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# Involvement of sapecin in embryonic cell proliferation of *Sarcophaga peregrina* (flesh fly)

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Addition of antibodies against sapecin to the culture medium of NIH-Sape-4 cells derived from a *Sarcophaga* embryo greatly inhibited cell proliferation, whereas addition of sapecin stimulated cell proliferation. These results suggest that sapecin is involved in the proliferation of embryonic cells of *Sarcophaga*. Sapecin is known to have potent antibacterial activity, so it seems to have two different biological functions: i.e. protection against bacterial infection and stimulation of embryonic cell proliferation.

Sapecin; Growth factor; Embryonic cell; *Sarcophaga peregrina*

## 1. INTRODUCTION

Sapecin is an antibacterial protein of *Sarcophaga peregrina* (flesh fly) consisting of 40 amino acid residues with three intramolecular disulfide bridges [1–3]. It has a more potent effect on Gram-positive bacteria than on Gram-negative ones [1]. Its primary target is thought to be the bacterial membrane, because it preferentially binds to cardiolipin in the membrane [4]. In fact, an *Escherichia coli* mutant with a defect in cardiolipin synthesis was found to be more resistant to sapecin than wild type *E. coli* [4]. Sapecin was originally purified from the culture medium of NIH-Sape-4 cells [1], an embryonic cell line of *Sarcophaga*, and was subsequently found to be induced in the larval hemolymph in response to body injury [2], like other *Sarcophaga* defense proteins such as *Sarcophaga* lectin [5] and sarcotoxin I [6]. Therefore, sapecin is also likely to be a defense protein protecting this insect from infection by pathogenic bacteria invading through a damaged body wall.

Northern blot hybridization using sapecin cDNA revealed that the sapecin gene is expressed twice during the life cycle of this insect without any outside stimulus: first in the embryonic stage and again in the pupal stage [2]. Thus its expression is similar to those of the genes for *Sarcophaga* lectin [7] and sarcotoxin I [8]. We studied the role of *Sarcophaga* lectin during the development of *Sarcophaga*, and found that this lectin is essential for the development of imaginal discs; namely, in the pupal stage, developing imaginal discs secrete *Sarcophaga* lectin and this stimulates further development of the discs in an autocrine manner [9]. From these findings, we proposed the new concept that some insect defense proteins play at least two independent roles, in defense and in development [10].

This paper describes the stimulating effect of sapecin on proliferation of NIH-Sape-4 cells. The results indicate that sapecin also may have dual functions, to kill invading bacteria and to stimulate cell proliferation during ontogeny.

## 2. MATERIALS AND METHOD

### 2.1. *Cells and culture media*

The *S. peregrina* embryonic cell line NIH-Sape-4 was used throughout. Usually the cells were cultured at 25°C in M-M medium [11], which contained yeast extract and lactoalbumin hydrolyzate. The cells proliferated well in this medium and released sapecin, as described before [1]. In experiments on the effect of exogeneously added sapecin on cell proliferation, we used Grace's insect medium (Gibco) supplemented with 3% fetal calf serum (FCS), 120 U/ml of penicillin G, 0.5 mg/ml of streptomycin and 120 U/ml of nystatin, because cells could not proliferate in this medium or produce sapecin, but remained viable.

### 2.2. *Sapecin, its antibody and sarcotoxin IA*

Sapecin was purified from the culture medium of NIH-Sape-4 cells as described before [1], about 1 mg of sapecin being obtained from 2.5 liters of culture medium. Antibody was raised against sapecin in male albino rabbits. As sapecin is a small protein, for this purpose we coated 2 mg of reverse-phase column packing material (Silica gel-$C_{18}$) with 200 μg of sapecin and injected it with complete Freund's adjuvant by the method of Flyg et al. [12]. A booster injection of 200 μg of sapecin in incomplete Freund's adjuvant was given 14 days later, animals were bled 7 days after the booster injection, and IgG was purified by the method of McCauley and Racker [13]. Sarcotoxin IA was synthesized chemically as described before [14].

### 2.3. *Electrophoresis and immunoblotting*

Electrophoresis on SDS-polyacrylamide slab gels was carried out by the method of Laemmli [15]. Samples were denatured by heating them

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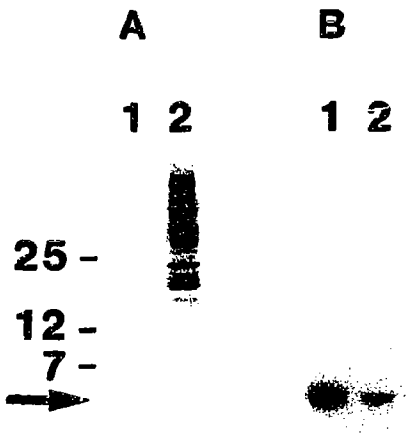


Fig. 1. Specificity of the antibody against sapecin. NIH-Sape-4 cells were cultured in M-M medium for 4 days. The culture medium was subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene difluoride membrane filters. The blotted filters were treated with anti-sapecin IgG, and then probed with radioiodinated second IgG to detect sapecin. A. Coomassie brilliant blue staining of the SDS polyacrylamide gel. B. Autoradiogram. Lane 1, authentic sapecin (50 ng); lane 2, protein in the culture medium (10 μg). Molecular mass standards (kDa) are shown on the left. The arrow indicates the position of authentic sapecin.

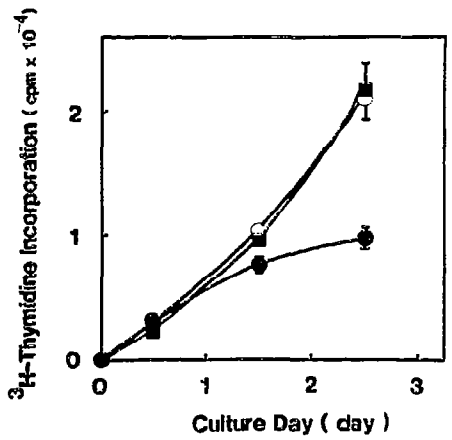


Fig. 2. Inhibition of cell proliferation by anti-sapecin IgG. NIH-Sape-4 cells were seeded into 500 μl of M-M medium at a density of $1.5 \times 10^5$ cells/ml. The medium contained 80 μg of anti-sapecin IgG (●) or normal IgG (○), or no addition (■). Culture was carried out at 25°C, and after 0, 24 h or 48 h of culture, 1 μCi of [$^3$H]thymidine was added to the medium. The cells were harvested 24 h later and the radioactivity incorporated into DNA was measured.

for 2 min at 100°C in 1% (w/v) SDS and 2% (v/v) 2-mercaptoethanol, and after electrophoresis, the gels were stained by the method of Fairbanks et al. [16].

Immunoblotting was performed essentially as described before [17]. Proteins separated by electrophoresis were transferred electrophoretically from the gel to polyvinylidene difluoride membrane filters. Then the filters were immersed in 5% skimmed milk solution for at least 1 h, transferred to the same solution containing antibody against sapecin (5 μg/ml) and kept at 4°C for 12 h. They were then washed well, transferred to 5% skimmed milk solution containing radioiodinated anti-rabbit IgG ($2 \times 10^6$ cpm) and kept for 4 h at 4°C. Finally they were washed well, dried and subjected to autoradiography.

### 2.4. *Measurement of cell proliferation*

Cell proliferation was measured as incorporation of [$^3$H]thymidine into DNA during culture for 24 h. For this, $1–4 \times 10^5$ NIH-Sape-4 cells were seeded in 500 μl of medium in wells and cultured at 25°C. DNA was labeled by adding 10 μl of the same medium containing 1 μCi of [$^3$H]thymidine (Amersham, 25 Ci/mmol) at an appropriate time and after 24 h, the medium was discarded and the cells were washed well with saline. Finally the cells were suspended in 0.5 ml of 0.5 N NaOH solution, 0.5 ml of ice-cold 60% trichloroacetic acid solution was added, and acid-insoluble radioactivity was trapped on a glass fiber filter (Whatman, GF/C) and measured.

## 3. RESULTS

### 3.1. *Effect of antibody against sapecin on growth of NIH-Sape-4 cells*

As the sapecin is activated transiently in the early embryonic stage of *Sarcophaga*, sapecin presumably plays some role in the embryonic development of *Sarcophaga*. NIH-Sape-4 cells are an established cell line derived from a *Sarcophaga* embryo [18], and the cells produce sapecin and secrete it into the medium when cultured in M-M medium [1]. As we are interested in the biological function of sapecin in the development of *Sarcophaga*, we tested if sapecin produced by NIH-Sape-4 cells is required for their growth. For this, we raised antibody against sapecin and tested if it inhibited the proliferation of NIH-Sape-4 cells. The specificity of the antibody against sapecin (IgG) used in this experiment is shown in Fig. 1. On immunoblotting, culture medium of NIH-Sape-4 cells containing sapecin gave a single band in the position of authentic sapecin, indicating that the antibody reacted specifically with sapecin. The effect of this antibody on proliferation of NIH-Sape-4 cells was examined by seeding $1.5 \times 10^5$ cells/ml in 500 μl M-M medium containing the antibody and adding [$^3$H]thymidine 0, 24 and 48 h later for 24 h periods. As shown in Fig. 2, DNA synthesis, measured as incorporation of [$^3$H]thymidine into the cells, was markedly repressed in the presence of the antibody against sapecin, but scarcely affected by normal IgG, as judged by comparison with that in control medium without additions.

### 3.2. *Effect of externally added sapecin on proliferation of NIH-Sape-4 cells*

As the antibody against sapecin inhibited cell proliferation, we next tested whether added sapecin restored proliferation. After being exposed to the antibody, no significant recovery of cell proliferation was observed in the presence of exogeneously added sapecin in M-M medium (data not shown). Therefore, we used Grace's insect medium. NIH-Sape-4 cells proliferate in this medium only in the presence of 5–20% FCS, and the extent of proliferation depended on the amount of added FCS; in the presence of 3% FCS, no significant cell proliferation is detected, but the viable cell number remains constant for at least 6 days.

Cells were cultured with various amounts of sapecin

in Grace's insect medium supplemented with 3% FCS for 24 h, and then [$^3$H]thymidine was added, and after culture for another 24 h, cell numbers in each well and radioactivities incorporated into the cells were determined. As shown in Fig. 3, both the cell number and the radioactivity incorporated into the cells increased significantly with increase in the amount of sapecin added to the medium. Thus addition of sapecin stimulated the proliferation of NIH-Sape-4 cells under these conditions. Probably, sapecin is essential for growth of NIH-Sape-4 cells, and is not synthesized in Grace's insect medium supplemented with 3% FCS, but is synthesized in this medium supplemented with higher concentrations of FCS that allow cell proliferation.

To demonstrate that the substance that stimulated cell proliferation was in fact sapecin, we fractionated the sapecin preparation by HPLC and examined which fraction stimulated incorporation of [$^3$H]thymidine. As is evident from Fig. 4, the activity to stimulate incorporation of [$^3$H]thymidine coincided with the peak of sapecin, and no other fraction contained activity. Thus, clearly sapecin itself has this activity.

A further possibility was that a small protein like sapecin has activity to stimulate cell proliferation nonspecifically. To examine this possibility, we tested whether sarcotoxin IA, another antibacterial protein of *Sarcophaga* consisting of 39 amino acid residues [19], stimulated cell proliferation. As shown in Fig. 5, sarcotoxin IA had no effect on cell proliferation under the conditions in which sapecin stimulated their proliferation significantly. Therefore, sapecin seems to have specific ability to stimulate proliferation of embryonic cells.

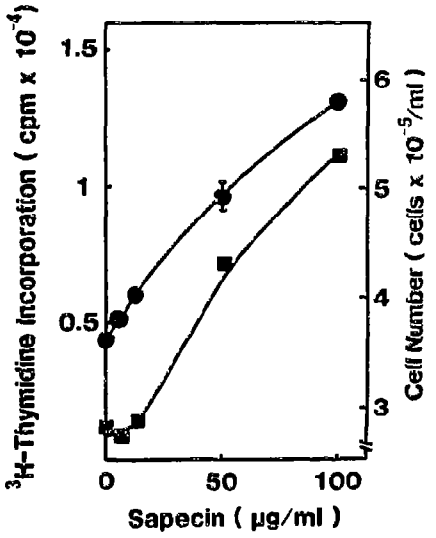


Fig. 3. Effect of sapecin on proliferation of NIH-Sape-4 cells. NIH-Sape-4 cells were cultured in 500 μl of Grace's insect medium containing 3% FCS serum and various amounts of sapecin. [$^3$H]thymidine (1 μCi) was added to the medium after 24 h and DNA was labeled for 24 h. Then the cell number and radioactivity incorporated into DNA were measured. (■), cell number; (●), radioactivity.

## 4. DISCUSSION

We showed in this paper that sapecin has activity to stimulate the proliferation of embryonic cells. Sapecin has been shown to have potent lethal effects on bacteria, so this is the second biological activity of sapecin demonstrated. Some insect proteins may function in two ways depending upon the physiological condition of the insects, because we previously demonstrated dual roles of *Sarcophaga* lectin [9]. Sapecin is the second defense protein of *Sarcophaga* found to have dual roles. These proteins may have had only one function, but have acquired the second function during evolution of insects.

Two points need discussion. (1) Although antibodies against sapecin significantly repressed the proliferation of NIH-Sape-4 cells, exogeneously added sapecin could not neutralize the effect of the antibody in M-M medium. As sapecin is an amphiphilic molecule, it may be integrated into the cell membrane as an intrinsic component. If this intrinsic sapecin is essential for cell proliferation, and if it is not easily replaced by exogeneously added sapecin, intrinsic sapecin blocked by the antibody may remain in the membrane irrespective of the presence of exogeneous sapecin.

(2) Although added sapecin stimulated cell proliferation in Grace's insect medium supplemented with 3% FCS, the concentration needed for this effect was very high compared with the effective concentrations of

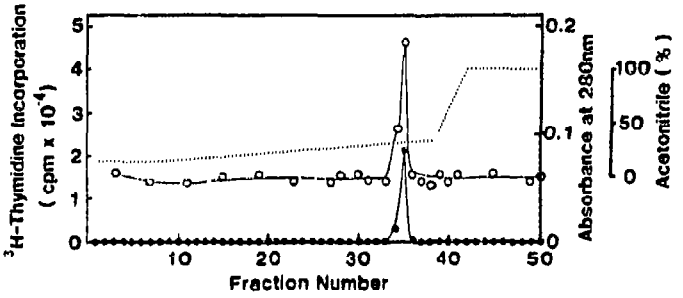


Fig. 4. Coincidence of sapecin and cell proliferation stimulating activity on HPLC. Purified sapecin was applied to a reverse-phase HPLC column ($C_{18}$) and eluted with a linear gradient of acetonitrile, as described before [1]. Absorbance at 280 nm was monitored. Each fraction was lyophilized, and the residues were dissolved in Grace's insect medium containing 3% FCS calf serum for assay of their effects on proliferation of NIH-Sape-4 cells. Cell proliferation was assayed by measuring DNA synthesis. (●), absorbance at 280 nm; ( ), radioactivity of [$^3$H]thymidine incorporated into DNA; (.....), concentration of acetonitrile.

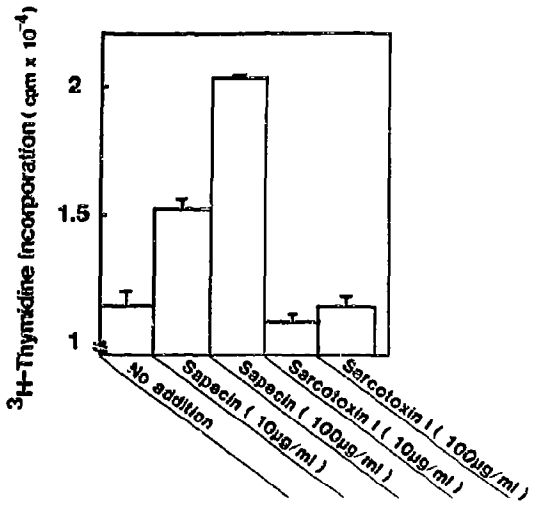


Fig. 5. Effect of sarcotoxin IA on cell proliferation. The effect of sarcotoxin IA on the incorporation of [$^3$H]thymidine into DNA was compared with that of sapecin. NIH-Sape-4 cells were labeled with [$^3$H]thymidine for 24 h in Grace's insect medium containing 3% FCS, in the presence or absence of sapecin or sarcotoxin IA at the indicated concentrations, and then incorporated radioactivity was measured.

mammalian growth factors. Probably, the mode of action of sapecin is different from those of mammalian growth factors. NIH-Sape-4 cells produce sapecin in M-M medium when they are growing, but the amount produced in much lower than the amount needed for stimulation of cell proliferation in Grace's insect medium. Possibly, intrinsic sapecin is present only in growing cells, and it is not present when the cells are in the resting condition in the Grace's insect medium. If the efficiency of integration of exogeneously added sapecin is much lower than that of endogeneous sapecin, a larger amount of sapecin may be needed for the cells to be competent for growth.

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