Molecular cloning of cDNA for sapecin B, an antibacterial protein of *Sarcophaga*, and its detection in larval brain

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Abstract **A cDNA clone for sapecin B, an antibacterial protein of *Sarcophaga*, was isolated. This cDNA encoded a precursor protein of sapecin B consisting of a signal sequence (24 residues), prosegment (30 residues) and mature sapecin B (34 residues). Sapecin B was synthesized almost exclusively in the fat body when the larval body wall was injured, but the brain of naive larvae was also demonstrated to contain a significant amount of sapecin B. These findings suggested that sapecin B is a bifunctional protein.**

Key words: Sapecin B; cDNA; Antibacterial protein; Brain; Potassium channel inhibitor

1. Introduction

Dipteran insects are known to have the ability to induce antibacterial proteins in response to bacterial infection or body injury [1–3]. Sapecin is one of these antibacterial proteins of *Sarcophaga peregrina* (flesh fly), and has been shown to have potent antibacterial activity against various bacteria [4]. Recently, we purified two sapecin homologues named sapecin B and C from this insect [5]. Sapecin and sapecin C each consisted of 40 amino acid residues, and show 78% sequence identity, whereas, sapecin B consisted of 34 residues, and its maximum sequence identity with sapecin is 41%, indicating that sapecin B is less similar than sapecin C to sapecin.

On the other hand, significant similarity was found between sapecin B and charybdotoxin (a scorpion venom toxin) [5]. On alignment to give maximum matching, 11 of its 34 residues were shown to be identical with those of charybdotoxin. Moreover, like charybdotoxin, sapecin B was found to be a potent inhibitor of calcium-activated potassium channels of rat cerebella Purkinje cells [6]. These findings suggested that sapecin B is a bifunctional molecule: one function is to kill infecting bacteria in the acute phase and the other to regulate potassium currents in the brain [7].

This paper reports molecular cloning of sapecin B cDNA. The structural organization of sapecin B cDNA was the same as that of sapecin cDNA, suggesting that these two genes are derived from a common ancestral gene [8]. However, only sapecin B was detected in the brain of naive larvae, although both sapecin and sapecin B are induced in response to larval body injury.

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2. Materials and methods

2.1. Experimental materials

Third instar larvae of *Sarcophaga* were used. Hemolymph and hemocytes were collected as described before [9]. The fat body and brain were excised from the larvae with fine forceps under a binocular microscope. For enhancement of defense mechanism, larvae were pricked with a hypodermic needle. An embryonic cell line of *Sarcophaga*, NIH-Sape-4, was routinely cultured in M-M medium [10]. Sapecin was purified from the culture medium of NIH-Sape-4 as described before [4]. Sapecin B was synthesized chemically [11].

2.2. Cloning of sapecin B cDNA

As NIH-Sape-4 cells are known to synthesize sapecin B constitutively, a cDNA library of NIH-Sape-4 cells was constructed by the method of Okayama and Berg [12]. The cloning strategy is shown in Fig. 1. Based on the amino acid sequence of sapecin B, two primers, N1 and C1, were synthesized. The polymerase chain reaction (PCR) was performed with these primers using the cDNA library of NIH-Sape-4 as template, and the nucleotide sequence of the resulting PCR product was determined. Based on this sequence, two other primers, N2 and C2, were synthesized. In addition to these primers, two primers derived from Okayama and Berg vector (F and R) were synthesized. PCR was performed with combinations of N2 and R, and C2 and F, respectively. From these PCR products, sequences of the 5′ and 3′ regions of sapecin B cDNA were determined. Finally, PCR was performed using two primers N (5′ACTCTTCTTGAAACTATTCA3′, sense strand) and C (3′TTTCAAACATCTACACATGT5′, antisense strand) that correspond to the 5′ and 3′ end of sapecin B cDNA, respectively. This PCR product was cloned and the complete sequence of sapecin B cDNA was determined.

2.3. Northern blot hybridization

This was done essentially as described before [8]. Electrophoresis of RNA (10 μg/lane) was performed in horizontal slab 1.2% agarose gel containing 2.2 M formaldehyde in 20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0). After electrophoresis, RNA was blotted onto nitrocellulose filters. Hybridization was performed in 50% (v/v) formamide/5 × SSC/5 × Denhaldt's solution/sonicated salmon sperm DNA solution (50 μg/ml) for 18 h at 42°C. Then the filters were washed and autoradiographed. Sapecin cDNA and sapecin B cDNA were used as probes.

2.4. Radioimmunoassay

We have reported radioimmunoassay of sapecin [13]. Radioimmunoassay of sapecin B was performed essentially according to our previous procedure. Antibody against sapecin B did not cross-react immunologically with sapecin and vice versa. However, for safety, we used affinity-purified antibody as described before [14]. Radioiodination of sapecin B was performed by a modification of the method of Hunter and Greenwood [15]. Labeled sapecin (10^4 cpm), antibody and serially diluted sapecin B were incubated. After precipitaiting the immune-complex, the radioactivity in the supernatant was measured. Radioactivity in the supernatant increased linearly with increase in the amount of unlabeled sapecin B. For determination of the amount of sapecin B in the sample, serially diluted samples were added to the reaction mixture instead of sapecin B. The amount of sapecin B was determined from the dose–response curve obtained with unlabeled sapecin B.

2.5. Other methods

DNA manipulations including restriction enzyme digestion, gel elec-

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trophoresis, DNA ligation, plasmid isolation and *E. coli* transformation were carried out by standard methods. Immunoblot analysis was performed as described before [16]. Protein was determined by the method of Lowry et al. [17].

3. Results

3.1. cDNA cloning of sapecin B

cDNA cloning of sapecin B was performed by repeated PCR using an NIH-Sape-4 cDNA library as a template. The eight primers and their orientations used for PCR are summarized in Fig. 1 with the determined structural organization of sapecin B cDNA. We first obtained cDNA corresponding to sapecin B. Then we performed two PCR experiments to determine the 5′- and 3′-untranslated regions of sapecin B cDNA. Finally, we performed PCR with primers of the 3′- and 5′-ends of sapecin B cDNA. This PCR product was ligated with a plasmid vector and transformed into *E. coli*. We selected hybridization-positive clones, isolated plasmids, and determined the sequences of their inserts. From these experiments, we determined the complete sequence of sapecin B cDNA to be as shown in Fig. 2 with the putative amino acid sequence.

This cDNA contained an open reading frame of 264 nucleotides corresponding to 88 amino acid residues. The amino acid sequence of sapecin B was located in the carboxyl terminal of this precursor protein. Judging from the hydropathy profile (data not shown) and specificity of signal peptidase of *Sarcophaga*, we concluded that the first 24 residues are a signal sequence. Thus, the intervening 30 residues correspond to a prosegment. A common potential cleavage signal, Lys–Arg, was located between sapecin B and the prosegment. This organization of sapecin B cDNA was the same as that of sapecin cDNA [8].

3.2. Expression of the sapecin B gene in an acute phase

Previously, we demonstrated that the sapecin gene was preferentially expressed in the hemocytes and fat body when the larval body wall was injured [8]. Under the same conditions, we examined expression of the sapecin B gene by Northern blot hybridization. As shown in Fig. 3A, sapecin B mRNA was detected almost exclusively in the fat body of injured larvae. Therefore, expression of the sapecin B gene is different from that of the sapecin gene (Fig. 3B).

To confirm the synthesis of sapecin B in the fat body, we measured the amount of sapecin B in the fat body and hemolymph with time after injury by radioimmunoassay. As shown in Fig. 4, the amounts of sapecin B in the fat body and hemolymph were very low at time 0, but increased rapidly to maxima after 20 h. Then they decreased gradually, but significant amounts of sapecin B were detected even after 80 h. Therefore, we concluded that sapecin B is synthesized in the fat body in response to body injury, and secreted into the hemolymph to prevent bacterial infection.

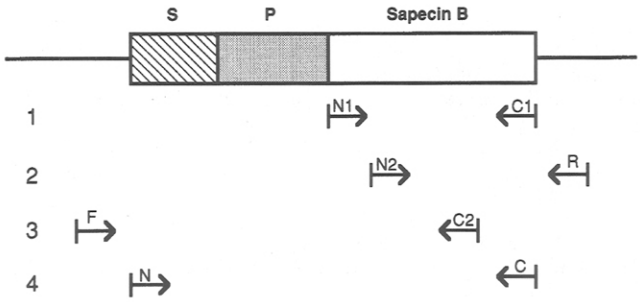

Fig. 1. Strategy of cDNA cloning of sapecin B by PCR. The structural organization of cloned cDNA is shown at the top. S and P indicate the signal sequence and prosegment, respectively. Horizontal lines show Okayama-Berg vectors. Sequential PCR was performed with the indicated primers. Arrows show primers with their orientations.

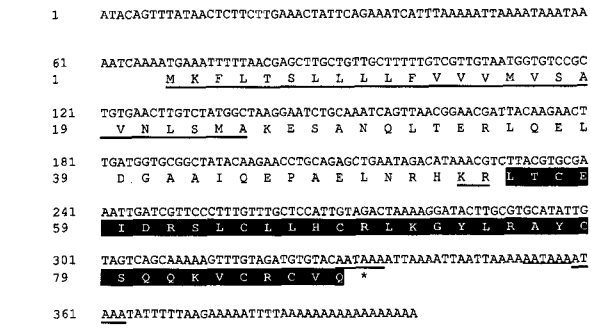

Fig. 2. Nucleotide sequence of sapecin B cDNA. The deduced amino acid sequence of the precursor protein is shown below the nucleotide sequence. The sequence of sapecin B is shaded. Amino acid residues are numbered from the first Met residue. The putative signal sequence, potential processing signal, and poly(A) addition signals are underlined. The asterisk indicates the termination codon.

3.3. Presence of sapecin B, but not sapecin, in the brain of normal larvae

Previously we demonstrated that, like charybdotoxin, sapecin B inhibited calcium-activated potassium channels of rat cerebella Purkinje cells [6]. These results suggested that sapecin B is a regulator of potassium channels in this insect. To examine this possibility, we tried Northern blot hybridization with brain of naive larvae. However, extraction of intact RNA from the brain was technically difficult. Therefore, we tested for sapecin B in the brain by radioimmunoassay. As summarized in Table 1, sapecin B, but not sapecin, was detected in the brain of naive larvae. A significant amount of sapecin was detected in the brain of injured larvae, but this may have been due to hemolymph contaminating the brain preparation, since sapecin is induced in the hemolymph when the body wall is injured.

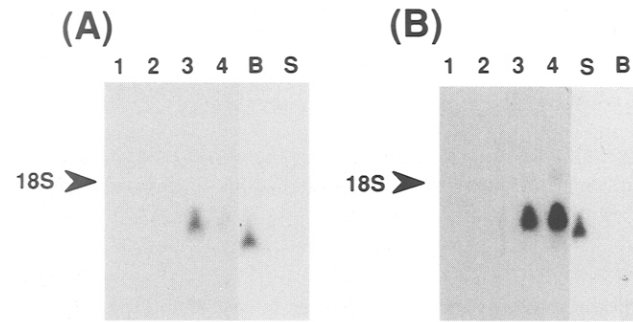

Fig. 3. Detections of sapecin B and sapecin mRNA. RNA was extracted from fat body and hemocytes, and subjected to Northern blot hybridization with sapecin B cDNA (A) and sapecin cDNA (B) as probes. Each lane contained 10 μg of RNA. Lane 1 = fat body of naive larvae; lane 2 = hemocytes of naive larvae; lane 3 = fat body of injured larvae; lane 4 = hemocytes of injured larvae. Sapecin B and sapecin cDNA (each 30 pg) were electrophoresed in lanes B and S, respectively. The fat body and hemocytes were collected 6 h after injury of larvae.

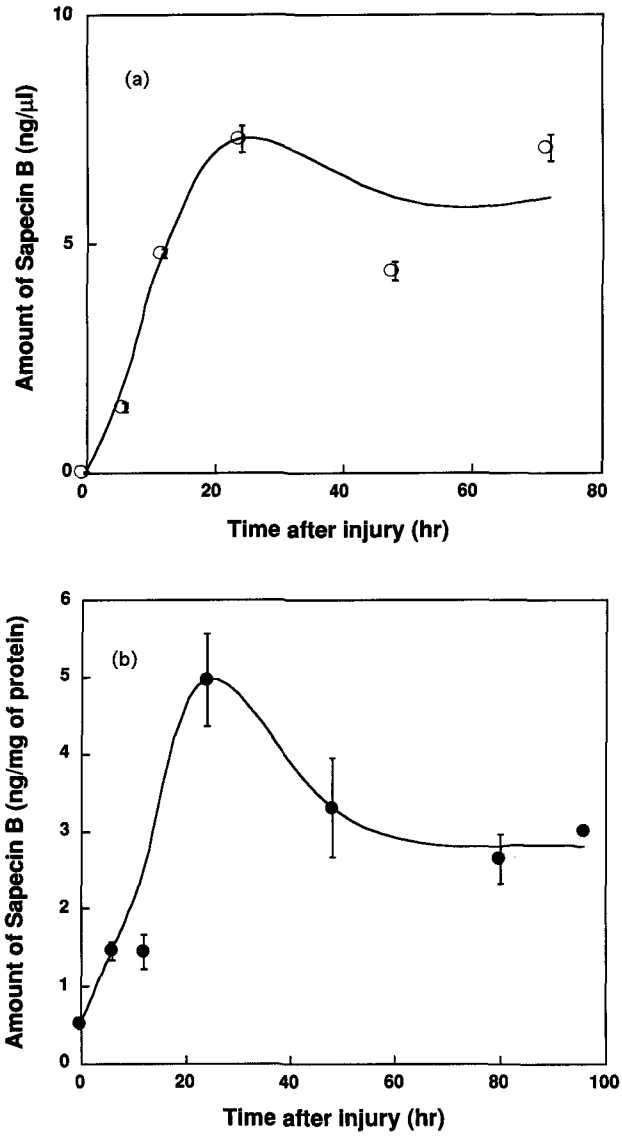

Fig. 4. Change in the amount of sapecin B in response to body injury. Larvae were injured at time 0. The fat body and hemolymph were collected at the indicated times after injury, and contents of sapecin B were measured by radioimmunoassay. Each point represents the mean of duplicate measurements with deviation. (a) Hemolymph; (b) fat body.

4. Discussion

In this study, we found that the structural organization of sapecin B cDNA was the same as that of sapecin cDNA. Namely, it consisted of a signal peptide, prosegment and sapecin B. When the sequence of signal peptide and prosegment of the precursor of sapecin B is compared with that of the same region of the precursor of sapecin, the maximal sequence identity was less than 20%, which was much less than identity between sapecin and sapecin B [5]. Thus, if sapecin, sapecin B and sapecin C are derived from a common ancestral gene, the sapecin B gene seems to have evolved differently from the genes for sapecin and sapecin C.

Table 1
Contents of sapecin B and sapecin in larval brain. Means of duplicate measurements are given with deviations

	Sapecin B (ng/mg)	Sapecin (ng/mg)
Normal larvae	4.24 ± 0.19	0.04 ± 0.01
Injured larvae*	2.49 ± 0.25	1.06 ± 0.09

*Brain was collected 24 h after injury.

We found that sapecin B, but not sapecin, is present in the brain of normal larvae. Thus sapecin B in the brain is likely to regulate potassium channels. Nothing is known about potassium channels in *Sarcophaga* brain, but recently potassium channels were identified in *Drosophila* [18,19]. We assume that sapecin B is a bifunctional molecule: in normal larvae, it may be expressed in the brain to regulate potassium channels, while when the body wall is injured, it may be rapidly synthesized in the fat body and secreted into hemolymph to prevent bacterial infection.

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