The fate of the prosegment in the acute-phase and programmed synthesis of sapecin, an antibacterial peptide of the flesh fly (*Sarcophaga peregrina*)

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The nucleotide sequence of sapecin cDNA suggested that this antibacterial peptide of the flesh fly (*Sarcophaga peregrina*) is produced from preprosapecin by post-translational processing. We examined the production of sapecin and its prosegment by radioimmunoassay under two different physiological conditions in which its gene is activated, assuming that the prosegment has some biological role. Results suggested that the prosegment is degraded selectively during production of sapecin. We also found that imaginal discs synthesize sapecin when cultured in the presence of 20-hydroxyecdysone.

INTRODUCTION

Sapecin is an antibacterial peptide purified from the culture medium of NIH-Sape-4, an embryonic cell line of the flesh fly, *Sarcophaga peregrina* [1]. This peptide consists of 40 amino acids, including six cysteine residues forming three intramolecular disulphide bridges, and has strong affinity to cardiolipin, which is a major phospholipid of Gram-positive bacteria [2,3]. On Northern-blot analysis with cDNA for this peptide we obtained the following results.

(i) The sapecin gene is inactive in third-instar larvae, but is markedly activated in haemocytes when the larval body wall is injured [4]. From these findings we concluded that sapecin is an acute-phase protein and is secreted into the haemolymph by haemocytes to prevent bacterial infection caused by body injury.

(ii) This gene is also activated transiently in embryos and pupae in the absence of any external stimulus, suggesting that this peptide plays another role in the development of this insect [4]. In fact, we found that sapecin has growth-factor-like activity on NIH-Sape-4 cells [5].

Unlike sarcotoxin I and II, which are also antibacterial proteins of *Sarcophaga* [6,7], sapecin was suggested to be produced from preprosapecin by post-translational processing [4]. The reading frame of sapecin cDNA encodes 94 amino acid residues with sapecin at the C-terminus of the sequence. As the signal peptide is supposed to be the first 23 amino acid residues from its N-terminus, an unidentified prosegment between sapecin and the signal peptide should be produced during the biosynthesis of sapecin.

In the present study we investigated the production of sapecin and the prosegment by radioimmunoassay with antibodies of the respective peptides. We found that accumulation of the prosegment was significantly less than that of sapecin, suggesting that the prosegment is degraded preferentially in both acute-phase and programmed syntheses of sapecin.

MATERIALS AND METHODS

Insects and preparation of haemolymph and tissues

Flesh flies (*Sarcophaga peregrina*) were reared by the method of Ohtaki [8]. The physiological age of third-instar larvae can be synchronized by dry-wet treatment. When the larvae were transferred from wet conditions to dry conditions at 27 °C, they started to pupate 16 h later. The anterior tip of third-instar larvae was cut off with fine scissors; the drop of haemolymph

that exuded was collected in a Petri dish on ice, and haemocytes were separated from the haemolymph by centrifugation. Embryos were collected by cutting the posterior tip of female flies and gently squeezing the abdomen, as described previously [9].

Radioimmunoassay of sapecin and its prosegment

Sapecin was purified as described previously [1]. The prosegment of sapecin, consisting of 29 amino acid residues (SPAAAAEESKFVDGLHALKTIEPELHGRY), was synthesized chemically, and purified, and its sequence was confirmed. As sapecin and its prosegment are small peptides consisting of 40 and 29 amino acid residues respectively, for raising antibodies against them we coated 2 mg of reverse-phase column-packing material (silica-gel C-18) with 200 µg of these peptides by the method of Flyg *et al.* [10] and injected the preparations with complete Freund's adjuvant into male albino rabbits. A booster injection was given 14 days later, and animals were bled 7 days after the booster injection. The antisera obtained were specific for sapecin and prosegment respectively and showed no significant cross-reactivity with the other peptide on immunoblotting [5].

Radioiodinations of sapecin and its prosegment were performed by the method of Hunter & Greenwood [11]. The specific radioactivities of the labelled peptides were about 10⁷ c.p.m./µg. Radioimmunoassay with antiserum and labelled sapecin or its prosegment was performed as follows. Labelled peptide (10⁴ c.p.m.), antiserum and various amounts of unlabelled sapecin or prosegment were incubated in 520 µl of 75 mM-phosphate buffer, pH 7.2, containing 75 mM-NaCl, 0.1 % gelatin, 1 % Triton X-100 and 0.02 % Na₂S₂O₃ for 12 h at 4 °C. Then 50 µl of *Staphylococcus aureus* Cowan I solution, prepared by the method of Kessler, [12] was added. The reaction mixture was centrifuged at 8800 g for 10 min, and the radioactivity in the resulting supernatant was measured. Radioactivity in the supernatant increased linearly with the increase in the amount of unlabelled sapecin or prosegment. For determination of the amount of sapecin or prosegment in tissues, samples were diluted serially and added to the reaction mixture instead of unlabelled sapecin or prosegment. The amount of sapecin or prosegment was determined from the dose-response curve obtained with unlabelled peptide.

Culture of *Sarcophaga* imaginal discs *in vitro*

Leg imaginal discs were excised from third-instar larvae under a binocular microscope and cultured in Grace's insect medium

(Gibco) in the presence of 20-hydroxyecdysone, exactly as described previously [13]. Usually 30 discs were cultured in a single well containing 350 μ l of medium at 25 °C.

Immunofluorescence study

Haemocytes adhering to a coverslip were fixed by immersing the cover slip in phosphate-buffered saline (8 mM- Na_2HPO_4 , 12 mM- H_2O /1.5 mM- KH_2P_4 /130 mM- NaCl /3 mM- KCl , pH 6.8) containing 2% (v/v) formaldehyde for 20 min. Then the cells were permeabilized by immersing the coverslip in the same phosphate-buffered saline containing 0.5% (w/v) Nonidet P40 for 1 min. The coverslip was then transferred to 50 mM-Tris/HCl buffer, pH 7.4, containing 130 mM- NaCl and 1% (w/v) BSA. It was then thoroughly washed and treated successively with antiserum for 1 h and fluorescein isothiocyanate-conjugated anti-rabbit sheep IgG for 30 min. After final washing the haemocytes were examined under a fluorescence microscope.

RESULTS

Measurements of sapecin and its prosegment during their acute-phase and programmed syntheses

As reported previously, the sapecin gene in haemocytes is markedly activated when the body wall of *Sarcophaga* larvae is pricked by a hypodermic needle [4]. Therefore it was suggested that sapecin was synthesized by haemocytes and secreted into the haemolymph under these conditions. Moreover, judging from

Fig. 1. Schematic illustration of preprosapecin

Numbers of amino acid residues in each segment are indicated in parentheses. Putative cleavage sites are shown by arrows.

Fig. 2. Standard curves of radioimmunoassay

Fixed amounts of radioiodinated peptide and specific antiserum were incubated with increasing amounts of unlabelled peptide. The relative amount of radioactivity in the immunocomplex is plotted against the amount of competitor, taking the radioactivity without competitor as 100%. Open circles indicate competition of the prosegment with sapecin and vice versa. Each point represents the mean of duplicate measurements; except where vertical bars are shown, deviation was within the symbol. Standard curves for (a) sapecin and (b) the prosegment are shown.

the structure of its cDNA, sapecin was supposed to be synthesized as a preprosapecin [4] and then to be converted into sapecin by a two-step post-translational process, as illustrated in Fig. 1. During this process, a prosegment of sapecin consisting of 29 amino acid residues should be liberated. The same may be the case on activation of the sapecin gene in embryonic and pupal stages, although we have not yet identified the cells that synthesize sapecin in these stages. Moreover, nothing is known about the biological function of the prosegment, if any, in the defence system and in the development of this insect.

To obtain information about these points, we tried to measure the amounts of sapecin and its prosegment with time after activation of the sapecin gene. For this, we first established a radioimmunoassay system for sapecin and its prosegment. The assays were based on determination of the amounts of peptide that inhibited formation of immunocomplexes between the respective radioiodinated peptides and their antibodies. The standard curves obtained with serial dilutions of sapecin and chemically synthesized prosegment respectively are shown in Fig. 2. By this method it was possible to detect these peptides at nanogram levels in sample solutions. As sapecin did not interfere with the measurement of its prosegment and vice versa, we concluded that these systems were valid for measuring the amount of each peptide in tissue samples.

We examined the changes in the amounts of sapecin and its prosegment in the haemolymph with time after injury of the body wall of third-instar larvae. As is evident from Fig. 3(a), after this treatment the amount of sapecin in the haemolymph increased significantly for at least 10 h and then decreased gradually, but remained at above 4 μ g/ml for nearly 50 h. Thus, as the minimum inhibitory concentration of sapecin for *S. aureus* and certain other Gram-positive bacteria is about 5 μ g/ml, sapecin should prevent infection with sensitive bacteria under these conditions. No appreciable amount of the prosegment was detected in the haemolymph for at least the first 10 h after body-wall injury, although later it became detectable at a much lower level than that of sapecin. These results suggested that although sapecin is secreted into the haemolymph, its prosegment may stay in the haemocytes.

To examine this possibility, we measured the amounts of sapecin and its prosegment in the haemocytes. Results showed that the amount of prosegment increased linearly for 50 h, but that its accumulation was much less than that of sapecin. As equimolar amounts of sapecin and prosegment should be produced simultaneously, and the amount of the prosegment was much less than the total amount of sapecin in the haemolymph and haemocytes, the prosegment seems to be degraded rapidly as soon as it is produced. No significant accumulation of sapecin in the haemocytes was detected in the first 10 h, but thereafter its amount increased with the decrease in its content in the haemolymph, as shown in Fig. 3(a).

Thus sapecin seems to be secreted from haemocytes as soon as it is synthesized soon after body-wall injury, but later its secretion seems to stop, resulting in its accumulation in the cells.

This situation was confirmed by an immunofluorescence study. Haemocytes were prepared 1 h and 30 h after body-wall injury, and the localization of sapecin and its prosegment in the haemocytes was investigated by indirect immunofluorescence microscopy using respective antibodies. Significant fluorescence due to sapecin was detected only in the haemocytes prepared 30 h after body-wall injury, but fluorescence in the haemocytes prepared 1 h after body-wall injury was very faint (almost background level), as shown in Figs. 4(a) and 4(c) respectively. As sapecin in the haemolymph becomes detectable soon after body-wall injury, these results again show the accumulation of sapecin in the haemocytes with time after body-wall injury.

Fig. 3. Changes in the amounts of sapecin and prosegment in acute-phase expression

Larvae were injured at zero time. Haemolymph and haemocytes were collected with time and their contents of sapecin and prosegments were determined separately by radioimmunoassay. Each point represents the mean of duplicate measurements; deviations greater than the symbol size are shown. Samples were from (a) haemolymph or (b) haemocytes. $\circ$, Sapecin; $\bullet$, prosegment.

Fluorescence due to the prosegment was at background levels in both haemocyte preparations (results not shown).

As the sapecin gene is activated in the embryonic stage and pupal stage [4], we examined the syntheses of sapecin and its prosegment at these stages. The specific contents of sapecin in embryos and pupae were one order of magnitude less than that in haemocytes from injured larvae, but clearly increased during development of embryos and pupae, as shown in Figs. 5(a) and 5(b). The content of the prosegment was much less than that of sapecin at both stages, suggesting that the prosegment is rapidly degraded in the embryos and pupae as soon as it is produced.

Therefore we concluded that, in the post-translational processing of sapecin, the prosegment is degraded selectively after its separation from the N-terminus of sapecin.

Production of sapecin by imaginal discs

Previously we demonstrated that sapecin stimulates the proliferation of NIH-Sape-4 cells in Grace's insect medium supplemented with 3% (v/v) fetal-calf serum [5]. Possibly it acts as a growth factor for certain cells in the embryos. However, nothing is known about the function of sapecin in the pupal stage. In the pupal stage, most larval tissues are decomposed and adult structures develop from the imaginal discs. Therefore, we examined whether imaginal discs synthesize sapecin during their differentiation. As reported previously, *Sarcophaga* imaginal discs can differentiate into adult structures *in vitro* in the presence of 20-hydroxyecdysone [13]. Using this system, we measured the amount of sapecin in the culture medium of leg imaginal discs by radioimmunoassay. As shown in Fig. 6, we found that leg discs

Fig. 4. Immunofluorescence study of sapecin

Haemocytes were prepared 1 h and 30 h after body-wall injury. Fixed haemocytes were treated successively with antiserum against sapecin and fluorescent-labelled second antibody. (a) Fluorescence microscopy of anti-sapecin-antibody-treated haemocytes prepared 30 h after body-wall injury; (b) bright-field microscopy of (a); (c) fluorescence microscopy of anti-sapecin-antibody-treated haemocytes prepared 1 h after body-wall injury; (d) bright-field microscopy of (c); (e) fluorescence microscopy of preimmune-serum-treated haemocytes prepared 30 h after body-wall injury; (f) bright-field microscopy of (e). The bars represent 30 μm .

Fig. 5. Changes in the amounts of sapecin and prosegment during programmed expression

Embryos were collected from female flies on the indicated days after emergence. Embryos on day 7 are mature oocytes just after fertilization and those on day 11 are almost first-instar larvae. Pupae were collected on the indicated days after pupation. Imagoes appear on day 10. The contents of sapecin and prosegment in the tissue samples were determined separately by radioimmunoassay. Each point represents the mean of duplicate measurements; deviations are shown where larger than the symbols. Samples were from (a) embryos or (b) pupae. $\circ$, Sapecin; $\bullet$, prosegment.

Fig. 6. Radioimmunoassay of sapecin in the culture medium of leg imaginal discs

Leg discs were cultured in Grace's insect medium in the presence (○) or absence (●) of 20-hydroxyecdysone. On the indicated days the amount of sapecin in the culture medium was measured. Each point represents the mean of duplicate measurements; deviations are shown where larger than the symbol.

secreted a significant amount of sapecin into the medium when cultured *in vitro* in the presence of 20-hydroxyecdysone, but not in its absence. Therefore production of sapecin in the pupal stage is partly due to its synthesis by imaginal discs.

DISCUSSION

Sapecin is a defensin-type antibacterial peptide of *Sarcophaga* [1] and is very similar to a peptide recently found in *Phormia terranova*, another dipteran insect [14]. Unlike other antibacterial proteins of *Sarcophaga*, sapecin seems to undergo post-translational processing, since there is a fairly long prosegment between the signal peptide and intact sapecin, and a processing signal Lys-Arg at the junction between the prosegment and sapecin [4].

It seemed possible that, besides sapecin, the prosegment might play a role in the defense or development of this insect. Accordingly we measured the contents of sapecin and its prosegment in various tissue samples by radioimmunoassay. We found that the content of prosegment was significantly lower than that of sapecin in all samples examined, suggesting that the prosegment is degraded selectively and has no biological function. However, it is noteworthy that the specific content of the prosegment in embryos was higher than that in the pupae, although the specific contents of sapecin at the two stages were similar. Furthermore, the embryos isolated from female flies of 7–8 days old seemed to contain about equimolar amounts of sapecin and prosegment, although their specific contents were below 10 ng/mg of protein. *Sarcophaga* is an ovoviviparous

insect and embryogenesis starts in the uterus of 6-day-old females. At present we cannot assess the significance of these results.

The present results suggested the existence of some mechanism regulating the secretion of sapecin from haemocytes. Although larval haemocytes secrete sapecin actively for at least 10 h after injury of the epidermis, later they seem to stop secreting sapecin, because at this time sapecin started to accumulate in the haemocytes with a concomitant decrease in its content in the haemolymph. Sapecin is probably gradually degraded in haemolymph. But why do haemocytes stop secreting sapecin although they continue to synthesize it? Possibly the sapecin content of the haemolymph is important. If it exceeds a certain level in the haemolymph, it may cause negative feedback of its secretion from the haemocytes.

Imaginal discs synthesize sapecin in response to 20-hydroxyecdysone. We have shown that imaginal discs secrete *Sarcophaga* lectin, another defence protein of *Sarcophaga* [15], during development and that this lectin stimulates their differentiation in an autocrine manner [13]. *Sarcophaga* lectin is essential for morphogenesis of imaginal discs. However, it remains to be elucidated whether sapecin is, like *Sarcophaga* lectin, needed for the differentiation of imaginal discs.

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