Antimicrobial Tachyplesin Peptide Precursor

cDNA CLONING AND CELLULAR LOCALIZATION IN THE HORSESHOE CRAB (TACHYPLEUS TRIDENTATUS)*

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The hemocytes of the horseshoe crab have been found to contain a new family of Arthropodous antibiotics, termed the "tachyplesin family." These peptides are composed of 17-18 amino acid residues with a carboxyl-terminal arginine amide. We report here the entire cDNA sequence coding for the tachyplesin precursors and their distribution in various tissues of the horseshoe crab. Sequence analysis of the cloned cDNAs revealed that the tachyplesin precursors consist of 77 amino acids with 23 residues in a presegment, and that there are two types of mRNAs corresponding to the isopeptides tachyplesins I and II. Both precursors contain a putative signal peptide, a processing peptide sequence and a carboxyl-terminal amidation signal "Gly-Lys-Arg" connected to the mature tachyplesin peptide. Moreover, an unusual acidic amino acid cluster, Asp-Glu-Asp-Glu-Asp-Glu-Glu-Glu-COOH, is present in the carboxyl-terminal portions of both precursors. These results suggest that the two types of tachyplesin precursors are first synthesized as preproproteins and are then incorporated into the intracellular organelle, accompanied by various processing events. Northern blot analysis on a total RNA from various tissues of the horseshoe crab revealed that the tachyplesin precursors are expressed mainly in hemocytes and cardiac and brain tissues. Tachyplesin was immunohistochemically localized in the smaller dense granules rather than the typical large granules present in abundance in the hemocytes.

The circulating hemolymph in the horseshoe crab contains various biologically active substances, including lectins (1-3), clotting factors (4-6), proteinase inhibitors (7, 8) and antithrombin of the Japanese horseshoe crab (Tachypleus tridentatus) (4). Tachyplesin I was isolated from acid extracts of hemocyte debris according to our method (12). All DNA-modifying enzymes were purchased from Bethesda Research Laboratories. [γ-32P]ATP (600 Ci/mmol) and [α-32P]dCTP (300 Ci/mmol) were from Du Pont-New England Nuclear. The multiprime DNA labeling system and the DNA labeling kits were from Amer sham-Japan and Nippon Gene Co., respectively. RNA size marker was obtained from Bethesda Research Laboratories. tRNA from brewer's yeast and proteinase K were purchased from Boehringer Mannheim. Agt11 lambda cDNA library from the hemocyte poly(A)+ RNA from T. tridentatus were the same as those described previously. 2

Synthesis of Oligonucleotide Probe—An oligonucleotide probe of 17 nucleotides (Fig. 1) was synthesized using an Applied Biosystems 380A DNA Synthesizer as a mixture of 64 sequences based on the amino acid replacements, named tachyplesins II and III (12, 13) and polyphemusins I and II (12), have been found in the hemocytes of the Southeast Asian horseshoe crabs (T. tridentatus, Tachypleus gigas, and Carcinorhopus rotundicauda) and in Limulus polyphemus. These peptides are present in abundance in the hemocytes of four species of horseshoe crabs living in various parts of the world (11-13) and inhibit the growth of not only Gram-negative and -positive bacteria but also fungi, such as Candida albicans (11, 12).

For a better appreciation of the biological significance of this tachyplesin family, the tissue distributions of the peptide and the entire amino acid sequence of its precursor protein synthesized in the hemocytes have to be examined. We cloned the DNA complementary to tachyplesin mRNA and examined the distribution of the mRNA expressed in various tissues of the horseshoe crab. We obtained evidence that tachyplesin exists in the pre-pro form consisting of 77 amino acid residues and that the precursor is processed by intracellular proteases and an amidation enzyme, and then incorporated into the small granules of the hemocytes.

EXPERIMENTAL PROCEDURES

Materials—Japanese horseshoe crabs (T. tridentatus) were collected in Imatsu Bay, Fukuoka prefecture, and bled by cardiac puncture (4). The hemocytes from the hemolymph were prepared as described (4). Tachyplesin I was isolated from acid extracts of hemocyte debris according to our method (12). All DNA-modifying enzymes were purchased from Nippon Gene Co., Toyama, Toyobo Co., LTD., Osaka, Takara Shuzo Co., Kyoto, or Bethesda Research Laboratories. [γ-32P]ATP (600 Ci/mmol) and [α-32P]dCTP (300 Ci/mmol) were from Du Pont-New England Nuclear. The multiprime DNA labeling system and the DNA labeling kits were from American-Japan and Nippon Gene Co., respectively. RNA size marker was obtained from Bethesda Research Laboratories. tRNA from brewer's yeast and proteinase K were purchased from Boehringer Mannheim. Agt11 lambda cDNA library from the hemocyte poly(A)+ RNA from T. tridentatus were the same as those described previously. 2

Screening of cDNA Library—1.5 x 10^6 plaques from the hemocyte cDNA library 2 were lifted onto duplicate nitrocellulose filters and hybridized with the 32P-labeled oligonucleotide probe in a solution of 6 x SSC (1 x SSC = 15 m M sodium citrate and 150 mM NaCl, pH 7.0), 10 x Denhardt's solution (1 x Denhardt's solution = 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% formaldehyde), and 0.3% sodium pyrophosphate.
col), 0.1% SDS, and 100 μg/ml of yeast trRNA at room temperature for 24 h. The filters were washed in a solution of 1.5 × SSC containing 0.1% SDS at 30 °C or 0.5 × SSC containing 0.1% SDS at room temperature, followed by autoradiography. After the positive clones had been plaque-purified, the insert DNAs were subcloned into pUC 118 (Takara Shuzo Co.) and sequenced.

The DNA fragment encoding the partial sequence of the tachyplexin I precursor was labeled with [35S]CTP, using a multiprime labeling system or a random primer DNA labeling kit. Filters carrying the plaques were hybridized with the 35S-labeled probe at 65 °C in the same solution as used for the previous screening. The filters were then washed in 0.1 × SSC and 0.1% SDS at 65 °C and autoradiography was performed.

**DNA Sequencing**—The inserts of positive clones were subcloned into pUC 118. A single-stranded DNA as the template was prepared using MVI184 as the host cell and M13K07 as the helper phage (15). Determination of the nucleotide sequence was made with an Applied Biosystems 370A DNA Sequencer using fluorescent oligonucleotide primers (16). Sequence (United States Biochemical) or Taq polymerase (Promega) were used for the extension reactions.

**Northern Blot Analysis of RNA from Several Tissues**—Organs and tissues used for Northern blot analysis were obtained from an adult male Japanese horseshoe crab, immediately after dissection, the hepatopancreas, heart, stomach, intestine, muscle, brain, and coxal gland were excised, washed extensively in sterile water and placed in liquid nitrogen. The RNAs were prepared from each tissue using the acid guanidinium thiocyanate/phenol/chloroform method (17).

**Southern Blot Analysis**—DNA used for the Southern blot analysis was prepared from hemocytes of an adult female Japanese horseshoe crab, according to Sambroids and Wilkie (18), but with some modification. Hemocytes in 2.5 ml of hemolymph were collected in a sterile tube by centrifuging at 30 g for 2 min. Two ml of 20 mM sodium acetate, pH 7.0, containing 8 mM guanidine-HCl, 50 mM EDTA, and 0.7 M 2-mercaptoethanol were added and the suspension was mixed several times by inverting the tubes, then leaving the tubes to stand for 2 h in ice water. After further incubation at room temperature for 2 h, 0.4 ml of 10% SDS was added, and the preparation was incubated at 65 °C for 15 min. The DNA precipitated after the addition of an equal volume of isopropanol alcohol was dissolved in 2 ml of 10 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, then was dialyzed extensively against the same solution. Further purification of the DNA was made by subsequent treatment with ribonuclease A, proteinase K, phenol, and ethanol precipitation. The DNA thus obtained was dissolved in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and was stored at 4 °C.

Ten μg of the DNA was digested with various restriction endonucleases, subjected to electrophoresis in 0.7% agarose gel, and transferred to a nitrocellulose membrane (19). The membrane was probed with a 32P-labeled probe at 65 °C for 15 min. The DNA precipitated after the addition of an equal volume of isopropanol alcohol was dissolved in 2 ml of 10 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, then was dialyzed extensively against the same solution. Further purification of the DNA was made by subsequent treatment with ribonuclease A, proteinase K, phenol, and ethanol precipitation. The DNA thus obtained was dissolved in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and was stored at 4 °C.

Preparation of Anti-tachyplesin Antibody—Tachyplesin I peptide was coupled to bovine serum albumin, using a bifunctional cross-linking reagent, N-(γ-maleimidobutyryloxy) succinimide (Dojindo Laboratories, Kumamoto), as described (20, 21). The conjugate was coupled with tachyplesin I. Elution was performed with 0.1 M glycine-HCl, pH 2.8.

**Subcellular Localization**—Hemolymph was collected into sterile tubes and immediately fixed in 0.1 M sodium cacodylate buffer, pH 7.4, containing 4% paraformaldehyde and 1.2% glutaraldehyde. 4.5 mM CaCl2 and 0.1% sucrose at 0 °C for 30 min. The hemocytes were collected by centrifugation and washed several times with 0.1 M sodium cacodylate, pH 7.4. The specimens were dehydrated with a gradual increase of methanol concentration and then embedded in LR Gold (Polaron Instruments Inc., Cambridge, MA), which was polymerized by ultraviolet irradiation. Ultrathin sections were prepared using a diamond knife. The sections were mounted on a stainless steel grid, and washed with distilled water. Following blocking with 10 mM Tris-HCl, pH 7.2, containing 1% (w/v) bovine serum albumin and 0.5 M NaCl, the sections were incubated for 1 h with anti-tachyplesin IgG (0.7 μg/ml) in the same albumin containing buffer at room temperature. The sections were washed six times with 10 mM Tris-HCl, pH 7.2, containing 0.5 M NaCl and then with distilled water, the sections were double stained with uranyl acetate and lead tartrate and examined under an electron microscope (Hitachi H-500).

**RESULTS**

**cDNA Cloning**—Based on the sequence from amino-terminal Lys-1 to Val-6 of tachyplesin I, an oligonucleotide of 17 nucleotides was synthesized as a mixture of 64 sequences (Fig. 1). With the 32P 5'-end-labeled probe, a hemocyte cDNA library constructed in λgt11 was screened. Of four positive clones isolated, one clone, XTPNI-A3, contained the sequence encoding tachyplesin I, although the 5' upstream region contained an unusual sequence, poly(T), presumably derived from cloning artifacts (data not shown). To remove the region of poly(T), a TaqI fragment containing the 3'-terminal of the XTPNI-A3 insert was prepared from a plasmid, pTPNI-A3, in which the insert was in an EcoRI site of pUC 118 used as a vector. With this probe, the cDNA library was again screened. This procedure yielded numerous positive plaques, estimated to be approximately one positive/200 of the plaques screened. Fifteen positive clones were isolated and the four longest were sequenced (Fig. 2). Three clones (XTPNI-B2, B3, and B11) of the four contained the same sequence as that shown in Fig. 3. When this sequence was compared with that of XTPNI-A3, the XTPNI-A3 contained a fragment corresponding to nucleotide 118-243 (Fig. 3) followed by a 64-base sequence of XTPNI-B4, which was an additional sequence at the 5' terminus. This sequence was not present in the other clones examined.

To confirm whether these discrepancies were due to cloning artifacts, a cDNA library was rescreened with XTPNI-B11 or the 5'-terminal fragment of XTPNI-B4 serving as probes. The clones hybridizing with the probe specific for XTPNI-B4 did not.

**FIG. 1. Synthetic oligonucleotide probe used for screening.** The amino acid sequence of tachyplesin I (amino acid 1-6), the corresponding codons, and the sequence of the synthesized oligonucleotide are shown. The oligonucleotide was synthesized using an Applied Biosystems 380A DNA Synthesizer and a mixture of 64 sequences. The bottom line shows the sequence of the isolated cDNA.
Fig. 2. Restriction map and sequencing strategy of the tachyplesin precursors cDNA. A box in the restriction map represents an open reading frame. The region encoding mature tachyplesin is shown by a black box. A+ (from nucleotides 1-567 in Fig. 3), XTPNI-B2 (1-520), XTPNI-B3 (93-528), and XTPNI-B11 (142-531) encode tachyplesin I and XTPNI-C3 (3-548) is for tachyplesin II. The nucleotide sequence analysis was carried out with an Applied Biosystems 370A DNA Sequencer in directions and distance indicated by arrows, after subcloning into pUC 118 vector.

Fig. 3. Nucleotide and deduced amino acid sequences of tachyplesin precursors. Nucleotide (upper) and deduced amino acid sequences (lower) of tachyplesin I precursor are shown. The amino acid sequences are numbered from the amino terminus of mature tachyplesin I and the sequence is underlined. The sequences for tachyplesin II encoded by XTPNI-C3 are shown below the sequence of tachyplesin I, in italics. The polyadenylation signal (AA-TAAA) is double-underlined.

FIG. 4. Northern blot analysis of RNAs. Twenty pg of total RNAs from various tissues were separated by 1.4% agarose-formamide gel electrophoresis, blotted to GVHP membrane, and hybridized with 32P-labeled XTPNI-C1 insert. The positions of RNA size markers are shown on the left. The numbers indicate size in kilobases. 1, hemocytes; 2, hepatopancreas; 3, heart; 4, stomach; 5, intestine; 6, muscle; 7, brain; 8, coxal gland. See "Experimental Procedures" for details.
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extraordinarily strong, this peptide was assumed to be abundantly expressed in hemocytes.

Among RNAs from various tissues of the horseshoe crab, (hepatopancreas, heart, stomach, intestine, muscle, brain, and coxal gland), RNA from the heart showed an intense band with the same mobility as that of the hemocytes. RNA from the brain also showed a relatively intense band. The other tissues examined showed a faint band with longer exposure of the film. It was not clear, however, whether these bands were derived from small amounts of contamination by hemocytes in the dissected tissues or whether they actually were expression of small amounts of tachyplesin.

Southern Blot Analysis—The gene of the tachyplesin precursor was examined by Southern blot analysis of the XTPNICT insert used for Northern blotting (Fig. 5). The genomic DNA was completely digested by four different restriction enzymes (EcoRI, HindIII, PstI, and XbaI), none of which digested the obtained cDNA. The relatively large bands strongly hybridized with the probe. In the EcoRI digest, three bands, approximately 11.0, 9.5, and 8.5 kbp, were observed. Three bands (17.0, 14.5, and 11.0 kbp) in the HindIII digest, two bands (29.0 and 11.0 kbp) in the PstI digest, and two bands (18.5 and 2.5 kbp) in the XbaI digest were also observed, respectively.

Subcellular Localization—Antibody to tachyplesin I conjugated with bovine serum albumin was raised in rabbits and the localization of tachyplesin in hemocytes was investigated. Sections of the fixed hemocytes were stained with anti-tachyplesin IgG and gold particle conjugated. When this preparation was analyzed by electron microscopy, numerous gold particles were present in the granules (Fig. 6). Although two types of granules, large but less dense, and smaller but dense, have been identified (23, 24), the gold particles were mainly found in the latter, as shown in Fig. 6. The former contained coagulation factors (25), such as pro clotting enzyme, factor III, and coagulin and are secreted from the cells following lipopolysaccharide stimulation (26). The function of the smaller granules containing the tachyplesin peptide remains to be determined.

DISCUSSION

Naturally occurring biologically active substances that exert inhibitory effects on growth of microbes are present in the hemolymph of invertebrate animals (27). However, most of the bactericidal activity so far described has not been characterized in terms of structure and function except for the cecropin family from Lepidopteran insects (28), the sarcotoxin family from Sarcophaga peregrina (flesh fly) larvae (29-31), and the phormin family from Diptera (32).

The tachyplesin family, including polyphemusins, are potent antimicrobial peptides detected in our laboratory in acid extracts of hemocytes from Asian and American horseshoe crabs (11-13). These substances are composed of 17 or 18 amino acid residues and are abundant in the hemocytes, estimated as approximately 10 mg in the total hemolymph of individual horseshoe crabs. Interestingly, in the tachyplesin family there is a characteristic structure with three tandem repeats of a tetrapeptide sequence, namely hydrophobic amino acid-Cys-aromatic amino acid-Arg(Lys), suggesting that the amphipathic nature is closely associated with biological activity (11). These interesting properties led to the cloning of the cDNA encoding the precursor, and we examined the tissue distribution of the mRNA in the horseshoe crab.

The amino acid sequence deduced from the cloned cDNA sequence for the tachyplesin precursor revealed that it consists of 77 residues, including the tachyplesin sequence, and that there are at least two types of mRNAs corresponding to tachyplesins I and II. The existence of two mRNA encoding slightly different tachyplesin sequences was unexpected as we first considered a tandem repeat of the tachyplesin peptide sequence in the precursor mRNA. Since the mRNA had been extracted from the hemocytes of several horseshoe crabs, we are not sure as to whether the different sequences we observed represent the mRNA species derived from a single crab. However, the Southern blot analysis using genomic DNA prepared from the hemocytes does suggest that the two mRNAs for tachyplesin precursors are encoded by multiple genes.

The tachyplesin precursor consists of a single peptide of 23 amino acid residues, a mature peptide followed by an amidation signal "Gly-Lys-Arg" and an additional carboxyl-terminal sequence of 34 residues, including an acidic amino acid cluster. Evidence of these structural segments led to the notion that mature tachyplesin may be generated through conventional processing mechanisms. First, release of the signal peptide; next, cleavage between Arg-Asn at positions 20 and 21 by a processing enzyme which recognizes the dibasic

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sequence (Lys-Arg) of the large precursor; third, release of Lys-19 and Arg-20 by a carboxypeptidase B-like enzyme; and finally, oxidative amidation catalyzed by an enzyme recognizing the carboxyl-terminal glycine residue of the precursor peptide. Another possibility is that the amino-terminal signal sequence of the tachyplesin precursor is processed in a stepwise fashion by a signal peptidase followed by a dipeptidyl aminopeptidase, since the Glu-Ala-Glu-Ala sequence preceding the mature tachyplesin peptide is present in the carboxyl-terminal portion of the signal peptide. While the functional significance of the carboxyl-terminal portion consisting of 34 amino acid residues in the precursor is unknown, this portion contains an unusual acidic amino acid cluster consisting of 9 residues. This acidic region may interact with a cationic part of the tachyplesin peptide to stabilize a conformation of the precursor suitable for proteolytic degradation. The acidic sequence may also function as a signal to transport tachyplesin to the smaller granules in the hemocytes.

Northern blot analyses of total RNA prepared from various tissues indicated that the tachyplesin precursor is expressed exclusively in the hemocytes, in accordance with the high levels of tachyplesin peptides recovered therefrom. The mRNAs from heart and brain tissues also contain a transcript identical in size to that found in the hemocytes, but the signals are relatively weak, at least under our experimental conditions. The amount of tachyplesin precursor mRNA in other tissues including hepatopancreas, stomach, intestine, muscle, and coxal gland is negligible. Although little is known of the hematopoietic production of hemocytes in horseshoe crabs, our observations suggest that cardiac tissue may be the site of formation of circulating hemocytes.

The subcellular localization of tachyplesin in the hemocytes proved to be in the smaller granules, as determined immuno histochemically. While the larger granules are known to be essential for hemolymph coagulation, little is known of the smaller but dense granules. Experiments are ongoing to determine if tachyplesin is discharged from the cell on lipopoly saccharide stimulation, since tachyplesin was detected by us in acid extracts of hemocyte debris (11–13). Studies on dynamic changes in the intracellular localization of molecules responsible for biological defense systems will contribute to elucidation of the functions of tachyplesin in vivo.

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REFERENCES
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