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Review

Origin of neurotoxins from defensins

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Abstract: There are at least three conserved protein folds shared by ion channel-targeted neurotoxins and antimicrobial defensins, including cysteine-stabilized α-helix and β-sheet fold (CSαβ), inhibitor cystine knot fold (ICK) and β-defensin fold (BDF). Based on a combined data of sequences, structures and functions, it has been proposed that these neurotoxins could originate from related ancient antimicrobial defensins by neofunctionalization. This provides an ideal system to study how a novel function emerged from a conserved structural scaffold during evolution. The elucidation of functional novelty of proteins not only has great significance in evolutionary biology but also will be helpful in guiding rational molecular design. This review describes recent progresses in origin of neurotoxins, focusing on the three conserved protein scaffolds.

Key words: neofunctionalization; cysteine stabilized α-helical and β-sheet fold; inhibitor cystine knot fold; β-defensin fold; ion channel

靶向离子通道的神经毒素起源于抗微生物防御肽

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摘 要：靶向离子通道的神经毒素和抗微生物防御肽(defensin)享有三个共同的蛋白折叠(fold)类型，即半胱氨酸稳定的α-螺旋和β-片层折叠(cysteine-stabilized α-helix and β-sheet, CSαβ)，抑制剂胱氨酸结折叠(inhibitor cystine knot, ICK)和β-防御肽折叠(β-defensin, BDF)。序列、结构和功能等多维证据显示靶向离子通道的神经毒素起源于相关的古老防御肽。毒素和防御肽系统正在成为研究保守结构支架功能新颖化(neofunctionalization)进化机制的理想体系。阐明蛋白质功能新颖化的进化机制不仅有利于回答进化生物学的基本科学问题，而且对于合理的药物设计具有重要的指导意义。本文综合阐述了神经毒素起源于抗微生物防御肽方面的最新研究进展。

关键词：功能新颖化；半胱氨酸稳定的α-螺旋和β-片层折叠；抑制剂胱氨酸结折叠；β-防御肽折叠；离子通道
中图分类号：Q594；Q951；R97

1 Introduction

The understanding of molecular events responsible for functional novelty of proteins has important significance to evolution-guided molecular design. Recognition of such events usually needs a large amount of comparative biochemical and functional data to reconstruct evolutionary processes leading to novel functions. Protein engineering and resurrection of ancient proteins are two popular approaches that have converged to outline structure-functional relationships in shaping the evolutionary process of some enzymes, vision proteins and receptor specificities [1–4]. Reconstructing ancestral proteins from modern molecular

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sequences is a novel and powerful tool in solving evolutionary issues. However, in some cases, its results might be altered when different taxonomic sampling is applied. This makes the outcome less reliable [5].

Studies of enzymes and evolutionarily related non-enzymes through protein engineering have contributed to our understanding of functional innovation of proteins. This model is of obvious advantages because of distinct functional boundaries between enzymes and non-enzymes and the availability of a large number of three-dimensional (3D) structures of enzymes. Despite this, most of these researches focus on the catalytic center of enzymes and thus lead to deficiency in overall vision; and large molecules may not be easily operated.

As an alternative, venom-derived toxins provide an emerging system to study functional novelty of proteins [6]. These toxins are produced by a diversity of venomous animals from various phyla. They are often small in size and contain multiple disulfide bridges for structural stability. These toxic peptides direct toward a variety of molecular targets (e.g. enzymes, receptors and ion channels) [7]. On the basis of phylogenetic analyses, Fry has proposed that snake toxins could have arisen from gene recruitment events, in which an ordinary body protein gene was firstly duplicated, and then the new one was selectively expressed in the venom gland. In addition, some snake toxins could also originate as a result of modifications of existing salivary proteins because snake venom glands have evolved from salivary glands [8]. It is remarkable that most of these snake toxins still retain some activities of the ancestral proteins. Notwithstanding the experimental evidence is lacking, this is the first glimpse on the origin of snake toxins from evolutionarily related body proteins. It was also found that some endogenous toxin-like proteins function in non-venom context. For example, Lynx1 and SLURP-1, two such proteins, can modulate nicotinic acetylcholine receptors (nAChRs), as snake α-neurotoxins [9–11]. Computational approaches also identified some novel families of toxin-like proteins in insects and mammals. For example, OCLP1, a protein expressed in bee brain, shows significant similarity to ion channel toxins from venoms of cone snails and assassin bugs [12]. This peptide could induce a strong yet reversible paralytic effect when injected to fish. A novel mammalian cluster of toxin-like peptides was also characterized to be expressed in the testis and probably involved in regulation of nAChRs to affect the acrosome reaction and sperm motility [12].

The evolutionary link between toxins and related body proteins is further strengthened by the observation that some ion channel-targeted neurotoxins and antimicrobial defensins share the same 3D folds [13,14]. This review describes recent views on evolutionary relationship between toxins and defensins, focusing on three shared structural scaffolds (cysteine-stabilized α-helix and β-sheet fold, CSαβ; inhibitor cystine knot fold, ICK; and β-defensin fold, BDF) (Fig. 1).

2 The CSαβ superfamily

The CSαβ fold contains one α-helix and a two antiparallel-stranded β-sheet stabilized by three intramolecular disulfide bridges. The α-helix containing an invariant motif (CXXXC, X is any amino acid) is connected to the second β-strand with a conserved motif (CXC) by two disulfide bridges (C_2-C_5 and C_3-C_6). The third disulfide bridge (C_1-C_4) joins the N-terminus to the first β-strand. Some CSαβ-type peptides have the fourth disulfide bridge in variable positions [14]. For drosomycin and most of scorpion toxins affecting Na^+ channels, the fourth disulfide bridge connects the N-terminus to C-tail. Peptides with this scaffold possess stable structure that can tolerate insertions, deletions and substitutions and thus become an attractive candidate for engineering design to create novel functions [15]. In addition, this versatile framework is also shared by a variety of peptides with diverse biochemical and biological functions [14].

The CSαβ peptides are the richest in arthropods, represented by scorpions, insects and ticks. They are functionally classified into neurotoxins targeting ion channels, such as K^+, Na^+, Ca^{2+} and Cl^- channels [14], and antimicrobial defensins [16]. Overall, scorpion venom-derived neurotoxins can be further divided into two distinct groups based on their molecular size: the long-chain toxins of approximately 60 residues and four disulfide bridges, which often target Na^+ and Ca^{2+} channels; and the short-chain toxins of approximately 30–40 residues and three to four disulfide bridges, being K^+ and Cl^- channel-specific blockers [17].

Based on a combined data of 3D structure, precursor and genomic organization as well as biological activity, it has been proposed that defensins and neurotoxins with a CSαβ fold could have originated from a common ancestor [18]. Subsequently, it was suggested that a position-specific deletion event could lead to the emer-

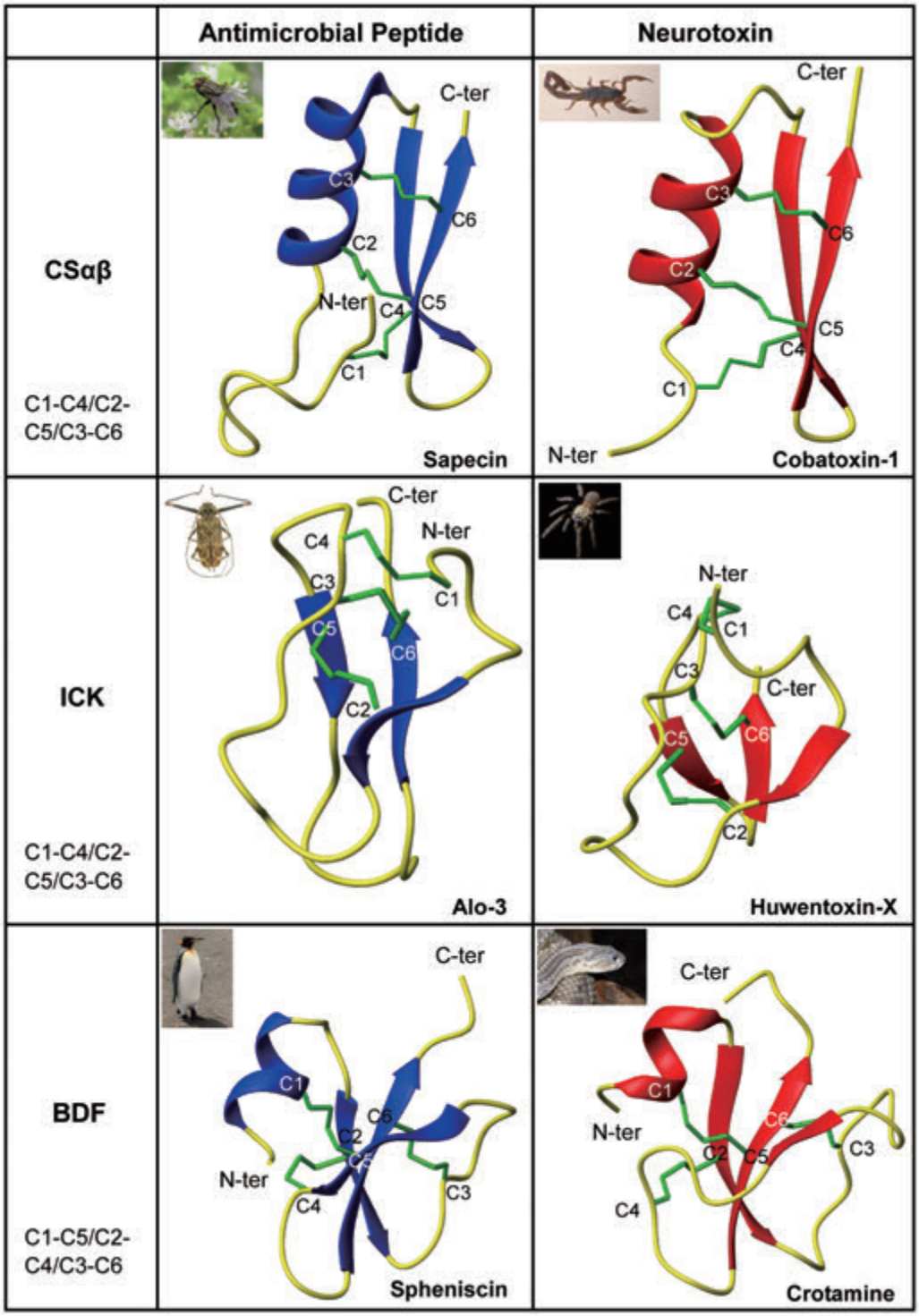

Fig. 1. Three classical fold types shared by ion channel-targeted neurotoxins and antimicrobial defensins. PDB entries for the structures listed here: Sapecin (1L4V); Cobatoxin-1 (1PJV); Alo-3 (1Q3J); Huwentoxin-X (1Y29); Spheniscin (1UT3); Crotamine (1H5O).

gence of short-chain toxins from an ancestral long-chain toxin in the scorpion lineage [19]. However, our recent studies have clearly indicated that these two classes of scorpion toxins could independently evolve from different types of ancestral defensins [6, 14, 20].

Firstly, it was found that the core of some scorpion Na^+ channel toxins shares high sequence similarity to the *Drosophila* CSαβ-type antifungal peptide drosomy-

cin (E-values from 1×10^{-3} to 4×10^{-6}) [20], although in comparison with drosomycin, these toxins possess an extension of 14-residues in the C-tail and an insertion of 5-residues in the N-turn, both comprising a structural subdomain (NC-domain) [14]; Secondly, drosomycin is able to bind to the *Drosophila* Na^+ channels [21]; and thirdly a scorpion Na^+ channel toxin with the NC-domain deleted exhibited a comparable antifungal activity to drosomycin [21]. More importantly, by grafting the NC-domain of a scorpion Na^+ channel toxin onto the drosomycin scaffold to mimic natural evolutionary process, we generated an engineered molecule (drosotoxin) that obtained an inhibitory activity on mammalian Na^+ channels [22]. C-terminal truncation combined with site-directed mutational analyses of drosotoxin highlighted the importance of the N-turn insertion in emergence of the neurotoxicity from the antifungal scaffold [23].

More recently, by using experimental evolution we converted a venomous insect defensin to a classical scorpion K^+ channel toxin (Fig. 2), which provides the first structural and functional evidence for their evolutionary relationship [6]. Scorpion K^+ channel toxins and insect defensins share a conserved 3D structure and related biological activities (defense against competitors or invasive microbes by disrupting their membrane functions). Based on the established scorpion toxin signature (STS), we searched all insect defensins derived from six insect Orders to find those likely containing STS as an evolutionary intermediate for experimental studies. The STS includes six cysteines for three disulfide bridges and two conserved functional residues in the motif (Lys-Cys4-Xaa-Asn) for direct interaction with the K^+ channel pore [6]. From two venomous insect Orders (Hemiptera and Hymenoptera), we found some evolutionary intermediates that could have potential in developing a scorpion K^+ channel toxin. By deleting a conformationally flexible amino-terminal loop (n-loop) of the venomous parasitic wasp *Nasonia vitripennis* defensin to remove the steric hindrance of peptide-channel interaction, we generated a peptide (named navitoxin) with similar structure, pharmacological activity and functional surface to scorpion K^+ channel toxins. This study provides not only new evidence for predictability of toxic origin, but also an approach for studying evolutionary relationship of two distantly related peptide families. They represent two typical examples of divergent evolution where small indel-mediated structural alterations in ancestral structural scaffolds lead to functional novelty of proteins from immunity against microbes to defense against competitors and prey capture [6, 22].

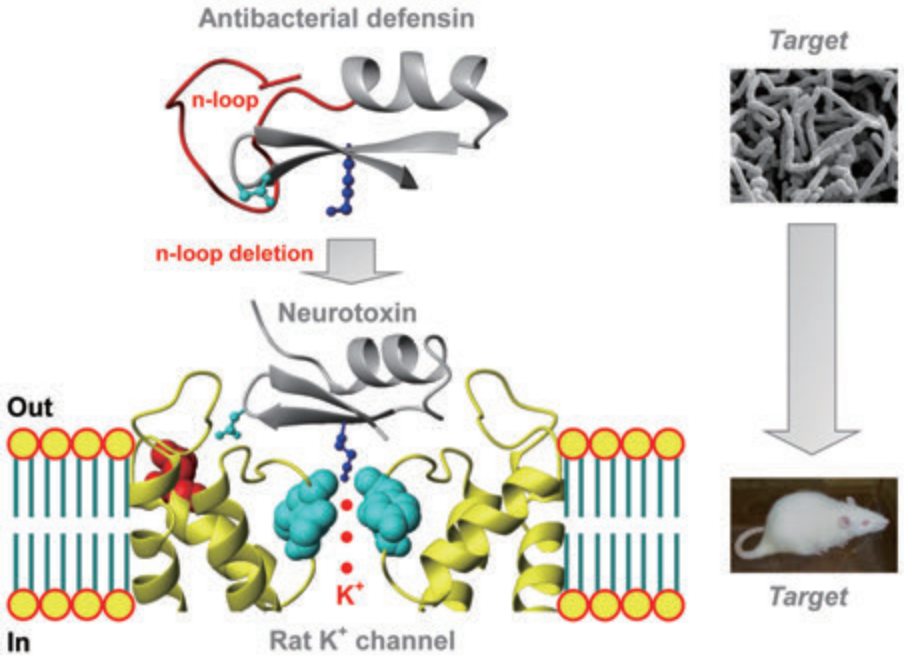

Fig. 2. Experimental conversion of an antibacterial insect defensin into a neurotoxin targeting mammalian K^+ channels. The deletion of n-loop is considered as a key event in functional conversion since it removes the steric hindrance of peptide-channel interactions [6].

3 The ICK superfamily

The ICK fold is another evolutionarily conserved structure shared by neurotoxins and antimicrobial defensins from a myriad of organisms, including plants, fungi, insects and mammals [24]. Most ICK peptides are composed of 30–40 residues with a consensus $C_1X_{[3-7]}C_2X_{[3-6]}C_3X_{[0-5]}C_4X_{[1-4]}C_5X_{[4-13]}C_6$, where X represents any amino acid except cysteine. The third and fourth cysteines are usually contiguous in animal ICK peptides while in most plants ICK peptides there are other residues between these two cysteines [25]. This structural motif consists of an anti-parallel, triple-stranded β-sheet stabilized by a cystine knot where a ring is formed by two disulfide bridges (C_1-C_4 and C_2-C_5) and the interconnecting backbone, and the third disulfide bridge (C_3-C_6) crosses the ring. Because of remarkable structural stability and the tolerance to loop mutations, this class of molecules is a robust scaffold for protein engineering to design peptides for a variety of biomedical targets [26–28].

Although found in animals, plants and fungi, ICK peptides are the richest in venoms of cone snails and spiders as neurotoxins to affect functions of various ion channels [29], such as Na^+, K^+, Ca^{2+}, and transient receptor potential (TRP) channels, indicating that functional diversification has occurred during evolution. By contrast, ICK peptides only represent minor components of scorpion venom as activators of sarcoplasmic reticulum Ca^{2+} release channels/ryanodine receptors (RyRs) of skeletal and cardiac muscles [30]. They target the plasma membrane of cells via their positively-charged residues to interact with negatively-charged lipids and penetrate the membrane [31, 32]. Such a mode action is also observed in many cationic antimicrobial peptides (AMPs) whose cationic charges and structural amphipathicity allow them to attach to and insert into membrane bilayers to form pores for microbial killing [33]. More recently, several scorpion venom ICK toxins were found to affect K^+ channels [34, 35]. Based on multidimensional evidence, it was proposed that K^+-channel blocking activity was firstly developed in its ancient form and then the Ca^{2+} release channel activating activity evolved [35].

Unlike the CSαβ peptides whose evolution has been traced to defensins, the origin of toxic ICK peptides remains unsolved. By analyzing the precursor and genomic organization, 3D structure as well as functional data of ICK peptides from snails, scorpions, plants, fungi and viruses, Zhu *et al.* proposed the concept of common origin for animal-derived ICK peptides while ICK peptides from plants and fungi might be independently evolved from different ancestors. Viral ICK peptides could be gained via ancient horizontal gene transfer (HGT) from arthropods [36]. In addition, more and more ICK peptides were also found in multiple insect species. It is remarkable that these new ICK peptides differ in their gene structure from the animal toxins [35–37], as identified by their phase-0 intron locating at the second cysteine of the mature peptide. Similar to viral ICK peptides, ants' ICK precursors are composed of signal peptide and mature peptide whereas ICK peptides from *N. vitripennis*, scorpions and *Conus* have a propeptide between the signal peptide and mature peptide.

Despite diverse gene structure, precursor organization as well as low sequence similarity hampering us from establishing a clear evolutionary relationship for animal-derived ICK peptides, structural information again provides insights into the origin of venomous ICK peptides from an antimicrobial ancestor. As shown in Fig. 3, a family of antifungal defensins with a typical ICK fold exhibits structural similarity to neurotoxins from cone snails and spiders [38–42]. The peptides are present in several major eukaryotic lineages, such as plants, fungi, horseshoe crabs and insects. In comparison with these toxins, the defensins have a longer loop 4. Since a smaller loop 4 is obviously a prerequisite for κ-PVIIA, an ICK toxin from *Conus purpurascens*, to interact with the K^+ channel pore via the dyad, a functional motif comprising a lysine (K^7) and an aromatic residue (F^9) in a distance of approximately 7 Å (Fig. 3), we assume that the deletion of this loop could remove steric hindrance between peptide-channel interaction to evolve to a neurotoxin, as observed previously in the origin of scorpion K^+ channel toxins [6].

Based on structural data, some authors have proposed that the evolution of ICK neurotoxins might have originated from a simpler structural motif, the disulfide-directed β-hairpin (DDH), through structural elaboration to form one additional disulfide bridge [43]. Indeed, LITX-Lw1, a DDH toxin from scorpion venom, adopts a similar action model to scorpion venom ICK peptide (e.g. maurocalcine) to interact with Ca^{2+} channels [44]. However, the restricted distribution of DDH in Arachnida (spider and scorpion) suggests that DDH could be a derivative of ICK through deletion of two cysteines of the ICK scaffold in the specific evolutionary lineage.

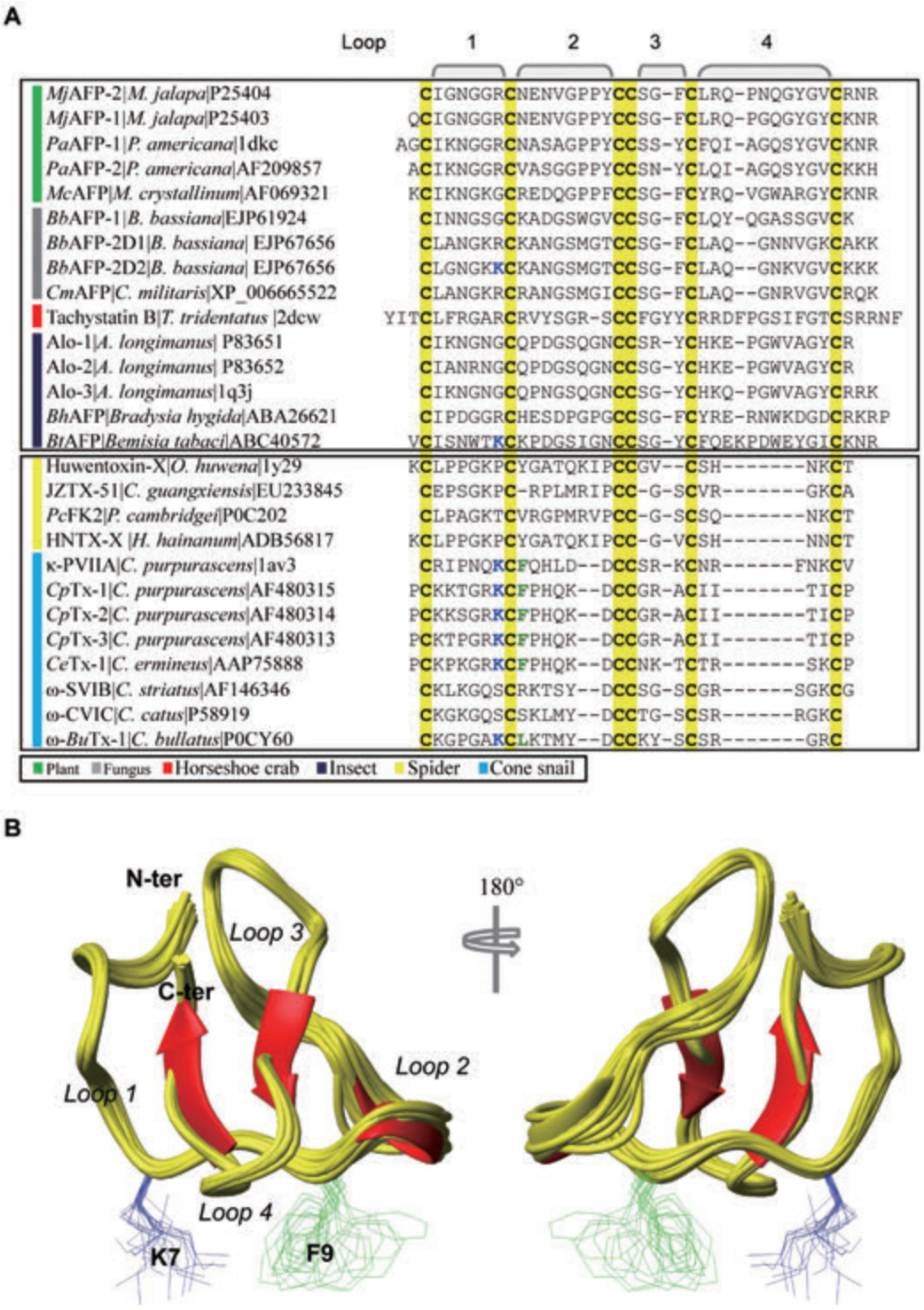

Fig. 3. Comparison of antifungal defensins and neurotoxins with an ICK fold. *A*: Multiple sequence alignment. Identical cysteines are shadowed in yellow and residues belonging to the dyad are shown in color. *B*: The structure of κ-PVIIA (PDB entry 1AV3) showing the position of the functional dyad (Lys^7 and Phe^9) and loop 4 that is obviously shorter than that of the antifungal ICK peptides in Fig. 3*A*.

4 The BDF superfamily

The BDF is also shared by defensins and neurotoxins [45]. Beta-defensins are a group of AMPs primarily found in vertebrates, which possess microbicidal and immune regulatory functions and whose ancestor has been traced to invertebrate big defensins [46]. The BDF toxins

are present in some snake venoms and they function as ion channel modulators [45]. This family of peptides contains about 35–50 amino acids with a distinctive molecular framework characterized by six cysteines paired as C_1-C_5, C_2-C_4, and C_3-C_6. Apart from these structural residues, other sites exhibit large variations among members. Structurally, BDF is generally characterized by the presence of a short α-helix, or a turn, juxtaposed to two or three anti-parallel β-strands and connected by three disulfide bridges [47].

In recent years, the evidence for origin of the BDF-type toxins from ancestral β-defensins is accumulating. Thanks to the genome sequencing project of the platypus, comparative data for the platypus BDF-type toxins and β-defensins are currently available, which allows to evaluate their evolutionary relationship in a specific species [48]. Firstly, β-defensins and BDF-type toxins (*Ov*DLPs, *Ornithorhynchus* venom defensin-like peptides) share similar 3D and gene structure. Secondly, there exists an evolutionary intermediate, DEFB-VL, between β-defensins and *Ov*DLPs. DEFB-VL is unique in that it exhibits more similarities in antibacterial property, 3D-structure characteristics and gene structure to β-defensins, however, its primary sequence is more similar to *Ov*DLPs. This finding suggests an evolutionary potential of DEFB-VL in toxic evolution. Thirdly, human β-defensin-2 (hBD-2) and crotamine, a BDF-type toxin from snake venom, have been suggested to function reciprocally: 1) They both have antimicrobial activity with similar antimicrobial spectra and pH optima; 2) They both have membrane disruptive activities, and the membrane potential alteration is consistent with their ion channel-perturbing activities [49].

5 Evolutionary events of toxic origin

Now it appears to be clear that some venom-derived neurotoxins originated from immune-related proteins. However, one open question is to identify key evolutionary events responsible for such functional switch. In spite of remarkable importance of point mutations in functional diversification of toxins, there are no data showing the same importance of such mutations in toxic origin from a non-toxic scaffold. Insertions and deletions (indels) are another common type of sequence mutations in protein evolution [50]. It has been reported that regulatory loops of serine proteases in shrew and lizard venoms all have small insertions and subsequent accelerated sequence evolution to create new chemical environment and functional changes from proteases to toxins [51]. This review presents data supporting a key role of indels in origin of ion channel-targeted neurotoxins. The discovery of removal of steric hindrance of peptide-channel interaction via small loop deletion inducing a dramatic functional switch of immune-related peptides could be helpful in enlarging the toxin family from non-toxic sources for therapeutic aims.

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