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Novel sequences encoding venom C-type lectins are conserved in phylogenetically and geographically distinct *Echis* and *Bitis* viper species

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Abstract

Envenoming by *Echis* saw scaled vipers and *Bitis arietans* puff adders is the leading cause of death and morbidity in Africa due to snake bite. Despite their medical importance, the composition and constituent functionality of venoms from these vipers remains poorly understood. Here, we report the cloning of cDNA sequences encoding seven clusters or isoforms of the haemostasis-disruptive C-type lectin (CTL) proteins from the venom glands of *Echis ocellatus*, *E. pyramidum leakeyi*, *E. carinatus sochureki* and *B. arietans*. All these CTL sequences encoded the cysteine scaffold that defines the carbohydrate-recognition domain of mammalian CTLs. All but one of the *Echis* and *Bitis* CTL sequences showed greater sequence similarity to the β than α CTL subunits in venoms of related Asian and American vipers. Four of the new CTL clusters showed marked inter-cluster sequence conservation across all four viper species which were significantly different from that of previously published viper CTLs. The other three *Echis* and *Bitis* CTL clusters showed varying degrees of sequence similarity to published viper venom CTLs. Because viper venom CTLs exhibit a high degree of sequence similarity and yet exert profoundly different effects on the mammalian haemostatic system, no attempt was made to assign functionality to the new *Echis* and *Bitis* CTLs on the basis of sequence alone. The extraordinary level of inter-specific and inter-generic sequence conservation exhibited by the *Echis* and *Bitis* CTLs leads us to speculate that antibodies to representative molecules should neutralise the biological function of this important group of venom toxins in vipers that are distributed throughout Africa, the Middle East and the Indian subcontinent.

Keywords: C-type lectins; *Echis ocellatus*; *Echis pyramidum leakeyi*; *Echis carinatus sochureki*; *Bitis arietans*; Sequence conservation

1. Introduction

Snake bites cause over 20,000 deaths annually in Africa (Chippaux, 2002). The majority of these deaths result from systemic envenoming by *Echis ocellatus* (West Africa), *E. pyramidum leakeyi* (East Africa) saw scaled vipers and *Bitis arietans* puff adders (pan-African distribution). Survivors of envenoming also suffer long-term disability, scarring and occasionally amputation as a consequence of local venom-induced damage to tissues extending from the bite site (Warrell, 1996). Despite their medical importance, there has been surprisingly little research performed on the composition and constituent functionality of venoms from these snakes. The systemic and local pathological effects of venoms from the African *Echis* and *Bitis* vipers show many parallels with venoms from Asian and American pit vipers. The extensive literature on the latter vipers indicates that of the 100 or more constituents of viper venoms, a relatively small number of venom toxins are responsible for the pathological effects of envenoming. A group of zinc-containing metalloproteinases degrade the vascular subendothelium to cause haemorrhage and, by virtue of a carboxyl disintegrin-like domain, interfere with the aggregation of platelets that would normally serve to repair the damage and thereby arrest bleeding (Kamiguti et al., 1996; Bjarnason and Fox, 1994). Similar metalloproteinases with prothrombin- and factor X-activating effects (Nishida et al., 1995) and thrombin-like serine proteases (Pirkle and Theodor, 1990) promote consumptive coagulation (Hutton and Warrell, 1993). This venom arsenal is reinforced by other toxins,

Abbreviations: CTL, C-type lectins; PLA_2, phospholipase A_2; spp, species in plural.

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including RGD-containing disintegrins, phospholipases A_2 (PLA_2) and C-type lectin (CTL) proteins (Markland, 1998) that prevent blood clotting by interference with platelet aggregation and distinct elements of the coagulation cascade.

One of our main research objectives is to develop toxin-specific antivenom to treat envenoming by the African *Echis* and *Bitis* vipers. This requires a more complete definition of the venom proteome of these vipers. We and others have previously cloned sequences encoding venom haemorrhagic (Paine et al., 1994) and prothrombin-activating (Nishida et al., 1995; Yamada et al., 1996) zinc metalloproteinases of *E.p. leakeyi*. These cDNAs exhibited high sequence similarity to analogous molecules in related Asian and American vipers. More recently, we isolated cDNAs encoding PLA_2 enzymes of *E. ocellatus*, *E.p. leakeyi* and *E.c. sochureki* and demonstrated that they also showed a high level of sequence conservation (Bharati et al., in press).

Here we report the cloning of *E. ocellatus*, *E.p. leakeyi*, *E.c. sochureki* and *B. arietans* cDNAs (termed *Eo*, *Epl*, *Es* and *Ba*, respectively) that encode several isoforms of the CTL proteins. In venoms of related vipers, these venom toxins exert a variety of haemostasis-disruptive functions including binding the IX and X coagulation factors to inhibit the clotting cascade and agonistic and antagonistic effects on platelet aggregation (Wisner et al., 2002). *E.c. sochureki* (Indian subcontinent) was included to evaluate whether the sequence conservation of viper venom toxins would extend to the phylogenetically close but geographically distant Asian viper. The *Echis* and *Bitis* viper CTL cDNAs isolated here showed considerable sequence identity with each other and to analogues of related vipers, including retention of specific amino acid substitutions used to distinguish CTL isomers.

2. Materials and methods

2.1. Animals

Adult *E.p. leakeyi* (Kenya), *E.c. sochureki* (Pakistan) and *E. ocellatus* (Nigeria) carpet vipers and *B. arietans* puff adders (Ghana and Saudi Arabia) used in this study were maintained in the herpetarium, Liverpool School of Tropical Medicine, Liverpool, UK.

2.2. Extraction of total venom gland RNA and construction of cDNA libraries

Venom glands were dissected from three snakes of each *Echis* species and from two *B. arietans* snakes. The vipers were sacrificed 3 days after venom extraction when toxin gene transcription rates are at a peak. Glands were homogenized under liquid N_2 and total RNA extracted using guanidinium thiocyanate-phenol-chloroform as described previously (Bharati et al., in press). Lambda phage cDNA libraries for *E. sochureki*, *E. ocellatus* and *B. arietans* were constructed by RT-PCR using the SMART cDNA library construction kit (Clontech, California, USA). The lambda ZapII vector was used to construct the *E.p. leakeyi* cDNA library (Stratagene, California, USA). The lambda phage of the four snake species was packaged using Gigapack III Gold Packaging Extract (Stratagene) and boiled for 5 min prior to being used as targets of polymerase chain reaction (PCR) amplification.

2.3. Isolation and analysis of cDNA sequences

A PCR strategy was used to isolate sequences encoding CTLs from the cDNA libraries. A sense primer (5′-**GGA-TCC**-ATG-GGG-CGA-TTC-ATC-TTC-3′) and an anti-sense primer (5′-**CTC-GAG**-CTA-TGC-CGG-GCT-CTT-GCA-GAC-GAA-3′) complimentary to highly conserved amino-terminal signal peptide (M-G-R-F-I-F) and to the less conserved carboxy-terminal (F-V-C-K-S-P-A) domains of published CTL DNA sequences of related viper species were synthesized commercially (Sigma-Genosys, UK). A TAG stop codon was inserted in the 3′ primer and *Bam*H1 and *Xho*1 restriction endonuclease sites (bold) were included in the 5′ and 3′ primers, respectively, to facilitate future subcloning.

PCR was performed using an initial denaturation (95 °C×6 min) and annealing (55 °C×1 min) step, followed by 35 cycles (1 min each) of extension (74 °C), denaturation (94 °C) and annealing (55 °C) and a terminal extension step (7 min) at 72 °C in a thermal cycler (Gene Cycler, BioRad, Hercules, CA, USA). The inclusion of water-only controls with each PCR reaction allowed us to monitor and prevent cross-over contamination. The amplicons were subcloned into the TA cloning vector, pCR 2.1-TOPO, (Invitrogen, Groningen, The Netherlands) and used to transform chemically competent *E. coli* cells (TOP10F', Invitrogen) under ampicillin selection. Plasmid DNA was extracted (Mini-spin prep kit, Qiagen, Hilden, Germany) and digested with *Bam*H1 and *Xho*1 at 37 °C to select plasmids containing inserts of the predicted size for DNA sequencing.

DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method in a Beckman Coulter CEQ™ 2000 XL DNA Analysis System. To confirm that the cDNA sequences encoded CTLs, the predicted amino acid sequences were subjected to BLAST searches of the GenBank, PDB, SwissProt, PIR and PRF databases. All the cDNAs exhibited significant sequence homology to CTLs of related vipers. The CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix was used to align deduced amino acid sequences representing each *Echis* spp and *B. arietans* CTL isoform with analogues in venoms from related *Viperidae* species: ECLV IX/X bp β (AAB36402) from *E. leucogaster* (Chen and Tsai, 1996), Echicetin β (P81996) from *E. carinatus* (Peng et al., 1994), Bitiscetin α (JC5058) and β (JC5059) subunits from *B. arietans* (Matsui et al., 1997), CHH-B α (P81508) and β (P81509) subunits from *Crotalus horridus horridus*

(Andrews et al., 1996), Convulxin β (CAA76182) from *C. durissus terrificus* (Leduc and Bon, 1998), Alboaggregin α subunit 4 (P81114) from *Trimeresurus albolabris* (Kowalska et al., 1998), α (D83331) and β (D83332) IX/X bp subunits from *Protobothrops flavoviridis* (Matsuzaki et al., 1996), Flavocetin-A β subunit (AAN72437) also from *P. flavoviridis* (Shin et al., 2000), Aggretin (JC7105) from *Calloselasma rhodostoma* (Chung et al., 1999), α (AF176420) and β (BAB20441) subunits from *Deinagkistrodon acutus* (direct submissions) and, finally, α (AF190827) and β (AF197915) subunits from *Gloydius halys* (direct submissions). The phylogenetic trees constructed from the above alignments were generated by a neighbour-joining (Saitou and Nei, 1987) algorithm in Lasergene software (DNASTAR, USA). The predicted antigenic profile (Jameson and Wolf, 1988) of the published and new *Echis* and *B. arietans* CTL isoforms analysed here was determined using Protean software (DNASTAR).

3. Results

3.1. Isolation of cDNAs encoding E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans CTLs

PCR screening of the *Echis* spp and *B. arietans* venom gland cDNA libraries resulted in a total of 11 *E.p. leakeyi* (*Epl*), 14 *E.c. sochureki* (*Es*), 9 *E. ocellatus* (*Eo*) and 11 *B. arietans* (*Ba*) cDNAs whose sequences matched (BLAST searches) those of published CTLs. Where two or more identical sequences were obtained from any one of these libraries, a single representative cDNA was used for subsequent analysis. The cDNAs consisted of 441–447 nucleotides (data not shown) and were predicted to encode open reading frame proteins of 147–149 amino acids (16.9–17.5 kDa). Accession numbers assigned to the new *Echis* and *B. arietans* CTL sequences are as follows: *Es CTL*-1 (AY254331), *Es CTL*-3 (AY254332), *Es CTL*-8 (AY254334), *Es CTL*-9 (AY254336), *Epl CTL*-1 (AY254337), *Epl CTL*-4 (AY254338), *Epl CTL*-5 (AY254339), *Epl CTL*-7 (AY254340), *Eo CTL*-1 (AY254330), *Eo CTL*-2 (AY254333), *Eo CTL*-27 (AY254335), *Ba CTL*-1 (AY254325), *Ba CTL*-2 (AY254326), *Ba CTL*-5 (AY254327), *Ba CTL*-6 (AY254328) and *Ba CTL*-8 (AY254329).

3.2. The CTLs of E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans exhibit extensive inter-specific and inter-generic sequence similarity or identity

Alignment of the predicted amino acid sequences of the 16 species-specific cDNAs encoding CTLs from the *Echis* spp and *B. arietans* (Fig. 1) revealed extensive sequence similarities. Phylogenetic tree analysis (Fig. 2) was used to catego-

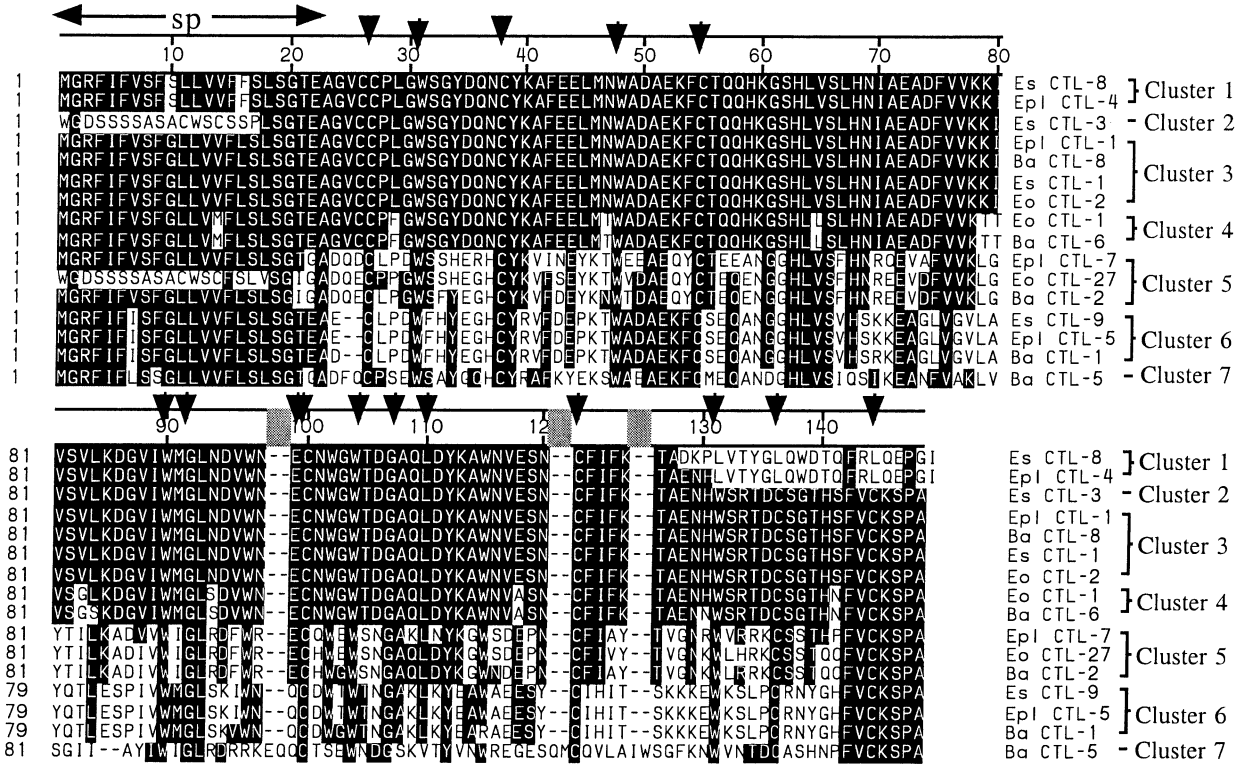

Fig. 1. Alignment and grouping of the *E. ocellatus, E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL deduced amino acid sequences. The horizontal arrows depict the signal peptide (sp) domain (residues 1–23). The vertical arrows identify amino acid residues referred to in the text that are associated with the carbohydrate recognition domain of mammalian CTL proteins. Residues shaded in black are identical to *Epl CTL*-1.

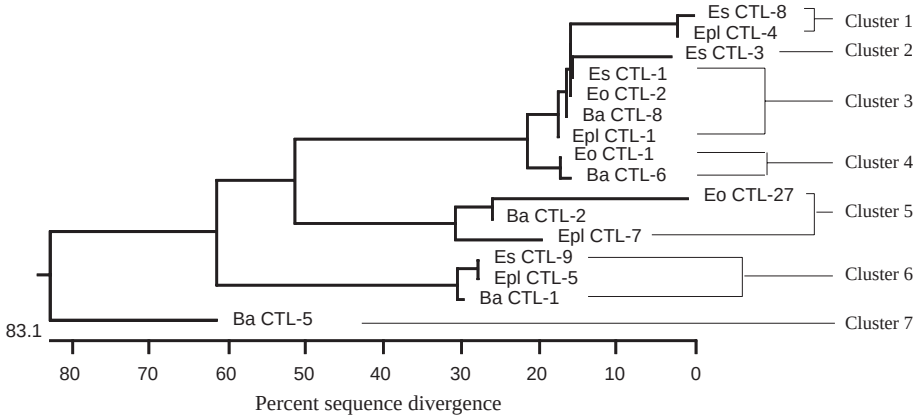

Fig. 2. Phylogenetic tree analysis of the *E. ocellatus, E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL deduced amino acid sequences. This neighbour-joining analysis was used to categorise the sequences into seven distinct clusters. Branch lengths (dark horizontal lines) are drawn to scale and represent the percent amino acid divergence for each sequence.

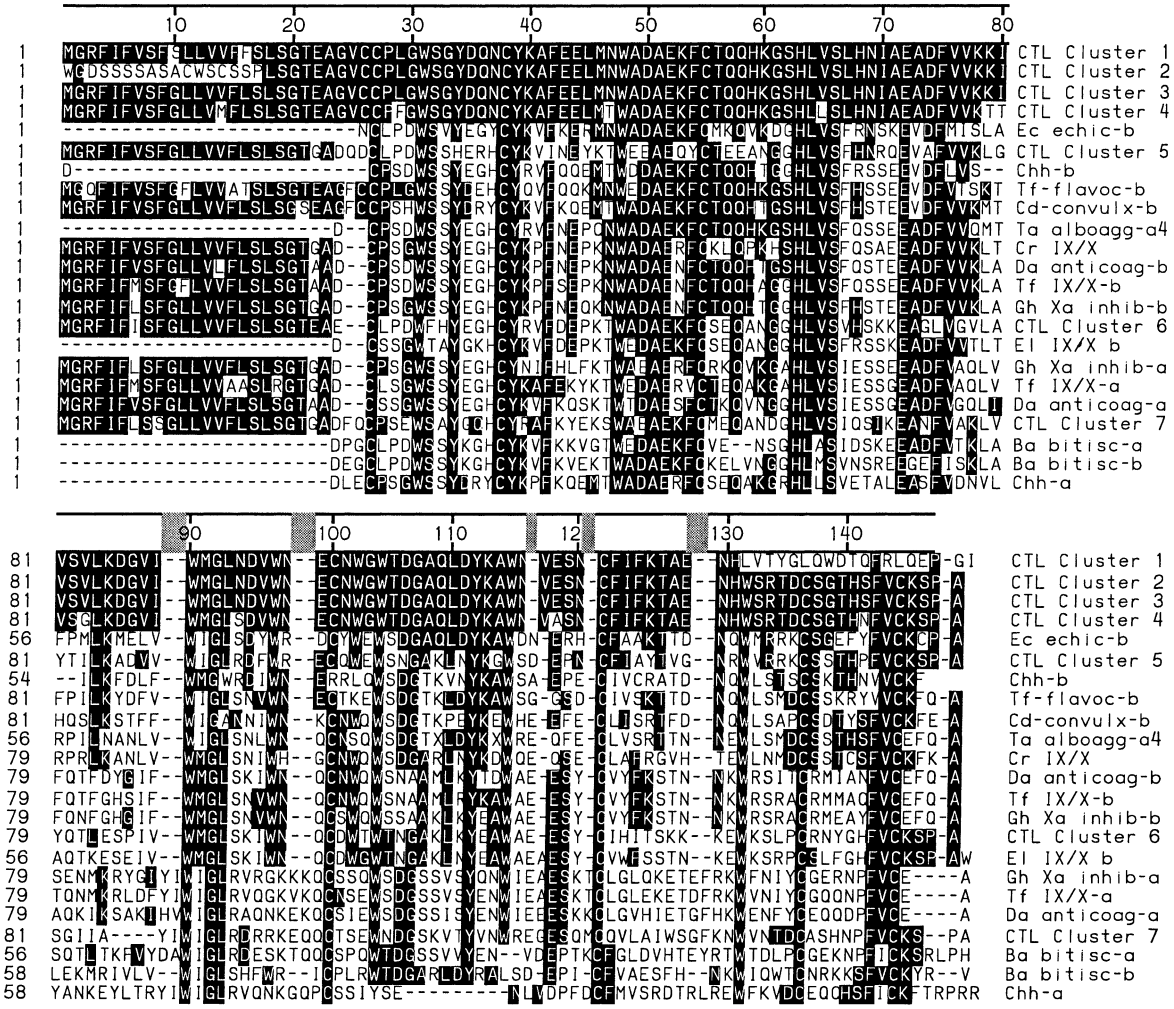

Fig. 3. Amino acid sequence alignment of the *E. ocellatus, E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL clusters with venom CTLs from related vipers. Ec echic-b=echicetin β from *E. carinatus*; Chh-a and -b=α and β CTL subunits from *C.h. horridus*; Tf-flavoc-b=β CTL subunit from *P. flavoviridis*; TfIX/X-a and -b=α and β subunits of the IX/X binding protein of *P. flavoviridis*; Cd-convulx-b=β subunit of convulxin from *C. durissus terrificus*; Ta-alboagg-a4=alboaggregin α subunit 4 from *T. albolabris*; Cr IX/X=aggretin, a factor IX/X binding protein from *C. rhodostoma*; Da anticoag-a and -b=α and β subunits of the anticoagulant CTL from *D. acutus*; Gh Xa inhib-a and -b=α and β subunits of the factor Xa inhibiting CTL from *G. halys*; El IX/X-b=β subunit of ECLV, the IX/X binding protein of *E. leucogaster*; Ba bitisc-a and -b=α and β subunits of bitiscetin from *B. arietans*. Residues shaded in black are identical to *Epl CTL*-1 of cluster 3.

rise the 16 CTL sequences into seven distinct clusters, based solely on sequence alignment. Cluster 1 contained *Es CTL*-8 and *Epl CTL*-4 which were 98.1% identical (differing by three residues) and, had they not contained a distinct carboxyl-terminus, would have retained this level of sequence identity to sequences in cluster 3. Cluster 2 was represented by a single cDNA, *Es CTL*-3, which did not contain the methionine start codon and its amino terminal sequence differed markedly from the signal peptide sequence (residues 1–23) conserved in most of the other cDNAs. Intriguingly, *Eo CTL*-27 had a similar amino terminal sequence to *Es CTL*-3 but the down stream sequence encoded a distinct mature protein. The distinct 5′ sequence composition of *Es CTL*-3 and *Eo CTL*-27 was not considered the result of cloning artefacts since four other *Es CTL* cDNAs were found with identical sequences.

Immediately downstream of the signal peptide domain, the sequences within each CTL cluster showed a remarkable degree of sequence conservation in most of the viper species examined. For instance, cluster 3 was represented in all four viper species by *Epl CTL*-1, *Ba CTL*-8, *Es CTL*-1 and *Eo CTL*-2, which were completely identical. Cluster 4 contained *Eo CTL*-1 and *Ba CTL*-6 that were 98.7% identical and contained identical substitutions of nine residues evenly distributed along the length of the sequence. Cluster 5 contained representatives from three of the four viper species, *Epl CTL*-7, *Eo CTL*-27 and *Ba CTL*-2, that showed a comparatively low sequence similarity of between 75% and 84%. Cluster 6 also contained representatives from three of the four viper species: *Es CTL*-9, *Epl CTL*-5 (100% sequence identity) and *Ba CTL*-1 (96.8%). The sequences in this group were less than 60% similar to those in the above groups. Cluster 7 contained a single representative sequence, *Ba CTL*-5, that showed less than 50% sequence similarity to all the preceding cDNAs.

The deduced amino acid sequences of the *Echis* spp and *B. arietans* CTLs possessed the cysteine scaffold that defines the carbohydrate-recognition domain (CRD) of mammalian CTLs (Drickamer, 1988) that bind sugars in a Ca^{2+}-dependent manner. Disulphide bridges formed between C27 and C38, C55 and C144 and between C122 and C136 (arrows in Fig. 1) were conserved in all the *Echis* spp and *B. arietans* CTL sequences, except in those of cluster 1 where the carboxyl cysteine residues were both substituted with leucine residues. The CRD-invariant residues W31, W48, W90-X-G92, W104-X-X-G107 and W131 (Wisner et al., 2002) were all represented in the *Echis* and *B. arietans* CTL clusters, except cluster 1 which showed an L for W substitution at position 131. In contrast to the conserved mammalian CRD sequences, all the above viper venom CTL clusters showed a G for W substitution at position 92 and an L for P substitution at position 110, except cluster 7 which had a V at position 110. Also, the viper sequences did not contain the E-P-N and Q-P-D triplets associated with CRD binding by mammalian CTLs to mannose and galactose, respectively. The *Echis* and *Bitis* CTL sequences appeared incapable of binding calcium, a feature common to venom CTLs that bind to coagulation factors IX and X (Atoda et al., 2002). Thus, unlike CTL subunits of *P. flavoviridis* (Shin et al., 2000) and *E. leucogaster* (Atoda et al., 2002) that contain the requisite S-66, E-68, E-72 and E-146 for calcium binding (Mizuno et al., 1997), all the *Echis* and *Bitis* CTL clusters, except cluster 7, showed an H for E-68 and an S for E-146 substitution. Cluster 1 sequences, however, retained the E-146 residue.

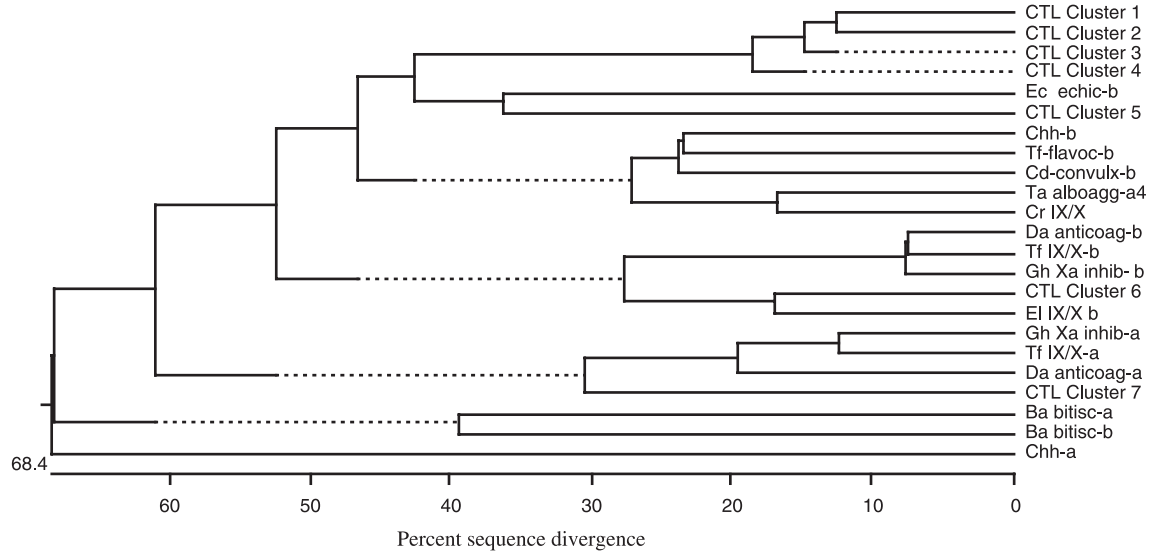

Fig. 4. Phylogenetic tree (neighbour-joining) analysis of the *E. ocellatus, E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL clusters and venom CTLs from related vipers. Branch lengths are drawn to scale and represent the percent amino acid divergence for each cluster or sequence.

3.3. Sequence alignment of the E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans CTL clusters with analogues in related viper species

Viper venom CTL sequences in the genetic databases were compared with the *Echis* spp and *B. arietans* CTL clusters (Fig. 3) by BLAST. Clusters 1–4 represent novel, highly similar, CTL isomers with less than 65% sequence similarity to analogues in related viper species. Phylogenetic tree analysis showed that clusters 1–4 were most closely associated with β-subunit CTLs (Fig. 4). Cluster 5 was assigned to the same clade as the β-subunit of echicetin from *E. carinatus* and generally was aligned with other CTL β-subunits. Cluster 6 showed the greatest sequence similarity (62%) to the CTL β-subunit of the West African *E. leucogaster* viper. Of all the *Echis* spp and *B. arietans* CTL clusters, only cluster 7 seemed to represent an α-CTL subunit sequence which showed the highest sequence similarity (59%) to the α-subunit of the Xa-binding CTL of the Asian *G. halys* viper. None of the clusters showed more than 50% sequence similarity (data not shown) to the partial peptide sequences for the α- and β-subunits of the EM 16, a CTL purified from the venom of the Asian *E. multisquamatus* viper (Marcinkiewicz et al., 2000). Similarly, the α- and β-subunits of bitiscetin from *B. arietans* venom showed no greater than 42% sequence similarity to any of the *Echis* spp or *B. arietans* CTL sequences.

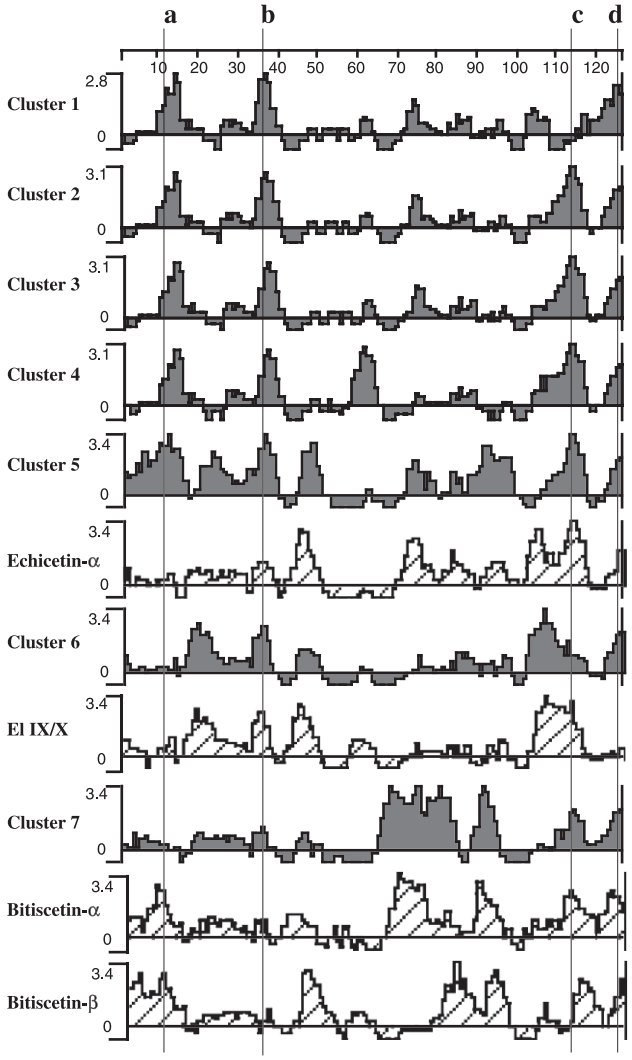

Fig. 5. Jameson–Wolf antigenic profiles of the *E. ocellatus, E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL clusters and all known CTLs from related African vipers. The top horizontal scale represents the number of amino acid residues (the 23 signal peptide residues are not presented here). The vertical scales represent comparative antigenic values. The four thin vertical lines (a–d) are a subjective assignation of antigenic domains that exhibit the greatest phylogenetic representation.

3.4. Predicted antigenic profiles of the E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans CTL clusters

Since the main focus of our research is to develop toxin-neutralising antibodies by immunisation with DNA encoding specific toxins in venoms of the most medically important African vipers (Harrison et al., 2000, 2002), we next compared the algorithm-predicted immunogenicity of the *Echis* spp and *B. arietans* CTL cluster cDNA sequences with those of all the published CTLs from vipers of African origin (Fig. 5). The deduced signal peptide domains of the *Echis* spp and *B. arietans* CTL were not represented here because they would be cleaved from the native proteins. The thin vertical lines are a subjective identification of the most immunogenic CTL domains that show the greatest phylogenetic conservation. Line (a) approximately corresponded to the residues C-Y-K (38–40; Fig. 3) and was common to clusters 1–5. The highly conserved domain between residues 48 and 55 (W-A-D-A-E-K-F-C) was not predicted to be immunogenic. Line (b) corresponded to the residues around H-L-V-S (63–66) and was common to clusters 1–6 and the *E. leucogaster* CTL that bind coagulation factors IX/X. Lines (c) (residues around C136) and (d) (V-C-K at positions 144–146) appeared to represent immunogenic domains common to all the new and published African viper venom CTL sequences.

4. Discussion

The *Echis* and *Bitis* CTL sequences described here contained all the cysteine residues and most of the associated amino acid motifs that define the CRD domain and overall architecture of the mammalian and viper CTL sequences (Drickamer, 1988). These results represent a significant addition to the venom gland transcriptome of the *Echis* saw scaled vipers and *Bitis arietans* and reveal the CTL composition of these vipers to be as complex as that of the better characterised New World vipers (Wisner et al., 2002). The latter review illustrates that viper venom CTLs possess a range of pharmacological functions that disrupt haemostasis by inhibiting platelet aggregation through interaction with several platelet receptors, including von Willebrand factor domains (e.g., Botrocetin; Bitiscetin), the glycoprotein receptors GPIa (e.g., Aggretin), GPIb

(e.g., Echicetin, Flavocetin, CHH-A and B) and GPVI (e.g., Convulxin). Venom CTLs also activate the coagulation cascade through cleavage of factor X (e.g., RVV-X), factor IX (e.g., IX activator) and factor II (e.g., Carinactivase). Conversely, other venom CTLs inhibit coagulation by binding to factors IX and X (e.g., IX/X binding proteins from venoms of *P. flavoviridis* and *E. leucogaster*).

The *Echis* and *Bitis* sequences grouped in CTL clusters 1–4 appear to be distinct but highly similar CTL subunits that are novel to the serpents genetic database. In common with CTL β-subunits of flavocetin-A and convulxin from venoms of *P. flavoviridis* and *C. d. terrificus*, respectively (Shin et al., 2000; Leduc and Bon, 1998), CTL clusters 1–4 contained a double cysteine motif at positions 26 and 27 that have been invoked to differentiate β- from α-subunits of CTLs (Shin et al., 2000). The N-terminal additional C26 of β-CTL subunits is thought to form a disulphide bridge with a C-terminal cysteine residue of α-subunits permitting the polymerisation of these peptides into heterodimeric molecules (Wisner et al., 2002). Although they lacked the additional C26 residue, the sequences in clusters 5 and 6 were also most closely associated with β-CTL subunits. Only *Ba CTL*-5 (cluster 7) showed a closer sequence similarity to the α-CTL subunits. Since it was the only sequence in this cluster, we presume it to be a low copy gene. We infer from the absence of an initiating methionine and non-signal peptide-like N-terminal sequence of *Es CTL*-3 (cluster 3) and *Eo CTL*-27 (cluster 5) that these sequences are derivatives from other genes, presumably the group IV metalloproteinases at either the carboxyl (Bjarnason and Fox, 1994) or amino terminal ends (Kini, 1996). We could not, however, find sequences similar to these domains in group IV metalloproteinases or in venom CTL sequences known to be associated with group IV metalloproteinases. It is tempting to use the sequence similarity between the *Echis* and *Bitis* CTL sequences and published venom CTLs of known biological activity to predict the functionality for the new CTL clusters. However, the sequence–function relationship of venom CTLs (and many other venom toxins) is still insufficiently understood to confidently assign a function to a molecule based solely on sequence characteristics.

The extraordinary degree of CTL sequence conservation between the geographically distant *E. ocellatus*, *E.p. leakeyi*, *E.c. sochureki* and *B. arietans* vipers indicates the biological importance of the multimeric nature of this group of venom toxins. Here, and as frequently observed in the snake venom literature (Deshimaru et al., 1996), the DNA sequences encoding these *Echis* and *Bitis* CTLs showed a higher level of sequence conservation than the deduced amino acid sequences (data not shown). Accelerated evolution of snake venom toxins has been mooted to confer an evolutionary advantage by increasing the range of prey susceptible to envenoming. The identical inter-specific and inter-generic sequences for cluster 3 CTLs and near identity of sequences in other clusters suggest that accelerated venom toxin evolution is regulated by a sophisticated control mechanism, rather than a system that simply accepts degenerative substitutions. While this is likely to be a fruitful area of research, it was not the purpose of this study.

Our objective was to evaluate the range of CTL molecules likely to be represented in venoms of *E. ocellatus*, *E.p. leakeyi*, *E. sochureki* and *B. arietans* vipers in order to guide the design of DNA-immunisation constructs required to generate toxin-neutralising antibodies. We have shown that DNA immunisation is an efficient means of generating specific antibodies with potent venom toxin-neutralising activity (Harrison et al., 2000, 2002) and that the toxin-specific antibodies exhibit immunological reactivity to venoms from a wide range of viper species (Harrison et al., 2003). Our utilisation of a low-stringency PCR approach was successful in amplifying several distinct CTL isoforms from the venom glands of these vipers. The predicted antigenic profiles of the *Echis* and *Bitis* CTLs illustrated in Fig. 5 indicate that neutralisation of this complex group of toxins will require antibodies with several specificities. Thus, while an antibody raised by immunisation with cluster 3 DNA is likely to be effective against the gene products of clusters 2, 3 and 4, additional antibodies will be required to neutralise the other clusters. Also, because of the medical importance of echicetin (Peng et al., 1994) and bitiscetin (Hamako et al., 1996), we have initiated experiments to isolate cDNAs encoding these molecules from the respective libraries. Therefore, to be confident of neutralising the function of this group of venom toxins, we speculate the requirement of between six and eight CTL antibody specificities. While this may seem excessive, our results indicate that such a panel of antibodies would neutralise this important group of functionally diverse toxins in venoms of the most important African vipers across their geographic range. Given (i) the basal status of the *Echis* genus in viperine lineages (Lenk et al., 2001) and (ii) the high degree of inter-specific and inter-generic CTL sequence conservation observed here, it is possible that the venom CTL-neutralising effectiveness of such a panel of antibodies may extend to other African vipers and also to phylogenetically related vipers in Asia.

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References

Andrews, R.K., Kroll, M.H., Ward, C.M., Rose, J.W., Scarborough, R.M., Smith, A.I., Lopez, J.A., Berndt, M.C., 1996. Binding of a novel 50 kDa alboaggregin from *Trimeresurus albolabris* and related viper ven-

om proteins to the platelet membrane glycoprotein Ib-IX-V complex. Effect on platelet aggregation and glycoprotein Ib-mediated platelet aggregation. Biochemistry 35, 12629–12639.

Atoda, H., Kaneko, H., Mizuno, H., Morita, T., 2002. Calcium-binding analysis and molecular modelling reveals *echis* coagulation factor IX/factor X-binding protein has Ca-binding properties and Ca ion-independent folding of other C-type lectin-like proteins. FEBS Lett. 531, 229–234.

Bharati, K., Hasson, S.S., Oliver, J., Laing, G., Theakston, R.D.G., Harrison, R.A., 2003. Molecular cloning of phospholipases A_2 from venom glands of *Echis* carpet vipers. Toxicon (in press).

Bjarnason, J.B., Fox, J.W., 1994. Haemorrhagic metalloproteinases from snake venoms. Pharmacol. Ther. 62, 345–368.

Chen, Y.-H., Tsai, I.-H., 1996. Functional and sequence characterization of coagulation factor IX/factor X binding protein from the venom of *Echis leucogaster*. Biochemistry 35, 5264–5271.

Chippaux, J.-P., 2002. The treatment of snake bites: analysis of requirements and assessment of therapeutic efficacy in tropical Africa. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 457–472.

Chung, C.-H., Au, L.-C., Huang, T.-F., 1999. Molecular cloning and sequence analysis of Aggretin, a collagen-like platelet aggregator. Biochem. Biophys. Res. Commun. 263, 723–727.

Deshimaru, M., Ogawa, T., Nakashima, K., Nobuhisa, I., Chijiwa, T., Shimohigashi, Y., Fukumaki, Y., Niwa, M., Yamashina, I., Hattori, S., Ohno, M., 1996. Accelerated evolution of crotalinae snake venom serine proteases. FEBS Lett. 397, 83–88.

Drickamer, K., 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. J. Biol. Chem. 263 (20), 9557–9560.

Hamako, J., Matsui, T., Suzuki, M., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y., Titani, K., 1996. Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* venom. Biochem. Biophys. Res. Commun. 226, 273–279.

Harrison, R.A., Moura-da-Silva, A.M., Laing, G.D., Wu, Y., Richards, A., Broadhead, A., Bianco, A.E., Theakston, R.D.G., 2000. Antibody from mice immunised with DNA encoding the carboxyl-disintegrin and cysteine rich domain (JD9) of the haemorrhagic metalloprotease, Jararhagin, inhibits the main lethal component of viper venom. J. Clin. Exp. Immunol. 121, 358–363.

Harrison, R.A., Richards, A., Laing, G.D., Theakston, R.D.G., 2002. Simultaneous GeneGun immunisation with plasmids encoding antigen and GM-CSF: significant enhancement of murine antivenom IgG1 titres. Vaccine 20, 1702–1706.

Harrison, R.A., Wüster, W., Theakston, R.D.G., 2003. The conserved structure of snake venom toxins confers extensive immunological cross-reactivity to toxin-specific antibody. Toxicon 41, 441–449.

Hutton, R.A., Warrell, D.A., 1993. Action of snake venom components on the haemostatic system. Blood Rev. 7, 176–189.

Jameson, B.A., Wolf, H., 1988. The antigenic index: a novel algorithm for predicting antigenic determinants. Comput. Appl. Biosci. 4 (1), 181–186.

Kamiguti, A.S., Hay, C.R.M., Theakston, R.D.G., Zuzel, M., 1996. Insights into the mechanism of haemorrhage caused by snake venom metalloproteases. Toxicon 34, 627–642.

Kini, R.M., 1996. Are C-type lectin-related proteins derived by proteolysis of metalloproteinase/disintegrin precursor proteins? Toxicon 34 (11/12), 1287–1294.

Kowalska, M., Tan, L., Holt, J., Peng, M., Karczewski, J., Calvete, J., Niewiarowski, S., 1998. Alboaggregin A and B. Structure and interaction with human platelets. Thromb. Haemost. 79, 609–613.

Leduc, M., Bon, C., 1998. Cloning of subunits of convulxin, a collagen-like platelet-aggregating protein from beta *Crotalus durissus terrificus* venom. Biochem. J. 333, 389–393.

Lenk, P., Kalayabina, S., Winl, M., Joger, U., 2001. Evolutionary relationships among the true vipers (*Reptilia*: *Viperidae*) inferred from mitochondrial DNA sequences. Mol. Phylogen. Evol. 19, 94–104.

Marcinkiewicz, C., Lobb, R.R., Marcinkiewicz, M.M., Daniel, J.L., Smith, J.B., Dangemarier, C., Weinreb, P.H., Beacham, D.A., Niewiarowski, S., 2000. Isolation and characterization of EM16, a C-lectin type protein from *Echis multisquamatus* venom, a potent and selective inhibitor of the $\alpha 2\beta 1$ integrin. Biochemistry 39, 9859–9867.

Markland, F.S., 1998. Snake venoms and the haemostatic system. Toxicon 36 (12), 1749–1800.

Matsui, T., Hamako, J., Suzuki, M., Hayashi, N., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y., Titani, K., 1997. Complete amino acid sequence of bitiscetin, a novel von Willebrand factor modulator protein purified from snake venom of *Bitis arietans*. Res. Commun. Biochem. Cell Mol. Biol. 1, 271–284.

Matsuzaki, R., Yoshiara, E., Yamada, M., Shima, K., Atoda, H., Morita, M., 1996. cDNA cloning of IX/X-BP, a heterogeneous two-chain anticoagulant protein from snake venom. Biochem. Biophys. Res. Commun. 220, 382–387.

Mizuno, H., Fujimoto, Z., Koizumi, M., Kano, H., Atoda, H., Morita, T., 1997. Structure of coagulation factors IX/X-binding protein, a heterodimer of C-type lectin domains. Nat. Struct. Biol. 4, 438–441.

Nishida, S., Gyuta, S., Kohno, N., Morita, T., Takeya, H., Kido, I., Paine, M.J., Kawabata, S., Iwanaga, S., 1995. cDNA cloning and deduced amino acid sequence of a prothrombin activator (Ecarin) from *Echis carinatus* venom. Biochemistry 34, 1771–1778.

Paine, M.J., Moura-da-Silva, A.M., Theakston, R.D.G., Crampton, J.M., 1994. Cloning of metalloprotease genes in the carpet viper (*Echis pyramidum leakeyi*). Further members of the metalloprotease/disintegrin gene family. Eur. J. Biochem. 224, 483–488.

Peng, M., Holt, J.C., Niewiarowski, S., 1994. Isolation, characterization and amino acid sequence of echicetin beta subunit, a specific inhibitor of von Willebrand factor and thrombin interaction with glycoprotein Ib. Thromb. Haemost. 67, 702–707.

Pirkle, H., Theodor, I., 1990. Thrombin-like venom enzymes: structure and function. Adv. Exp. Med. Biol. 281, 165–175.

Saitou, N., Nei, M., 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Shin, Y., Okuyama, I., Hasegawa, J., Morita, T., 2000. Molecular cloning of glycoprotein Ib-binding protein, Flavocetin-A, which inhibits platelet aggregation. Thromb. Res. 99, 239–247.

Thompson, J.D., Higgins, D.J., Cibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Warrell, D.A., 1996. Clinical features of envenoming from snake bites. In: Bon, C., Goyffon, M. (Eds.), Envenomings and their Treatments. Foundation Marcel Merieux, Lyon, pp. 63–76.

Wisner, A., Leduc, M., Bon, C., 2002. C-type lectins from snake venoms: new tools for research in thrombosis and haemostasis. In: Menez, A. (Ed.), Perspectives in Molecular Toxinology. Wiley, Chichester, pp. 357–376.

Yamada, D., Sekiya, F., Morita, T., 1996. Isolation and characterization of carinactivase, a novel prothrombin activator in *Echis carinatus* venom with a unique catalytic mechanism. J. Biol. Chem. 271, 5200–5207.