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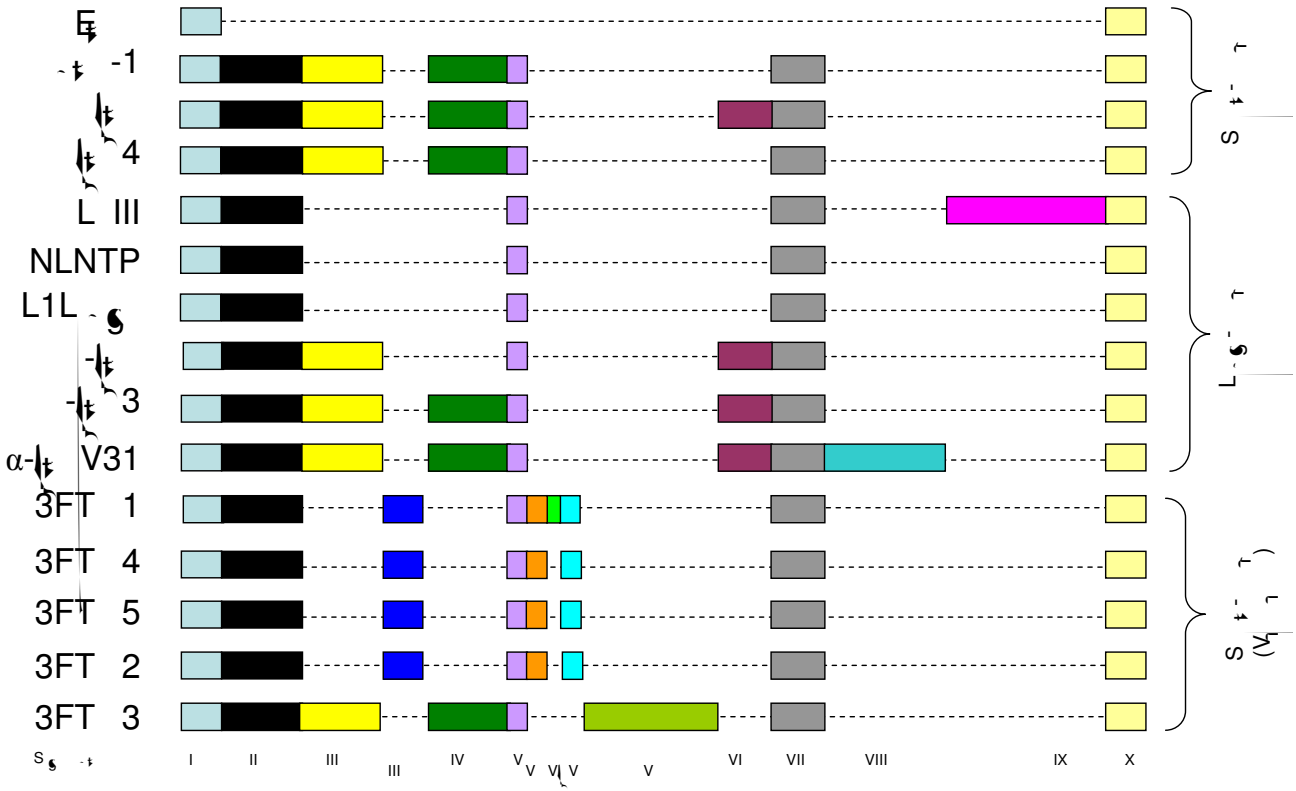
of this family target various receptor/ion channel proteins such as α1-nAChRs, L-type calcium channels and integrin $\alpha_{IIb}\beta_3$ (Table 1). Such a wide diversity in their molecular targets is due to changes in their primary sequences, while keeping the basic molecular scaffold intact. Analysis of amino acid sequences and gene structures will help elucidate the molecular evolution of these functionally important toxins.

As with other snake venom proteins [4-6], three-finger toxins are also encoded by a multigene family [7-10] and contain functionally diversified isoforms. These venom protein families have evolved through a process of gene duplication followed by accelerated point mutations in

3

Phylogenetic relationship of representative Viperidae, Colubridae and Elapidae three-finger toxins. Tree was constructed using the software DNAMAN. The gene sequences used were obtained from GenBank and are represented by their name. alpha btx V31 (Y17693), kappa-btx (Y11768) and kappa-btx3 (Y11769) are from *Bungarus multicinctus*; LsIII (AB098531), NLNTP



Comparison of intron I structure. The gene sequences were obtained from GenBank and are represented by the gene name (and accession number): Etxc (X51410), ntx1 (AF096999), cbtx (Y12492), cbtx4 (Y12493), LsIII (AB098531), NLNTP (AB098532), L1Long (AB098533), κ-btx (Y11768), κ-btx3 (Y11769) and α-btxV31 (Y17693). Source species are as in Fig. 3. 3FTx 1–5 are gene structures of *S. catenatus edwardsii* three-finger toxins from the present study. The nucleotide sequences were aligned using the online software DIALIGN Multiple Sequence Alignments tool at BiBiServ and then were divided into segments. The nucleotide segments are represented with boxes, and gaps are indicated with a dash. The additional segments in 3FTx 1, 4, 5, 2 and 3 are named IIIa and Va-Vd.

thereby maintaining similar disulfide pairing, folding architecture and the overall three-finger fold of the mature protein.

There are several possibilities that could explain the observed switching of segments.

1. Splicing variations: The difference in isoforms of some proteins due to change of segments can be easily explained based on splicing variation [30]. However, unlike these proteins, the segment switching in viperid 3FTx occurs within exon II and exon III. Among three-finger toxins, long-chain neurotoxins arose from short-chain neurotoxins through an error in the splicing site [31]. Such an error leads to insertion of a short segment with the fifth disulfide in the second loop. In viperid toxin genes, however, the insertions of segments do not occur at the intron-exon boundaries, so the mechanism of insertions/deletions or switching of segments does not occur due to errors in splicing.

2. Recombination: Distinct genes encoding isoforms of proteins are also generated through recombination of two related/unrelated genes [32,33]. In general, the segments involved in such recombination events are fairly large (700 to 2500 bp), and the segments that are exchanged in exons of 3FTxs are probably much too small. Therefore, canonical recombination may not be involved in these exchange events.

3. Accumulation of point mutations: Accelerated point mutations in three-finger toxins are common and they lead to the evolution of several isoforms [10,11,14]. Although these point mutations occur in exon segments, they may not explain such a distinct change in the sequences of segments. This possibility requires the

repeated occurrence and accumulation of multiple point mutations within each segment. Further, all the intermediates have to be selected through evolution. The absence of intermediate isoforms, however, contradicts this possibility; and

4. Independent recruitment events: Venom protein genes are thought to be recruited to the venom gland genome by gene duplication of a normal physiologically important gene and recruitment of the duplicated gene for expression in the venom gland [34]. It is possible, but not probable, that each of these isoforms has an independent origin and their ancestral three-finger toxin genes were recruited at different times. High similarity across numerous 3FTx genes in exon I, and introns I and II, supports instead a single recruitment and lineage of these genes and not multiple recruitment events. In the unlikely events of independent recruitments, introns will have to undergo convergent evolution to explain the high similarity while the exons will be undergoing divergent evolution. Therefore, segment switching results in divergence of functional regions of exons (see below) while maintaining the basic fold, rather than convergence upon a single scaffold motif during independent recruitment.

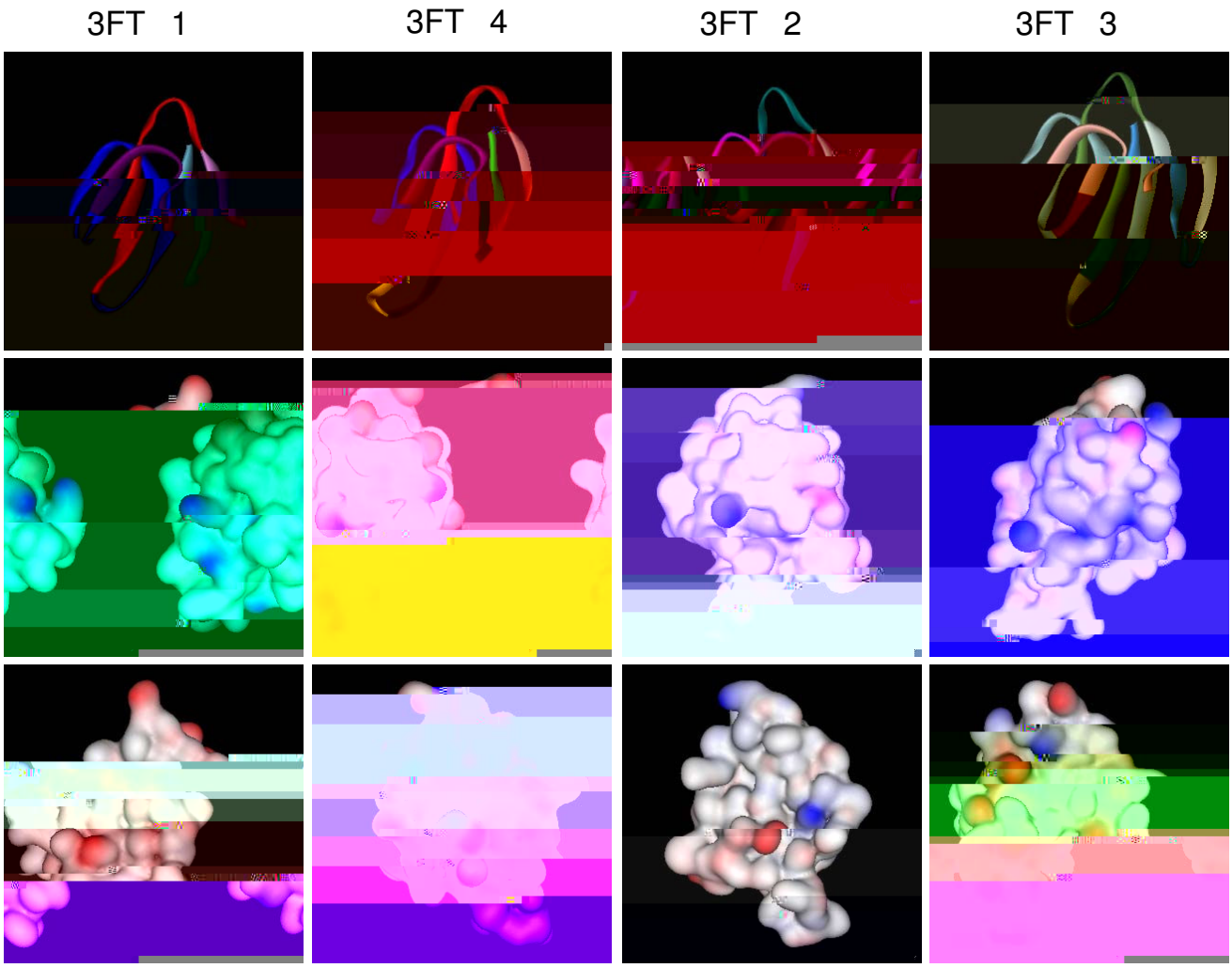
Although the mechanism of the exchange of segments in exons is unknown, it is apparent that these events play important roles in the evolution of these toxins, in addition to the role that accelerated point mutations in the exons plays in toxin evolution [10,11,14]. These point

alter targeting (ASSET) is a phenomenon which plays an important role in "remodeling" a toxin toward a different and novel receptor target (see below).

ASSET and Molecular Surface

To understand the impact of switching of segments on the molecular surfaces of three-finger toxins in [illegible], we modeled all four distinct three-finger toxins. As shown in Figure 7, the three β-sheeted loops in these toxins are distinctly different from one another, as most of them are replaced by segment switching (Figure 7, top row). Further, the electrostatic potentials of these toxins indicate that the charge distributions on their molecular surfaces are also different. 3FTx 1 and 4 have more acidic residues on the surface as compared to 3FTx 2 and 3 (Figure 7, middle and bottom rows). This drastic difference in the charge residues on the surface is due to the exchange of segments (see Additional file 1), but retention of the similar molecular fold. Such a change in the charged residues might play an important role in switching the molecular targets. Since most of the functional sites are located on these β-sheeted loops (for a review see, [36]), it is logical to propose that all of these novel viperid toxins have distinct pharmacological properties. Therefore, ASSET phenomena affect the molecular surfaces of three-finger toxins significantly and alter their molecular targets, playing a crucial role in the evolution of the three-finger toxins.

Systematic analyses of gene sequences of [illegible] (Desert Massasauga) three-finger toxins indicate that short segments in exons II and III are changed more rapidly compared to intron segments. We propose that such a phenomenon (ASSET) of accelerated segment switching in exons has the effect of rapidly altering the molecular surface properties. This mechanism of rapid change can provide a selective advantage to venomous snakes in predator/prey coevolutionary arms races, resulting in a diversity of structurally similar toxins in a single venom and allowing the venom toxins repertoire to stay a step ahead of prey defensive responses [1,17]. Thus ASSET



7

Three-dimensional models of three-finger toxins from *S. catenatus edwardsii* venom gland transcriptome. Top row shows the solid ribbon models. Segments are color coded as in Figure 1. Middle and bottom (180° rotation) rows show the electrostatic potential of both the surface. The positively and negatively charged residues are shown in blue and red colors, respectively, and the hydrophobic residues are shown in white color.

ABI PRISM® BigDye® terminator cycle sequencing ready reaction kit was purchased from Perkin Elmer (Foster City, CA, USA). Oligonucleotides were custom synthesized by 1st BASE (Singapore). All other chemicals and reagents used were of the purest grade available.

Ge c DNA e ac

Genomic DNA (gDNA) was extracted from the . liver tissue (30 mg, previously stored in RNAlater) using the DNeasy Tissue kit (Qiagen, USA) according to the manufacturer's instructions. RNaseH was used during the extraction to remove contaminating RNA. The integrity of the isolated gDNA was confirmed by 0.8% agarose gel electrophoresis and DNA was quantified spectrophotometrically.

DNA PCR

gDNA PCR was performed to obtained the gene sequence of 3FTx 3 using a gene-specific forward primer 5'-ATGAAAACTCTGCTGTTGATCCTGGGGGT-3' and gene-specific reverse primer 5'-GCCAATAGTCACTTTTAGAAC-TATTTGTTGCAGTTGTCTG-3'. The PCR reaction contained 1.0 unit of Long PCR Enzyme Mix, 1 μg of gDNA. 0.2 mM dNTP, 0.2 μM primers and 1 × Long enzyme mix polymerase buffer in a total of 50 μL. The thermal cycling reaction involved 35 cycles of one step each at 95°C for 15

s, 60°C for 15 s, 68°C for 3 min followed by a final extension step at 68°C for 10 min. The amplified PCR products were extracted and cloned as mentioned below.

C .c Ge eWa b a e

The GenomeWalking libraries were constructed using the Universal GenomeWalker™ kit (Clontech Laboratories Inc, USA) according to the manufacturer's instructions. Briefly, libraries were constructed with 3 μg of gDNA restriction digested with DraI, EcoRV, PvuII and StuI. The 'genome walk' involved two sets of primers: adaptor primer 1 (AP1-sense) 5'-GTAATACGACTCACTATAG-GGC-3' and nested PCR adaptor primer 2 (AP2-sense) 5'-ACTATAGGGCACGCGTGGT-3', both provided in the kit, and 25-mer and 27-mer gene-specific primers designed from the signal peptide regions of cDNAs of all the three-finger toxins. Primary and nested PCRs were performed as recommended by the manufacturer (BD Genome-Walker™) using Advantage Polymerase 2 Mix obtained from Clontech Laboratories Inc (Palo Alto, CA, USA). The 50.0 μl reaction mixture consisted of 1 μl of DNA template (0.1 μg) (either from each library or from primary PCR products), 1× PCR buffer (provided in the kit), 0.2 mM dNTPs, 0.2 μM appropriate adaptor primers, 0.2 μM of appropriate gene-specific primers, 1× Advantage™ 2 polymerase mix. The thermal cycling profile used was as follows: 7 cycles of 94°C for 2 s, 72°C for 3 min; 32 cycles

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