Evidence for a new class of scorpion toxins active against K^+ channels

Christian Legros, Brigitte Céard, Pierre E. Bougis, Marie-France Martin-Eauclaire*

Laboratoire de Biochimie, Unité Mixte de Recherche 6560 du Centre National de la Recherche Scientifique, Institut Fédératif de Recherche Jean Roche, Université de la Méditerranée, Faculté de Médecine Secteur Nord, 15 Bd Pierre Dramard, F-13916 Marseille Cedex 20, France

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Abstract cDNAs encoding novel long-chain scorpion toxins (64 amino acid residues, including only six cysteines) were isolated from cDNA libraries produced from the venom glands of the scorpions *Androctonus australis* from Old World and *Tityus serrulatus* from New World. The encoded peptides were very similar to a recently identified toxin from *T. serrulatus*, which is active against the voltage-sensitive 'delayed-rectifier' potassium channel, but they were completely different from the long-chain and short-chain scorpion toxins already characterised. However, there was some sequence similarity (42%) between these new toxins, Aa TX Kβ and Ts TX Kβ, and scorpion defensins purified from the hemolymph of *Buthidae* scorpions *Leiurus quinquestriatus* and *A. australis*. Thus, according to a multiple sequence alignment using CLUSTAL, these new toxins seem to be related to the scorpion defensins.

Key words: Scorpion; Toxin cDNA; Potassium channel; Defensin

1. Introduction

Scorpion venoms are mixtures of many polypeptide components, of which only a small proportion are lethal to mammals. They are long-chain toxins containing 60 to 70 amino acid residues and four disulphide bridges [1]. Their targets are the voltage-sensitive Na^+ channels of excitable nerve and muscle cells and they can be classified into α-toxins, which slow down Na^+ channel inactivation [2], and β-toxins, which affect the channel activation process [3]. Other long-chain toxins are specifically lethal to either insects or crustaceans [4]. Short-chain toxins have also been found using an electrophysiological approach [5]. They act by blocking various voltage-dependent or Ca^{2+}-activated K^+ channels. These toxins contain 31 to 38 amino acid residues and three or four disulphide bridges [6–29]. The two classes of toxins share a common structural framework consisting of a double-stranded antiparallel β-sheet linked to a short α-helix. This basic configuration including disulphide bridges is also found in the defensins, produced by insects in response to body injury and to prevent bacterial infection [30], and in plant γ-thionins [31].

A new scorpion toxin has been previously described, which is active against a voltage-gated non-inactivating K^+ channel, but its full amino acid sequence has not yet been determined [19,32]. It was purified from the South American scorpion *Tityus serrulatus* and it is called tityustoxin Kβ (Ts TX Kβ). Herein, a cDNA encoding Ts TX Kβ was amplified by PCR and sequenced from a venom gland cDNA library of the Brazilian scorpion *T. serrulatus*. Then, an analogue of Ts TX Kβ was also characterised from a venom gland cDNA library of the North African scorpion *Androctonus australis*. The original primary structures depicted show an unusual feature since these 'long-chain' scorpion toxins (8 kDa instead of 3.5 to 4.5 kDa for the other scorpion toxins active on K^+ channels) are cross-linked by only three disulphide bridges.

2. Materials and methods

2.1. Materials

The following reagents were obtained from the indicated sources: restriction enzymes from Bethesda Research Laboratories and New England Biolabs; standard molecular biology reagents from Sigma and Prolabo; K12 MC1061 and JM 109 *E. coli* strains from Pharmacia Biotech.; Sequenase from Amersham; agaroses from FMC; dNTP from Perkin Elmer; Bio*Taq* from Eurobio; Gene Clean kit from Bio 101; Wizard PCR Prep DNA Purification System from Promega.

2.2. Construction of the cDNA library

Standard recombinant DNA techniques were used [33]. The scorpions of the species *T. serrulatus*, collected near Santa Barbara, were a gift from Dr. C.R. Diniz, Instituto E. Diaz, Belo-Horizonte, M.G Brazil. The construction of the λgt 10 cDNA library from telsons has already been described in detail [34]. Five μg of mRNA was purified on an oligo (dT)-cellulose column (Clontech Laboratories) after extraction from the venom gland by the guanidinium hot phenol method [33]. The mRNA was inserted into *Eco*RI-cut λgt 10 (Promega). The library contained 2.5×10^6 independent phage clones. The Okayama-Berg cDNA library from telsons of *A. australis* scorpions was constructed as previously described [35].

2.3. Polymerase chain reaction (PCR)

PCR was used as described [16] to screen the two libraries. Template cDNA was purified on a Tip 100 Quiagen column from an overnight 50 ml culture seeded with 1 ml of the library. Oligonucleotide primers were synthesised using an Applied Biosystems model 391 DNA synthesiser and purified by ethanol precipitation. PCR products were subjected to electrophoresis in a 3% Nusieve GTG/1% Seaplaque agarose gel in TAE buffer and were purified using the Wizard PCR Prep DNA Purification System. We obtained the 5′ region of the Ts TX Kβ cDNA by PCR using a degenerate reverse primer (degenerate primer 1) 5′-CC(AG)AA(CT)TG(ACGT)GT(TC)TT(ACGT)GC(ACGT)AC(TC)TT-3′, corresponding to the residues Lys-21 to Gly-28 of N-terminal amino acid sequence of Ts TX Kβ [19], and the forward primer 5′-GAATTGGTCCGA CTTGAACGAGTT TTC-3′ on the λgt 10 phage. The PCR product obtained was sequenced and a second PCR was performed using the forward primer (primer 2) 5′-AAG TTG GTG GCT TTA ATT CC-3′ corresponding to residues Lys-1 to Pro-7 and the reverse primer 5′-ATGGGACCTTCTTTATGAGTATTCGGTGGAG-3′ on the λgt 10 phage. We obtained the 5′ part of the Aa TX Kβ cDNA, by PCR with the forward primer 5′-GCTCTAAAAGCTGCTGCAGGG-3′ on the Okayama-Berg plasmid and the degenerate reverse primer (degenerate primer 3) 5′-(AG)TC(CT)TT(ACGT)GC(AG)CA(CT)TT(AG)CA(CT)TT-3′, the sequence of which was based on the amino acid sequence of the Ts TX Kβ precursor (residues Lys-54 to Asp-60; this work). This PCR product (PCR 3) obtained was directly sequenced and two new PCR (PCR 4 and 5) were carried out with the following sets of homologous primers: (i) forward 5′-GCTCTAAAAGCTGCTGCAGGG-3′ on the

*Corresponding author. Fax: +33 4 91 69 88 39.

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Okayama-Berg and reverse (primer 4) 5′-AAATCCGTGACAAAAACCTTCTTC-3′ on the Aa TX Kβ insert 3′ end; (ii) forward (primer 5) 5′-ACAAATGCTGTTCCAGTAGGTACT-3′ on the Aa TX Kβ insert 5′ end and reverse 5′-CTGGATCCCTCGAGGCTGGTTCTTT-3′ on the Okayama-Berg plasmid. PCR was carried out as follows: total volume 50 μl, 1 min denaturation at 94°C, 1 min 30 annealing at 60°C, 2 min 30 elongation at 72°C, 40 cycles. The PCR fragments were directly sequenced. A final PCR (PCR 6) was performed, using the library as template and homologous primers corresponding to the 5′ and 3′ non-coding regions obtained by direct sequencing: (i) forward (primer 6) 5′-CATTTACAGCTGGTTAACAGTCAACATGC-3′; (ii) reverse (primer 7) 5′-GTAATTGAAATTAAAATCCCAACCACTCGC-3′. The amplified product was inserted into pBluescript SK^+ and sequenced.

2.4. DNA sequence analysis

For direct sequencing of the PCR products, DNA was denaturated at 100°C for 3 min and rapidly cooled in liquid N_2, then sequenced with Sequenase, adding 1 μl DMSO to the first 10 μl reaction mixture and diluting the labelling mix in DMSO. When required, the PCR products were inserted into pBluescript SK^+ using *E. coli* XL1-Blue for plasmid propagation. The ends of the PCR products were blunted with dNTPs, using the Klenow fragment. The PCR products were then inserted into the *Pst*I and *Eco*RV restriction sites of pBluescript SK^+, and were sequenced [36] with Sequenase and the 17-base universal M13 primer.

2.5. Preparation of scorpion genomic DNA

Scorpion genomic DNA was purified from the muscle tissue of the *A. australis* scorpion (three animals from Beni-Khedache and one from Tozeur were used separately) as previously described [33,35].

2.6. Sequence analysis

Sequence analysis was carried out with PC/GENE and sequence alignments with BISANCE [37]. CLUSTAL W (1.7) was used to generate multiple sequence alignment. First, the four Def sequences were aligned, then the profile obtained used as input to the alignment of the other sequences. The following parameters were used: for pairwise alignments, a gap open penalty (gop) of 10, a gap extension penalty (gep) of 0.1; for multiple alignments, in the case of Def sequences, a gop of 1 and a gep of 0; and in the case of the other sequences, a gap of 1 and a gep of 0.05; protein weight matrix BLOSUM30. The phylogeny inference package (PHYLIP, version 3.5 C), was used to generate a distance matrix data using PROTDIST and Dayhoff PAM matrix; FITCH and DRAWTREE were used on the distance matrix data to generate an unrooted tree, the input order of species was randomised 30 times and the power was set to 2.

2.7. Mass spectrometry analysis

On-line Liquid-Chromatography ElectroSpray Mass Spectrometry (LC-ES-MS) analysis of toxic fraction from *T. serrulatus* venom was performed by Atheris Laboratories, Geneva, Switzerland.

2.8. Purification of Ts TX Kβ and determination of its N-terminal sequence

After gel filtration through Sephadex G-50, the toxic fraction was loaded onto a Merck 4×250 mm analytical high pressure liquid chromatography (HPLC) column (Lichrospher, 5 μm, 100 RP-18), as already described [11]. Automatic sequencing was as described [11].

3. Results and discussion

3.1. Sequences of cDNAs

The PCR strategy used to obtain the oligonucleotide sequences of Ts TX Kβ and Aa TX Kβ is described in Fig. 1. The first PCR amplified a 250 bp fragment from the *T. serrulatus* cDNA library. This was inserted between the *Sma*I and *Eco*RI sites of pBluescript SK^+. Seven clones were sequenced. The PCR insert encompassed the N-terminal part of the mature Ts TX Kβ, including part of a putative signal peptide (amino acid residues −25 to +28). The fragment amplified by the second PCR (315 bp) was inserted between the *Eco*RV

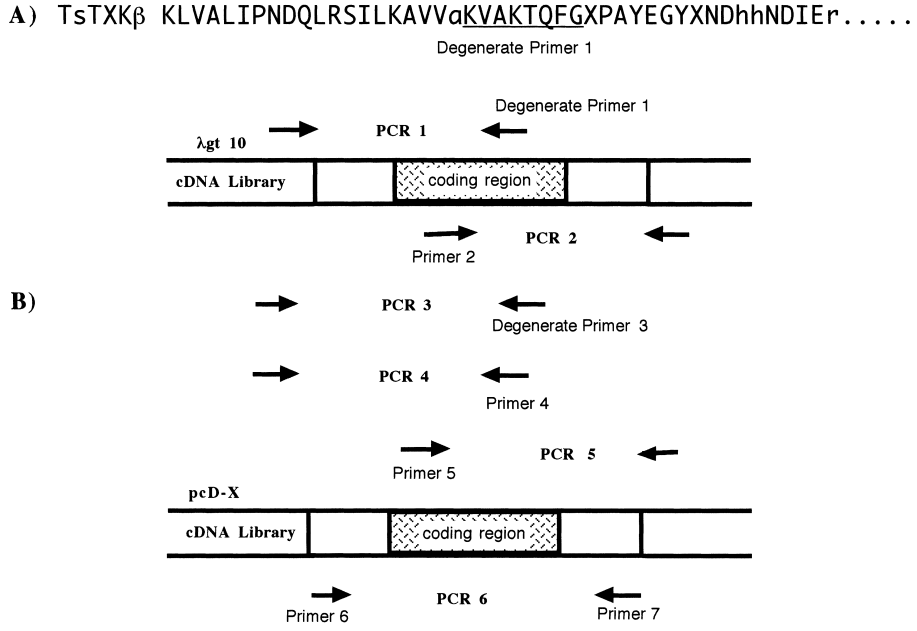

Fig. 1. Strategy used to obtain the Ts TX Kβ and Aa TX Kβ cDNAs. A: PCR amplification of Ts TX Kβ cDNA. The arrows indicate the direction of PCR amplification. The degenerate primer 1 is based on the partial Ts TX Kβ amino acid sequence (first 45 amino acids) obtained by Edman degradation [19] and is underlined above. X, unidentified amino acid; lowercase, confirmation required. Primer 2 is based on the nucleotide sequence of the Ts TX Kβ cDNA determined by PCR 1. The cDNA was inserted into λgt 10. B: PCR amplification of Aa TX Kβ cDNA. The arrows indicate the direction of PCR amplification. Degenerate primer 3 corresponds to residues Lys-54 to Asp-60 of the Ts TX Kβ cDNA as determined in A. Primers 4 and 5 are based on the oligonucleotide sequence of the fragment of the Aa TX Kβ cDNA obtained by PCR 3. Primers 6 and 7, used to amplify the complete cDNA, are based on the nucleotide sequence of the 5′ and 3′ non-coding regions of the Aa TX Kβ cDNA, as determined by PCR 4 and 5. The cDNA was inserted into the Okayama-Berg plasmid pcD-X. See Section 2 for more details.

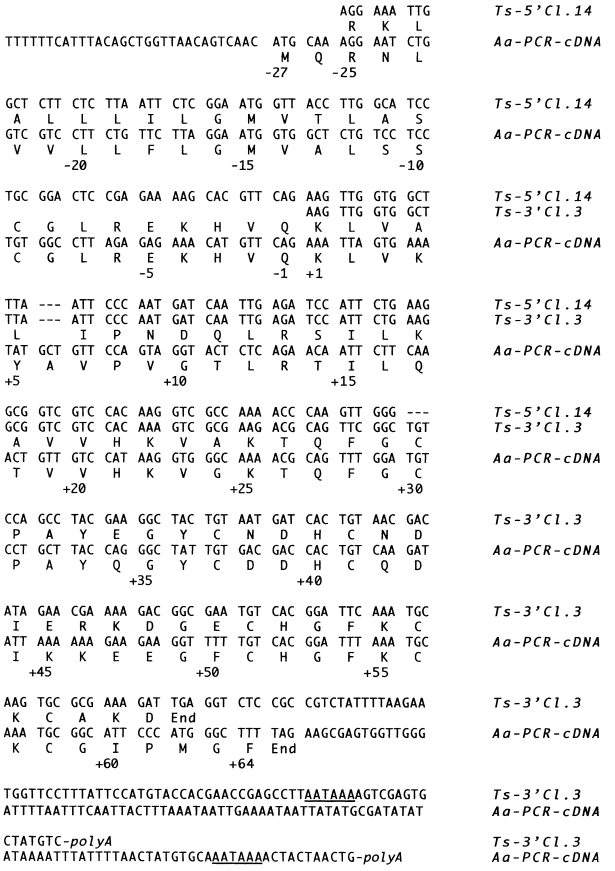

Fig. 2. Nucleotide sequences of cDNAs and predicted protein sequences of Ts TX Kβ and Aa TX Kβ: Ts-5′Cl.14, sequence of the 5′ part of clone 14 of the Ts TX Kβ cDNA of *Tityus serrulatus* obtained by PCR 1 (see Fig. 1A); Ts-3′Cl.3, sequence of the 3′ part of clone 3 of the Ts TX Kβ cDNA of *Tityus serrulatus* obtained by PCR 2 (see Fig. 1A); Aa-PCR cDNA, full sequence of the Aa Ts Kβ cDNA (four clones sequenced) obtained by PCR 6 (see Fig. 1B); sequences are aligned for maximum identity. The putative poly(A) signal AATAAA is underlined.

and *Not*I restriction sites of pBluescript SK$^+$. Four clones were analysed. These fragments together comprised a gene encoding an 85 amino acids long Ts TX Kβ precursor (Fig. 2). Even in the longest insert analysed, no in-frame Met initiation codon was found, probably due to incomplete elongation during the cDNA synthesis by reverse transcriptase. The deduced N-terminal amino acid sequence of the mature Ts TX Kβ was completely consistent with the N-terminal sequence previously obtained by Edman degradation [19]. The complete sequence was 60 amino acids long and was very different from those of the 60 to 70 residue toxins active against Na$^+$ channels or those of the 30 to 40 residue toxins active against K$^+$ channels. Only the positions of the cysteines were similar, suggesting a common disulphide bridge pattern. The first Cys was at position +29, which is unusual for scorpion toxins. The C-terminal residue should be not amidated because no Gly was found before the stop codon [33,34]. We checked whether this molecule was the first member of a new class of toxins by looking for a possible analogue of Ts TX Kβ in a cDNA library made from the *A. australis* venom gland. No primary structure information was available in this case. However, the Aa TX Kβ cDNA was amplified by PCR as described in Section 2 and in Fig. 1. The PCR fragments obtained were directly sequenced. Then the nucleotide sequence data were used to design primers 6 and 7 for the amplification of the total cDNA encoding Aa TX Kβ. PCR 6 products were subcloned into pBluescript SK$^+$ and sequenced (Fig. 2). There were 27 amino acid residues between the first Met initiation codon and the N-terminal part of the mature Aa TX Kβ protein, as determined by comparison with the sequence of Ts TX Kβ [19]. The mature toxin should be composed of 64 amino acid residues, cross-linked by six cysteine residues, with no Gly for amidation at the C-terminus. PC/GENE predicted that the precursor would have a putative signal peptide, different from those of the precursors of other scorpion toxins active against Na$^+$ or K$^+$ channels [34]. This putative peptide of 19 residues would be followed by a propeptide of eight residues. The possible existence of a short propeptide in the Ts TX Kβ and Aa TX Kβ precursors emphasises the similarity of these molecules to insect defensins, which have a propeptide in their precursor. Moreover, preliminary data from the PCR amplification of the Aa TX Kβ gene from genomic DNA of four different animals suggested that there are no introns present, based on the sizes of the amplified fragments (data not shown). The insect defensin genes are intronless [38].

On the 3′ end, a putative polyadenylation site was identified 11 to 16 bp before the poly(A) track (not shown). The nucleotide sequences of Ts TX Kβ and Aa TX Kβ were 66% identical.

3.2. Biochemical characterisation of Ts TX Kβ

On-line LC-ES-MS analysis of *T. serrulatus* venom (to be published) was efficiently used to detect Ts TX Kβ on the basis of its atomic mass. A polypeptide with an experimental mass of 6716.15 Da was detected. This was consistent with the amino acid sequence predicted from the cDNA encoding Ts TX Kβ, which gives a theoretical mass of 6716.76 Da. The N-terminal sequence (eight amino acid residues) of this polypeptide, obtained by Edman degradation, was the same as that of Ts TX Kβ already described [19].

3.3. Amino acid sequence analysis

The amino acid sequences of these two new toxins were compared to each other (61% similarity between Ts TX Kβ and Aa TX Kβ) and with those of other scorpion toxins able to block various types of K$^+$ channels [6–29], defensins purified from the hemolymph of the *Buthidae* scorpions *Leiurus quinquestriatus* or *A. australis* [39,40] and of two insect orders (Diptera and Odonata) [41]. Surprisingly, these two new toxins have some segments similar to those of scorpion defensins. Sixteen residues could be aligned between these defensins and the 38 residues of the C-terminal part of the Aa TX Kβ, giving 42% identity (Fig. 3A). Phylogeny clustered the sequences into three groups: (i) the toxins of about 4.5 kDa in size, blockers of various voltage-activated K$^+$ channels and of the high conductance Ca^{2+}-activated K$^+$ channels; (ii) the toxins <4 kDa in size, specific blockers of the apamine-sensitive Ca^{2+}-activated K$^+$ channels; (iii) Ts TX Kβ, Aa TX Kβ and the scorpion defensins, related to insect defensins [41]. There were no apparent differences between toxins of different geographical origins (Old versus New World). So, the phylog-

A)

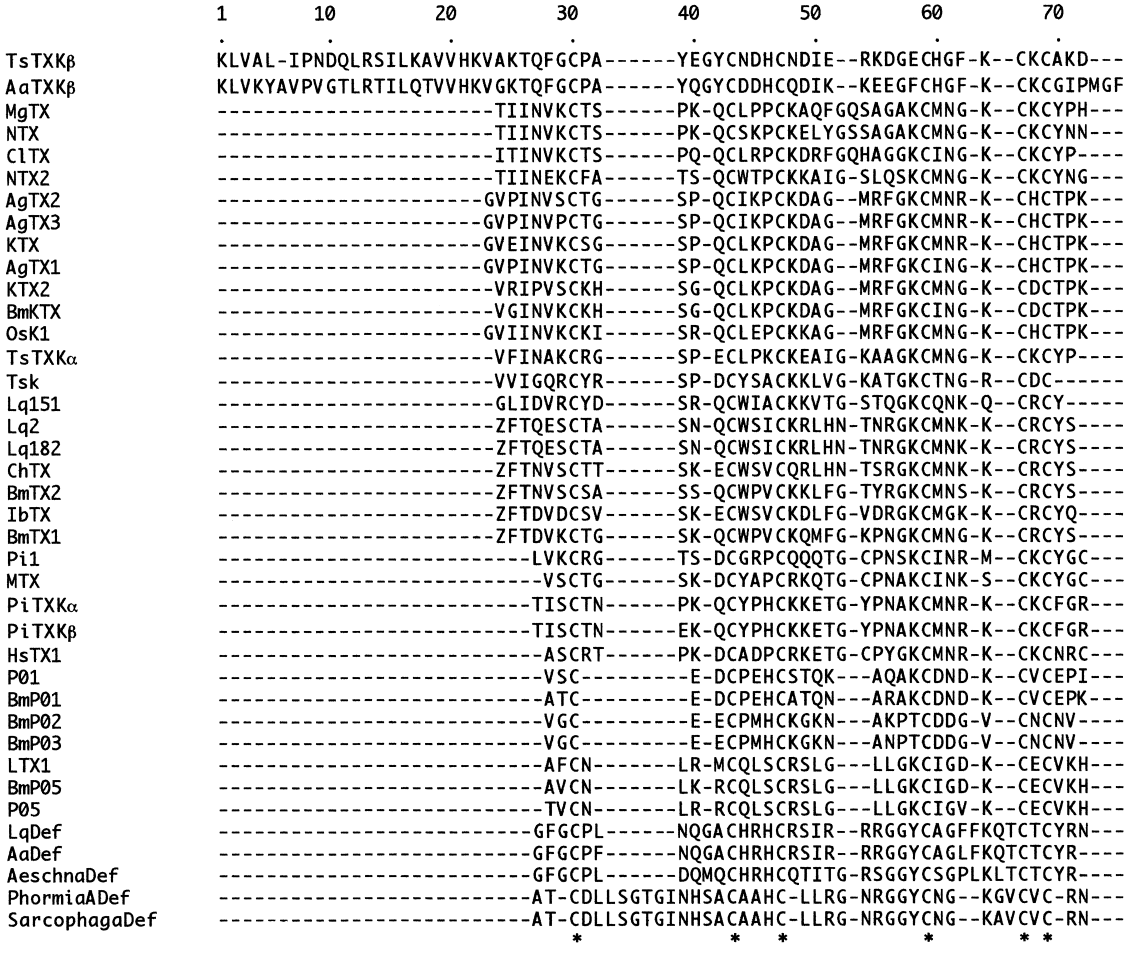

Fig. 3. Comparison of the amino acid sequences of short-chain toxins active against various types of K^+ channel [6–29], the Ts TX Kβ and Aa TX Kβ toxins (this work), the scorpion defensins from *Leiurus quinquestriatus* [39] and *Androctonus australis* [40] and defensins from two insect orders (Diptera and Odonata) [41]. A: Multiple sequence alignment. Ligands of the apamine binding site and their analogues: LTX1, Leiurotoxin 1 (ScTX, Scyllatoxin) from *Leiurus quinquestriatus hebraeus* [6,7]; P05 and P01 from *Androctonus mauretanicus mauretanicus* [8,9]; BmP01, BmP02, BmP03, BmP05 from *Buthus martenzi* [10]; Ts κ from *Tityus serrulatus* [11]. Short toxins cross-linked by four disulphide bridges: Pi 1 from *Pandinus imperator* [12]; MTX, Maurotoxin from *Scorpio maurus palmatus* [13]; HsTX1 from *Heterometrus spinnifer* [14]. Ligands of others types of potassium channels: KTX, Kaliotoxin from *Androctonus mauretanicus mauretanicus* [15]; KTX2, Kaliotoxin 2 from *Androctonus australis* [16]; BmKTX, BmTX1 and BmTX2, Kaliotoxin and toxins 1 and 2 from *Buthus martenzi* [17]; AgTX1, AgTX2, AgTX3, Agitoxin 1, 2 and 3 from *Leiurus quinquestriatus hebraeus* [18]; Ts TX-Kα from *Tityus serrulatus* [19]; MgTX, Margatoxin from *Centruroides margaritus* [20]; NTX, Noxiustoxin from *Centruroides noxius* [21]; NTX2, Noxiustoxin 2 from *Centruroides noxius* [22]; PiTX-Kα and PiTX-Kβ from *Pandinus imperator* [23]; ClTX from *Centruroides limpidus limpidus* [24]; IbTX, Iberiotoxin from *Buthus tamulus* [25]; ChTX, Charybdotoxin, Lq2 and Lq18-2 or Lq15-1 from *Leiurus quinquestriatus hebraeus*, respectively [26–28]; OsK-1 from *Orthochirus scrobiculosus* [29]. Ts TX Kβ and Aa TX Kβ (this work). Lq Def and Aa Def, sequences of the scorpion defensins from *Leiurus quinquestriatus* and from *Androctonus australis* respectively [39,40]. Aeschna Def, PhormiaA Def, Sarcophaga Def, insect defensins from Aeschna (Odonata), Phormia and Sarcophaga (Diptera) [41]. Z, pyroglutamic acid. B: Phylogeny. Input distance matrix data correspond to sequence alignment of A.

eny analysis shows that Ts TX Kβ, Aa TX Kβ and insect defensins belong to a monophyletic group (Fig. 3B).

Taking into account the sequence alignment of the six cysteines as reference and because these residues are consensual between the scorpion toxins and insect defensins, it may be speculated that the conformation of the residues 24 to 61 core of Aa TX Kβ is the same as for charybdotoxin, with an α-helix (two-and-an-half turns) and a triple-stranded anti-parallel β-sheet [30]. Therefore, the scorpion toxin core might be perfectly conserved in Aa TX Kβ. The new sequence data suggest that scorpions produce toxin homologues in their venoms, which are structurally diverse due to their N- and C-terminal extensions protruding from a common structural core. Toxins are mostly minor venom components, so the question arises as to how such structural diversity is of positive evolutionary value? The Ts TX Kβ and Aa TX Kβ are the first scorpion toxins described with more than 60 amino acid residues but only three disulphide bridges. Are they a link between the long-chain toxins active against voltage-sensitive Na^+ channels and the short-chain scorpion toxins active against K^+ channels? A way of evolution from three to four disulphide bridges and, thus, from one specificity to another? One thing seems clear to us: these two new toxins have sequences very similar to those of scorpion defensins, supporting the idea that scorpion toxins and defensins may be derived from a common ancestral molecule.

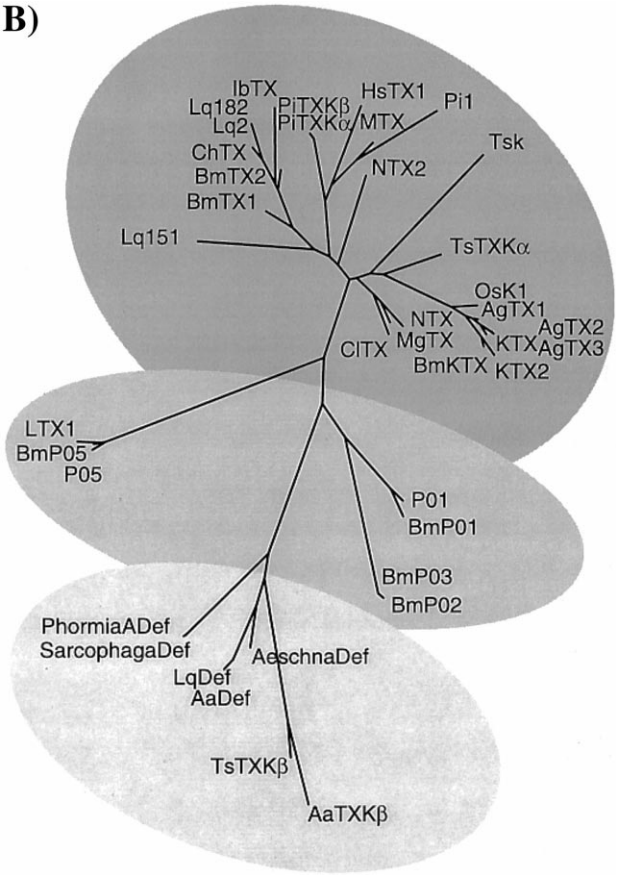

Fig. 3. (*continued*)

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