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Short Communication

Localization of antimicrobial peptides in the tunic of *Ciona intestinalis* (Ascidiacea, Tunicata) and their involvement in local inflammatory-like reactions

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ABSTRACT

Tunicates comprising a wide variety of different species synthesize antimicrobial peptides as important effector molecules of the innate immune system. Recently, two putative gene families coding for antimicrobial peptides were identified in the expressed sequence tag database of the tunicate *Ciona intestinalis*. Two synthetic peptides representing the cationic core region of one member of each of the families displayed potent antibacterial and antifungal activities. Moreover, the natural peptides were demonstrated to be synthesized and stored in distinct hemocyte types. Here, we investigated the presence of these natural peptides, namely Ci-MAM-A and Ci-PAP-A, in the tunic of *C. intestinalis* considering that the ascidian tunic is a body surface barrier exposed to constant microbial assault. Furthermore, as the tunic may represent a major route of entry for pathogen invasion after its damage we monitored the location of these peptides upon a local inflammatory-like reaction induced by injection of foreign cells. Using immunocytochemistry and electron microscopy both peptides were localized to the tunic and were massively present in granulocytes of inflamed tissue. Conclusively, antimicrobial peptides may constitute a chemical barrier within the tunic of urochordates.

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1. Introduction

Tunicates, one of the most evolved invertebrate taxa, are marine organisms considered to be a sister group of vertebrates being classified in the phylum Chordata, subphylum Tunicata [1]. Owing to their phylogenetic position they represent significative animal models when invertebrates and vertebrates are compared. Like all invertebrates, tunicates lack an adaptive immune system and rely on a robust innate immunity to defend themselves against micro-organisms [2,3]. This innate immune system consists of both cellular and humoral components. Humoral responses include proteolytic cascades leading to melanization by the prophenoloxidase-activating system as well as the production of various killing factors such as antimicrobial peptides (AMPs) [4–6]. AMPs are in fact crucial and evolutionarily conserved effector molecules of the immune system with a broad spectrum of activities against bacteria, both Gram-positive and Gram-negative, viruses, and fungi.

AMPs are defined as short peptides that are often cationic and have the ability to adopt an amphipathic structure [7–9]. They are produced by bacteria [10], fungi [11], protozoa [12], metazoa and plants [7]. More than 1700 AMPs have been identified to date ([13,14]; http://aps.unmc.edu./AP/main.php). Several of these were characterized from different marine invertebrate taxa including tunicates [15–24]. All antimicrobial peptides described from tunicates so far have been isolated from circulating hemocytes that are considered to be responsible for most of the defense reactions in these organisms.

Recently, two novel gene families coding for putative AMPs were identified in the EST database of the solitary ascidian *Ciona intestinalis* (Tunicata, Ascidiacea). Peptides corresponding to the cationic core region of two of the deduced precursor molecules were synthesized and used as antigens to produce specific antibodies. By using these antibodies in immunocytochemical analyses it became evident that the natural peptides are synthesized and stored in a defined subpopulation of hemocytes [25,26]. The synthetic peptides, Ci-MAM-A24 and Ci-PAP-A22, displayed potent antimicrobial activity against various bacterial pathogens both Gram-positive and Gram-negative, and against the yeast *Candida albicans* [25,26]. Moreover, Ci-MAM-A24 was shown to be effective against multidrug-resistant pathogenic bacteria of humans and mycobacteria [27,28].

Abbreviations: AMPs, antimicrobial peptides; Ci-MAM, *Ciona*-molecule against microbes; Ci-PAP, *Ciona*-putative antimicrobial peptide; EST, expressed sequence tag; KLH, keyhole limpet hemocyanin

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Most tunicates are characterized by the presence of the tunic, an outer protective specialized tissue, covering the mantle epithelium or epidermis. The tunic consists of a leathery or gelatinous matrix containing microfibrils of polysaccharides linked to proteins, and free living cells randomly distributed within it [29–31]. These cells are involved in various biological functions such as tunic synthesis, wound healing, immunological and excretory activities ([32], and references therein; [33]). The origin of tunic cells is not entirely clear; in general, they are thought to originate from the hemocytes or connective tissue. In *C. intestinalis* it has been shown that during inflammatory-like reactions [34] hemocytes migrate by diapedesis from the hemolymphatic lacunae trough the mantle epithelium into the tunic leading to a subsequent increase of the tunic cell population [35]. Apart from its role as a support and an adhesive to the substratum, the tunic is considered as a protective barrier of the soft body against mechanical damage and infection, and a site of self/non-self recognition [36,37].

Here, we search for the presence of the natural molecules Ci-MAM-A and Ci-PAP-A in the tunic from naïve *C. intestinalis* by using immunocytochemistry and employing specific antibodies against these antimicrobial peptides. Moreover, to investigate whether these peptides are actually involved in immune defense, we also analyzed tissue samples of specimens where local inflammatory-like reactions in the tunic have been experimentally induced. The present study aims at extending the understanding of the functions of AMPs in tunicates by investigating their significance in local immune responses aside from their role as potent effector molecules of circulating hemocytes in the hemolymph.

2. Material and methods

2.1. Animals

C. intestinalis specimens about 10–12 cm in size were collected from Termini Imerese harbor (Sicily, Italy). Animals free of encrusting marine matter were maintained at 15–18 °C in aerated sea water.

To provoke an inflammatory reaction, sheep erythrocytes (1×10^7 suspended in 0.2 ml phosphate buffered saline (PBS), pH 7.4) were injected into the tunic tissue. Four days later, the specimens showing an immune reaction in the tunic (macroscopically seen as a circular or elliptical whitish area visible through the transparent tunic) were chosen for further analyses. *Ciona* specimens injected with 0.2 ml PBS served as a control.

2.2. Microscopy

For routine microscopy, cubes of tunic fragments, 1–3 mm^3 in size, cut off from different regions of the animal body and from the oral siphon, as well as excised from the injection site were processed by standard techniques which can be summarized as follows: fixed with 1.5% glutaraldehyde (Sigma Chemical Co, St. Louis, Missouri, USA) buffered in 0.05 M sodium cacodylate, pH 7.3, post-fixed in 1% OsO_4, and dehydrated in a graded series of ethanol solutions, and subsequently embedded in epoxy resin. Ultrathin sections (50–70 nm thick) stained with uranyl acetate and lead citrate solution were examined using a Hitachi S7000 transmission electron microscope (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 antisera, either anti-Ci-PAP-A22 or anti-Ci-MAM-A24. 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 (Anti Bcl-xL, H5 mouse IgG1, Santa Cruz Biotechnology, Santa Cruz, CA, USA, no. 8392) 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 [25,26], antisera were preincubated with KLH prior to their use to exclude the possibility that the staining was due to anti-KLH antibodies with cross-reactivity to *C. intestinalis* hemocyanin-like proteins.

2.3. Photomicrographs

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

3. Results

By performing immunoelectron microscopy with the specific antibodies against Ci-PAP-A22 and Ci-MAM-A24 on fixed samples from the naïve *Ciona* body and the oral siphon, the natural peptides were localized to the tunic tissue (Fig. 1). Among the different cell types that are dispersed within the entire tunic (Fig.1A), the Ci-PAP-A and Ci-MAM-A positive cells belong to the granulocyte population of "tunic large granule cells" and "tunic morula cells", previously described by De Leo [30] on the basis of their morphology, and "tunic compartment cells". The word "tunic" is included to emphasize that these cells are permanently resident in the tunic and to avoid confusion with the names applied to the hemocytes of the hemolymph.

Tunic large granule cells are characterized by possessing a single, large compartment occupied by homogeneous fibrogranular content. The large inclusion inside the unique granule is surrounded by a thin peripheral rim of cytoplasm which contains a small nucleus, some vesicles and free ribosomes. The large granules immunoreacted with anti-Ci-MAM-A (Fig. 1B and E) and anti-Ci-PAP-A (data not shown).

Particularly abundant in some areas of the oral siphon are tunic morula cells and tunic compartment cells (Fig. 1C). Tunic morula cells are usually roundish shaped cells with a berry-like appearance under the light microscope; they possess several tightly packed globular vacuoles partially or completely filled with masses of granular dense material (Fig. 1C and D). Their nucleus is eccentrically located.

Tunic compartment cells are characterized by the presence of two or more cytoplasmic large globules giving the cells a compartmentalized appearance and containing electron-dense granular inclusions of different size and shape, often surrounded by a translucent halo (Fig. 1C), and nucleus centrally located.

Because only slight morphological differences distinguish compartment and morula cells, we here collectively refer to them as "tunic compartment/morula cells".

Using the anti-Ci-MAM-A antibody the immunostaining was observed inside several small granules, present among the globules or at the periphery of the cytoplasm in both of the latter cell types. These granules appear oval to spindle-shaped, they are

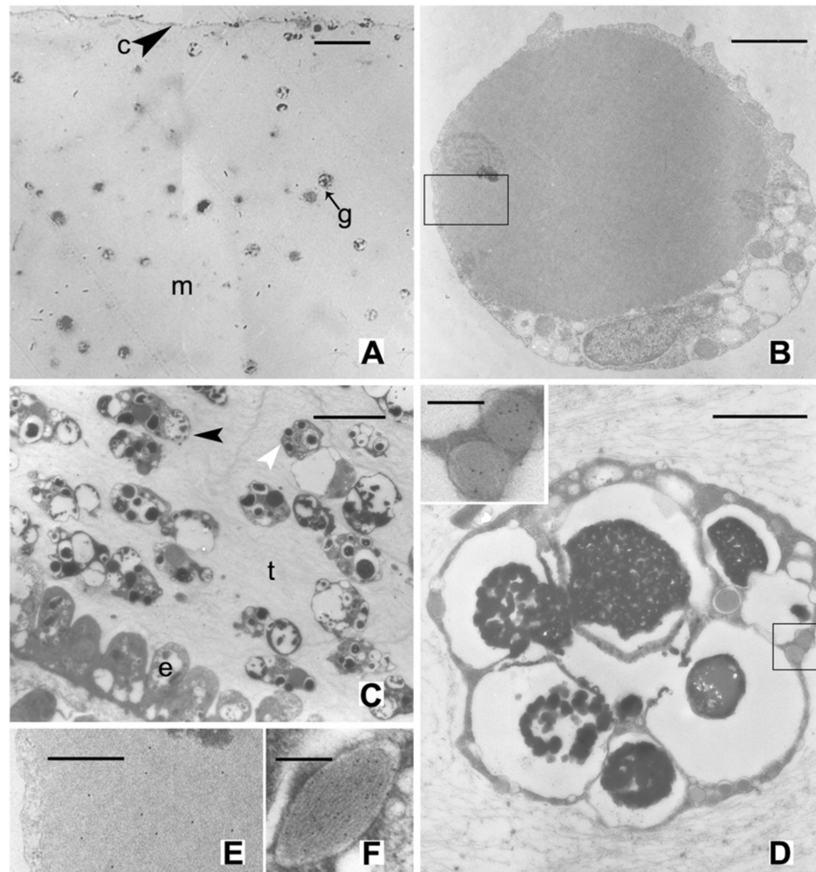

Fig. 1. Immunolocalization of Ci-MAM-A in the tunic of *Ciona intestinalis*. The peptide was detected and visualized by using a specific antiserum and a secondary antibody conjugated with colloid gold. (A) Light micrograph of tunic fragment (c—cuticle; m—matrix; g—granulocytes) where free cells are scattered. Semithin sections were stained with toluidine blue. (B) Tunic large granule cell characterized by a single granule in the cytoplasm. The nucleus and free ribosomes are confined to the periphery of the cytoplasm. The peptide Ci-MAM-A is localized inside the large single inclusion (box enlargement: E). (C) Electron micrograph surveying a portion of *Ciona* tunic in the region of oral siphon (e—epidermis; t—tunic). Note the abundant cells scattered in the matrix (*black arrowhead*: compartment cell, *white arrowhead* morula cell). (D) A tunic morula cell in which vacuolar roundish contents are heavily electron-dense. A clear space is observed around them. Within the cell, the peptide Ci-MAM-A is associated with small granules scattered among the globules. Square highlights granules shown at greater magnification in inset. (F) Detail of a granule from a tunic compartment cell showing colloid gold particles. *Scale bars*: 60 μm (A); 2.5 μm (B, D); 8 μm (C); 0.5 μm (E); 0.15 μm (F); and 0.25 μm (inset).

surrounded by a membrane and contain granular or floccular material which is moderately dense (Fig. 1D and F).

To investigate whether an inflammation state alters the distribution of AMPs in the tunic cell population, the immunolocalization was performed on samples during an experimentally induced inflammatory-like reaction. It has been shown previously that, following the initiation of an inflammation by the application of an elicitor, the cell number is massively increased in the inflamed area of the tunic as hemocytes infiltrate from the hemocoel or the mesenchymal space to encapsulate foreign material and release substances in order to destroy it [38,39]. Thus, considering that most of the cells present in the tunic during an inflammatory response are hemocytes (the classification of which is a controversial issue), and in order to facilitate their identification, cells are here termed according to the nomenclature reported by De Leo [40]. He suggested to distinguish four types of granulocytes: clear, clear vesicular, micro- and vacuolar granulocytes (with unilocular and globular subtypes).

The infiltrating granulocytes observed in the inflamed area are identified as

(i) "globular granulocytes", cell types closely resembling compartment/morula cells; (ii) "unilocular granulocytes" both possessing a single electron-dense large granule, and with a large electron-transparent granule occupying entirely the cytoplasm, like "signet ring cells"; (iii) "microgranulocytes"; (iv) "clear granulocytes and clear vesicular granulocytes".

The cells are often in close contact to one another and appear frequently to be in a degranulating active state, releasing vesicles and showing drastic structural changes so that many cellular ghosts are observed in the tunic matrix (Fig. 2A).

Unilocular granulocytes in the inflamed area were immunostained with both anti-Ci-MAM-A (Fig. 2C) and anti-Ci-PAP-A (data not shown) antibodies in their large granule.

Many more gold particles were observed with the anti-Ci-PAP-A and anti-Ci-MAM-A antibodies in the cytoplasmic small granules (Fig. 2E and G) adjacent to the globules of tunic compartment/morula cells. When the material inside the granule was lost owing to the discharging process, the gold particles decorated the remnant of the content or appeared attached at its margin (Fig. 2F and G).

Moreover, gold particles were also seen scattered over the tunic matrix where cells in response to the stimuli degranulate and discharge their content (Fig. 2B and D).

Notably, immunostaining was still observed in the tunic sections when antisera were pretreated with KLH, confirming the specificity of the staining, whereas no positive staining was observed in negative controls.

4. Discussion and conclusion

Recently, the transcripts of two putative antimicrobial peptide genes of the *Ci-mam* and *Ci-pap* gene families as well as the

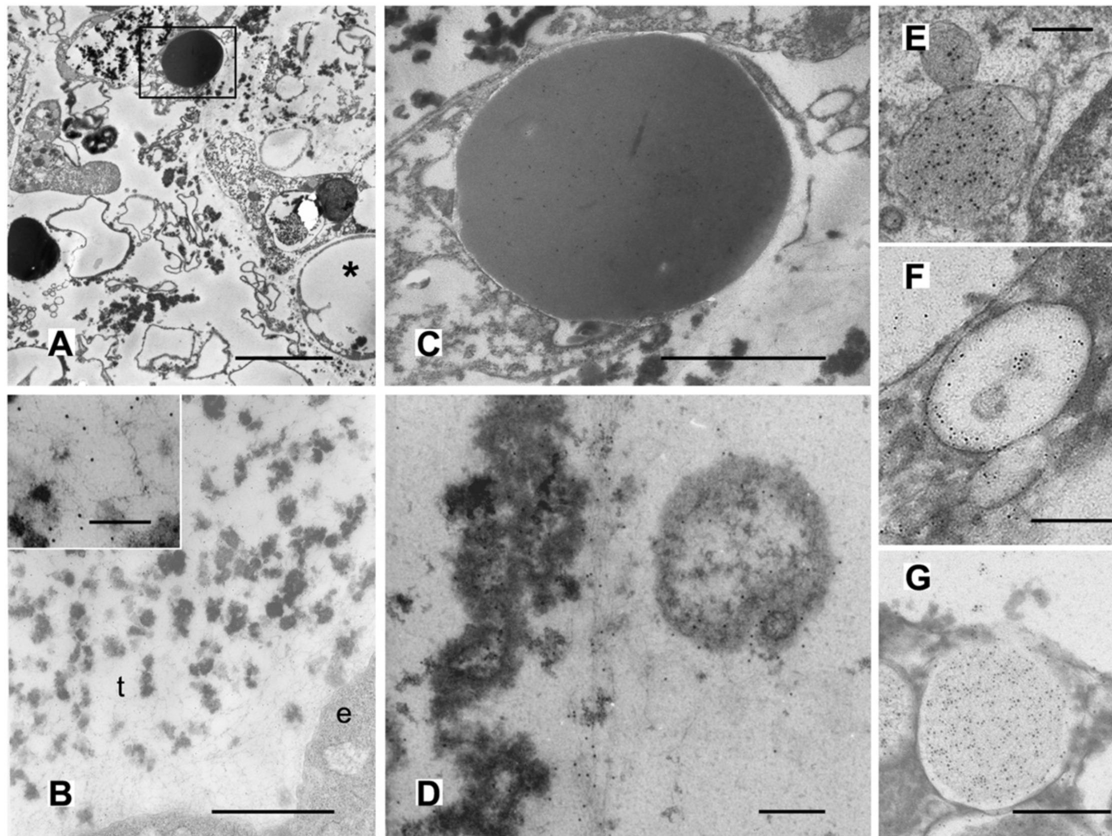

Fig. 2. Immunolocalization of Ci-MAM-A and Ci-PAP-A in the *Ciona* injured tunic after the inoculation of foreign materials. (A) Section of inflamed tunic area with different cells in a degranulating state; cell ghosts (*), granular and fibrillar material, strongly electron-dense particles in close contact with membrane debris can be seen. (B) Labeling for Ci-MAM-A is also localized within the tunic matrix among the remnants of cells (e—epidermis; t—tunic). *Inset*: a magnification of tunic matrix area. (C) Enlargement of a large granule cell shown in (A) (*black box*) immunolabeled exclusively inside the Ci-MAM-A positive inclusion that occupies the entire cell. (D) Gold particles representing Ci-PAP-A are seen associated with electron-dense particles, membrane debris, and fine fibrils in the tunic matrix. (E) Higher magnification of small granules from compartment/morula cells where the peptide Ci-PAP-A accumulates. Labeling is found to be significantly increased compared to the granules from the same cell types of naïve ascidians. (F,G) Gold particles representing Ci-MAM-A appear to be associated to the content inside the granules or extruded by granule discharge of compartment/morula cells. *Scale bars*: 4 μm (A); 2 μm (B); 1.2 μm (C); 0.25 μm (D,E,G, inset); and 0.15 μm (F).

corresponding natural peptide molecules have been localized to distinct hemocyte types in *C. intestinalis* [25,26]. Using the antibodies generated against the corresponding synthetic peptides Ci-MAM-A24 and Ci-PAP-A22, we extended the results of the previous study showing that the natural peptides are present in the granulocyte population resident in the tunic of *C. intestinalis* adults. The presence of these AMPs emphasizes the protective role of the tunic tissue as an important barrier against microbial invasion particularly around the oral siphon. The result reported here provides also evidence that these peptides are utilized as part of the antimicrobial repertoire of inflammatory cells in injured animals.

Tunic large granule cells show morphological features similar to a particular type of circulating hemocytes, the unilocular granulocytes. These cells were recently also termed as "unilocular refractile granulocytes" (URG) by Parrinello [41] because they appear refractile when observed under contrast microscopy. The present findings showing the labeling in the sole large inclusion of these cells in the tunic from both naïve and immune-stimulated ascidians are consistent with the previous report on the presence of Ci-PAP-A in a URG hemocytic subpopulation from naïve ascidians [25]. These cells appear to be particularly immune competent as they have been shown to be involved in different defense reactions. URGs have been found to have a strong PO activity; the prophenoloxidase (proPO) activating system is a very sophisticated cascade reaction involved in immune reaction and probably a molecular cross-talk takes place between the proPO system and other cellular defense responses which are activated by microbial products signals [41,42].

Notably, these AMPs are not only found inside the tunic large granule cells but also within other granulocyte subtypes residing in the tunic. As evidenced by electron microscopy Ci-MAM-A and Ci-PAP-A are stored in the cytoplasm of tunic morula/compartment cells exclusively in the small granules found among the globules or vacuoles containing the material of various electron-density. Even if morula and compartment cells may be considered as intermediate forms of cell differentiation, or may represent different stages of maturation of a cell lineage, they have been described as being involved in the allorejection reaction between colonies, in cytotoxic reactions [43–45], and in the melanization process via tunichrome oxidation by phenoloxidase [46]. Accordingly, our results can reinforce the idea of the protective role of tunic morula/compartment cells. Microscopic observations on *C. intestinalis* indicate the presence of intratunical bacteria and alga-like cells [30,47]. Unicellular algae seem to be restricted to certain periods and to be related to the environment in which animals live. As regards bacteria, it is still unclear whether they are symbiotic or pathogenic, or whether they are stable and seasonally dependent. Since they are found both in the matrix and within phagocytic features in some tunic cells, there may be a defense system against bacterial infection or against some groups of bacteria. At present there is no ecological and taxonomic information on the bacteria found in the *Ciona* tunic, and it is still unclear by which mechanisms they can survive in the tunic.

Further investigations of their fine structure and further efforts to clarify their role are needed.

The present observations disclose an involvement of particular cell types in the production of AMPs in the inflamed tunic. Infiltration of hemocytes induced in *C. intestinalis* upon injection of sheep erythrocytes into the tunic increases significantly the number of cells in the area of entry; most of them are in a degenerative state and undergo drastic changes so it is difficult to identify all cell types on the basis of their ultrastructural aspects. The release of their content which could take part in the destruction of the foreign cells, may be judged by the presence of gold particles labeling the cellular remnants and granules significantly positive to Ci-MAM-A and Ci-PAP-A. AMPs are released into the extracellular space, upon the activation of degranulation processes. Variously distributed in the tunic matrix of the inflamed area are electron-dense particles which can be clustered and packed, or be interspersed among degranulating cells to form irregular masses; associated with them we localized the natural molecules Ci-MAM-A and Ci-PAP-A.

Concerning 'inflammatory hemocytes' in the hemocoel we expect an increased expression of AMP genes. The next major objective is the elucidation of the distribution and the rate of synthesis of these molecules in circulating hemocytes during an inflammation.

In conclusion, our data clearly demonstrate for the first time that AMPs are also synthesized in the tunic cells of *C. intestinalis*, and give further evidences that these peptides constitute an important part in host defense against invading microbes. However, the complex interactions leading from recognition of invaders via signal transduction to induction of AMP genes needs further investigation.

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