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cDNA Cloning of Two Novel T-superfamily Conotoxins from *Conus leopardus*

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Abstract The full-length cDNAs of two novel T-superfamily conotoxins, Lp5.1 and Lp5.2, were cloned from a vermivorous cone snail *Conus leopardus* using 3′/5′-rapid amplification of cDNA ends. The cDNA of Lp5.1 encodes a precursor of 65 residues, including a 22-residue signal peptide, a 28-residue propeptide and a 15-residue mature peptide. Lp5.1 is processed at the common signal site -X-Arg- immediately before the mature peptide sequences. In the case of Lp5.2, the precursor includes a 25-residue signal peptide and a 43-residue sequence comprising the propeptide and mature peptide, which is probably cleaved to yield a 29-residue propeptide and a 14-residue mature toxin. Although these two conotoxins share a similar signal sequence and a conserved disulfide pattern with the known T-superfamily, the pro-region and mature peptides are of low identity, especially Lp5.2 with an identity as low as 10.7% compared with the reference Mr5.1a. The elucidated cDNAs of these two toxins will facilitate a better understanding of the species distribution, the sequence diversity of T-superfamily conotoxins, the special gene structure and the evolution of these peptides.

Key words *Conus leopardus*; T-superfamily; conotoxin; cDNA cloning

Cone snails are predatory marine animals belonging to gastropod mollusks. There are more than 500 species of cone snails living mainly in the tropical marine area around the world. These venomous creatures have evolved a highly sophisticated neuropharmacological strategy based on small peptides (conotoxins). They are usually composed of 10–50 amino acids and are rich in disulfide bonds with a few widely shared structure motifs [1]. However, individual peptides are selectively targeted to a specific isoform of ion channel or receptor. A variety of such targets have been identified, including voltage- and ligand-gated ion channel subtypes, as well as G protein-linked receptors and norepinephrine transporters [2]. Conotoxins have great potential as a tool for neuroscience and as therapeutic agents. In addition, each Conus species has its own distinct repertoire of 50–200 different venom peptides [3,4]. Thus, there are tens of thousands of different active peptides in Conus venoms. However, only 0.2% of them have been elucidated.

According to the disulfide framework and the sequence of signal peptides, conotoxins can be grouped into several superfamilies, such as the A-, O-, M-, P-, I-, S- and T-superfamily. It is well known that, although members of each superfamily share a similar cysteine pattern, their molecular targets can be quite different. For example, in the A-superfamily, αA- and κA-conotoxins share the same Cys pattern (CC-C-C-C-C), but they are totally different in physiological activity. αA-conotoxins act as the specific blocker of nicotinic acetylcholine receptor (nAChR), whereas κA-conotoxins target the potassium ion channel [5]. Conotoxins are also characterized by the ability to discriminate the different subtypes of their molecular targets [6]. In addition, there are also conotoxins of different superfamilies which have an effect on the same target. For example, the α-conotoxins in the A-superfamily and the ψ-conotoxins in the M-superfamily both target

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the nAChR. The only difference is that α-conotoxins compete for the binding site of ACh, whereas ψ-conotoxins inhibit the activity of nAChR in a noncompetitive manner [7,8].

Compared with conotoxins from other superfamilies, T-superfamily conotoxins found in all three major feeding types of cone snails are extremely small peptides (10–17 amino acids). The widespread distribution of T-superfamily conotoxins suggests that they might have an important physiological function for the genus [9]. Among the T-superfamily, many members are reported to have a highly post-translationally modified sequence. An unusually high degree of post-translational processing, including L-6-bromination of tryptophane hydroxylation of proline and O-glycosylation of threonine, was found in tx5a, which was also reported as ε-TxIX and may target presynaptic Ca^{2+} channels or act on G protein-coupled presynaptic receptors [10,11]. However, no such modifications have been observed in other members, including mr5a and au5a. Considering that T-superfamily conotoxins share low sequence identity, the function of these peptide toxins might also be divergent.

In this report, the full-length cDNA sequences of two novel T-superfamily conotoxins, Lp5.1 and Lp5.2, have been cloned from *Conus leopardus*. The successful gene cloning of these two conotoxins will facilitate better understanding of the species distribution and sequence diversity of T-superfamily conotoxins, and of the special gene structure and evolution of these peptides.

Materials and Methods

Materials

The specimens of *C. leopardus* for gene research were obtained from Sanya (China) near the South China Sea. The venom ducts were dissected from living snails, then immediately frozen in liquid nitrogen and stored at −80 ºC. The 3′-rapid amplification of cDNA end (RACE) kits and Trizol reagent were purchased from Invitrogen (Carlsbad, USA), Taq DNA polymerase and the pGEM-T Easy Vector System from Promega (Madison, USA), and restriction enzymes from NEB. 5-Bromo-4-chloro-3-indolyl β-*D*-galactopyranoside, isopropyl β-*D*-thiogalactopyranoside and other reagents were of analytical grade.

Preparation of total RNA

One venom duct frozen in liquid nitrogen was ground into fine powder and homogenized. Using the Trizol reagent kit, the total RNA extraction was carried out according to the instruction manual.

cDNA cloning and sequencing

Approximately 5 μg of total RNA from the *C. leopardus* venom duct was taken to convert mRNA into cDNA using Superscript II reverse transcriptase (Invitrogen) with a universal oligo(dT)-containing adapter primer 5′-GGC-CACGCGCGTCGACTAGTAC$(dT)_{17}$-3′. For the polymerase chain reaction (PCR) amplification of the genes encoding T-superfamily conotoxins, 5′ forward primer 1 (5′-ATGCGCTGTGTCCCAGTCTTC-3′) based on the conserved signal peptide sequence of T-superfamily conotoxins was used, paired with an abridged universal amplification primer devoid of the poly dT tail. In the case of Lp5.1, an additional 3′ forward primer 2 (5′-CTG-AAATATTTAATC-3′) was used. PCR was performed as follows: an initial denaturation of 94 ºC for 2 min; 10 cycles of 94 ºC for 45 s, 55 ºC for 45 s, decreasing 1 ºC per cycle, 72 ºC for 2 min; 25 cycles of 94 ºC for 45 s, 45 ºC for 45 s, 72 ºC for 2 min; then terminated with a final extension at 72 ºC for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gel. The PCR product band was excised from the gel and purified with a Gel Extraction Mini Kit. The purified PCR products were inserted into the pGEM-T Easy Vector System by TA cloning. Transformed colonies were screened with white-blue identification for sequence analysis.

Results

PCR amplification with 5′ forward primer 1 paired with an abridged universal amplification primer was carried out to screen the *C. leopardus* cDNA library. In the case of Lp5.1 an additional 3′ forward primer 2 was used. A prominent PCR product band of approximately 700 bp was obtained in both cases. At least 12 clones were sequenced each time. The precursor peptide sequences of two novel T-superfamily conotoxins, Lp5.1 and Lp5.2, were deduced according to the cDNA sequences (**Figs. 1** and **2**), both of which include a long 3′-untranslated region of approximately 500 bp. The deduced precursor of Lp5.1 consists of a 22-residue signal peptide, a 28-residue propeptide and a 15-residue mature peptide (**Fig. 1**). In the case of Lp5.2, the cleavage site is uncertain, but the highly conserved prepeptide sequences and the same cysteine pattern (CC-CC) of both peptides clearly indicate that they should belong to the T-superfamily conotoxins.

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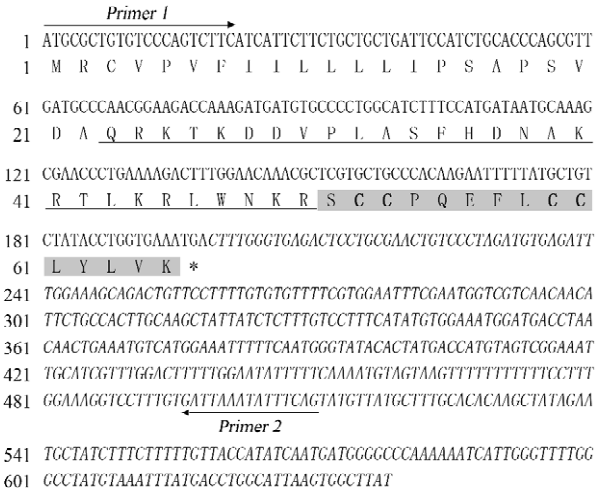

Fig. 1 cDNA and deduced sequences of T-conotoxin Lp5.1

The untranslated regions are in italics; the pro-region underlined; the mature peptide region shadowed. The primers for 3′/5′-rapid amplification of cDNA ends are indicated with arrows. GenBank accession number of the coding region of Lp5.1 is AY591769.

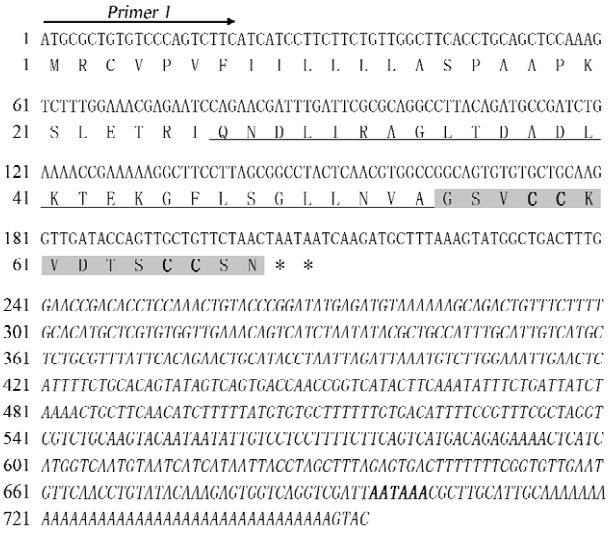

Fig. 2 cDNA and deduced sequences of T-conotoxin Lp5.2

The untranslated regions are in italics and the polyadenylation signal "AATAAA" bold. The primer for 3′-rapid amplification of cDNA end is indicated with an arrow, the pro-region underlined and the mature peptide region shadowed. GenBank accession number of the coding region of Lp5.2 is AY591770.

Discussion

As most conotoxins, the prepropeptide of Lp5.1 is cleaved at the signal site -X-Arg- to yield the mature toxin, whereas in the sequence of Lp5.2 there is no apparent cleavage site that contains alkaline amino acids. Our assumption is that precursor Lp5.2 is possibly cleaved at the site -X-Ala-, as previously characterized in conotoxin BtX [12]. Consequently, there must be an unusual proteinase cleaving at such a different site to yield mature toxins. But this is still to be identified by isolating and characterizing the mature toxin from venom. We also noted that the identity of the pro-region between Mr5.1a (as reference) and Lp5.2 is 10.7%, which is by far the lowest of all known T-superfamily conotoxins [9]. However, the pre-regions of both conotoxins are, relatively, much more highly conserved. The pre-region identities of Lp5.1 and Lp5.2 are 90.1% and 69.2%, respectively (**Table 1**).

Sharing no obvious homology, the mature peptide sequences of T-superfamily conotoxins are more diversified than those of other known superfamilies, whose identities range from 25.0% to 86.7%, taking Mr5.1a as a reference. Except Mr5.1b, each toxin has a low identity below 50% (**Table 2**). Lp5.1 and Lp5.2 elucidated in this work have quite differing identities of 40.0% and 28.6%, respectively. In the sequences of Lp5.1 and Lp5.2, there is no conserved glutamic acid residue preceded by the second two adjacent Cys, unlike the conotoxins found in *Conus marmoreus* [9]. Because the mature toxins of Lp5.1 and Lp5.2 have not yet been isolated and characterized from venom, it is still unknown whether they undergo post-translational modification. But the T-superfamily is still a family endowed with fairly abundant post-translational modification, as shown in **Table 2**. Of all the T-superfamily conotoxins, tx5a is the most complicated. An unparalleled degree of post-translational processing including bromination, hydroxylation, and glycosylation were found in one toxin for the first time [10,11]. It is worth noting that some post-translational modifications have been shown to be indispensable for the specific biological activities of several conotoxins [17].

From the deduced precursor sequences we cannot be sure whether there is post-translational processing in Lp5.1 and Lp5.2, however there are some possible sites that may undergo post-translational modification. For example, the O-glycosylation of the serine or threonine residue, the hydroxylation of Pro residue and the γ-carboxylation of Glu residue.

The elucidated full-length cDNAs of two novel T-conotoxins from *C. leopardus* in this work again provide evidence that T-superfamily Conus peptides share a relatively conserved signal sequence, but rather diversified pro-region and mature peptides. The high variability of mature Conus peptides and mechanism of post-translational modification are intriguing. As with the discovery

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Table 1 Comparison of entire peptide sequences between T-superfamily conotoxins Lp5.1/Lp5.2 and Mr5.1a

Conotoxin	Sequence	Identity (%)	
		Pre	Pro
Mr5.1a	MRCVPVFVILLLLIASAPSVDARLKTKDDMPLPSSHANIKRTLQIHRNKRCCPGWELCCEWDEW	100.0	100.0
Lp5.1	MRCVPVFIILLLLIPSAPSVDAQRKTKDDVPLASFHDNAKRTLKRLWNKRSCCPQEFLCCLYLVK	90.1	65.4
Lp5.2	MRCVPVFIILLLLASPAAPKSLETRIQNDLIRAGLTDADLKTEKGFLSGLLNVAGSVCCKVDTSCCSN	69.2	10.7

The cysteine residues are in bold; propeptides are underlined; mature peptides are shadowed. The percentages indicate the sequence identity of the pre-region and pro-region of Lp5.1 and Lp5.2 (Mr5.1a as reference).

Table 2 Mature peptide sequence comparison of T-superfamily conotoxins

Conotoxin	Sequence	Identity (%)	Conus species	Reference
Mr5.1a	**CC**PGWEL**CC**EWDEW	100.0	*C. marmoreus*	[9]
Mr5.1b	**CC**PGWEL**CC**EWDDGW	86.7	*C. marmoreus*	[9]
Mr5.2	F**CC**RTQγV**CC**γAIKN†	40.0	*C. marmoreus*	[9,13]
Mr5.3	**CC**ITFγS**CC**γFDLK	50.0	*C. marmoreus*	[9,13]
Mr5.4a	**CC**QVMPQ**CC**EWN	42.9	*C. marmoreus*	[9]
Lp5.1	S**CC**PQEFL**CC**LYLVK	40.0	*C. leopardus*	This work
Lp5.2	GSV**CC**KVDTS**CC**SN	28.6	*C. leopardus*	This work
Gm5.1	L**CC**VTEDW**CC**EWW	46.7	*C. gloriamaris*	[10]
Tx5.1	**CC**QTFYW**CC**VQ	28.6	*C. textile*	[10]
mr5a	NA**CC**–IVRQ**CC**	25.0	*C. marmoreus*	[9]
p5a	G**CC**PKQMR**CC**TL†	33.3	*C. purpurasceus*	[10]
au5a	F**CC**PFIRY**CC**	33.3	*C. aulicus*	[10]
tx5a	γ**CC**–γDGW‡**CC**T§AAO	37.5	*C. textile*	[10,11]
MrIA	NGV**CC**GYK–L**C**HP**C**	29.4	*C. marmoreus*	[14–16]

† C-terminal amidation; ‡ bromination; § O-glycosylation. γ, gamma-carboxyglutamic acid, E, predicted to be gamma-carboxyglutamic acid.

of the presence of a γ-carboxylation recognition signal in the pro-region of the precursor of tx5a [10,18], there may also be recognition signals in the prepropeptide for bromination and O-glycosylation enzymes. Thus, tx5a and other members of the T-superfamily might provide good model substrates for studying post-translational modification of Conus peptides. The high diversity of Conus peptides could be an optimum evolutionary strategy of the genus; the relatively conserved signal sequences and the pro-regions are necessary and may take on certain functions during the course of folding, post-translational modification and secretion [19,20]. The cDNA cloning and analysis of cone snails is an efficient method to discover novel toxin peptides, and it also facilitates a better understanding of the complicated post-translational processing and evolution of conotoxins.

It has already been confirmed that the T-superfamily conotoxins are extremely diversified. The highly variable toxin peptides, as well as their abundant post-translational modification, may lead to the exhibition of different functions. Therefore, the T-superfamily conotoxins may serve as a good library for studying the structure-function relationship of conotoxins. Given that the specific functions of many already identified T-superfamily conotoxins are still unknown, more research in this area is needed to reveal their physiological activities.

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