Purification, characterization and cDNA cloning of a phospholipase A₂ inhibitor from the serum of the non-venomous snake *Elaphe quadrivirgata*

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The serum of a non-venomous striated snake, *Elaphe quadrivirgata*, was found to contain phospholipase A₂ (PLA₂) inhibitory proteins (PLIs). One of these inhibitors was purified by Sephadex G-200 gel filtration, Q-Sepharose FF ion-exchange chromatography and Butyl Sepharose 4FF hydrophobic chromatography. The purified PLI inhibited the enzymic activities of all PLA₂ groups, including *Elapidae* venom (group-I), *Viperidae* venom (group-II) and honeybee PLA₂s (group-III). The inhibitor was a 130 kDa glycoprotein consisting of two distinct subunits, A and B, of 30 and 29 kDa respectively; each of which was glycosylated with N-linked oligosaccharide chains. The cDNAs encoding the respective inhibitor subunits were isolated from a liver cDNA library by the use of probes, prepared by PCR, based on the partially determined amino-acid sequences of the corresponding

subunits. The respective nucleotide sequences encoded 19-amino-acid-residue signal sequences, followed by 183- and 181-residue protein sequences for the A and B subunits respectively. The amino-acid sequences revealed that the *E. quadrivirgata* inhibitor corresponded to PLI γ , one of three kinds of inhibitors purified from venomous snakes. The existence of PLI γ in the serum of this non-venomous snake suggested that, besides having a protective role against the venom PLA₂s of other venomous snakes, PLI γ has other important physiological functions in regulating local PLA₂ activities; and thus it raises the possibility that PLI γ occurs in other animals, including mammals.

Key words: phospholipase A₂, serum, cDNA cloning.

INTRODUCTION

Phospholipase A₂ [EC 3.1.1.4] (PLA₂) catalyses the hydrolysis of the acyl-ester bond at the *sn*-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. Snake venom is one of the most abundant sources of secretory PLA₂s. Secretory PLA₂s have been classified into three groups, I, II and III, according to their primary structures [1]. *Elapidae* venom contains group-I PLA₂s and *Viperidae* venom group-II PLA₂s. These venom PLA₂s exhibit a wide variety of toxicity, such as neurotoxicity and myotoxicity [2]. Venomous snakes have PLA₂ inhibitory proteins (PLIs) in their blood serum, which protect them from the leakage of their own venom PLA₂s into the circulatory system. Previously, we purified three distinct types of PLIs (PLI α , PLI β and PLI γ) from the blood plasma of the Chinese mamushi, *Agkistrodon blomhoffii siniticus* [3]. PLI α , which inhibited the group-II acidic PLA₂s, was found to be a 75 kDa glycoprotein [4] and a trimer of 20 kDa subunits having sequence homology with the carbohydrate-recognition domain of C-type lectins [5]. This type of inhibitor also had been purified previously from the blood plasma of the Habu, *Trimeresurus flavoviridis* [6,7]. PLI β , which specifically inhibits some group-II basic PLA₂s, was shown to be a 160 kDa glycoprotein composed of three identical 50 kDa subunits [3]. The 50 kDa subunit had leucine-rich repeats and a 33% identical sequence with that of human leucine-rich α_2 -glycoprotein [8]. The PLI γ , which exhibited a broad inhibition spectrum against various PLA₂s, was found to be a similar type of PLI to those purified from the plasma of *Naja naja kaouthia* [9] and *Crotalus durissus terrificus* [10,11]. It was a 100 kDa glycoprotein containing 25 kDa and 20 kDa subunits,

and their amino-acid sequences were characterized by two tandem patterns of cysteine residues as found in Ly-6 related proteins [12].

Some non-venomous snakes have been reported to be resistant to a wide variety of snake venoms and to contain neutralizing substances in their blood [13]. In order to explore the possibility of the existence of PLIs in non-venomous snakes, we sought to isolate them from the serum of the non-venomous *Elaphe quadrivirgata*. In the present study, a PLI, corresponding to PLI γ , was purified from *E. quadrivirgata* serum and its amino acid sequence was determined by cDNA cloning.

EXPERIMENTAL

Materials

The Japanese striated snake, *E. quadrivirgata*, and its serum were obtained from Ueda Trading Co. (Gifu, Japan). *A. blomhoffii siniticus* acidic PLA₂ and other snake venom PLA₂s were purified as described previously [4]. Honeybee *Apis mellifera* PLA₂ was purchased from Sigma. All experiments were carried out at 4 °C unless otherwise stated.

Purification of PLI

The serum was dialysed against 50 mM Tris/HCl (pH 7.5), centrifuged at 10000 *g* for 10 min and the supernatant was fractionated on a Sephadex G-200 column (3.4 cm × 93 cm) which had been equilibrated with 50 mM Tris/HCl (pH 7.5). The fractions exhibiting inhibitory activity against *A. blomhoffii siniticus* acidic PLA₂ were then applied to a Q-Sepharose FF column (1 cm × 10 cm) equilibrated with the same buffer, and the

Abbreviations used: PLA₂, phospholipase A₂; PLI, PLA₂ inhibitor; EqPLI γ -A, the A subunit of *E. quadrivirgata* PLI; EqPLI γ -B, the B subunit of *E. quadrivirgata* PLI.

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The nucleotide sequences reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB021425 for EqPLI γ -A and AB021426 for EqPLI γ -B.

adsorbed proteins were eluted with a gradient of 0–0.4 M NaCl. The major fractions exhibiting inhibitory activity were dialysed against 50 mM Tris/HCl (pH 7.5) containing 0.5 M $(\text{NH}_4)_2\text{SO}_4$, and loaded on to a Hi-Trap Butyl Sepharose 4FF column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The PLI was eluted with a decreasing linear gradient of 0.5–0 M $(\text{NH}_4)_2\text{SO}_4$.

PLA₂ assays

PLA₂ activities were assayed fluorimetrically, with 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine (Molecular Probes) as substrate, according to the method of Radvanyi et al. [14] with modifications as described previously [5]. During the course of purification, the inhibitory activity was monitored by the inhibition toward *A. blomhoffii siniticus* acidic PLA₂. In order to determine the inhibition constant (K_i), the enzymic activities of various PLA₂s were measured as described previously [4] in the presence of various concentrations of the inhibitor (10^{-11} to 10^{-5} M).

Subunit separation and deglycosylation

The two subunits of the inhibitor were separated by reverse-phase HPLC (Cosmosil C4 column; Nacalai Tesque) with 0.1 % (v/v) trifluoroacetic acid containing a linear gradient of 0–80 % acetonitrile. The separated subunits were deglycosylated by *N*-Glycosidase F (Boehringer Mannheim) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 24 h. The deglycosylated samples were separated by SDS/PAGE, using the method of Laemmli [15], and analysed by the decrease in the apparent molecular mass. The protein bands were stained with Coomassie Blue.

Amino-acid sequence determination

Each subunit was pyridylethylated in the presence of 6 M guanidine hydrochloride by the method described previously [16]. The pyridylethylated subunit was digested with lysyl endopeptidase (Wako Pure Chemicals), and the resultant fragments were separated by reverse-phase HPLC (Vydac C4 column; The Separations Group) with 0.1 % (v/v) trifluoroacetic acid containing a linear gradient of 0–48 % acetonitrile. The N-terminal amino-acid sequences of the subunits and the peptides were determined using a protein sequencer (model 477A; Applied Biosystems) equipped with a 120 A phenylthiohydantoin (PTH) analyser.

Construction of a cDNA library from striated snake liver

Total RNA was isolated from freshly dissected striated snake liver using a total RNA extraction kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Poly(A)-enriched RNA was obtained using a oligo(dT)-cellulose column (Amersham Pharmacia Biotech) and oligo(dT)-primed cDNA synthesis was carried out by means of a Superscript choice system (Life Technologies, Inc.). cDNAs were ligated to λ MOSElox arms (Amersham Pharmacia Biotech), and packaged via a λ -*in vitro* packaging module (Amersham Pharmacia Biotech).

cDNA cloning of PLI

To obtain the probe for cDNA screening, reverse transcriptase-PCR using *E. quadrigata* liver total RNA as template was performed. On the basis of the determined N-terminal and

internal peptide sequences of each subunit, two sets of degenerate oligonucleotide primers were designed. EQPR11 (sense: 5'-TGYGARATHTGYCAYAAAGT-3', where R, H and Y denote G/A, A/T/C and T/C respectively, corresponding to the N-terminal residues 3–9) and EQPR12 (antisense: 5'-ACRCANC-CYTDDATRTTSTA-3', where N denotes G/A/T/C, corresponding to residues 141–147) were designed for amplification of a cDNA encoding the A-subunit. EQPR13 (sense: 5'-ATHGAY-TGYGARCAAYTGYGT-3', corresponding to N-terminal residues 1–7) and EQPR14 (antisense: 5'-CKCCARAAAYTTYTT-YTCRAA-3', where K denotes G/T, corresponding to residues 155–161) were designed for amplification of a cDNA encoding the B-subunit.

The total RNA was subjected to first-strand cDNA synthesis using Superscript reverse transcriptase (Life Technologies, Inc.). The single-stranded cDNA obtained was used as a template for PCR amplification with *Ex Taq* DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan) under the following thermal cycling conditions: 5 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. After removal of the primers, the amplified products were labelled with horseradish peroxidase using an ECL direct nucleic-acid labelling system (Amersham Pharmacia Biotech). Using the labelled fragments as probes, plaques formed from the *E. quadrigata* liver cDNA library were screened. The positive phages were located with ECL detection reagents (Amersham Pharmacia Biotech).

DNA sequencing and analysis

Both DNA strands were sequenced with 7-deaza-dGTP using a Thermo Sequenase core sequencing kit (Amersham Pharmacia Biotech) on an SQ-3000 DNA sequencer (Hitachi, Japan) after subcloning into pBluescript (Stratagene). Sequence analysis was carried out (GENETYX program; Software Development Co. Ltd., Tokyo, Japan, and DNASIS program; Hitachi Software Engineering Co. Ltd., Tokyo, Japan).

RNA and DNA analyses

For Northern-blot analysis, 10 μ g of total RNAs prepared from various tissues of *E. quadrigata* were electrophoresed in a 1.2 % (w/v) agarose gel containing 2.2 M formaldehyde, and transferred on to a Hybond N+ membrane (Amersham Pharmacia Biotech). The peroxidase-labelled probes were prepared from the PCR-amplified products with an ECL direct labelling system (Amersham Pharmacia Biotech). The transcripts for the two subunits of PLI were detected with a SuperSignal NA chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

For Southern-blot analysis, genomic DNA from striated snake liver was prepared using a standard procedure [17]. The DNA was completely digested with various restriction endonucleases, electrophoresed and transferred on to a Hybond N+ membrane. The DNA fragments were then hybridized with a peroxidase-labelled probe and detected with an ECL direct nucleic-acid detection system (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

RESULTS

Purification and characterization of PLI

The inhibition of acidic PLA₂ activity of *A. blomhoffii siniticus* was examined using the serum of the non-venomous snake *E. quadrigata*. Unexpectedly, the *E. quadrigata* serum strongly inhibited this PLA₂, with an IC₅₀ value corresponding to a 700-

Figure 1 Gel filtration of *E. quadrivirgata* serum

The serum was applied to a Sephadex G-200 column equilibrated with 50 mM Tris/HCl (pH 7.5) and 10 ml fractions were collected. The vertical arrows show the elution positions of: A, Blue Dextran; B, γ -globulin (150 kDa); C, BSA (67 kDa). Absorbance at 280 nm (○), relative inhibition of *A. blomhoffii siniticus* acidic PLA₂ (●).

Figure 2 Q-Sepharose FF chromatography of the gel filtration sample

The sample was applied to a Q-Sepharose FF column equilibrated with 50 mM Tris/HCl (pH 7.5). The column was washed and the adsorbed proteins eluted with the same buffer containing a linear gradient of 0–0.4 M NaCl. Absorbance at 280 nm (○), relative inhibition of *A. blomhoffii siniticus* acidic PLA₂ (●).

fold dilution of the serum (results not shown), which was 30-fold lower than that determined previously for *A. blomhoffii siniticus* serum [3]. Since this suggested that a PLI existed in *E. quadrivirgata* serum, an attempt was made to isolate the inhibitor by using the inhibitory activity toward *A. blomhoffii siniticus* acidic PLA₂ as a marker.

Figure 1 shows an elution profile of *E. quadrivirgata* serum after Sephadex G-200 column chromatography. The inhibitory activity was present in two peaks, one of high molecular mass (300 kDa; fractions 33–39) and one of low molecular mass (130 kDa; fractions 40–47). The 130 kDa fractions were further purified by Q-Sepharose chromatography, as shown in Figure 2. Fractions 57–70, which exhibited inhibitory activity, were pooled and further chromatographed on a Hi-trap Butyl Sepharose column. The inhibitor was adsorbed on to the column in the presence of buffer containing 0.5 M (NH₄)₂SO₄ and eluted with the same buffer lacking (NH₄)₂SO₄ (results not shown). The

Figure 3 SDS/PAGE of the two subunits of the purified inhibitor

The purified inhibitor and the separated subunits were digested with *N*-glycosidase F and subjected to SDS/PAGE. Lane 1: purified *E. quadrivirgata* inhibitor; lane 2, A subunit of the inhibitor; lane 3, A subunit digested with *N*-glycosidase F; lane 4, B subunit of the inhibitor; lane 5, B subunit digested with *N*-glycosidase F; M, molecular-mass standards. The molecular masses (kDa) of the markers are indicated on the left.

Table 1 Apparent inhibition constant [$K_i(\text{app})$] of the PLI of various groups of PLA₂

Group-I PLA₂s used in the present study were purified from the venom of *L. semitasciata*, *Pseudechis australis*, *N. naja kaouthia* and *N. naja atra*. Group-II PLA₂s were from the venom of *T. flavoviridis*, *A. blomhoffii siniticus*, *A. halys blomhoffii* and *Vipera russelli russelli*. The group-III enzyme was from the honeybee, *A. mellifera*.

Group-I PLA ₂	PLA ₂	$K_i(\text{app})$ (nM)
<i>L. semitasciata</i>	PLA-I	11.0
	PLA-III	12.3
	<i>P. australis</i>	
	Pa-12A	7.55
	Pa-9C	16.9
<i>N. naja kaouthia</i>	CM-II	31.1
	<i>N. naja atra</i>	
	PLA ₂	29.2
Group-II PLA ₂	PLA ₂	$K_i(\text{app})$ (nM)
<i>T. flavoviridis</i>	Acidic PLA ₂	6.44
	Acidic PLA ₂	13.5
	Neutral PLA ₂	7.84
	Basic PLA ₂	11.5
<i>A. halys blomhoffii</i>	Acidic PLA ₂	13.2
	Neutral PLA ₂	9.05
	Basic PLA ₂	5.26
<i>V. russelli russelli</i>	PLA ₂ -III	8.20
	PLA ₂ -V	10.0
Group-III PLA ₂	PLA ₂	$K_i(\text{app})$ (nM)
<i>A. mellifera</i>	PLA ₂	13.0

inhibitor preparation so obtained produced one protein band with pI 4.5 on an isoelectrofocusing gel. A volume of 20 ml of serum yielded 3 mg of purified inhibitor.

When the 300 kDa protein obtained by Sephadex G-200 column chromatography was further purified using the method described for the 130 kDa fractions, the elution profiles of the

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1 GCAGAGTCCAGAACTTCACTGAGGTCAACATATCCTATCCAGCTCTGATCTTCACTTGA 60
61 GCCATGAAATCTCTACAGATCATCTGCTCTTTTCACTTTTGTAGCCAGAGGAAGCTGC 120
-19 M K S L Q I I C L L F I F V A R G S C -1
121 CGCTCATGTGAAATTTGTACAACTGGGAAATGATTGTGGCTATGATTACGTAGAAGAG 180
1 R S C E I C H N V G N D C G Y D Y V E E 20
181 TGTCACCTCCAGAAAGATCAATGCGGCAAGGTGTGTGGAGATTTCGTGAGCACTT 240
21 C H S P E D O C G K V L L E I S S A P L 40
241 TCCATCCGATCCAGTCAATAGGAAGTGTTCATCCAGCCTCTGCAAACTTGAGCACTTC 300
41 S I R S S H R N C F S S S L C K L E H F 60
301 GATGTCAATACTGGACAGGAGACCTATCTGAGAGGAAGAATCCATTGTTGTGATGAAAA 360
61 D V N T G Q E T Y L R G R I H C C D E K 80
361 AAGTGTGAAGGCGCCATTTCTTGGAGTGCCTCTCCATCCAAATGGATACGTCTGT 420
81 K C E G R P F P G L P L S H P N G Y V C 100
421 CCTGGCGTCTTGGTCTATTCTCAGAGGACAGCAGTGAATCTGAAGCTGCTTGAAGAAG 480
101 P G V L G L F S E D S S E S E A A C K G 120
481 GACGAAACCAATGCATTAACATTGTGGGATACAGAAAGAAAGATTCTGGCGACATC 540
121 D E T K C I N I V G Y R K E R F P G D I 140
541 GCTTATAATCAAGGATGCGTTTCTTCTGTCCAGAACTGAGATTGAGCAATAGAACG 600
141 A Y N I K G C V S S C P E L R L S N R T 160
601 CACGAAGAAGCTAGAAATGATCTGATAAAGTTGAATGTAGAGACGCGCTCAAAATTACA 660
161 H E R R N D L I K V E C R D A V K I T 180
661 CCTTCTGAATAATTCGTCACCAATTTTGAATCTGAAGTGACGCGTTTCATCCAATAGA 720
181 P S E * 183
721 ACCATGGGTATGGCTCTGAATGGAAGAACTTACATTTTAACTTCTGATTCTCTGCCAA 780
781 CTAATTTTTCAGAAATAAAAAAGTAAAAATAAAAGAAGCAAGAGGCTGAGTTGGA 840
841 AAAAAAAAAAAAAAAAAAAAAAAAAA 867

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Figure 4 cDNA sequence and deduced protein sequence of the A subunit of the inhibitor

The nucleotide sequence (upper) and the deduced amino acid sequence (lower) are numbered. N-glycosylation site is shown in bold type. The N-terminal and internal sequences elucidated by sequencing of the lysyl endopeptidase digests are underlined.

inhibitory activity were similar to those for the 130 kDa fractions. Therefore the inhibitor contained in the 300 kDa fractions could be identical with that of the 130 kDa fractions. Further purification and characterization of the inhibitor from the 300 kDa fractions are in progress.

The purified inhibitor could be separated into two fractions that were eluted at 53 min and 68 min by reverse-phase HPLC. Neither of these fractions exhibited any inhibitory activity toward PLA₂ (results not shown). SDS/PAGE of these fractions revealed that the former fraction contained mainly a 30 kDa protein and the latter fraction a 29 kDa protein (Figure 3). In the present study, the 30 kDa protein was designated subunit A and the 29 kDa protein as subunit B. When the two subunits were treated with N-glycosidase F, their apparent molecular masses were decreased to 25 kDa, indicating that both possess N-linked carbohydrate chains.

Specificity of the inhibitor toward various PLA₂s

The inhibitory activity of the purified inhibitor toward various venom PLA₂s was investigated. The inhibitor strongly inhibited all the PLA₂s used in the present study, irrespective of the groups (I, II or III). The apparent inhibition constants [K_i (app)] of the inhibitor against PLA₂s from various species are shown in Table 1. A broad inhibition spectrum of the inhibitor was found, which was characteristic of PLI γ , one of the three PLIs purified from the plasma of venomous snakes. The inhibitor inhibited all groups of PLA₂s with K_i (app) values from 10⁻⁹ to 10⁻⁸ M, which were one order of magnitude lower than those of PLI γ s from *N.*

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1 CTACAAAAGGAAACACAAAAGGAGAAG 29
30 GAGCTGCGCCCGTTGACTGGTAGCATCTGGCAGCTCTCTGAGAAAAATTACTGGCTGCAGC 89
90 TTCTTGAAGCTCTACGTATTTCTATCTAGATTGGCCCTGGGCATCAATCATCGTTCTCC 149
150 ACCATGAAGTTCTCTTATCTGTGTCTCTTTGGCACTTTCTTAGCTACAGGCATGTGT 209
-19 M K F L L F C C L F G T F L A T G M C -1
210 ATTGACTGTGAACATTGTGTGTGGTGGGGCCAGAAATGTACCGCTGGAAAGAACCTGT 269
1 I D C E H C V V W G O N C T G W K E T C 20
270 GGAGAGAATGAAGATACTTGTGTACCTATCAAACTGAAGTGATAAGACCTCTCTGTCC 329
21 G E N E D T C V T Y O T E V I R P P L S 40
330 ATCACTTTCACGTGCAAAAACCTGTGGCACCTCTGACACTTGGCATCTTACTACGTGGAG 389
41 I T F T A K T C G T S D T C H L D Y V E 60
390 GCAATCCACATACTGAACCTAACACTGAGGGCCAAACGACCTGCTGTACGGGAGATGAA 449
61 A N P H T E L T L R A K R A C C T G D E 80
450 TGTCAAACTGCCACCTCTGTGCTTGAACCCCAAGTCAACCGACCAATGGACTTCAG 509
81 C O T L P P P V L E P Q V N R P N G L Q 100
510 TGTCTGGATGCATTGGACTACCTCAACTGAATGCAATGAATACCTGGTTTCCTGCCAQ 569
101 C P G C I G L T S T E C N E Y L V S C Q 120
570 GGATCTGAAACCCAGTGTGTTGACTATAATCCTGAAGAAACCTGACTTCTCATGAGTGA 629
121 G S E N Q C L T I I L K K P D F S L S E 140
630 ATGCTCTTCAAAGGATGCGCTTCTGAGAATCTGTGCCTTTTATTTGAGAAGAAGTTCTGG 689
141 M S F K G C A S E N L C L L F E K K F W 160
690 AGATTTTGTAGAGCCTCTGAAGTAGACGTAATAATGCACCCAGCTGTCCCGCAGACTTCC 749
161 R F L E A S E V D V K C T P A V P O T S 180
750 CAGTGTATTAAGTGCACGTCAAGGCCATCGCTCCACCTTACTAGAACTTAGCGCCAA 809
181 Q * 181
810 CATGGCTCATCTCTGTTTGTAGTCACTTCAACAGAAATTCGTCTTCTGGATGTGTCAA 869
870 AGTTTCCACGATTCGTGTGAAATTAAGTACGACCATGGTCCAATCAATAAGCAGTAAC 929
930 TTTAAAAAGAAAAA 947

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Figure 5 cDNA sequence and deduced protein sequence of the B subunit of the inhibitor

See legend to Figure 4.

naja kaouthia, *A. blomhoffii siniticus*, and *Laticauda semifasciata* [4,18].

Amino-acid sequence and cDNA cloning of the two inhibitor subunits

The N-terminal sequences of the S-pyridylethylated A and B subunits were: RSCEICHNVGNDGCGDYVEECHSPEDQ-CGKVLLEI and IDCEHCVVWGXCTG (where X denotes an unidentified amino acid) respectively. Amino-acid sequencing was also performed on the peptides derived from the subunits by treatment with lysyl endopeptidase. On the basis of these sequences, two pairs of degenerate oligonucleotide primers were synthesized and the cDNA fragments were amplified by reverse transcriptase-PCR using striated snake liver total RNA as a template. The respective 450-bp products were used as probes for the A and B subunits to screen the *E. quadricolor* liver λ MOSElox cDNA library. The clones, pEQ303 and pEQ414, which contained the largest inserts encoding the A and B subunits respectively, were selected and sequenced. The complete nucleotide sequences of the cDNAs encoding the inhibitor A and B subunits and their deduced amino-acid sequences are shown in Figures 4 and 5. The open reading frames encoded 19-residue signal sequences, followed by 183- and 181-residue protein sequences for the A and B subunits respectively. The deduced amino-acid sequences contained all the sequences determined for the lysyl endopeptidase peptides of the respective A and B subunits. The molecular masses of the mature A and B subunit proteins were calculated to be 20421 and 20041 Da respectively. Each subunit contained one potential N-linked glycosylation

Figure 6 Comparison of the amino-acid sequences of *E. quadrivirgata* PLI γ (*Eq*PLI γ) subunits with those of *A. blomhoffii* siniticus PLI γ (*Abs*PLI γ), *N. naja kaouthia* PLI γ (*Nnk*PLI γ), *L. semifasciata* (*Ls*PLI γ), *C. durissus terrificus* CNF (*Cdt*CNF), and *T. flavoviridis* PLI-I (*Tf*PLI-I)

Cysteine residues are indicated in white-on-black type. Residues conserved in at least five of the ten sequences are shown in boxes.

Figure 7 Southern blot of PLI γ

E. quadrivirgata genomic DNA was digested with various restriction endonucleases (shown above the gel), electrophoresed on 0.7% (w/v) agarose gel and transferred to a nylon membrane. The membrane was hybridized with the peroxidase-labelled probe of the portion of the coding region corresponding to (A) nt 127–563 of the A subunit or (B) nt 210–691 of the B subunit. DNA size markers (kb) are indicated on the right of each panel.

site, at Asn-158 for the A subunit and at Asn-12 for the B subunit. These residues would be actually glycosylated as expected from the results of *N*-glycosidase F digestions of each subunit (see Figure 3). The deduced amino-acid sequence of the A subunit revealed approx. 70% identity with that of the A subunits of PLI γ s from venomous snakes, and the sequence of the B subunit revealed approx. 60% identity with that of their B subunits; whereas there was only 30% sequence identity between A and B subunits (Figure 6). Therefore the PLI purified from the serum of *E. quadrivirgata* in the present study was concluded to correspond to PLI γ . The *E. quadrivirgata* PLI γ is, therefore, the first example of the existence of PLI γ in animals other than venomous snakes.

RNA and genomic DNA blotting analysis

Northern-blot analysis revealed that respective single hybridizing bands of approx. 1.5 kb for the A and B subunits were observed only in the liver but no hybridizing bands were detected in 12 other tissues from this snake, including oesophagus, stomach, pancreas, spleen, gallbladder, small intestine, rectum, kidney, trachea, lung, testis and body fat (results not shown).

Genomic Southern-blot analysis, using the corresponding PCR-amplified products as probes for the subunits, revealed that the respective genes encoding the A and B subunits were single copies (Figure 7). When genomic DNA was digested with various restriction endonucleases, several identical hybridized bands were observed with the two probes (shown by arrows in Figure 7). Since the probes for neither subunit cross-hybridized, the genes encoding each subunit might be located very close to each other.

DISCUSSION

PLIs so far have been characterized only in venomous snakes. In the present study, we found that the serum of the non-venomous striated snake unexpectedly inhibited acidic PLA₂ from the venom of the Chinese mamushi, *A. blomhoffii siniticus*. Furthermore, this inhibition was surprisingly one order of magnitude greater than that of the Chinese mamushi serum. The inhibitory 130 kDa protein was purified from serum by sequential chromatography on Sephadex G-200 (Figure 1), Q-Sepharose (Figure 2), and Butyl Sepharose columns. The purified inhibitor was composed of two distinct subunits A and B, of 30 kDa and 29 kDa respectively. On the basis of the amino-acid sequences of the subunits, the purified inhibitor seemed to correspond to the PLI γ s purified from venomous snakes, and was thus regarded as the *E. quadrivirgata* PLI γ (*Eq*PLI γ). Like other PLI γ s, this inhibitor showed a broad spectrum of inhibition with various venom PLA₂s of groups I, II and III (Table 1). As had been discussed previously [4], the inhibition mechanism of PLI γ might include binding to PLA₂ via a common structural element on the PLA₂ molecule, such as the Ca²⁺-binding loop, which is conserved among all of the PLA₂s groups.

In Figure 6 the deduced amino-acid sequences of the A subunit (*Eq*PLI γ -A) and B subunit (*Eq*PLI γ -B) are compared with those of the two respective subunits of the *A. blomhoffii siniticus* [19], *N. naja kaouthia* [12] and *L. semifasciata* PLI γ s [18], and with the sequences of the *C. durissus terrificus* CNF [10] and *T. flavoviridis* PLI-I [20]. The amino-acid sequence of *Eq*PLI γ -A showed maximum homology with that of the A subunits, and the sequence of the *Eq*PLI γ -B showed maximal homology with that of the B subunits of other PLI γ s. The amino-acid differences of 11–38% among the PLI γ -A subunits and of 35–48% among

the PLI γ -B subunits were much smaller than those between the PLI γ -A and PLI γ -B subunits (67–72%), suggesting that the genes encoding the respective subunits were produced by gene duplication of a common ancestral gene, which had occurred before the divergence of snakes.

There were 27 amino acid residues conserved in the PLI γ -A and PLI γ -B subunits. Although a few of these residues might contribute directly to the inhibitory activity toward PLA₂, we suggest that most of them are essential for the tertiary structure; i.e. 16 residues were cysteine residues. As described previously, both PLI γ subunits contained two intramolecular repeats of the cysteine-rich domains, which are commonly found in a structurally related family of proteins including urokinase-type plasminogen activator receptor (uPAR), CD59, Ly-6, and snake venom neurotoxins [12]. PLI γ -B subunits were characterized by the existence of one additional disulphide bridge between Cys-104 and Cys-112. The N-glycosylation site of PLI γ -A, Asn-158, was conserved in all of the PLI γ -A subunits, although such glycosylation is not essential for inhibitory activity [18].

Some non-venomous snakes have Duvernoy's glands, which secrete various enzymes including proteases, esterases, and phospholipases [13]. One of these snakes, *Malpolon monspessulanus*, was reported to secrete a toxic PLA₂ from its Duvernoy's gland [21]. Some PLIs are probably contained in the serum of this snake to protect itself from the leakage of toxic PLA₂ into the circulatory system. However, *E. quadrivirgata*, has neither venom nor Duvernoy's glands and, therefore, the abundance of PLI γ in the serum is not necessarily for protection against venom PLA₂s. Recently, a novel bone-specific protein, RoBo-1, was found in rat tibia and has been shown to have significant sequence similarity with PLI γ [22]. Although PLA₂ inhibitory activity of RoBo-1 has not been confirmed, this protein might play an important role in the control of bone growth by regulating local PLA₂ activity.

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