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The *Ciona intestinalis* immune-related galectin genes (CiLgals-*a* and CiLgals-*b*) are expressed by the gastric epithelium

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ABSTRACT

The transcription of two *Ciona intestinalis* galectin genes (CiLgals-*a* and CiLgals–*b*) is uparegulated by LPS in the pharynxis (hemocytes, vessel epithelium, endostilar zones) which is retained the main organ of the immunity. In this ascidian, for the first time we show, by immunohistochemistry and *in situ* hybridization methods, that these two immune-related genes are expressed in the gastric epithelium of naïve ascidians, whereas the galectins appear to be only contained in the intestine columnar epithelium. In addition, according to previous results on the pharynx, the genes are also expressed and galectins produced by hemocytes scattered in the connective tissue surrounding the gut. The genes expression and galectin localization in several tissues, including the previous findings on the transcription upregulation, the constitutive expression of these genes by endostylar zones and by the gastric epithelium suggest a potential multifunctional role of these galectins. In this respect, it is of interest to define where the CiLgals are normally found as related to the tissue functions. Such an approach should be a starting point for further investigations.

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1. Introduction

Galectins constitute a family of ß-galactoside-binding proteins. They are soluble proteins characterized by a conserved Carbohydrate Recognition Domain (CRD), no divalent cation requirement for binding, a conserved primary structure motif and a unique structural fold [1–3]. They are present in the cytosol, the nucleus, and in the extracellular matrix, forming a complex repertoire for recognizing and cross-linking glycan groups of glycoconjugates of cells and extracellular matrix, involved in several biological processes including immunity [4–9]. These proteins lack of signal sequence and glycosilation, therefore they escape the ER/Golgi apparatus and are released extra-cellularly by a non classical mechanism [7,9,10]. In mammals, multiple galectin family members are expressed in the organism, in some cases, they are expressed in a cell/tissue-specific manner, or, otherwise, a same galectin is widely distributed among different cells and organs, and can be involved in various functions [2,4,7,9]. The discovery of a galectin fold in the protistan parasite *Toxoplasma gondii*, of galectin-like proteins in the fungus *Coprinopsis cinerea*, in the sponge *Geodia cydonium* [11–13], and in both protostome and deuterostome lineages of metazoans [14–17], indicate the early emergence and evolutionary structural conservation of these proteins.

The molecular subfamily of the bi-CRD galectins is characterized by two distinct carbohydrate-binding domains, joined by a random-coil linker on a single chain. The activity and multiple roles of this galectin type result from the constitutive bivalency, the ability to form oligomers and the wide distribution in several tissues [2,8,18]. In *Ciona intestinalis*, two bi-CRD galectins (CiLgals-*a* and CiLgals-*b*) have been identified [17]. Sequence homology and gene organization have revealed that the CiLgals-a exhibits the F4-CRD-linker-F3-CRD gene arrangement typical of the amphioxus and vertebrate bi-CRD galectins genes, whereas the CiLgals-b presents a specific F4-CRD-linker-F4-CRD gene organization. Presumably, a tandem duplication of an F4-CRD gene organization would have given rise to the ancestral F4-CRD-linker-F3-CRD of chordates. The homology modeling process has shown that the N-CRD and C-CRD of CiLgals-*a* or the CiLgals-*b* domains have structural properties for the binding to β -galactosides, and they share a structural model with human N-CRDs and C-CRDs [19]. The interest in the study of sea squirts has been amplified by the recent molecular

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approach to chordate phylogenesis that enable us to retain the ascidians as the sister group of vertebrates [20,21]. In addition, the genome analysis of *C. intestinalis* has provided a detailed picture of expressed genes, including galectins [19,22,23].

The pharynx is retained the main organ of *C. intestinalis* immunity [24], and the CiLgals genes are immune-related being their transcription upregulated by lipopolysaccharides (LPS) in vessel epithelium and hemocytes that populate the vessels [19,23]. In addition, they are expressed by the endostyle that is the first trait of the digestive apparatus producing mucus for filter feeding [23]. Now, the question arises whether they are expressed by the stomach and intestine epithelia of naïve ascidians, also taking into account that the gut is in continous contact with seawater microbiota and it is a site of immunological interaction [25–27]. In this regard, the first step in gaining the understanding of galectin functions is to determine where they are normally found as related to the tissue functions. Therefore, in the present paper, the expression of these immune-related genes in the ascidian alimentary canal is examined, and it can be a starting point for further investigations. To achieve this goal, the antibodies and riboprobes, that previously showed the gene transcription upregulation on histological sections of LPS-challenged pharynx, are tested on gut histological section by using the same methods [19,23]. Our observations on gut histology have benefited from the study on the morphology (light and transmission electron microscopy) and histochemistry of stomach and intestine epithelial walls, primarily described in details by Yonge [28], Thomas [29,30] and Ermak [31].

For the first time, we show that both immune-related *Ciona* galectin genes are expressed in the gastric epithelium of naïve specimens, meanwhile galectins are contained in the intestine epithelium. Although the CiLgals functions in the gut are not shown, the findings here reported suggest a potential multifunctional role of these galectins.

2. Materials and methods

2.1. Animals and tissue preparation

Ascidians were gathered from Termini Imerese marinas (Sicily, Italy), maintained in aerated sea water at 15 °C and dissected on the second day. Before dissection the tunic surface was accurately cleaned with marine solution (12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0). The stomach and intestine from 10 specimens were excised. The study was performed in full compliance with the national (D.Lgs n.116/1992 and n.26/2014) and international European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). Although the systematic of this species has been reexamined and the Mediterranean specie defined as *Ciona robusta* [32] we used the *Ciona intestinalis* until definitive clarification of Mediterranean species had been established.

2.2. Histological methods

The excised tissues were fixed in Bouin's fluid (saturated picric acid: formaldehyde: acetic acid 15:5:1) for 24 h, paraffin embedded and serially cut at 6 μm (Leica RM2035 microtome, Solms, Germany). Serial sections of paraffin-embedded stomach and intestine were alternatively examined by immunohistochemistry and *in situ* hybridization under a Leica DMRE microscope. To display the histological organization of the tissues, sections were stained with Mallory's trichrome stain [33].

2.3. Antibody specificity and immunohistochemical assays

According to previous studies [19,23], specific antibodies against CiLgals-a and CiLgals-b galectin subtypes were prepared. As already reported, the antibodies were selectively purified, and tested for their specificity. Polyclonal antibodies were raised in rabbit against peptides selected from the deduced mature CiLgals-*a* and CiLgals-*b* sequences, and provided of immunogenic properties (GenScript USA Inc., Piscataway, NJ). CiLgals-*a* DTGIEIPKPAVDTL-C and CiLgals-*b* MFRTQRKLNRPAI-C peptides were synthesized and used as an antigen. The specific antibodies were isolated by affinity chromatography on CNBR-activated Sepharose column coupled with the antigen peptide (GenScript USA Inc.), dyalized versus PBS, and stored at −80 °C until to be used. The antibody titer (1:25,000) was recorded by ELISA on plates (Nunc, Denmark) coated with the peptide used as an antigen (10 μg/well). Then, the wells were separately incubated in blocking solution, with: 1. anti-CiLgals-*a* or anti- CiLgals-*b* antibody preparations (1:1000–1:50,000); 2. Preimmune rabbit serum (1:50–1:200). The antibody-peptide reaction was disclosed with peroxidase-conjugated anti-rabbit IgGs (1:10,000) in blocking solution, and then incubated with *o*-phenylenediamine and the peroxidase product quantified (492 nm). The antibody specificity, previously checked by indirect peptide ELISA, was further supported by competitive peptide ELISA as described by Plagemann [34] and previously reported in detail [19,23]. In brief, diluted antibody preparations containing a suitable amount of CiLgals-*a* or −*b* peptides, were incubated in peptide-coated wells. In these assays, no peptide-antibody reaction was disclosed by the peroxidase-conjugated anti-rabbit IgG preparations.

Immunohistochemistry assays were performed on histological sections incubated with 3% BSA in PBS containing 0.1% (v/v) Tween 20 (PBS-T) for 2 h at r.t., and then (overnight at 4 °C) with the primary antibody (0.0170 mg/ml anti-CiLgals-*a* or 0.026 mg/ml anti-CiLgals-*b*) in PBS-T/1%BSA. Anti-rabbit IgG phosphatase alkaline conjugated antibody was used as secondary antibody (1:10,000; 90 min at r.t.). The sections were rinsed with PBS-T and stained with BCIP/NBT liquid substrate system.

2.4. In situ hybridization (ISH)

The ISH method has been previously reported in detail [19,23]. In brief, histological sections were treated with CiLgals-*a* and CiLgals-*b* digoxigenin-11-UTP-labeled riboprobes (1 mg/ml final concentration) (Roche Diagnostics). The riboprobes contained the C-CRD (C terminus) sequence including 621–1223 residue positions of the Ci-Lgals-*a* cDNA, or the 610-1331 C-CRD residue positions of the CiLgals-*b* cDNA. After digestion with proteinase K (10 mg/ml) in PBS-T (phoshate buffer saline, pH 7.4, containing 0.1% Tween 20) for 5 min, washed sections were treated with hybridization buffer containing 50% formamide, 5X SSC (1X SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50 mg/ml heparin, 500 mg/ml yeast tRNA and 0.1% Tween 20, at 37 °C overnight. After 1 h incubation with anti-DIG-Fab-AP conjugate (Roche Diagnostics) diluted 1:100, the sections were washed in PBS-T and finally incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma-Aldrich, Germany). Color development was stopped after 30 min at r.t. The prehybridization was carried out in the hybridization buffer for 1 h at 37 °C. Preliminary hybridization assays at higher temperatures showed that the incubation at 37 °C of the histological sections allowed to obtain cleaner controls by treating them with the sense probes. In addition, the same incubation temperature that showed the riboprobe labels in hemocytes and pharynx in naïve and LPS challenged ascidians, was used.

3. Results

3.1. Histological observations of the stomach and intestine epithelium

To better identify the tissue localization of the CiLgals in the gut, preliminary histological observations were performed. Fig. 1 shows histological sections (Mallory's stain) of the simple epithelial walls of stomach and intestine. The stomach is a sac-like enlargement of the digestive tube, formed by ciliated epithelium which is folded into a series of furrows-ridges separated by unfolded stretches (Fig. 1A–C). The cells of the straight epihelium were arranged in a columnar shape and appeared to be "gland cells" or "secretory cells" as previously identified by Yonge and Thomas [28–30] The photomicrographs show lengthened cells rich in granular material in their apical area, and vesicles in the middle and lower parts of the cells (Fig. 1B). In the magnifications 1 and 2 vesicles and smaller vesicles containing a single granule can be seen. The elongated "vacuolated cells" described by Thomas [29] appeared to be restricted to the upper side wall and top of a stomach ridge (Fig. 1C) where they were characterized by vesicles containing unstained material, and small vesicles containing a single granule (Fig. 1 B, magnification 3). Moreover, the magnification 2 shows that these vesicles can be released from the outer cell surface. At the buttom of the forrow, vacuolated cells had narrow and elongated features, and appeared to be connected to a bulk of vesicles (Fig. 1C). In the epithelium fold, the tissue was expanded because it was filled with vesicles of varying size which extended up to its apex where small vesicles accumulated (Fig. 1C).

The intestine extended in a curve (U shape) from the stomach to the rectum. The simple intestine epithelium appeared to be composed of ciliated columnar cells characterized by cytoplasmic granular material and small vesicles (Fig. 1D, and inset 3). The histological preparations and Mallory's stain did not allow to identify the gastric and intestinal epithelium-associated mucus as well as undifferentiated cells.

The connective tissue was associated to the outer surface of the digestive canal, outside which the testicular follicles were located (Fig. 1A). This tissue appeared to be composed by a network of fibers to which packed or scattered populations of hemocyte-like cells were associated (Fig. 1B and C). Although, detailed observations on these cells were not performed, their morphological features were similar to those of circulating hemocyte types. Hemocyte-like cells enriched the connective tissue (Fig. 1B and C), whereas the inset 4 in Fig. 1D shows that, in some traits, hemocyte-like cells could be less abundant and scattered in the tissue.

3.2. Specific antibodies identified the CiLgals-a and CiLgals-b in the stomach epithelium

Fig. 2A–G shows that both CiLgals have similar tissue localization in the stomach or intestine epithelium, and Fig. 2H displays a histological section after the treatment with the secondary antibody alone, as a control. Along the stomach epithelium, traits more or less marked by both antibodies were observed. Between the furrows-ridges, traits of simple columnar epithelium ("gland cells") presented a high galectin content in the form of numerous positive

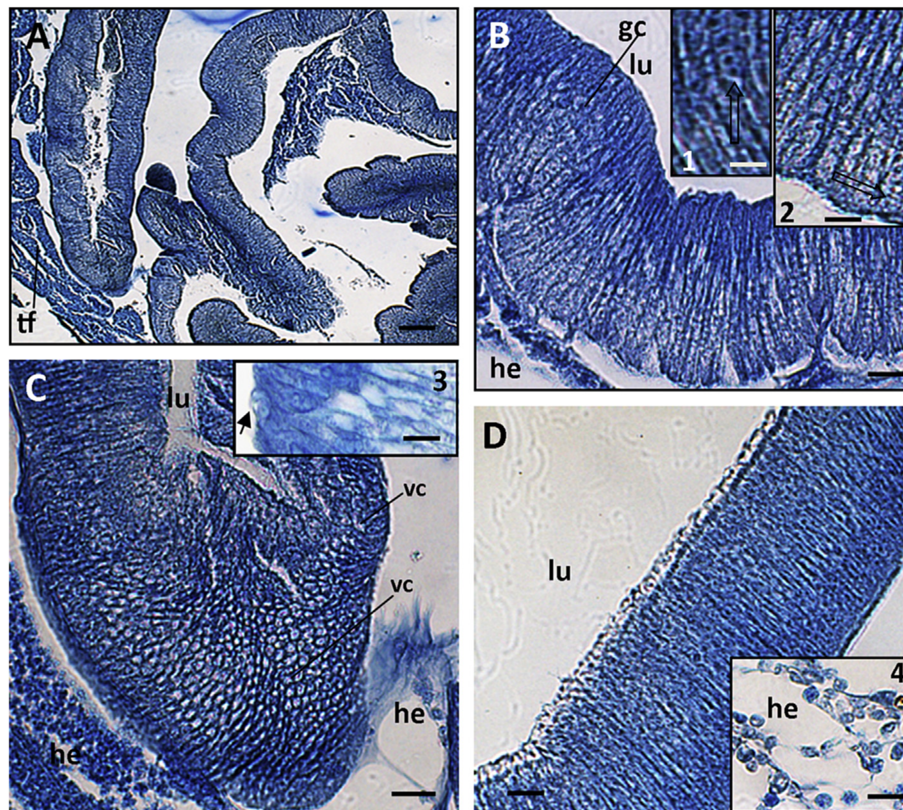

Fig. 1. Histological sections of *Ciona intestinalis* stomach and intestine epithelia (Mallory's stain). A: Broad view of the stomach and mid-gut. B: Stomach gland cells; magnification (inset 1) shows cytoplasmic vesicles and small vesicles containing a single granule; C: A stomach epithelium furrow full of vesicles; "vacuolated cells" and "gland cells" respectively, at the buttom and at the ridges of a groove, can be seen. In the inset 2, small vesicles appear to be released into the gut lumen. D: Intestine; magnification (inset 3): columnar cells in which cytoplasmic vesicles, containing a single granule, are seen; the inset 4 shows hemocyte-lke cells associated with the connective tissue. lu: gut lumen; gc: "gland cells"; vc: "vacuolated cells"; he: hemocyte-like cells; tf: testicular follicles. Bars, Bars: B, B1, D = 10 μm; B2, C, C3, D4 = 20 μm; A = 50 μm.

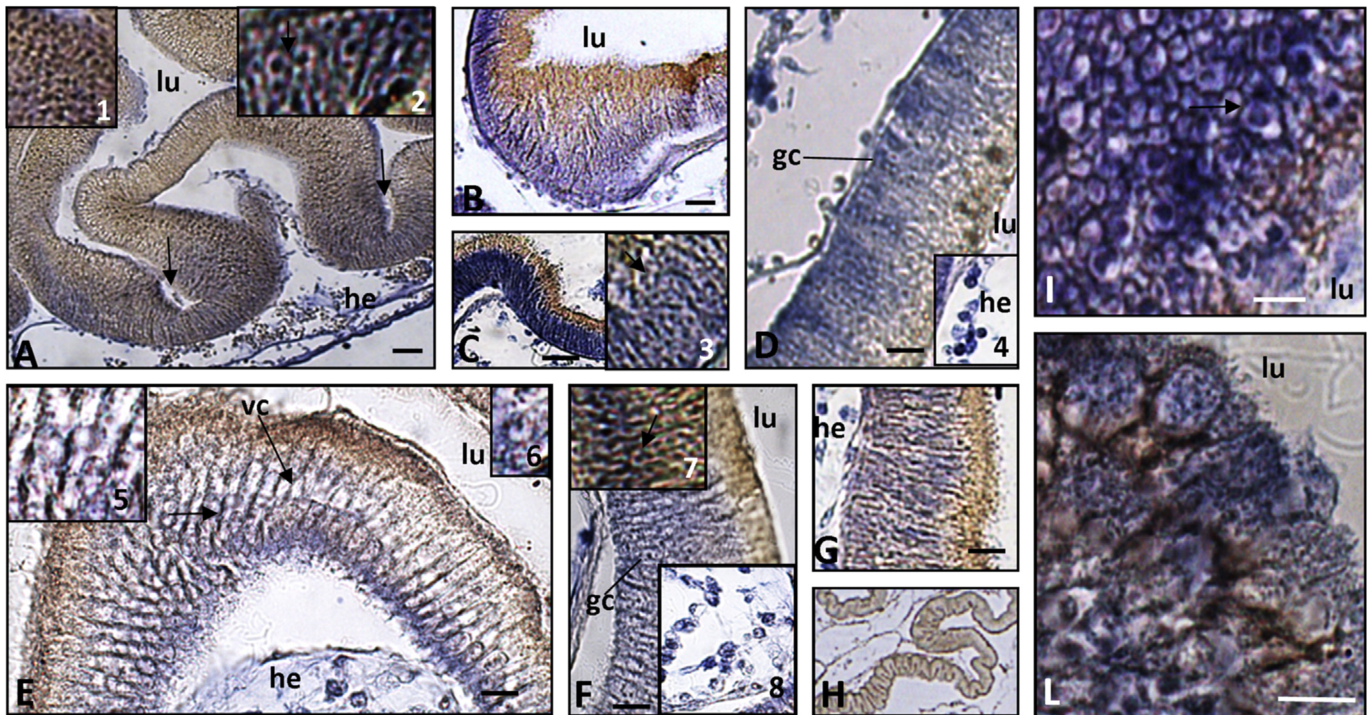

Fig. 2. Immunohistochemistry with anti-CiLgals-*a* (A-D, I,L) or CiLgals-*b* (E–G) antibody on histological sections of *Ciona intestinalis* stomach and intestine epithelium. A–C: Stomach epithelium, immunohistochemistry with anti-CiLgals-*a* antibody. A: stomach furrow-ridge full of small vesicles containing a single positive granule (magnifications 1 and 2). B: another view of a furrow-ridges full of CiLgals-*a* granules and small vesicles containing a single positive granule. C: Positive epithelium between furrow-ridges; the enlargement in the inset 3 shows small vesicles containing a single granule. D: Intestine; inset 4: hemocyte-like cells associated with the connective. E–F: Stomach epithelium, immunohistochemistry with anti-CiLgals-*b* antibody. E: stomach furrow-ridge, CiLgals-*b* on the outer surface of elongated vesicles (magnification 6); the magnification 7 shows small vesicles containing a single positive granule. F: Positive epithelium between furrow-ridges; the magnification 8 shows small vesicles containing a single granule; hemocyte-like cells associated with the connective (inset 9). G: Intestine; inset 4: hemocyte-like cells associated with the connective. I,L: Magnification of vesicles on the luminal side of a stomach epithelium furrow; the granular positive content can be released on the luminal surface of the epithelium. Arrows indicate small vesicles containing a single labeled granule. lu: gut lumen; gc: "gland cells"; vc: "vacuolated cells"; he: hemocyte-like cells. Bars: D, E, F, G, I,L = 10 μm; A; B = 20 μm; C: 60 μm; H = 300 μm.

granules (Fig. 2C,F). In these regions small vesicles with an internal positive granule can also be seen (Fig. 2C,F insets 3,7). The density of the positive material was high in a large part of the basal zone of the section and extended to the midsection.

Fig. 2(A–C, E) shows various features of furrows in the folds along the stomach epithelium. The anti-CiLgals-*a* or the anti-CiLgals-*b* antibody localized the protein in granules that occupied the basal and midsection zone of the furrows where, as shown by Mallory's stain (Fig. 1C), vesicles were located (Fig. 2A). The content of these vesicles was unlabeled by the immunohistochemistry method, so "vacuolated cells" could not be distinguished, whereas positive (with both antibodies) granules were contained inside small vesicles (Fig. 2A, magnifications 1, 2; Fig. 2C, magnification 3; Fig. 2E, magnification 6). The anti-CiLgals-*b* antibody detected another feature of the cytolocalization showing packed and elongated vesicles bordered by positive material, while their content was unmarked (Fig. 2E, magnification 5). In the same area, small vesicles containing a single positive granule were also present (Fig. 2E, magnification 6). In a furrow, the density of the granules decreased from the cell basis to the apex (Fig. 2A–C,E). The magnification of a histological section treated with anti-CiLgals-*a* antibody shows the luminal side of the epithelium in a furrow (Fig. 2I), where a bulk of vesicles contained labeled granules while some of them, larger in size, discharged their content on the luminal surface of the stomach (Fig. 2L).

In the connective tissue that lined the outer side of the stomach, hemocyte-like cells were marked by the CiLgals-*a* or CiLgals-*b* antibody (Fig. 2A,C,E, inset 8).

3.3. CiLgals-a and −b galectins are contained in the intestine epithelium

The reaction with anti-CiLgals-*a* (Fig. 2D) or anti-CiLgals−*b* antibody (Fig. 2G), showed that the galectins were spreaded in the columnar cells of the intestine epithelium. As already seen in the stomach epithelium histological sections, the intensity of the positive reactions decreased from the basal side up to the luminal one. Labeled hemocyte-like were components of the connective tissue (Fig. 2D inset 4; Fig. 2G).

3.4. CiLgals-a and -b riboprobes mark the stomach epithelium

Along the stomach epithelium, traits more or less marked by both riboprobes were observed. The CiLgals-*a* and the CiLgals-*b* transcripts were mainly found in "gland cells" of the stomach epithelium that linked the furrows, where those transcripts were mainly localized in the inner side of the tissue (Fig. 3A,E). The CiLgals-*a* transcript was scarcely present in the folded epithelium where the "vacuolated cells" were located (Fig. 3A), whereas the hybridization assay with the CiLgals-*b* riboprobe showed that, in some cases, the "vacuolated cells" located in the basal area of a furrow were marked (Fig. 3, inset 2). The thin layer marked by the riboprobe found in some traits of the outer or luminal surface of stomach epithelium, could merely depend on the passive capture of the probes. In the connective tissue associated to the stomach, hemocyte-like cells were marked by the CiLgals-*a* or CiLgals-*b* riboprobe (Fig. 3A, inset 1; E, inset 3). Fig. 3 D and G show the controls with the sense riboprobes.

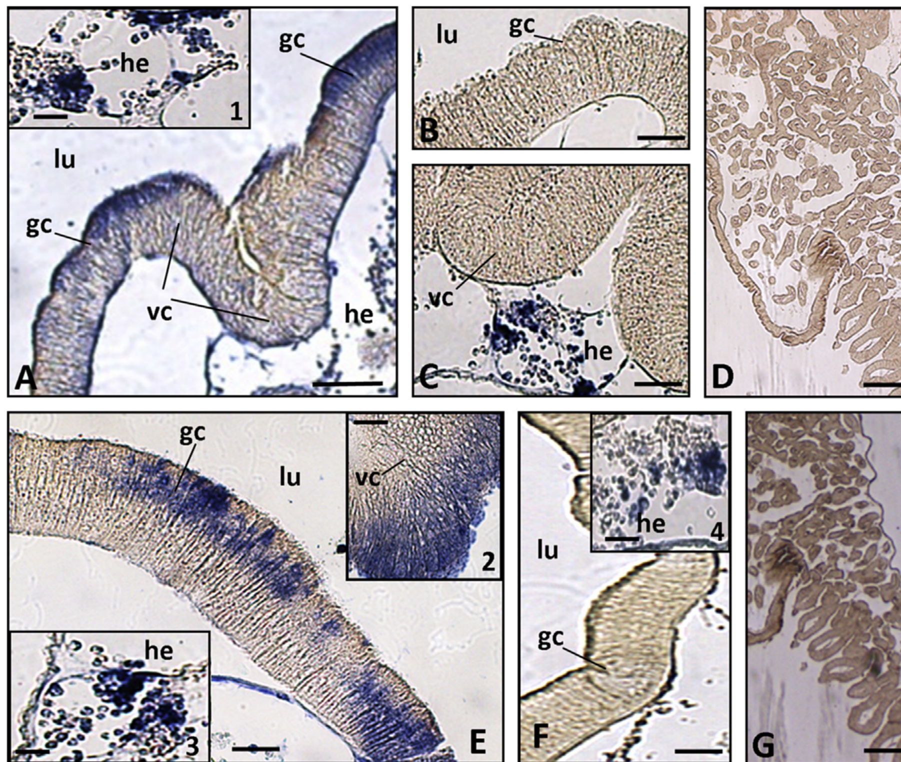

Fig. 3. ***In situ*** **hibridization with CiLgals-*a* and CiLgals-*b* riboprobes on histological sections of *Ciona intestinalis* stomach and intestine epithelium**. A: Stomach epithelium treated with CiLgals-*a* riboprobe. B–C: unmarked intestine epithelium and labeled hemocyte-like cells in the connective tissue. D: Control section treated with the sense probe; E: Stomach epithelium between furrow-ridges treated with CiLgals-*b* riboprobe; inset 2: labeled cells in a furrows; inset 3: labeled hemocyte-like cells in the connective tissue. F: unmarked intestine epithelium treated with CiLgals-*b* riboprobe; inset 4: labeled hemocyte-like cells in the connective tissue. G.: Control section treated with the CiLgals sense probe. lu: gut lumen; gc: "gland cells"; vc: "vacuolated cells"; he: hemocyte-like cells. Bars, Bars: A, A1,B, C, D, E, E3, F4, D1, E2 = 20 μm; D,G = 300 μm.

3.5. Riboprobe labels were not found in the intestine epithelium

Neither the CiLgals-*a* (Fig. 3B and C) or the CiLgals–*b* (Fig. 3F) riboprobe marked the intestine epithelium. Conversely, both riboprobes labeled hemocyte-like cells scattered in the surrounding connective tissue (Fig. 3A inset 1; Fig. 3E inset 3). Since in a same histological section unmarked epithelium and labeled hemocyte-like cells were found, the accuracy of the hybridization method was recognized (Fig. 3C; Fig. 3F, inset 4).

4. Discussion

Since the main aim of the present paper was to search the constitutive expression of the immune-related CiLgals-*a* and -*b* genes in *C. intestinalis* stomach and intestine tissues, histochemical tests were not carried out to identify every cell type that characterizes these tissues, as previously reported in detail by other Authors [28–31]. The Mallory's staining distinguishes the folded (furrow-ridges) and unfolded gastric epithelium, while a dynamic succession of functional phases of these two regions along the epithelium can be hypothesized. In the unfolded tissue, the columnar cells have the morphological features of "gland cells/ secretory cells" as described by Thomas [29,30] and Yonge [28]. As reported by these Authors, cytoplasmic fine structures characterize synthesizing cells and can reflect a phase of the secretory process, as also indicated by many small vesicles filling the cytoplasm. The histochemical reactions [29] had shown that "gland cells" produce a seromucous secretion particularly rich in proteins. In addition, the fine structure of "gland cells" further up the side wall of a furrow, suggested that they are no longer actively secreting (post-synthesizing cells) [29]. Although the present observations cannot distinguish "vacuolated cells" as described by Thomas [29,30], cells similar to them could be recognizable at the upper side wall and top of a stomach ridge, while vacuoles which enrich a forrowed epithelium accumulate apically, and they appear to be swelled and released from the cell apex. Moreover, undifferentiated cells [29,31], at the bottom of a ridge, could not be recognized by using the Mallory's stain.

Immunohistochemical analysis with the anti-CiLgals-*a* and -*b* antibodies show several features of the epithelium involved in galectins production. Various aspects of galectins localization are presumably dependent on the examined sections and physiological *status* of the epithelium, i.e. producing and releasing pathways. The columnar cells of the epithelium between the furrow-ridges and the cells in the grooves contain the galectins mainly associated with small vesicles containing a single positive granule. The observation of a luminal region of the epithelium shows that small vesicles, accumulated apically, could fuse to form larger vesicles full of smaller positive granules which could be released on the luminal epithelium surface.

Although we were not able to demonstrate the association galectin-mucus, the possibility exists that the released galectins are associated to the mucus (carbohydrate-protein complexes and sulphated acid mucopolysaccharides) [29,30]. It is reasonable that the treatments of the histological procedure has eliminated the mucus, therefore that association cannot be observed.

Another feature of the galectin localization is shown by an epithelium fold in which the CiLgals-*b* appears to be mainly associated with the outer surface of elongated vacuoles that occupy the cytoplasm of the cells in the furrow. Such a cytolocalization could

be dependent on the site of galectin synthesis that bypasses the classical biosynthetic ER-Golgi pathway. In this respect, the galectin-containing small vesicles, observed in all the epithelium sections regardless of the used primary antibody, may be components of an exosomal pathway that could provide an alternative route for galectin secretion into the extracellular milieu [35]. It can be hypothesized that the figures, here showed, represent synthesizing (epithelium between the grooves) and post-syntesizing/releasing (epithelium grooves full of small vesicles) phases of the stomach epithelium activity.

In situ hybridization assay results coincide in good part with the immunohistochemical galectins cyto-localization, while the thin layer marked by the riboprobe found in some traits of the outer or luminal surface of stomach epithelium, could merely depend on the passive capture of the probes. The CiLgals-*a* and CiLgals-*b* transcripts were mainly found in the stomach "gland cells" of the epithelium between the folds. In the stomach ridges, where the galectin-containing small vesicles were abundant, the CiLgals-*a* transcript was almost absent, However, this result cannot be generalized, and the CiLgals-*b* riboprobe labels the transcript in the cells at the basis of a furrow.

All in all, the observed differences between CiLgals-*a* and CiLgals-*b* mRNAs and proteins localization in the tissue, as well as the differences in the cell features in several histological sections [27], could be mainly imputed to mRNA transcription and protein production phases, and their turnover levels along the tissue as well as to differences between sampled individuals. In this respect, we could speculate that "gland cells" of the gastric epithelium and the furrow tissue rich in vesicles may represent two subsequent phases of the epithelium activity that includes seromucous production [27].

The present findings show that, in agreement with the constitutive expression in the pharynx [19,23], both immune-related galectin genes are transcribed in the stomach epithelium while the potential roles of the CiLgals remains to be examined. Likewise, differences between CiLgals-*a* and CiLgals-*b* immunolocalization in the examined histological sections, cannot be retained significant to suggest different roles.

It is of interest that, in mammals, multifunctional galectins are produced by the epithelium of the alimentary canal [36–39]. This localization has suggested the galectin contribution to host-pathogen interaction, epithelial homeostasis and trafficking in the gut. Recently, some papers reported the role of *C. intestinalis* gut in the immunity, controlling bacteria that colonize this apparatus [25–27]. Outstanding research by using real-timePCR analysis on the gut of naïve and LPS-inoculated ascidians, could contribute in disclosing the potential defence role of CiLgals in the gut. However, based on the known properties of the galectins, the CiLgals may also be involved in mucopolysaccharides processing, intracellular functions, and proliferation pathway of undifferentiated cells [2,6,7].

Our findings on the intestine are intriguing. The immunohistochemistry analysis showed columnar cells containing both the galectins, whereas the riboprobes, in spite of the repeated assays, never label these cells. Although the effect of the used methodoly cannot be entirely excluded, the outcome on the lack of riboprobe labels in this tissue seems to be independent on method troubles, because in a same histological section in which intestine cells were negative, the riboprobes mark hemocyte-like cells [40] associated to the surrounding connective tissue. Formerly, Ermak [31] has shown that the cells, associated to the connective tissue around the digestive canal, are differentiated hemocytes. In addition, Vizzini et al. [19] reported that, in the hemolymphatic connective *lacunae* associated to the pharynx, hemocytes were marked by antibodies or riboprobes that characterize them as CiLgals-*a* and −*b* producing cells, while it is known that separated hemocyte populations release *in vitro* galectin-like lectins with opsonizing properties [41]. The present observations cannot explain the difference between stomach and intestine behavior, and further experiments on the physiology of the gut, related to the CiLgals gene expression, could answer the question. According to Yonge [28] and Thomas [29,30], the possibility exists that the mere galectin content in the intestine may be related to its absorption activity which, conversely, is scarce in the stomach. On the other hand, the absorption from the hemocyte-like cells, as galectins-producing and releasing cells, associated to the connective tissue cannot be excluded.

In conclusion, the findings here reported on the CiLgals localization and gene transcription in the gut show that the immune-related CiLgals genes are involved in the gut physiology, presumably exerting a multifunctional role that could include the immune function. In this respect is relevant that the CliLgals are components of the inflammatory response, as well as they are constituvely expressed by the endostyle which contributes in filter feeding [19,23], and are involved in oogenesis (unpublished data). In mammals, galectins participate in a wide variety of intracellular functions [42] and are versatile modulators of cell adhesion, cell proliferation, and cell death [43].

Some properties of the galectin molecular structure could be consistent to the CiLgals multifunctional role [8,18]. Although they share common affinity for basic β-galactosides of glycosilated ligands, each CiLgals may display glycan-binding properties which allow the binding to different glycan structures (either branched, repeated, substituted) that characterize the geometry of multivalent carbohydrate ligands located in cell compartments or extracellular spaces. In addition, the ability of galectins to form oligomers suggests a certain degree of plasticity in sugar binding, so many glycan-binding-proteins oligomerize and interact with multivalent array of glycans leading to increased affinity for various and complex glycan structures. On the other side, the potential multiple role of galectins may be directly associated to the glycome (that includes glycans and glyconjugates) that characterize each cell type [8,44,45], and it is influenced, along with the glycan-binding protein genes expression, by numerous factors thus creating a system highly diverse and dynamic.

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