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Molecular cloning of phospholipases A_2 from venom glands of *Echis* carpet vipers

K. Bharati, S.S. Hasson, J. Oliver, G.D. Laing, R.D.G. Theakston, R.A. Harrison*

Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK

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Abstract

Venom toxin-specific antibodies offer a more rational treatment of snake envenoming than conventional antivenom. Here, we describe novel cDNAs encoding phospholipase A_2 (PLA_2) isoforms from venom gland RNA of *Echis pyramidum leakeyi* (Epl), *Echis sochureki* (Es) and *Echis ocellatus* (Eo). The deduced amino acid sequences of these cDNAs encoded proteins with high overall sequence identity to the viper group II PLA_2 protein family, including the 14 cysteine residues capable of forming seven disulphide bonds that characterize this group of PLA_2 enzymes. Comparison of the PLA_2 sequences from *Echis* with those from related vipers failed to make significant geographic, taxonomic or PLA_2-function distinctions between these *Echis* PLA_2 isoforms. However, their deduced hydrophilicity profiles revealed a conserved tertiary structure that we will exploit, by epidermal DNA immunization, to generate PLA_2-neutralizing antibodies with polyspecific potential.

Keywords: Phospholipases A_2; *Echis pyramidum leakeyi*; *Echis sochureki*; *Echis ocellatus*; cDNA cloning

1. Introduction

The *Echis* genus of carpet vipers constitutes one of the most medically important groups of snakes and is responsible for the majority of snakebite deaths and morbidity across Africa and the Indian subcontinent (Warrell and Arnett, 1976). The extreme haemorrhagic and coagulopathic manifestations of *Echis* envenoming are effected by zinc metalloproteinases that cause bleeding by degradation of the vascular subendothelium and a variety of other toxins that operate in distinct ways to prevent blood from clotting (e.g. disintegrins, prothrombin activators and factor IX/X activators; (Kamiguti et al., 1998)). It is well established that group II phospholipase A_2 (PLA_2) enzymes in venoms of related vipers in Asia, Europe, the Middle East and the Americas contribute to venom-induced pathology through a broad spectrum of toxic activities, including neurotoxicity (Habermann and Breithaupt, 1978), oedema-inducing activity (Vishwanath et al., 1987), anticoagulant activity (Boffa et al., 1976), haemorrhagic activity (Vishwanath et al., 1985; Yamaguchi et al., 2001) and activities that cause initiation or inhibition of platelet aggregation (Kini and Evans, 1990).

Comparatively little is known about the contribution of PLA_2 activity to the pathology of *Echis* viper envenoming. Kemparaju et al. (1994) isolated a 14 kDa basic (pI 7.2–7.6) PLA_2 (EC-IV-PLA_2) from Indian saw-scaled viper (*Echis carinatus*) venom that produced neurotoxicity and oedema in mice but had no direct haemolytic, myotoxic, cytotoxic or anticoagulant activities. Kemparaju et al. (1999) isolated a 16 kDa acidic (pI 4.2–4.8) PLA_2 (EC-I-PLA_2) from *E. carinatus* venom that was non-lethal to mice and devoid of neurotoxicity, myotoxicity, anticoagulant activity and cytotoxicity, but induced mild oedema in the foot-pads of mice. The purified PLA_2 inhibited ADP, collagen and adrenaline-induced aggregation of human platelets, that was both dose and time-dependent. Desmond et al. (1991) purified and partially sequenced the primary structure of two

* Corresponding author. Tel.: +44-151-705-3164; fax: +44-151-705-3371.

E-mail address: robharr@liverpool.ac.uk (R.A. Harrison).

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16 kDa PLA_2's (Ec1 and Ec2) from the venom of Kenyan *Echis pyamidum leakeyi* that exhibited sequence similarity with other toxic PLA_2's, but their role in the toxicity of the venom was not established. Polgar et al. (1996) purified and characterized an enzymatically active Ser-49 basic (pI 7.9) PLA_2, Ecarpholin S, from the venom of *Echis sochureki*. A PLA_2 complexed with another venom protein has been purified and crystallized from Indian *E. carinatus* venom (Nagpal et al., 1999). Only two *Echis* PLA_2 DNA sequences have been submitted to the genetic databases (*E. coloratus* AF253049/50; direct submission; Kordis, 2001).

A major research initiative of our laboratory is to generate venom toxin-specific antibodies by DNA immunization, as a means of developing rational immunotherapy (Harrison et al., 2000, 2002), particularly for vipers of greatest medical importance in Africa. We have included PLA_2s in this strategy, despite the paucity of data on their clinical importance in *Echis* envenoming, because it seemed imprudent to ignore a group of bioactive molecules with potential to disrupt haemostasis at several distinct levels and that comprise between 6 and 13% of the total venom protein of *E. p. leakeyi* (Desmond et al., 1991). Here, we report the isolation of three novel PLA_2-encoding cDNAs from *Echis* species with distinct residential boundaries in West Africa (*Echis ocellatus*), East Africa (*E. p. leakeyi*) and the Indian subcontinent (*E. sochureki*). The conserved secondary structure of all the PLA_2 isomers, across species and genus boundaries, augers well for the generation of neutralizing antibody with polyspecific cover.

2. Materials and methods

2.1. Animals, vector and cells

Adult carpet vipers, *E. p. leakeyi* (Kenya), *E. sochureki* (Pakistan) and *E. ocellatus* (Nigeria) used in this study were maintained in the herpetarium, Liverpool School of Tropical Medicine, Liverpool, UK. The TA cloning vector (pCR 2.1-TOPO) and chemically competent *E. coli* cells (TOP 10F′) were purchased from Invitrogen, Groningen, The Netherlands.

2.2. Isolation of total RNA and construction of cDNA library

Venom glands were dissected from three sacrificed snakes of each species (three days after venom extraction), homogenized under liquid N_2 and total RNA extracted using guanidinium thiocyanate–phenol–chloroform (Chomczynski and Sacchi, 1987) following standard protocols (Sambrook et al., 1989). Lambda phage cDNA libraries for the snakes were constructed by RT-PCR using the SMART cDNA library construction kit (CLONTECH, CA, USA) and Gigapack III Gold Packaging Extract (Stratagene, CA, USA) following the manufacturer's instructions.

2.3. Isolation and analysis of PLA_2 toxin cDNAs

A Polymerase Chain Reaction (PCR) strategy (Israel, 1993) was used to screen the cDNA libraries. A sense primer (5′-**GGA-TCC**-ATG-AGG-ACT-CTC-TGG-ATA-3′) and an antisense primer (5′-**CTC-GAG**-TCA-TCA-GCA-TTT-CTC-TGA-CTC-CTC-3′) complimentary to highly conserved amino (M-R-T-L-W-I) and carboxyl (E-E-S-E-K-C) regions of published group II PLA_2 DNA sequences of related viper species were designed and synthesized (Sigma-Genosys Ltd, UK). Two stop codons (TGA) and restriction endonuclease sites for *Bam* H1 and *Xho* 1 were included in the 5′ and 3′-primers, respectively, to facilitate future subcloning into mammalian expression plasmids.

PCR was performed using a thermal cycler (Gene Cycler, BioRad Hercules, CA, USA) programmed for an initial denaturation (95 °C × 6 min) and annealing (55 °C × 1 min) step, followed by 35 cycles (1 min duration each) of extension (74 °C), denaturation (94 °C) and annealing (55 °C). A final extension step (7 min) at 72 °C was also included in the program to ensure that all the products were double stranded. The PCR-amplified products were subcloned into the TA cloning vector, pCR 2.1-TOPO (Invitrogen), and used to transform chemically competent *E. coli* cells (TOP 10F′) under ampicillin selection, following the manufacturer's instructions. Plasmid DNA was extracted using a commercially available kit (Qiagen, Hilden, Germany) and digested with *Bam* H1 and *Xho* 1 at 37 °C to select plasmid containing inserts of the predicted size for DNA sequencing. DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method (Sanger et al., 1977) in a Beckman Coulter CEQ™ 2000 XL DNA Analysis System. The predicated amino acid sequences were compared to sequences in the GenBank, PDB, SwissProt, PIR and PRF databases using the BLAST program (Altschul et al., 1997).

The amino acid sequences of the three *Echis* cDNAs were aligned to PLA_2 isoform sequences from related *Viperidae* species: *E. coloratus* (AF253049), *Vipera ammodytes* (AF253048), *V. palaestinae* (U60017), *Trimeresurus flavoviridis* (D10720), *Calloselasma rhodostoma* (AF104067), *Bothrops asper* (AF109911), *B. jararacussu* (X76289) and *Crotalus atrox* (AF269131) using Lasergene software (DNASTAR, Madison, USA) and a phylogenetic tree constructed for the latter sequences and for peptide sequences of native PLA_2 from *E. p. leakeyi* (EplpLA_2-Ec1, (Desmond et al., 1991)) and *E. sochureki* (Ecarpholin S, P48650) using the CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix. The predicted isoelectric point of each PLA_2 isoform examined here was

determined using Lasergene software (Protean, DNASTAR).

3. Results and discussion

PCR amplification of *E. p. leakeyi*, *E. sochureki* and *E. ocellatus* venom gland libraries produced several cDNA sequences for each species (designated Epl, Es or Eo, respectively) that were identified (BLAST) as belonging to the PLA_2 enzyme family. Here, we show that single cDNA sequences for each snake species $EplPLA_2$-5, (Genbank submission-AF539920), $EsPLA_2$-4, (Genbank submission-AF539919) and $EoPLA_2$-5 (Genbank submission AF539921) were highly similar at the DNA and predicted amino acid levels (Fig. 1a and b). $EplPLA_2$-5 and $EsPLA_2$-4 (417 nucleotides) encoded an open reading frame of 139 amino acids (15.9 kDa) and $EoPLA_2$-5 (414 nucleotides) encoded a protein of 138 amino acids (15.7 kDa). Consistent with the secretory nature of venom PLA_2 proteins, the initiating 16 amino acid residues of all three *Echis* PLA_2 cDNAs encoded a conserved signal peptide domain (hatched arrow). The location of the less-conserved anticoagulant domain identified by Kini and Evans (1987) lies between residues 68–83 (solid arrow). Despite the geographical separation of *E. p. leakeyi* and *E. sochureki* species, the predicted amino acid sequences of $EplPLA_2$-5 and $EsPLA_2$-4 show only two amino acid substitutions, the first within the signal peptide domain at position 12 ($EplPLA_2$-5:Met; $EsPLA_2$-4:Val) and the second at position 62 ($EplPLA_2$-5:Met; $EsPLA_2$-4:Leu). These amino acid substitutions are, however, unlikely to affect the tertiary structure because the positions of the 14 cysteine residues are completely conserved in both molecules and likely reflect a common lineage (Moura-da-Silva et al., 1995).

The predicted amino acid sequences encoded by $EplPLA_2$-5, $EsPLA_2$-4 and $EoPLA_2$-5 were aligned with the PLA_2 sequences of vipers showing the highest similarity (Fig. 2a) during the BLAST search. The near-identical $EplPLA_2$-5 and $EsPLA_2$-4 showed a high percentage sequence identity with PLA_2 sequences from *E. coloratus* (72%; 71%) *V. palaestinae* (71%; 70%), *V. ammodytes* (69%; 69%), *T. flavoviridis* (60%; 61%), *B. jararacussu* (60%; 61%) and *C. rhodostoma* (58%; 59%). A phylogenetic tree of the above sequences and peptide sequences of

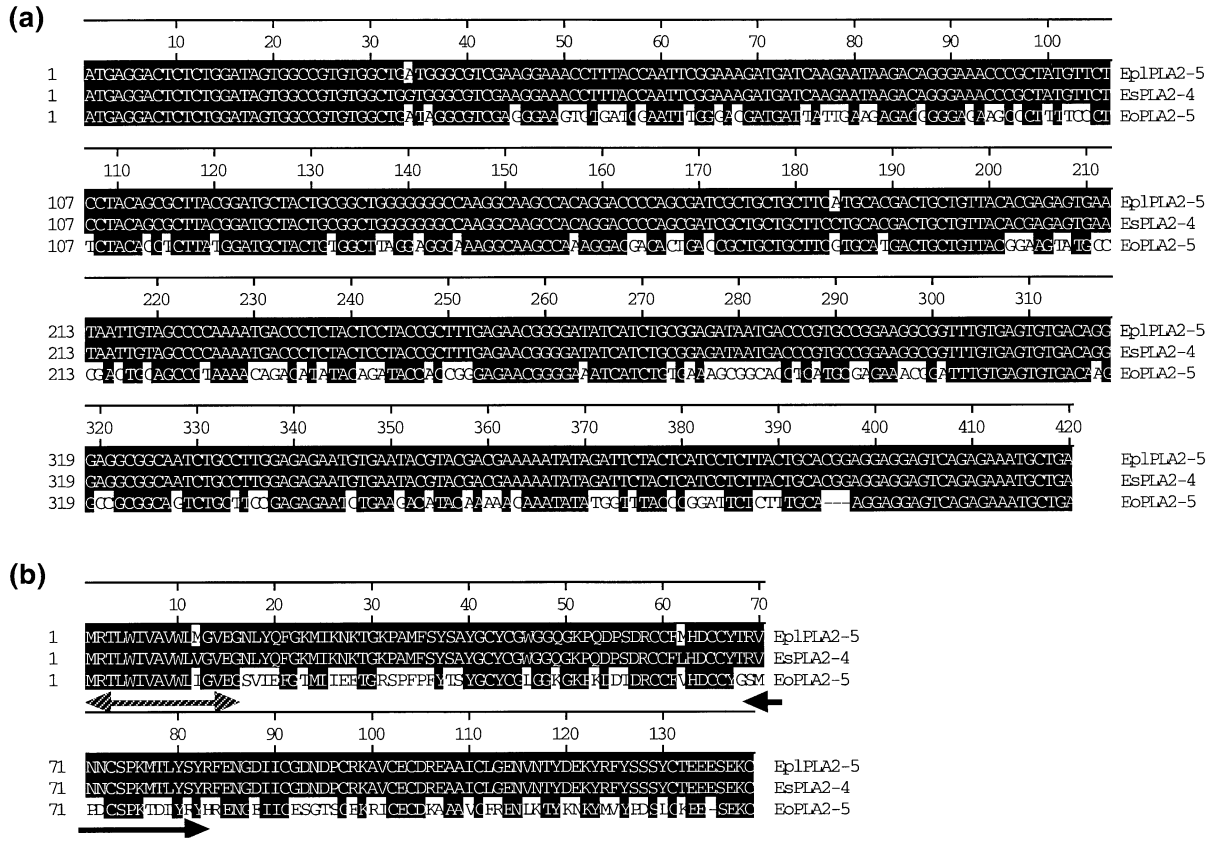

Fig. 1. (a) Complete cDNA and (b) deduced amino acid sequences of PLA_2's from *E. p. leakeyi* ($EplPLA_2$-5), *E. sochureki* ($EsPLA_2$-4) and *E. ocellatus* ($EoPLA_2$-5). The signal peptide (hatched arrow) and anticoagulant domain (solid arrow) are indicated.

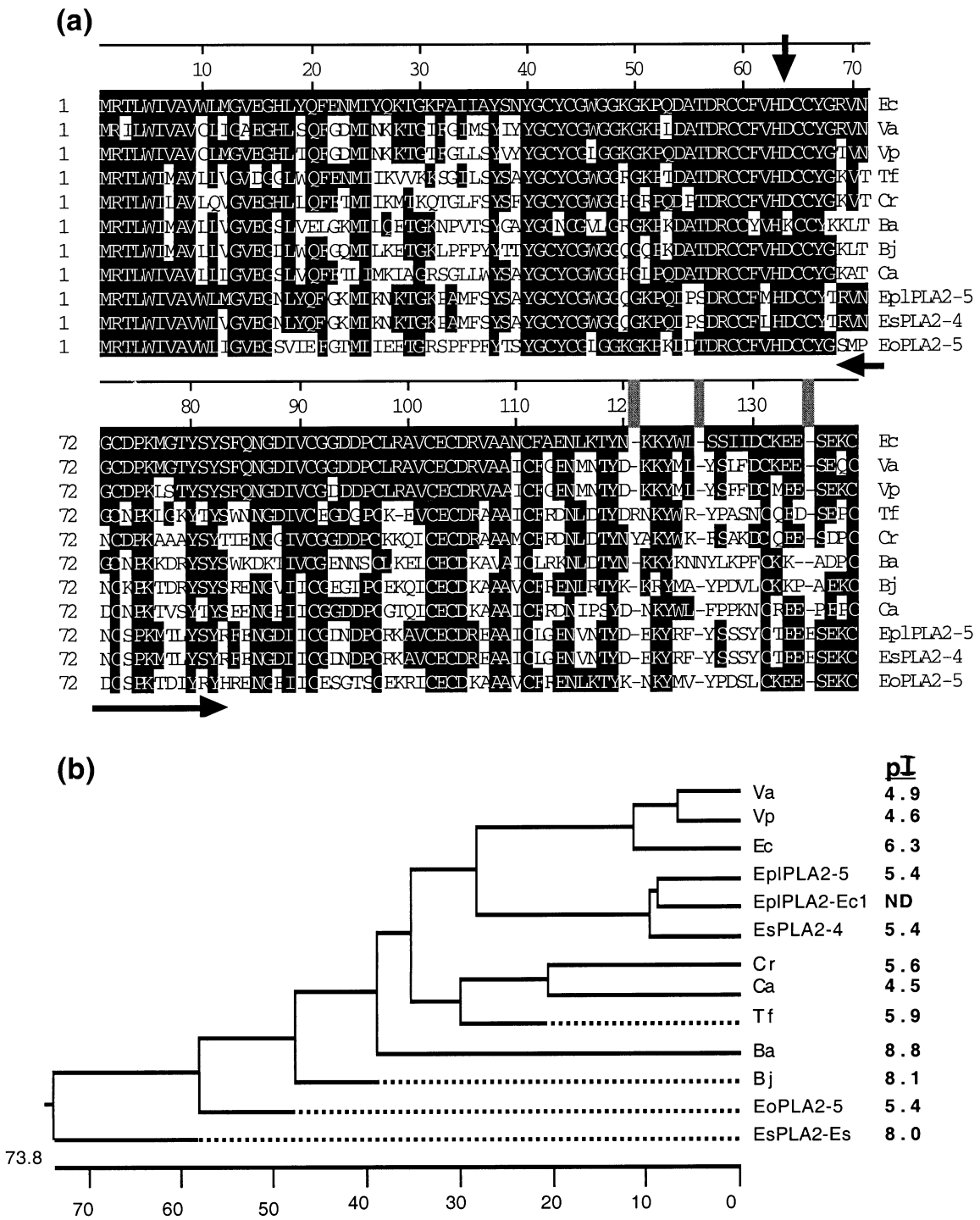

Fig. 2. Analysis of the amino acid sequences of EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5 with PAL$_2$'s from related *Viperidae* snake species by (a) sequence identity (residues shaded in black match *E. coloratus*; the vertical and horizontal arrows indicate the conserved calcium-binding aspartate residue and anticoagulant domain, respectively) and (b) phylogeny and predicted isoelectric point (pI). The tree was constructed from deduced amino acid sequences of group II snake venom PLA$_2$'s shown in Fig. 2a and include Ecarpholin S (EsPLA$_2$-Es) and EplPLA$_2$-Ecl. The scale beneath the tree measures the distance between sequences (in millions of years). Ec: *E. coloratus*; Va: *V. ammodytes*; Vp: *V. palaestinae*; Tf: *T. flavoviridis*; Cr: *C. rhodostoma*; Ba: *B asper*; Bj: *B. jararacussu*; Ca: *C. atrox*; EplPLA$_2$-Ecl, Desmond et al. (1991); EsPLA$_2$-Es: *E. sochureki* Ecarpholin S; ND: not determined.

native PLA_2 from *E. p. leakeyi* (EplPLA$_2$-Ecl, (Desmond et al., 1991) and *E. sochureki* (Ecarpholin S, P48650) was constructed (Fig. 2b) using the CLUSTAL W program with PAM250 residue weight matrix. The amino acid sequence of EplPLA$_2$-5 cDNA matched with near identity (Fig. 2b) to the sequence of a native PLA_2 from *E. p. leakeyi* venom (EplPLA$_2$-Ecl) suggesting a direct genomic relationship. In contrast, the predicted amino acid sequence of EsPLA$_2$-4 showed only 42% identity (Fig. 2b) to the peptide sequence of a native PLA_2, Ecarpholin S from *E. sochureki* (Polgar et al., 1996). This clearly indicates that EsPLA$_2$-4 encodes a distinct *E. sochureki* PLA_2 isoform. The amino acid sequence encoded by EoPLA$_2$-5 showed comparatively lower overall identity to viper PLA_2 isoforms than EplPLA$_2$-5 and EsPLA$_2$-4: *B. jararacussu* (65%), *C. atrox* (54%), *T. flavoviridis* (50%), *V. ammodytes* (51%), *E.*

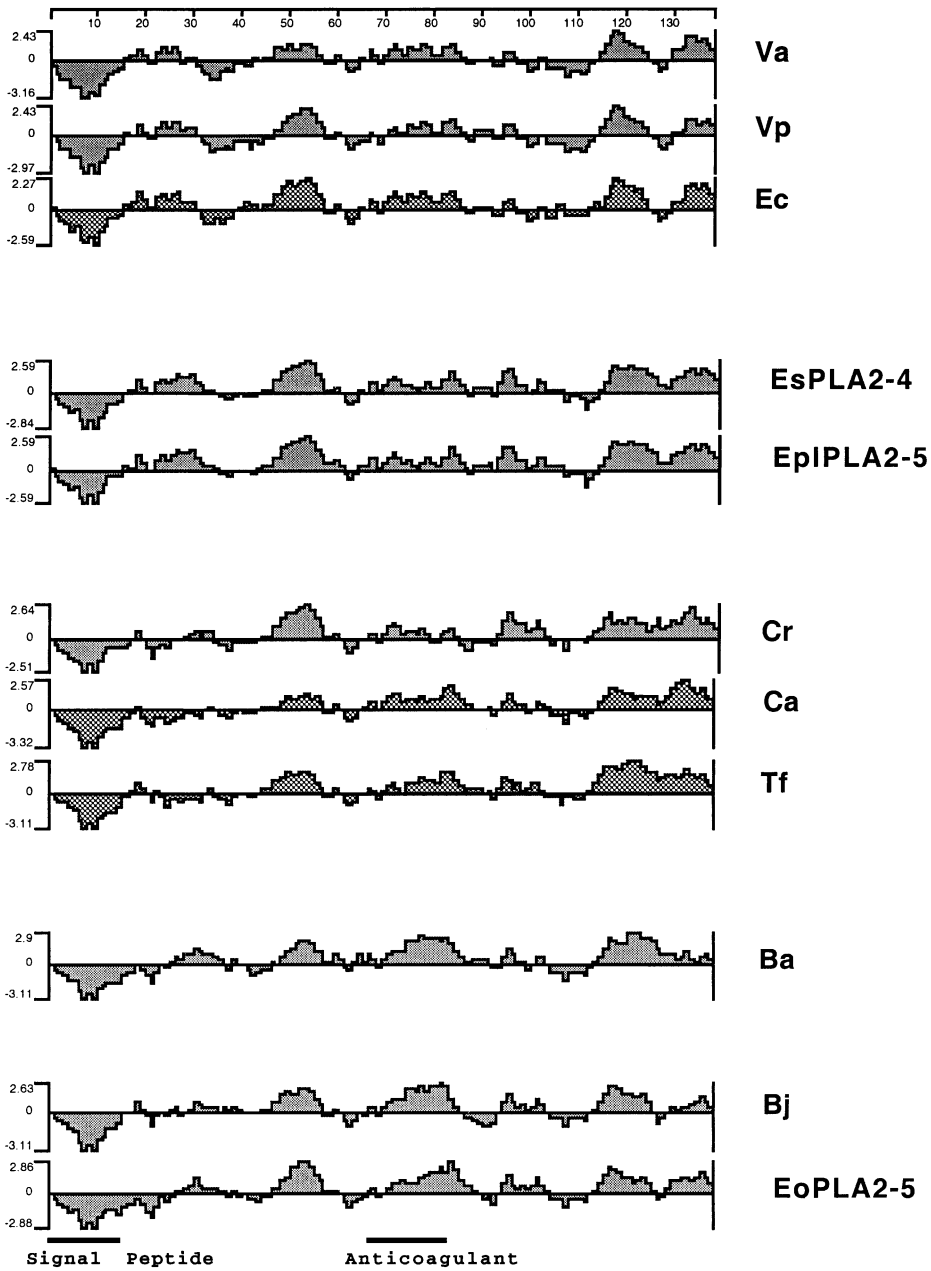

Fig. 3. Kyte–Doolittle hydrophilicity profile of viper PLA_2 isoforms used in Fig. 2a. The top horizontal scale represents the number of amino acid residues. The vertical scale represents the hydrophilic (+) and hydrophobic (−) domains, respectively. The conserved signal peptide and anticoagulant domains are indicated.

coloratus (50%), *B. asper* (50%), *V. palaestinae* (50%) and *C. rhodostoma* (48%).

In an effort to assign potential functional attributes to EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5, their deduced amino acid sequences were examined (i) for the expression of lysine or aspartate residue at position 64 (49 in sequences lacking the signal peptide) known to confer calcium-mediated catalytic activity, (ii) for the number of lysine residues within the anticoagulant domain and (iii) to identify sequences with a basic isoelectric point (pI). The latter two attributes have been correlated with anticoagulant activity of native PLA$_2$ enzymes (Kini and Evans, 1987). All the viper PLA$_2$ isoforms examined here, including EplPLA$_2$-5, EsPLA$_2$-4, EoPLA$_2$-5, expressed an aspartate residue at position 64 (with the exception of *B. asper*; lysine) indicating that they all possessed catalytic potential. Only *B. asper* PLA$_2$ isoform contained more than one lysine residue in the anticoagulant domain (Fig. 2a). Only the PLA$_2$ isoforms from *B. asper*, *B. jararacussu*, and Ecarpholin S from *E. sochureki* showed a basic pI (Fig. 2b). These indications suggest that EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5 encode PLA$_2$ isoforms with little, if any, anticoagulant activity. These observations illustrate the problems of using cDNA-derived amino acid sequence data to predict functional characteristics of proteins that exist as multiple isoforms, each with the potential to effect distinct functions. Current research is focused on linking sequence data for the existing (and remaining) PLA$_2$ cDNAs extracted from *E. p. leakeyi*, *E. sochureki* and *E. ocellatus* to isoforms recovered from two dimensional gel electrophoresis of whole venom.

The diverse functional capabilities of PLA$_2$ isoforms whose amino acid sequences differ only slightly does not, however, have a negative impact on the achievement of our objectives—the development of toxin-neutralizing antibodies by DNA immunization. The deduced primary structures of EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5 include the requisite, highly conserved, 14 cysteine residues that form the seven disulphide bonds responsible for the characteristic tertiary structure of group II PLA$_2$. It is therefore predicted that, transfection of mammalian cells with PLA$_2$-encoding DNA is likely to present to the immune system molecules that faithfully represent native venom PLA$_2$. The hydrophilic profile (Fig. 3) (Kyte and Doolittle, 1982) of all the PLA$_2$ molecules showed remarkable conservation, irrespective of their geographical distribution, pI, taxonomic designation and toxicity. The predicted Jameson–Wolf antigenic profiles (DNASTAR, USA) of these PLA$_2$ isoforms aligned with near identity to the Kyte–Doolittle hydrophilic profile plots (data not shown). This observation strongly suggests that an antibody developed to an amino acid domain that (i) has a high antigenic index and (ii) is conserved across snake species has potential for viper polyspecific neutralization of viper venom PLA$_2$ pathology.

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