    LECEFCFTPALQCD--NSRTKTCDANQDTCVTSQTEVIRAPVSLTFISKSCGTSDTCHLNYLETSPHNELTVKTKRTCCTGEECKTLPPPVLGDKVNPPN
PLI-II    IECEVCLRPGETC---NGTMMTCKDNEDTCVTFKTELIRAPLSFTFISKMCSTSDTCHLDYVETNLPHELAVRSKRACCVGDECKTMPPVVLEHHDNRYN
PLI-III   MECEVCVMPGETC---NGTMMTCKDNEDTCVTFQTELIRAPLSFTFISKMCSTSDTCHLDYVETNLPHELAVRSKRACCVGDECKTMPPVVLERHDNRYN
gPLI-Ib   LECEVCMQPGKRC---NGSMMTCKDNEDTCVMFQTEVIRAPLSFTFTSKMCSTSDTCHLDYVETNLPYELAVRSRRACCVGDECKTMPPVVLEHHDNRYN
PLI-I     RSCDFCHNIGADC---EGFQHECSSPEDECGKVGLEISSASLSVRTVHKNCFSSSVCKLRHFDVNIGHDSYIRGRINCCEKEPCEDQSFPGLP--LSQPN
gPLI-Ia   RSCDYCHNIGKDC---DGYEHECSSPEDVCGKVFLEISSASLSVRTVHKNCFSSSVCKLGHFDINIGHHSYIRGRINCCEKEPCEDQPFPGLP--LSQPN
            *  *      *         *      *                    *     *                    **    *
                 110       120       130       140       150       160       170       180       190
PLI-VI    SLRCPSCYAEDVDSCEADRKVTCVGEEDQCVTVTSTTVAYGNFTEKHTFQGCSTNTFCSYPLGESQMGDGLFQFNVTTLKCRSAHLPDPDYMW
E1BFZ1    GLRCPSCITAFTETCTATAEALCVGEETHCVAMSG-LMRP--AGDKFAVQGCGTKTACHSKPG-TLVPSGARLFTIKKTSCL
Q9PTC7    GLQCPGCLGLSSKECT-EHPVSCRGSENQCLSIIGKEFGLFFRALSY--KGCATESLCTLFEKRFWN---VLEDVEVDFKCTPALPKSSQ
PLI-II    GLHCPGCIGLASTECN-EKLVSCRDTENQCLSLTGKNLDFLVDDITM--KGCATESLCTLLQKKIFS---PIAEWELDVKCTTAFPQASQ
PLI-III   GLHCPGCIGLASTECN-EKLISCRDTENQCLSLTGKNLYFLVNDISI--KGCATESLCTLLQKKIFS---PIAEWELDVKCTPAFPQASQ
gPLI-Ib   GLHCPGCIGFGSHECN-EKLVSCRDTENQCLSLIGKNFDFVADDITI--KGCATESLCSLLQKKIFS---AIGEFDLDVKCTPVFPQSSQ
PLI-I     GYYCPGSLGLFTKDST-EFRAICKGTETKCINIVGHRYEHYPGDIAYNLKGCIS-S-CPLLSLSNATHE-ENRNYLEKVECKDALQFEKQ
gPLI-Ia   GYYCPGALGLFTEDST-EYEAICKGTETKCINIVGHRHENYPGDISYNLKGCVS-S-CPLLSLSNSTHE-ENRNYLEKVECKDAFKIASH
             *  #       #       *      *                     *     *                      *
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Fig. 4. Comparison of the amino acid sequences of PLIs and the related proteins. E1BFZ1, *Bos taurus* protein; Q9PTC7, *O. microlepidotus* protein; PLI-I, *T. flavoviridis*; gPLI-Ia, *G. blomhoffi siniticus*; PLI-II, *T. flavoviridis*; PLI-III, *T. flavoviridis*; gPLI-Ib, *G. blomhoffi siniticus*. Conserved Cys residues are marked by asterisks. Additional Cys residues found in 18-Cys type proteins are marked by #. Sugar-attached Asn residues are underlined.

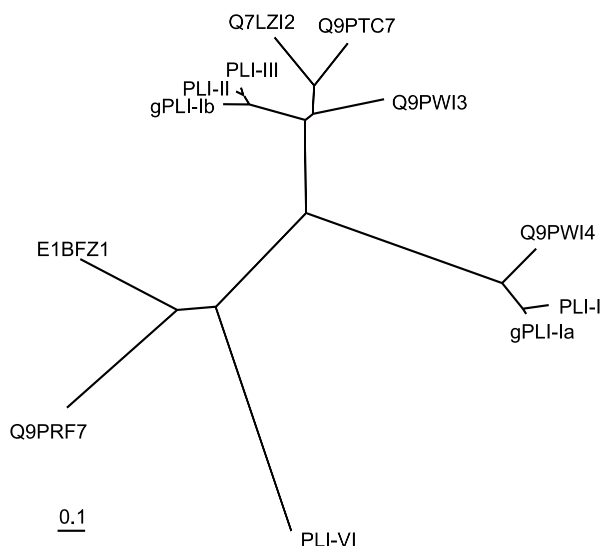

Fig. 5. The phylogenetic tree for the protein-coding regions of PLIs and the related proteins. The accession numbers are cited except PLIs. The tree was constructed by the neighbor-joining method[32] based on the number of nucleotide substitutions per site.

thought to be the components of heterodimeric PLIγ together with PLI-I in the snake serum[17, 25]. We could not yet determine the targets of PLI-VI.

Phylogenetic tree of PLIs was constructed by the neighbor-joining method[32]. Analysis of snake serum PLIs has revealed that PLI-VI belongs to a new subgroup of PLIγ subunits. We could expect other function of this protein. On the basis of primary structures, PLI-VI belongs to a clan uPAR/Ly6/CD59/snake toxin-receptor superfamily, which contains the following 5 members: activin receptor[33], BMP and activin membrane-bound inhibitor[34], PLA_2 inhibitor, neurotoxins[35], and uPAR/Ly6[29, 36]. Since proteins in this family exhibit a variety of functions, PLI-VI may also possess other unknown function.

References

[1] E. A. Dennis, *J. Biol. Chem.* **269**, 13057-13060 (1994).

[2] D. L. Scott and P. B. Sigler, in "Advances in Protein Chemistry" **45**, 53-88 (1994).

[3] G. A. Boffa, M. C. Boffa, and J. J. *Winchenne, Biochim. Biophys. Acta* **429**, 828-838 (1976).

[4] J. E. Fletcher, B.E. Rapuano, E. Condrea, C. C. Yang, and P. Rosenberg, *Toxicol. Appl. Pharmacol.* **59**, 375-388 (1981).

[5] H. Kihara, R. Uchikawa, S. Hattori, and M. Ohno, *Biochem. Int.* **28**, 895-903 (1992).

[6] S. Tanaka, N. Mohri, H. Kihara, and M. Ohno, *J. Biochem.* **99**, 281-289 (1986).

[7] S. -Y. Liu, K., Yoshizumi, N. Oda, M. Ohno, F. Tokunaga, S. Iwanaga, and H. Kihara, *J. Biochem.* **107**, 400-408 (1990).

[8] K. Yoshizumi, S. -Y. Liu, T. Miyata, S. Saita, M. Ohno, S. Iwanaga, and H. Kihara, *Toxicon* **28**, 43-54 (1990).

[9] R. D. Dunn and K. W. *Broady, Biochim. Biophys. Acta* **1533**, 29-37 (2001).

[10] S. Inoue, H. Kogaki, K. Ikeda, Y. Samejima, and T. Omori-Satoh, *J. Biol. Chem.* **266**, 1001-1007 (1991).

[11] S. Lizano, B. Lomonte, J. W. Fox, and J. M. Gutierrez, *Biochem. J.* **326**, 853-859 (1997).

[12] N. Ohkura, S. Inoue, K. Ikeda, and K. Hayashi, *J. Biochem.*, **113**, 413-419 (1993).

[13] N. Ohkura, S. Inoue, K. Ikeda, and K. Hayashi, *Biochem. Biophys. Res. Common.* **200**, 784-788 (1994).

[14] J. Perales, C. Villela, G. B. Domont, V. Choumet, B. Saliou, H. Moussatche, C. Bon, and G. Faure, *Eur. J. Biochem.*, **227**, 19-26 (1995).

[15] S. Inoue, A. Shimada, N. Ohkura, K. Ikeda, Y. Samejima, T. Omori-Satoh, and K. Hayashi, *Biochem. Mol. Biol. Int.* **41**, 529-537 (1997).

[16] H. Kogaki, S. Inoue, K. Ikeda, Y. Samejima, T. Omori-Satoh, and K. Hayashi, *J. Biochem.* **106**, 966-971 (1991).

[17] N. Ohkura, H. Okuhara, S. Inoue, K. Ikeda, and K. Hayashi, *Biochem. J.* **325**, 527-531 (1997).

[18] C. L. Fortes-Dial, Y. Lin, J. Ewell, C. R. Diniz, and T. -Y. Liu, *J. Biol. Chem.* **269**, 15646-15651 (1994).

[19] I. Nobuhisa, S. Inamasu, M. Nakai, A. Tatsui, T. Mimori, T. Ogawa, Y. Shimohigashi, Y. Fukumaki, S. Hattori, H. Kihara, M. Ohno, *Eur. J. Biochem.* **249**, 838-845 (1997).

[20] N. Aoki, M. Deshimaru, and S. Terada, *Fukuoka Univ. Sci. Rep.* **38**, 47-57 (2008).

[21] M. Deshimaru, C. Tanaka, A. Tokunaga, M. Goto, and S. Terada, *Fukuoka Univ. Sci. Rep.*

33, 45-53 (2003).

[22] U. K. Laemmli, *Nature* **227**, 680-685 (1970).

[23] H. Schagger, G. von Jagow, *Anal. Biochem.* **166**, 368-379 (1987).

[24] M. Friedman, L. H. Krull, J. F. Cavins, *J. Biol. Chem.* **245** 3868-3871 (1970).

[25] K. Okumura, K. Masui, S. Inoue, K. Ikeda, and K. Hayashi, *Biochem. J.* **341**, 165-171 (1999).

[26] K. Drickamer, J. Biol. Chem. **263**, 9557-9560 (1988).

[27] H.-J. Gabius, Eur. *J. Biochem.* **243**, 543-576(1997).

[29] Y. Wang, J. Dang, L. K. Johnson, J. J. Selhamer, and W. F. Doe, *Eur. J. Biochem*, **227**, 116-122 (1995).

[30] T. J. Fleming, C. O'hUigin, and T. R. Malek, *J. Immunol.* **150**, 5379-5390 (1993).

[31] R. Shirai, R. Gotou, F. Hirano, K. Ikeda, and S. Inoue, *J. Biol. Chem.* **285**, 21607-21614 (2010).

[32] N. Saitou and M. Nei, Mol. *Biol. Evol.* **4**, 406-425 (1987).

[33] J. L. Wrana and L. Attisano, *Cytokine Growth Factor Rev.* **7**, 327-339 (1996).

[34] D. Onichtchouk, Y. G. Chen, R. Dosch, V. Gawantka, H. Delius, J. Massague, and C. Niehrs, *Nature* **401**, 480-485 (1999).

[35] N. Fuse, T. Tsuchiya, Y. Nonomura, A. Menez, and T. Tamiya, *Eur. J. Biochem.*, **193**, 629-633 (1990).

[36] J. G. Petranka, D.E. Fleenor, K. Sykes, R. E. Kaufman, and W. F. Rosse, *Proc. Natl. Acad. Sci. U. S. A.*, **89**, 7876-7879 (1992).