A preliminary study on antimicrobial peptides in the naturally damaged tunic of *Ciona intestinalis* (Tunicata)

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Antimicrobial peptides are components of the innate immune systems, produced by a wide variety of different species such as animals, plants, microorganisms. Recently, two putative gene families coding for antimicrobial peptides were identified in the expressed sequence tag database of the tunicate *Ciona intestinalis*, and the natural peptides were demonstrated to be synthesized and stored in distinct hemocyte types. Here we report on the presence of one of these natural peptides in the tunic of *Ciona intestinalis* considering that the ascidian tunic is a body surface barrier exposed to constant microbial assault. Often damages caused by tunic invading pathogens can produce a simple defence reaction leading to wound healing and neutralization of foreign organisms. We monitored the location of the Ci-PAP-A peptide concluding that AMPs may constitute a chemical barrier within the tunic of tunicates.

**Keywords:** AMPs; Ciona intestinalis; ascidians; Innate immunity

1. Introduction

AMPs are crucial and evolutionary conserved effector molecules of the innate immune system with a broad spectrum of activities against bacteria both Gram-positive and Gram-negative, viruses, and fungi [1-2]. They are small peptides generally positively charged, and despite their high number and their great diversity, they share the ability to adopt amphipathic conformations [3-5]. More than 1,700 AMPs have been isolated to date from different sources as protozoa, metazoa including animals and plants [6,7] [3], and several originate from marine invertebrate taxa including urochordates (tunicata).

Recently, two putative gene families coding for antimicrobial peptides were identified in the expressed sequence tag database of the tunicate *Ciona intestinalis* (Tunicata, Asciidea). *C. intestinalis* is a reference species of the solitary ascidians, the marine cosmopolitan invertebrates that occupy a key phylogenetic position being considered a sister group of vertebrates, classified in the phylum Chordata, subphylum Urochordata [8]. As all invertebrates, tunicates rely on a robust innate immune system to defend themselves against invading pathogens [9].

Peptides corresponding to the cationic core region of two of the deduced precursor molecules were synthesized. The synthetic peptides, named Ci-MAM-A and Ci-PAP-A, have been shown to kill bacteria both Gram-positive and Gram-negative and to display activity against the yeast *Candida albicans* and mycobacteria. They were used as antigens to produce specific antibodies. By using these antibodies in immunocytotoxicological analyses, the natural molecules were immunolocalized in a defined subpopulation of hemocytes [10,11].

Most tunicates are characterized by the presence of the tunic, an outer specialized tissue covering the epidermis or mantle epithelium. The tunic constitutes a support to the soft body of the animal and it is an efficient protective barrier against mechanical assaults and infection. The tunic tissue consists of an amorphous matrix containing a fibrous network of polysaccharides associated to proteins, and free living cells randomly distributed within it. Cells are involved in various biological functions such as tunic synthesis, recognition of self and non-self, wound healing [12-15].

Here we report the presence of the natural molecule Ci-PAP-A in the tunic from naive *C. intestinalis* when damages caused by encrusting organisms produce a defence reaction leading to wound healing, neutralization of foreign bacteria and repair of the tunic architecture.

Thus the observations reported in the present study aims at extending the understanding of the function of AMPs in tunicates focusing their significance as effector molecules in local defence reactions.

2. Material and Methods

*C. intestinalis* specimens about 10-12 cm long were collected from Termini Imerese harbour (Sicily, Italy). Animals free of encrusting marine matter were maintained at 15-18°C in aerated sea water. Cubes of tunic fragments, 1 to 3 mm long and cut off from different regions of the animal body, were processed using the following procedure.

For conventional transmission electron microscopy (TEM) they were fixed with 1.5% glutaraldehyde, buffered with 0.05 M sodium cacodylate (pH 7.3 plus 1.7% sodium chloride). After brief rinsing, they were postfixed for 1 hr at 4°C with 1% osmium tetroxide in 0.05 M sodium cacodylate at pH 7.3.
All specimens were rinsed briefly and dehydrated in a graded series of ethanol solutions, cleared in propylene oxide and embedded in Epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (Philips CM 10), at 80 kV.

Immunostaining was carried out by placing thin sections on nickel grids, oxidizing them with sodium metaperiodate to restore specific labeling, rinsing and floating them on drops of 1% BSA/PBS to block non-specific staining. The grids were then incubated on drops of the primary antiserum anti-Ci-PAP-A22. After washing, the sections were exposed to protein A-conjugated colloidal gold particles of either 10 or 5 nm diameter (Sigma Chemical Co, St. Louis, Missouri, USA). Finally, sections were counterstained with uranyl acetate prior to examination in the electron microscope. As a negative control the first antibody was omitted or an irrelevant one was used. As for the production of antisera against Ci-MAM-A and Ci-PAP-A the synthetic peptides were coupled to keyhole limpet hemocyanin (KLH) and these conjugates were used as antigens to immunize rabbits [10,11]. Antiserum anti-Ci-PAP-A was preincubated with KLH prior to its use to exclude the possibility that the staining was due to anti-KLH antibodies with cross-reactivity to C. intestinalis hemocyanin-like proteins.

Negatives of photomicrographs were scanned on an Epson Perfection 2480 Photo scanner and acquired as TIFF files at 800 ppi. All TIFF files were resampled at 300 ppi and subsequently re-sized and optimised for brightness and contrast by using Photoshop (Adobe Systems).

3. Results

Observations at EM reveal the tunic of C. intestinalis as an inner layer, the ground substance, overlaid by a rather thin outer compact sheet called cuticle; the inner layer is mainly composed of loose fibrous material embedded in an amorphous matrix. In the cuticle fibrils result in a closely interwoven network as shown in Fig.1A. Dispersed within the entire tunic are different cell types that can move freely (Fig. 1B). Most of them belong to the granulocyte population. The tunic cuticle is sufficiently dense to prevent microorganisms from invading the tissue. In fact it is not unusual to observe as in Fig. 1B the external surface of the cuticle covered with a dense coat of encrusting organisms such as algal symbionts or cyanobacteria. Apart from the symbionts present in the tunic [16], other microorganisms can be found in the tunic matrix, where some tunic cells exhibit phagocytic activity (Figs. 1C and D).

The cuticle is thought to be often damaged by sand grains or by other organisms. Figure 2A shows the way through which microorganisms can reach the underneath of the cuticle and the inner layer of the tunic: breaks of the cuticle and interruptions of its continuity enable encrusting agents to be inside the tunic, and sometimes a massive presence of foreign agents is observed in the subcuticular areas as in Fig. 2B.

The cuticle damages produce a simple defence reaction leading to wound healing, neutralization of foreign bacteria and algae, and maintenance of tunic tightness.

**Fig. 1** Different aspects of the *Ciona* tunic; (A) Electron micrograph showing the outer cuticle sheet (e) consisting of packed electrondense fibrillar material and the ground substance or matrix (m) mainly composed of a network of fibrils. (B) The cuticular layer shows a waving profile and a biological film of numerous encrusting agents (e) adhering to its surface. Under the cuticle, bacteria are scattered in the matrix (b). Several tunic cells present in the subcuticular area actively phagocytize bacteria as visualized in enlargements (C and D): an epibiont (e) is seen on the cellular surface (arrowhead) and an other one inside the cell (C); a bacterium (*) is engulfed inside a cell vacuole (D). Magnification: (A) 7,000x; (B) 2,500x; (C and D) 17,700x.
In the damaged tissue the architecture of the cuticle is lost. Fig. 2C shows the outermost layer that appears very thin and the subcuticle consists of very loose fibrillar material. The cell number is massively increased in the subcuticular area. Cells often contain phagosomes, indicating the presence of phagocytic activity, or contain remains of disintegrating organisms and debris. Frequently, cells appear to be in a degranulating active state, releasing their contents and showing drastic structural changes becoming simple cellular ghosts as in Figs. 3A and B.

To investigate whether AMPs are involved in this inflammation-like state, using the anti-Ci-PAP-A antibody, immunolocalization was performed on samples of damaged tunic. Via electron microscopy immunostaining is seen inside degranulating cells. Fig. 4 shows immunoreactivity consistently present in the vesicles released by cells and gold particles associated to the material extruded in the surrounding matrix area after the membrane dissolution.

In the area of tunic injury, clumped tunic cells are seen beneath the wound-site, they are tightly adherent to one another forming aggregates that could seal the wound inducing clot formation (Fig. 2C).

Moreover, Fig.3B shows filaments often present around the discharging tunic cells observed in the injured area. These filaments may represent bundles of tunic matrix as regeneration of the tissue occurs.

Fig. 2 Electron micrographs of cross sections through the Ciona tunic. (A) Section showing openings of the cuticle layer (c) which has foreign material on its external surface. Under the cuticle a granulocyte (g) can be observed. (B) The biological film (e) usually found on the cuticle surface, is seen in the subcuticular layer where numerous bacteria, algae are entrapped. (C) The architecture of the cuticle and its compactness is lost. The outermost layer appears very thin and the subcuticle consists of loose fibrillar material. An encrusting organism (arrow) is hammered into the tunic producing break of the cuticle continuity. In the healing area aggregation of numerous granulocytes (g) can be observed; these cells contain fibrogranular material with different density. Magnification: (A) 4,400x; (B) 3,300x; (C) 2,400x.

Fig 3 Aspects of tunic cell degranulation. Many cells observed in the injured tunic undergo to drastic changes releasing dense materials, fibrils; they have several clear vacuoles, a lot of vesicles, and lysosomal figures. Cells are destructing in the tunic and discharging their contents they are contributing to the neutralization of foreign agents and the repair of the tunic architecture as filaments appear around in the matrix (B). Magnification: (A) 4,400x; (B) 5,500x.
**Fig 4** Immunolocalization of Ci-PAP-A peptide in the *Ciona* tunic. (A,B) Different cells are in a degranulating state and cellular ghosts can be observed. Note the presence of bacteria (*) in the surrounding matrix area. (C-E) enlargements of cells in A and B showing labeling localized within vesicles, within the tunic matrix among the remnants of cells, on the membrane debris, and electron dense particles. Some grids were not treated with primary antibody and the samples in the sections were negative (F). Magnification: (A, B) 3,000x; (C) 10,000x; (D) 8,000x; (E) 30,000x; (F) 8,000x.

### 4. Discussion

Antimicrobial peptides are considered very promising drug candidates as potential replacement to antibiotics. The marine organisms are probably the major source of bioactive molecules, and several antimicrobial compounds have been described in them including ascidian species [17,18].

Recently, the transcripts of two putative antimicrobial peptide genes of the *Ci-mam* and *Ci-pap* gene families as well as the corresponding natural peptide molecules have been localized to distinct hemocyte types in *C. intestinalis* [10,11].

Using the antibody generated against the corresponding synthetic peptide Ci-PAP-A, we extended the study showing that the natural peptide is present in damaged tunic of *C. intestinalis*.

As a protective structure the tunic cuticle hardens the tunic surface and prevents microorganisms from invading it, but it can be frequently damaged. Any break of the cuticle must be quickly and efficiently repaired to prevent intrusion of pathogens. When microbes have gained access to the tunic matrix through cuticle interruptions, a simple defence reaction is performed: cellular and chemical defences as the production of AMPs, should be available in the tunic. The number of cells increases significantly in the area of entry, some cells show phagocytic aspects and entrap bacteria, but most of them are in a degenerative state and undergo drastic changes releasing their contents. The massive presence of cells reinforces the idea of their protective role and their involvement in the defence reaction performed to protect the animal body from predators and/or infectious organisms. After stimulation by microbial products and upon the activation of degranulating processes, apparently followed by cell lysis, AMPs are released into the extracellular space as evidenced by the presence of gold particles scattered in the tunic matrix and labeling of the cellular remnants. Differently, AMPs have been found only in a defined subpopulation of tunic cells when other regions of the tunic were observed [19].
The features here observed remind experimentally induced acute inflammation in *Ciona* when particular cell types seem to be involved in the production of AMPs in the inflamed tunic [19].

Although it is not know how initiates this reaction, how the antimicrobial factors are released and which molecules are involved in the whole healing phenomenon, this process takes part in the destruction of foreign cells. Cell migration and aggregation in the wound sites participate to destroy foreign organisms. In the same time the restoration of the tunic fibrous structure and tissue functionality need to be performed.

These preliminary findings on the presence of the Ci-PAP-A peptide in the tunic of *C. intestinalis* during natural injuries confirm its protective effect against microbial attacks and its contribute in an active form to the clearance of bacterial infection, giving further interest in the potential development of AMPs as therapeutics. Further investigation needs to understand the complex interactions leading from recognition of invaders to induction of AMP gene.

Acknowledgements This work has been supported by grants from the Italian Ministero della Università e della Ricerca and the University of Palermo research grant to M.A.D. and G.D.L.

References


