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Arthropod Defensins Illuminate the Divergence of Scorpion Neurotoxins

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Abstract: Defensins are phylogenetically ancient antibacterial polypeptides found in plants and animals. Isolation of the cDNA and genomic sequences encoding the scorpion (*Leiurus quinquestriatus hebraeus*) defensin revealed similarity to scorpion neurotoxins in gene organization (two exons and a phase I intron) and intron characteristics (conserved acceptor, donor and putative branch sites). This commonality, alongside a similar core structure, protein sequence and bioactivity suggest that arthropod defensins and scorpion neurotoxins share a common ancestor. Interestingly, phylogenetic analysis of defensins and scorpion neurotoxins illuminates for the first time a putative evolutionary trajectory for scorpion sodium and potassium channel neurotoxins. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: scorpion; defensin; neurotoxin; evolution; ancestry

INTRODUCTION

Innate immunity, a component of the immune system, is phylogenetically ancient and is found in plants and animals. It confers broad protection against a wide variety of pathogens, and most multicellular organisms depend upon it to combat microbial infections. In vertebrates and invertebrates, the innate immunity consists in part of defensins, which are small (35–50 amino acids) cationic proteins secreted in response to bacterial or septic injuries [1].

Two defensins, isolated from the scorpions *Leiurus quinquestriatus* and *Androctonus australis* [2,3] show a high degree of sequence similarity to defensins from the dragonfly, mussel and tick [4–9]. Within arachnids (scorpions and ticks) mature defensins share 67%–94% identity [10]. Arthropod defensins have been shown to have antimicrobial activity against a wide range of Gram-positive and a few Gram-negative bacteria [2–9,11]. Their antibacterial activity is ascribed to their ability to disrupt the permeability barrier of the cytoplasmic membrane by interacting with the phospholipids and forming complexes that are not miscible in the lipid phase [12–14].

Scorpion neurotoxins modulate membrane potential by modifying ion channel conductance in excitable tissues. They are divided into two major categories: (1) 'long-chain' toxins (61–76 amino acid residues), which are constrained by four disulfide bridges and modulate sodium channel function [15]; and (2) 'short-chain' toxins (less than 40 amino acid residues), which are constrained by three disulfide bridges and affect potassium channels [16]. However, 'long-chain' potassium channel toxins and

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sodium channel toxins that are constrained by only three disulfide bridges have also been isolated [17,18].

Despite the commonality in the core structure of scorpion sodium and potassium channel toxins [16,19], their divergence route has remained elusive due to vast variations in sequence and length [19]. However, isolation of a defensin gene from the scorpion *Leiurus quinquestriatus hebraeus* with significant sequence similarity to scorpion 'short-chain' toxins illuminated a putative evolutionary trajectory for scorpion neurotoxins.

MATERIALS AND METHODS

Defensin Gene Isolation

The cDNA clone was isolated from a *Leiurus quinquestriatus hebraeus* (Lqh) cDNA library [20] using a degenerate oligonucleotide primer (5′-TAYCANGTYCANGTRTGRTTYAA-3′) designed according to the *C*-terminal amino acid sequence (accession number P41965) [2] and KS primer via polymerase chain reaction. The genomic DNA was isolated as was described [21]. The clone encoding Lqh defensin was isolated using oligonucleotides designed according to the 5′ and 3′ ends of the cDNA clone (5′-ATGAAAACCATTGTACTTCTTTTCATGTT-GG — 3′; 5′-TCCTGCGCAATATCCTCCTC-3′). Polymerase chain reaction was performed in a thermocycler (MJ Research Inc., USA) as follows: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and polymerization at 72 °C for 90 s.

Phylogenetic Analysis

The phylogenetic tree of scorpion defensin and neurotoxins was generated using the ClustalW 1.75 program, available at www.genebee.msu.su/services/phtree_reduced.html.

RESULTS AND DISCUSSION

Common Ancestry of Arthropod Defensins and Scorpion Neurotoxins

A clone was isolated encoding a scorpion defensin (Lqh-def) from a *Leiurus quinquestriatus hebraeus* (Lqh) cDNA library [20] using a degenerate oligonucleotide primer designed according to the *C*-terminal amino acid sequence (accession number P41965) [2] via polymerase chain reaction. The genomic defensin clone was isolated on the basis of the cDNA sequence using a previously described method [21]. Interestingly, the defensin sequences revealed similarities to scorpion sodium and potassium channel neurotoxins at five levels.

Sequence identity. Mature defensins from arachnids (scorpions and ticks), molluscs and insects share high similarity in amino acid sequence [22]. In addition to the similarity among arthropod and mollusc defensins, scorpion defensins share high protein sequence similarity with some scorpion neurotoxins. For example, a long potassium channel toxin, from the scorpion *Buthus Martensii* Karsch [17], shares 42% similarity with Lqh-def (Figure 1).

Bioactivity. Arthropod and mollusc defensins have antimicrobial activity against a wide range of Gram-positive and a few Gram-negative bacteria [11]. In addition to their antibacterial activity, some defensins have been shown to affect ion channels [23–25], cf. the modulation of sodium, potassium or chloride channels by scorpion neurotoxins [19].

Three-dimensional structure. To date, the structures of mussel (*Mytilus galloprovincialis*) and fly (*Phormia terranovae* and *Sarcophaga peregrina*) defensins (MGD1, Defensin A and sapecin, respectively) have been solved [26–29]. All three structures

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AaHII       ------VKDG---YIVD----DVNCT-YFC---GRNAYCNEECTKL--KGESGYCQWASPYGNACYCYKLPDH--VRTKGPGRCH 64
Cn2         -------KEG---YLVDK---NTGCK-YECLKLGDNDYCLRECKQQYGKGAGGYC-----YAFACWCTHLYEQAIVWPLPNKRCS 66
Lqh-def     ----------------------G---FGCP--LNQGACHRHCRSI--RRRGGYCAGF--FKQTCTCYRN-------------- 38
BmTXK       KLVKYAVPEGTLRTIIQTAVHKLGKTQFGCP--AYQGYCDDHCQDI--KKEEGFCHG---FK--CKCGIPMGF------------ 64
Birtoxin    -----ADVPG--NYPLD----KDGNT-YKCFLLGGNEECLNVCKLH--GVQYGYC-----YASKCWCEYLEDDKDVSV------- 59
ChTx        --------------------QFTN--VSCT---TSKECWSVCQRLH-NTSRGKC-----MNKKCRCYS---------------- 37
                                       *        *   *         * *         * *
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Figure 1 Sequence alignment of scorpion defensins and neurotoxins. Asterisks designate identical residues. AaHII (a four-disulfide-bridge sodium channel α-toxin from *Androctonus australis* hector; accession number P01484); Birtoxin (a three-disulfide-bridge sodium channel β-toxin from *Parabuthus transvaalicus*; accession number P58752); BmTXK (a three-disulfide-bridge long potassium channel toxin from *Buthus martensii* Karsch; accession number AAF29466); ChTx (Charybdotoxin, a three-disulfide-bridge short potassium channel toxin from *Leiurus quinquestriatus hebraeus*; accession number B60963); Cn2 (a four-disulfide-bridge sodium channel β-toxin from *Centruroides noxius*; accession number AAB36086); Lqh-def (a three-disulfide-bridge defensin from *Leiurus quinquestriatus*; accession number P41965).

consist of an α-helix and two antiparallel β-strands stabilized by three or four disulfide bonds forming the so-called common cystine-stabilized alpha-beta motif [26–29]. This structural motif, which constitutes the core of all these polypeptides, is also found in scorpion neurotoxins affecting sodium, potassium and chloride channels, as well as in other ion channel modulators (Figure 2; for comparison see [30]).

Genomic organization. The genomic defensin sequence contains a phase I intron that splits a codon toward the end of the leader sequence, so that the first nucleotide resides upstream of the intron, whereas the following dinucleotide is downstream of the intron boundary (not shown). In addition, the intron has remarkable resemblance with that of all distinct categories of scorpion 'long-chain' (sodium channel) and 'short-chain' (potassium and chloride) toxins [21]. The intron is characterized by a high A + T (80%) content and has a consensus GT/AG splice junction with the splice donor 5′-G|GTGAG-3′ (not shown) (5′-G|GTAAG-3′ in scorpions) [21]. Also, the intron contains a putative branch point, 5′-TAAT-3′ (not shown), located within the ideal distance upstream of the 3′ splice site [31].

Gene families. The cDNA and genomic sequences exhibited 97% and 77% similarity to the known defensin (accession number P41965) [2]. The finding of three different gene members of defensin indicates that, like scorpion neurotoxins [21], defensins are also clustered into gene families. This variability could be the result of continuous evolutionary 'pressure' and, hence, need for different antibacterial polypeptides.

To study the ancestry of neurotoxins and defensins a phylogenetic tree was generated using the three-disulfide-bridge scorpion defensin (Lqhdef) and representatives of the different neurotoxin categories (Figure 3). The neurotoxin representatives included a three-disulfide-bridge long potassium channel toxin (BmTXK) [17], a three-disulfide-bridge short potassium channel toxin (charybdotoxin, ChTx), a three-disulfide-bridge sodium channel toxin (Birtoxin) and four-disulfide-bridge sodium channel α- (AaHII) and β- (Cn2) toxins (Figure 3). From the phylogenetic tree it is clear that an ancestral gene developed in parallel into scorpion defensins and ion channel toxins (Figure 3). Furthermore, the finding that defensins originated relatively early in evolution is also corroborated by the existence of highly similar defensins in ticks and molluscs [7–9]. Altogether, the phylogenetic tree and the resemblance in protein sequence, structure, bioactivity and genomic organization of invertebrate defensins and scorpion neurotoxins suggest that they all originated from a common ancestor.

Phylogeny of Scorpion 'Long- and 'Short-chain' Neurotoxins

Up until now it has been suggested that sodium and potassium channel toxins share a common ancestor [19,21,30], but tracing their phylogeny has been limited. An attempt to construct an evolutionary tree of scorpion toxins affecting sodium, potassium, chloride and calcium channels resulted in a tree that had no root and it was difficult to discern the divergence of the different toxin categories [19]. Surprisingly, an evolutionary tree, which included defensins and scorpion neurotoxins, clearly indicates that 'short-chain' potassium channel toxins bearing three disulfide bridges, developed from an

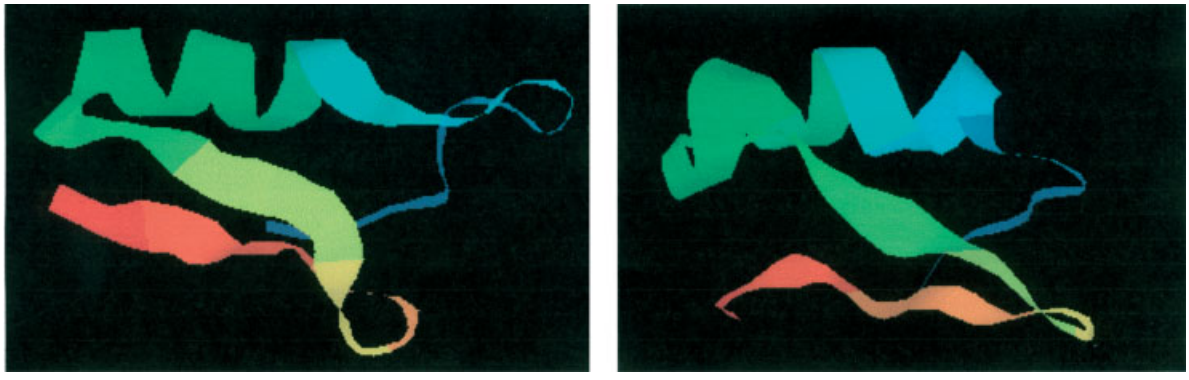

Figure 2 The structural core common to all scorpion neurotoxins and arthropod defensins. The structure of the fly defensin sapecin (left) and the scorpion potassium channel blocker PiTX-K (right) (PDB entries 1L4V and 2PTA, respectively) are presented.

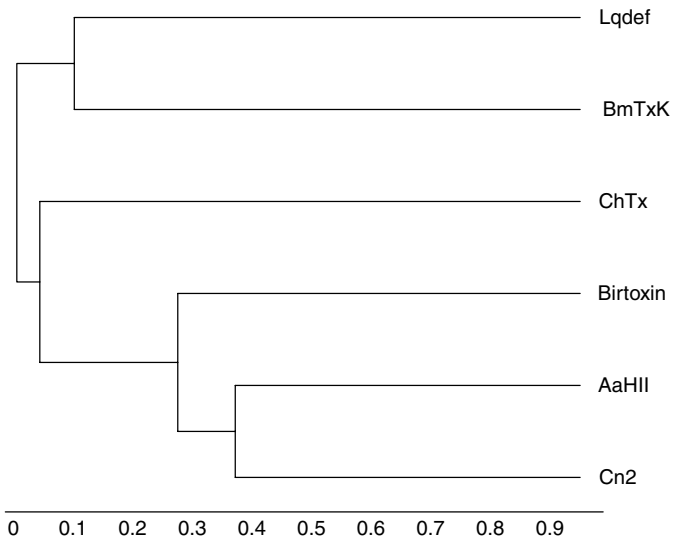

Figure 3 Phylogenetic tree of scorpion defensin and neurotoxins. Protein names are as in Figure 1.

ancestor that gave rise to three-disulfide-bridge sodium channel toxins, e.g. Birtoxin (Figure 3). Furthermore, α- and β-sodium channel neurotoxins, which have four disulfide bridges, probably diverged from the Birtoxin ancestor (Figure 3), as recently suggested [22].

Our findings delineate for the first time the evolutionary trajectory of scorpion defensins and neurotoxins. Since defensins with high homology are found in molluscs and ticks, it is feasible that the gene encoding a three-disulfide-bridge polypeptide was the ancestor from which defensins and potassium channel toxins developed (Figure 3). On the basis of our recent suggestion that defensins have evolved via exon-shuffling [10], it is possible that the short exon encoding the mature defensin or potassium channel toxin integrated at different locations in the genome. Accelerated evolution together with additional flanking sequences around the integration site, led to the generation of the three-disulfide-bridge 'long-chain' potassium (e.g. BmTXK; Figure 3) or sodium channel (e.g. Birtoxin; Figure 3) toxins. Later on, the three-disulfide-bridge 'long-chain' toxins affecting sodium channels gave most probably rise to the four-disulfide-bridge α- and β-toxins (Figure 3).

CONCLUSION

In summary, the isolation of the scorpion defensin gene enables us to suggest common ancestry for scorpion neurotoxins and arthropod defensins based on structure, bioactivity, gene organization and sequence similarity. Furthermore, phylogenetic analysis, which combines scorpion neurotoxins and defensins, sheds new light on the evolution of scorpion 'long-' and 'short-chain' neurotoxins, a task that has been impossible thus far, when only sodium and potassium channel toxins were compared.

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