Volume 302, number 3, 220–222 FEBS 11029 May 1992

Molecular cloning and nucleotide sequence analysis of a cDNA encoding the main β-neurotoxin from the venom of the South American scorpion *Tityus serrulatus*

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Received 22 February 1992; revised version received 31 March 1992

A cDNA encoding the main *Tityus serrulatus* β-neurotoxin was isolated from a venom gland cDNA library by using an oligonucleotide probe. The amino acid sequence deduced from the cDNA nucleotide sequence indicated that the toxin is the processed product of a precursor containing: (i) a signal peptide of 20 residues; (ii) the amino acid sequence of the mature toxin; and (iii) an extra Gly-Lys-Lys tail at the C-terminal end before the termination codon. Thus, in addition to the removal of the signal peptide by a signal peptidase, the generation of the mature toxin requires both a post-translational cleavage by a carboxypeptidase specific for basic residues and the action of an α-amidating enzyme. These results also show that the biosynthetic pathway for β-toxins of 'New World' scorpion venoms is similar to that already described for α-toxins of 'Old World' scorpion venoms.

Scorpion β-neurotoxin; cDNA

1. INTRODUCTION

Two types of toxins (α and β) have been characterized in the venom of the Brazilian scorpion, *Tityus serrulatus* (Ts), according to their specific binding to site 3 or 4 on the voltage-dependent sodium channel of rat brain synaptosomes, respectively [1,2]. The amino acid sequence of the main β-toxin, Ts VII, has been determined [3]. Ts VII appears to be identical with some other toxins isolated from the same venom by other authors, such as toxins 2 [4], Ts-γ [5–7] and T_1-VIII [8]. Ts VII exhibits the highest affinity known so far for sodium channel site 4, and it also acts on the insect nervous system sodium channel [1,9,10]. Recently, a genetic approach has been used to better elucidate the molecular basis for scorpion toxin specificity, i.e. α- and β-type for toxins active on mammals and the selectivity for insects. The cDNA encoding the precursors of α-toxins and 'excitatory' insect toxins from *Androctonus australis* Hector (AaH) have been cloned [11], as well as the precursor of a 'depressant' insect toxin (Bj IT2) from *Buthotus judaicus* [12]. These two scorpion species belong to the 'Old world'. In the work reported here the cDNA encoding Ts VII was cloned and its nucleotide sequence was determined. Lastly, the precursor of Ts VII and those of α-toxins and 'excitatory' or 'depressant' insect toxins were compared.

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2. MATERIALS AND METHODS

Standard recombinant DNA techniques were used [13]. Enzymes were from Boehringer (Mannheim, Germany). *E. coli* strains C600hfl [14] and JM109 [15] were used for phage production.

2.1. *Construction of the venom gland cDNA library*

Scorpions (100 animals) of the species *Tityus serrulatus* were collected in the area of Santa Barbara, MG, Brazil. They were sacrificed 2 days after extraction of their venom to allow the toxin-producing cells of the venom glands to enter the secretory phase. Total RNA was extracted from their venom-gland segments (telson) by using the guanidinium hot-phenol method [13]. Poly(A)$^+$ mRNA was purified using an oligo(dT)–cellulose column (Clonatech Laboratories Inc., Palo Alto, CA, USA). The intactness and ability of mRNA to serve as template for full-length reverse transcription was checked as already described [11]. Double-stranded cDNA was synthesized from 5 μg of mRNA by using the cDNA synthesis kit from Pharmacia (Uppsala, Sweden) and cloned into *Eco*RI-cut λgt10 (Promega, Madison, WI, USA). The library contained 2.5×10^6 independent phage clones.

2.2. *Synthesis of oligonucleotide*

The oligonucleotide probe used to screen the library was synthesized using an Applied Biosystems Model 391 DNA synthesizer. The sequence of the probe was 5' ATG GA[CT] CA[CT] GA[AG] GGT TG[CT] AAA and corresponded to the amino acid sequence of Ts VII from residues 6 to 12. The probe was ^{32}P-end-labelled using T_4 polynucleotide kinase.

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CGAAGAACAATCGATCTGAACG ATG AAA GGA ATG ATC TTG TTT ATT AGC TGC TTA TTG     58
                       M   K   G   M   I   L   F   I   S   C   L   L     -9

CTG ATC GGC ATT GTC GTA GAA TGT AAA GAA GGT TAT CTC ATG GAT CAC GAA GGT   112
L   I   G   I   V   V   E   C   K   E   G   Y   L   M   D   H   E   G     10

TGC AAA CTT AGT TGC TTT ATC AGA CCA TCG GGA TAC TGC GGC AGA GAA TGC GGA   166
C   K   L   S   C   F   I   R   P   S   G   Y   C   G   R   E   C   G     28

ATT AAA AAG GGC TCA TCG GGC TAT TGC GCC TGG CCC GCG TGT TAC TGC TAC GGG   220
I   K   K   G   S   S   G   Y   C   A   W   P   A   C   Y   C   Y   G     46

CTT CCA AAT TGG GTG AAA GTT TGG GAT AGA GCG ACG AAC AAA TGT GGC AAA AAA   274
L   P   N   W   V   K   V   W   D   R   A   T   N   K   C   G   K   K     64

TAA  ATTTGTTTCGCTGAAAATCCTTTACAAATGAACTGTAATAAGTTTGGCAAAAATAAAAAAATGTTC   343
*                                                                         65
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Fig. 1. Nucleotide sequence of the cDNA encoding the Ts VII precursor. The predicted protein sequence is given below the nucleotide sequence and is numbered starting from the NH_2-terminal amino acid residue of the toxin; the signal peptide sequence is underlined; the stop codon is designated by an asterisk; a potential polyadenylation signal of AATAAA is underlined twice. BISANCE [22] was used for sequence analysis.

2.3. *Screening of the cDNA library*

750,000 clones from the cDNA library were analysed as described [13].

2.4. *Subcloning and DNA sequencing*

The cDNA inserts excised from positive phage DNA with *Eco*RI were fractionated on 0.8% low-melting temperature Nusieve GTG agarose gel (FMC BioProducts, Rockland, ME, USA) recovered using the Gene Clean kit (Bio 101 Inc., La Jolla, CA, USA), and then subcloned into M_{13} mp 18 for sequencing according to the method of Sanger [16].

3. RESULTS AND DISCUSSION

Starting with 100 fresh telsons giving 1 g of tissue we obtained 17 μg of poly(A)$^+$ mRNA. To both characterize and assay the mRNA, single strand cDNA were reverse-transcribed and electrophorezed on a 1.5% alkaline agarose gel. Only one class of transcripts corresponding to an average size between 330 and 550 bp was observed. Two major classes (about 360 and 1,100 bp) were found for AaH [11]. Therefore, the mRNA from venom gland of Ts are related to short polypeptides only. The presence in the AaH venom of a large amount of proteins exhibiting a higher molecular weight than those found in the Ts venom had been found by molecular filtration of their respective venoms through Sephadex G-50 [17]. The initial screening of the Ts cDNA library with the probe yielded more than 2,000 positive clones. On the final screening, 24 clones were selected on the basis of the strength of the autoradiographic signal. Restriction analysis revealed size variation of the insert between 220 and 380 bp. Four inserts, the longest ones, were subjected to sequence analysis. The nucleotide sequences obtained were identical and displayed an open reading frame of 252 bp encoding a polypeptide of 84 amino acids. This polypeptide depicts the precursor of Ts VII (Fig. 1). The first 20 residues have a net hydrophobicity and they satisfy the minimal requirements for a functional signal sequence: (i) an initiation Met; (ii) a positively charged amino acid (Lys), which is known to improve the export efficiency of all prokaryotic and of most eukaryotic signal peptides [18]; (iii) a hydrophobic segment with a high Leu content, which is supposed to adopt an α-helical conformation, interrupted by an helice breaker residue (Gly) in position −6; and (iiii) a proteolytic cleavage site at a small neutral residue (Cys) [19,20]. In Fig. 2a the amino acid sequences of the signal peptides are compared, deduced from the depicted cDNA, of the precursors of diverse *Buthidae* toxins. These primary structures showed a low degree of similarity. However, it has previously been proposed that the residues at positions −1 and −3 are important for the interaction with the signal peptidase [19,20]. It is striking to find a Val in position −3 for all the signal peptides described here. After the signal peptide, 61 amino acid residues match the sequence of Ts VII. Finally, the Ts VII precursor sequence is extended by Gly-Lys-Lys on the COOH-terminal, as compared with the mature Ts VII. Accordingly, the Lys residue have to be removed by a basic residue-specific carboxypeptidase (CPAse B-like activity), after which the remaining Gly-extended peptide will be converted into a des-Gly peptide amine by an α-amidating enzyme [21]. The resulting Cys-amide residue correlates with that chemically determined on the native Ts VII [3]. No clear answer can be given for the existence of these basic residues at the C-terminal end of almost all of the scorpion toxin precursors depicted up to now (Fig. 2b). Moreover, a discrepancy is to be noted in the amidation processing steps of the different toxins. Mature AaH II and Ts VII are α-amidated and accordingly their precursors possess an extra Gly residue. In the case of BJ IT2, despite the presence of the Gly-Arg-Lys-Lys se-

a

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                                    ▼
Ts VII:              MKGMILFISCL-LLIGIVVEC...
AaH I, II, III:      MNYLVMISLAL-LLM-IGVES...
Lqh α:               MNHLVMISLAL-LLL-LGVES...
AaH IT1, IT2:        MKFLLLFLVVLPIMG---VLG...
Bj IT2:              MKLLLLLVISASMLLECLVNA...
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b

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Ts VII:              ...CGKK*
AaH II:              ...CHGR*
AaH I:               ...CTR
AaH III:             ...CHSR
AaH IT1, IT2         ...IN
Bj IT2:              ...CGRKK
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Fig. 2. Amino acid sequence comparisons for (a) the signal peptide and (b) the C-terminal end of scorpion toxins precursors. The amino acids have been aligned using the CLUSTAL program with fixed gap penalty=10 and varying gap penalty=10 [23]. The arrow indicates the site of cleavage of the precursor by the signal peptidase. The additional residues at the C-terminal end of precursors by comparison to native toxins are in bold; * indicates the sequences where the Gly residue will give a C-terminal amidated residue. Ts VII is the *main β-toxin of Tityus serrulatus*, active both on mammals and insects (this work, 9,10); AaH I, II and III are all α-toxins of *Androctonus australis* Hector, active on mammals [11]; Lqh α-IT is an α-toxin of *Leiurus quinquestriatus hebreus*, active on insects [24]; AaH IT1 and IT2 are 'excitatory' toxins of *Androctonus australis* Hector, active on insects; Bj IT2 is a 'depressant' toxin of *Buthotus judaicus*, active on insects [12].

quence, the mature toxin is not α-amidated and ends with a Gly-COOH.

In conclusion, the biosynthetic pathway for scorpion toxins seem not to be unique with respect to the maturation of their precursors. The necessity for the presence of basic residues at the COOH-terminal of their precursors is still an open question.

Acknowledgements: We wish to thank Mrs C. Roussarie for typing the manuscript. This work was supported by the Délégation à la Recherche et aux Etudes Techniques.

REFERENCES

[1] Barhanin, J., Giglio, J.R., Leopold, P., Schmid, A., Sampiero, S.V. and Lazdunski, M. (1982) J. Biol. Chem. 257, 12553–12558.
[2] Martin, M.F., Bechis, G., El Ayeb, M., Sampieri, F., Brando, T., Diniz, C.R. and Rochat, H. (1985) Toxicon 23, 594.
[3] Bechis, G., Sampieri, F., Yuan, P.M., Brando, T., Martin, M.F., Diniz, C.R. and Rochat, H. (1984) Biochem. Biophys. Res. Commun. 122, 1146–1153.
[4] Toledo, D. and Neves, A.G. (1976) Comp. Biochem. Physiol. 55B, 249–253.
[5] Possani, L.D., Martin, B.M., Mocha-Morales, J. and Svendsen, I. (1981) Carlsberg Res. Commun. 46, 195–205.
[6] Possani, L.D., Martin, B.M., Svendsen, I., Rode, G.S. and Erikson, B.W. (1985) Biochem. J. 229, 739–750.
[7] Possani, L.D., Martin, B.M., Fletchner, M.D. and Fletchner, P.L. (1991) J. Biol. Chem. 266, 3178–3185.
[8] Sampaio, S.V., Laure, C.J. and Giglio, J.R. (1983) Toxicon 21, 265–277.
[9] De Lima, M.E., Martin, M.F., Diniz, C.R. and Rochat, H. (1986) Biochem. Biophys. Res. Commun. 139, 296–302.
[10] De Lima, M.E., Martin, M.F., Hue, B., Loret, E., Diniz, C.R. and Rochat, H. (1989) Insect Biochem. 19, 413–422.
[11] Bougis, P.E., Rochat, H. and Smith, L.A. (1989) J. Biol. Chem. 264, 19259–19264.
[12] Zilberberg, N., Zlotkin, E. and Gurevitz, M. (1991) Toxicon 29, 1155–1158.
[13] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[14] Huynh, T.V., Young, R.A. and Davis, R.W. (1985) in: DNA Cloning 1 (Glover, D.M. ed.) pp. 56–110, IRL Press, Oxford, England.
[15] Yanish-Perron, C., Viera, J. and Messing, J. (1985) Gene 33, 103–199.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] Martin-Eauclaire, M.F. (1987) Thèse d'Etat Sciences, Université d'Aix-Marseille II.
[18] Boyd, D. and Beckwith, J. (1990) Cell 62, 1031–1033.
[19] von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21.
[20] Perlman, D. and Halporson, H.O. (1983) J. Mol. Biol. 167, 391–409.
[21] Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) Nature 298, 686–688.
[22] Dessen, P., Fondrat, C., Valencien, C. and Mugnier, C. (1990) CABIOS 6, 335–356.
[23] Higgins, D.G. and Sharp, P.M. (1988) Gene 73, 237–244.
[24] Gurevitz, M., Urbach, D., Zlotkin, E. and Zilberberg, N. (1991) Toxicon 29, 1270–1279.