Genomic organization of three novel toxins from the scorpion *Buthus martensi* Karsch that are active on potassium channels

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The cDNA and genomic DNA of three novel toxins from the scorpion *Buthus martensi* Karsch that are active on K⁺ channels, designated BmKTX (where KTX is kaliotoxin), BmTX1 and BmTX2, were cloned and sequenced. On the basis of their known amino acid sequences, gene-specific primers for 3' and 5' rapid amplification of cDNA ends (RACE) were designed and synthesized. By overlapping the two partial cDNA sequences obtained by 3' and 5' RACE, their full-length cDNA sequences were completed. BmKTX encodes a signal peptide of 22 amino acid residues and a mature toxin of 38 residues, whereas BmTX1 and BmTX2 encode signal peptides of 20 and 21 residues respectively and a mature toxin of 38 residues for each. Their cDNA-deduced amino acid sequences were totally consistent with those determined except that the C-terminus of BmKTX had an additional Gly residue, which was removed

during post-translational processing and was indispensable for the amidation of its C-terminal Lys residue. In addition, the first deduced amino acid for both BmTX1 and BmTX2 is Gln instead of pyro-Glu in the reported toxins, which obviously also undergoes post-translational processing. The genomic DNA species of these three toxins were also amplified by PCR, then cloned and sequenced. They all consisted of two exons disrupted by a small single intron. All of these introns were inserted within the signal peptides at position –6 for BmKTX and at position –5 for both BmTX1 and BmTX2 upstream of the mature toxins, and consisted of 87, 87 and 80 bp respectively.

Key words: nucleotide sequence, post-translational processing, rapid amplification of cDNA ends (RACE).

INTRODUCTION

Scorpion venom contains a diversity of neurotoxins, composed of two major polypeptide populations. One consists of several classes of long-chain toxin affecting Na⁺ channels (60–70 amino acid residues) [1–3], and the other one includes short-chain toxins (less than 40 residues) affecting K⁺ [4–9] or Cl[–] [10] channels. All short-chain toxins exhibit a similar overall three-dimensional structure with an α -helix and a three-stranded β -sheet constrained by three or four disulphide bridges [7,9,11]. The K⁺ channel toxins can further be classified into at least five subgroups sharing a low sequence similarity and having their own comparative selectivities towards each corresponding subtype of the target channels [4,8,9,12,13]. They therefore prove to be useful probes for investigating the diverse subtype of K⁺ channels. From the Chinese scorpion *Buthus martensi* Karsch, toxins named BmP01, BmP02, BmP03 and BmP05 acting on apamin-sensitive small-conductance Ca²⁺-activated K⁺ channels were first separated and purified [4], and a further three novel toxins, named BmKTX (where KTX is kaliotoxin), BmTX1 and BmTX2 acting on the voltage-gated K⁺ channels were subsequently characterized [12]. As BmTX1 and BmTX2 were also shown to compete with [¹²⁵I]-charybdotoxin (ChTX) and shared 65–70 % identity with its related toxins, they should be considered to belong to the ChTX subgroup, which is characterized by the activity towards high-conductance Ca²⁺-activated K⁺ channels, despite their broad specificity [12]. To answer the question of whether all these toxins come from a common ancestor, it seems

important to find a genetic link between these diverse toxins in addition to comparing their amino acid sequences. In a previous paper we described the genomic organizations of *B. martensi* toxins acting on small-conductance Ca²⁺-activated K⁺ channels [13]. Here we describe further the cDNA and genomic DNA sequences of BmKTX, BmTX1 and BmTX2, belonging to two further different subgroups. Comparing their gene sequences with other K⁺-channel toxins [13–16], it was indicated that despite only low amino acid sequence similarity, their gene organizations and intron features are conserved. In all toxins there are two exons and one intron splitting a codon at a similar location, namely close to the end of the signal peptide. In addition, their introns have similar sizes of approx. 80–90 bp and are rich in A+T. The results imply that they all might have evolved from an ancestral progenitor.

MATERIALS AND METHODS

Materials

B. martensi scorpions were collected in Henan Province, China. Their tails were cut off and immediately frozen in liquid nitrogen. The 3' and 5' rapid amplification of cDNA ends (RACE) kits and TRIzol reagent were purchased from Life Technology Co.; restriction enzymes, *Taq* DNA polymerase and pGEM-T Easy Vector System were from Promega. The DNA sequencing kit and [α -³²P]dATP were from Amersham. Acrylamide, bis-acrylamide, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside,

Abbreviations used: ChTX, charybdotoxin; KTX, kaliotoxin; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank®, DDBJ and GSDN Nucleotide Sequence Databases under the accession numbers AF208298 (BmKTX), AF208299 (BmTX1) and AF208300 (BmTX2).

isopropyl β -D-thiogalactoside and other chemical reagents were of analytical reagent grade.

Preparation of total RNA

Venous glands (50 mg) frozen in liquid nitrogen were ground into fine powder. By using TRIzol reagent kit, the total RNA extraction was performed in accordance with the instructions of the supplier.

3' RACE

Total RNA (5 mg) was taken to convert mRNA into cDNA species by using a 3' RACE kit provided with Superscript II reverse transcriptase and a universal oligo(dT)-containing adapter primer with a *Sal*I restriction site (5'-GGCCACGC-GTCGACTAGTACdT₁₇-3'). The synthesized cDNA species were then used as a template for PCR amplification with a pair of primers, namely the gene-specific primer and the abridged universal amplification primer containing an additional *Hind*III restriction site (5'-CGAAGCTTGGCCACGCGTCGACTAG-TAC-3') similar to the adapter primer but devoid of the poly(dT) tail. Referring to the preferential codon usage in other *B. martensi* scorpion toxins, and using the degenerate codons at the 3' end of the primer [13,17–20], gene-specific primer 1, with a *Bam*HI restriction site, was designed and synthesized for BmKTX (5'-CGGGATCC CAA TGT TTG AAA CCG/A/T/C TGC/T AA-3'), corresponding to the known amino acid sequence of residues 12–18 (Gln-Cys-Leu-Lys-Pro-Cys-Lys). On the same principle gene-specific primer 2, with an *Eco*RI restriction site, was synthesized for BmTX1 (5'-GCGAATTC TTT ACA GAT GTG/A/T/C AAG/A TG-3'), corresponding to the known amino acid sequence of residues 2–7 (Phe-Thr-Asp-Val-Lys-Cys). Gene-specific primer 3, with a *Bam*HI restriction site, was synthesized for BmTX2 (5'-CGGGATCC TTC GGA ACG TAT AGG GG-3'), corresponding to the known amino acid sequence of residues 21–26 (Phe-Gly-Thr-Tyr-Arg-Gly). The amplified products were precipitated and cloned into the pGEM-T Easy Vector for sequencing.

5' RACE

On the basis of the partial cDNA sequences of BmKTX, BmTX1 and BmTX2 determined by 3' RACE, their anti-sense primers were designed and synthesized for 5' RACE as follows: gene-specific primer 4, with a *Hind*III restriction site and a stop codon, for BmKTX (5'-CGAAGCTT TCA CCC CTT TGG TGT ACA GTC-3'), corresponding to residues 38–33 (Gly-Lys-Pro-Thr-Cys-Asp) and its nested primer 5, with a *Hind*III restriction site (5'-CGAAGCTT GCA TTT TCC AAA TCT CAT-3'), corresponding to residues 27–22 (Cys-Lys-Gly-Phe-Arg-Met); gene-specific primer 6, with a *Bam*HI restriction site, for BmTX1 (5'-CGGGATCC GTT AGG CTT TCC AAA CA-3'), corresponding to residues 25–20 (Asn-Pro-Lys-Gly-Phe-Met) and its nested primer 7, with an *Eco*105I restriction site (5'-GCTACGTA CGG CCA ACA TTG TTT-5'), corresponding to residues 15–11 (Pro-Try-Cys-Gln-Lys); gene-specific primer 8, with a *Hind*III restriction site and a stop codon, for BmTX2 (5'-CGAAGCTT TTA CGA ATA ACA ACG GCA-3'), corresponding to residues 37–33 (Ser-Trp-Cys-Arg-Cys), and its nested primer 9, with a *Hind*III restriction site (5'-CGAAGCTT TTT ACT ATT CAT ACA TTT-3'), corresponding to residues 32–27 (Lys-Ser-Asn-Met-Cys-Lys). With the same strategy as described previously [13,19,20], the 5'-end cDNA species of BmKTX, BmTX1 and BmTX2 were cloned and sequenced by using their own gene-

specific primers and nested primers respectively. The amplified products were then precipitated and cloned into the pGEM-T Easy Vector for sequencing.

Amplification of genomic DNA

Total genomic DNA was isolated from scorpion-tail glands by the method described in [20a]. With 5 μ g of total genomic DNA as a template, for amplification of the genomic DNA of BmKTX, gene-specific primer 10, with an *Eco*RI restriction site (5'-CGGAATTCATATATCTTTATTCGTCGG-3'), corresponding to the sequence of the 5' untranslated region at position –3 upstream of the initial codon ATG was paired with its anti-sense primer 4 used in 5' RACE. For amplification of the genomic DNA of BmTX1, gene-specific primer 11, with an *Eco*RI restriction site (5'-CGGAATTCGTATATATATGAGAGAA-3'), corresponding to the sequence of the 5' untranslated region at position –1 upstream of the initial codon ATG was paired with its anti-sense primer 12, with a *Bam*HI restriction site (5'-CGGGATCCATTATAAATCTGTAGAG-3'), corresponding to the sequence of the 3' untranslated region at position 2 downstream of the stop codon. For amplification of the genomic DNA of BmTX2, gene-specific primer 13, with an *Eco*RI restriction site (5'-GCGAATTCGGCTGGTAGAGAAAAA-TGA-3'), corresponding to the sequence of the 5' untranslated region extended with an initial codon and an additional adenosine nucleotide was paired with its anti-sense primer 8 used in 5' RACE. The amplified products were then precipitated and cloned into the pGEM-T Easy Vector for sequencing.

RESULTS

cDNA and genomic DNA of BmKTX

The cDNA sequence of BmKTX was completed by overlapping two fragments amplified by 3' and 5' RACE, its open reading frame encoded a signal peptide of 22 residues and a mature peptide of 38 residues (Figure 1). An additional Gly residue was found at the C-terminus of the mature peptide before the stop codon, which was removed during post-translational processing and was absolutely required for the C-terminal amidation of the mature toxin. The deduced amino acid sequence of BmKTX was consistent with that determined [12]. The polyadenylation signal, aataaa, was found in the 3' untranslated region at position 18 upstream of the poly(A). The genomic DNA of BmKTX consisted of two exons disrupted by an intron of 87 bp. The intron, with a consensus splice site of GT at the 5' end and AG at the 3' end, was inserted within the signal peptide at position –6 upstream of the mature toxin.

cDNA and genomic DNA of BmTX1

The cDNA of BmTX1 encoded a signal peptide of 20 residues and a mature peptide of 37 residues (Figure 2). The polyadenylation signal, aataaa, was found in the 3' untranslated region located at position 15 upstream from the poly(A). The deduced amino acid sequences of both BmTX1 and BmTX2 were consistent with those determined, except that the first deduced amino acid for both mature toxins is Gln instead of pyro-Glu in the reported toxins [12]. Obviously the residue Gln undergoes post-translational processing via condensation of the α -amino group of Gln with its γ -carboxy group to form the N-terminal residue pyro-Glu. The genomic DNA consisted of two exons disrupted by an intron of 87 bp. The intron was inserted near the C-terminus of the signal peptide at position –5 upstream of the mature toxin.

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                                atcaattc aagccgtaat tcacttttgt agatataaat
                                ^
Primer10
atatctttat tcgtcggaaa   -20   ATG AAG GTG TTT TTC GCA GTT TTA ATA ACT CTC
                                M   K   V   F   F   A   V   L   I   T   L

-10   -6
TTC ATC TGT TCA ATG A   gtaattagga attttcatta atttgtatat tttatatgaa
F   I   C   S   M

aaaacttaat gatccattaa ttaagcatat gttttcttat atttttag   TT ATT GGA ATT
                                I   I   G   I

-1   +10   Primer1
CAT GGA GTG GGA ATA AAT GTG AAA TGT AAG CAT TCT GGT CAA TGT TTG AAA
H   G   V   G   I   N   V   K   C   K   H   S   G   Q   C   L   K

+20   +30   Primer5
CCA TGC AAG GAT GCT GGA ATG AGA TTT GGA AAA TGC ATA AAT GGC AAA TGC
P   C   K   D   A   G   M   R   F   G   K   C   I   N   G   K   C

Primer4   +37
GAC TGT ACA CCA AAG GGG TGA   tttcttcttt aataaaacct attaaatatg taaaa
D   C   T   P   K   G   end

tctaa atattgaaaa taaaaactta tttcggaaac at .

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Figure 1 Genomic DNA sequence and deduced amino acid sequence of BmKTX

The exon is written in capital letters. Amino acids are denoted by one-letter symbols. The deduced mature toxin sequence is shown in bold. The primers for 3' and 5' RACE and genomic gene PCR are indicated with arrows. The polyadenylation signal, aataaa, is underlined.

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                                attcttaaag agaagtagat ttatcagata
                                ^
Primer11
tagtatatat atgagagaaa   -20   ATG AAA ATT TCA TTC CTT CTG CTA GCT CTC GTC
                                M   K   I   S   F   L   L   L   A   L   V

-5
ATT TGT TCA ATA G gtgtgttttc ttgtcccggt tatttttatt atttaaatat tatat
I   C   S   I

aggga attctaataa cgaatatggc aactattttt tttccag   GT TGG AGT GAA GCT
                                G   W   S   E   A

Primer2   +10   Primer7
CAG TTT ACA GAT GTA AAA TGC ACT GGT TCT AAA CAA TGT TGG CCG GTT TGT
Q   F   T   D   V   K   C   T   G   S   K   Q   C   W   P   V   C

+20   +30   Primer6
AAA CAA ATG TTT GGA AAG CCT AAC GGA AAG TGC ATG AAT GGT AAA TGC CGC
K   Q   M   F   G   K   P   N   G   K   C   M   N   G   K   C   R

+37   Primer12
TGC TAT TCG TAA   atctctacag atttataatg aaaatatgta atgcttaatt agtta
C   Y   S   end

taaataa aaatttttggc aaaaatct

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Figure 2 Genomic DNA sequence and deduced amino acid sequence of BmTX1

The exon is written in capital letters. Amino acids are denoted by one-letter symbols. The deduced mature toxin sequence is shown in bold. The primers for 3' and 5' RACE and genomic gene PCR are indicated with arrows. The polyadenylation signal, aataaa, is underlined.

cDNA and genomic DNA of BmTX2

The cDNA of BmTX2 encoded a signal peptide of 21 residues and a mature peptide of 37 residues (Figure 3). The polyadenylation signal, aataaa, was found in the 3' untranslated region at position 15 upstream from the poly(A), as in BmTX1. Comparing the determined gene sequence (TTT GGA ACG TAC AGG GG) with the designed primer 3 (TTC GGA ACG TAT AGG GG), only two bases were mismatched as underlined. The genomic DNA also consisted of two exons disrupted by an

intron of 80 bp. The intron was inserted at position -5 upstream of the mature toxin, as in BmTX1.

DISCUSSION

K⁺ channels are widespread among living creatures, where they have a key physiological role [21]. These channels are divided into many subtypes; for each subtype a corresponding scorpion neurotoxin can be found. As a result, scorpion K⁺ channel

Figure 3 Genomic DNA sequence and deduced amino acid sequence of BmTX2

The exon is written in capital letters. Amino acids are denoted by one-letter symbols. The deduced mature toxin sequence is shown in bold. The primers for 3' and 5' RACE and genomic gene PCR are indicated with arrows. The polyadenylation signal, aataaa, is underlined.

toxins can also be classified into at least five subgroups [12,13]. The toxins in the same group share similar amino acid sequences and physiological or pharmacological functions. Though over 40 different scorpion K^+ channel toxins have been reported [22], there has been little information on their gene structures. Only the genes of KTX from *Androctonus australis* [14], ChTX from *Leiurus quinquestriatus hebraeus* [16] and toxin Ts κ from *Tityus serrulatus* (only the cDNA sequence is available) [15] have been elucidated. In our previous paper we described the genomic organization of Bmp01, Bmp03 and Bmp05 acting on small-conductance Ca^{2+} -activated K^+ channels [13]. In the present paper we describe the gene structures of BmKTX, BmTX1 and BmTX2, which belong to two different K^+ -channel toxin groups, namely acting on voltage-dependent and high-conductance Ca^{2+} -activated K^+ channels. On the basis of these accumulated data, some genetic link between these diverse toxins has been found; a better understanding of the structure–function relationships of these toxins could be obtained by using mutagenesis to generate different mutants for the study of the interaction of variants with their corresponding target channels [23–25].

Comparing all of the gene structures of the scorpion toxins active on K^+ channels, we can come to some conclusions. (1) All precursors of toxins have a signal peptide with a size of 20–28 residues. The toxins active on small-conductance Ca^{2+} -activated K^+ channels have a larger signal peptide, of 28 residues, than those belonging to other groups, which usually have approx. 20 residues [13]. (2) All the genomic DNA sequences of the toxins consist of two exons disrupted by an intron of 80–130 bp inserted within the signal peptide region near its C-terminus. The introns agree with the GT/AG splice junctions and are rich in A+T content (more than 80%), in contrast with their exons (less than 60%) [26]. The intron sequences share high similarity with toxins from the same subgroup, but have no similarity with toxins from different subgroups. For example, the intron sequence of 87 bp of BmKTX has a similar size and approx. 90% identity with that of KTX [14] and both of them are voltage-dependent K^+ -channel toxins, although BmKTX is delivered from the

Asian scorpion *B. martensi*, whereas KTX is from the north African scorpion *Androctonus mauretanicus mauretanicus*. Considering the wide geographical distribution, this high similarity might imply that these two species of scorpions might have evolved from the same ancestor. However, the gene structures of KTX and BmKTX have no similarity with either BmTX1 and BmTX2 (active towards high-conductance Ca^{2+} -activated and also voltage-gated K^+ channels) or Bmp01, Bmp03 and Bmp05 (active towards low-conductance Ca^{2+} -activated K^+ channels). (3) In the deduced amino acid sequences of BmKTX and Bmp05, there are one or two additional residues (Gly or Gly-Lys) at the C-terminus of the mature peptide, which are removed during post-translational processing. The residue Gly is a donor of the C-terminal amide group of the mature toxin, which is absolutely required for peptidylglycine α -amidating monooxygenase [27]. It has been predicted that the N-terminus and the C-terminus are important in the interaction of these toxins with their target channels [28–30], and the C-terminal amidation will strengthen this interaction in some cases [31]. (4) In all toxins belonging to the ChTX subtype, the N-terminal residue is pyroglutamate, which is modified during post-translational processing [6,12]. In their cDNA deduced sequences, the corresponding residue is Gln, as found in both BmTX1 and BmTX2 and also in ChTX [16]. All the above observations suggest a genetic link between the diverse populations of all scorpion neurotoxins, which might come from a common ancestor.

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