Fish & Shellfish Immunology 42 (2015) 171–176

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Ciona intestinalis galectin (*Ci*Lgals-a and *Ci*Lgals-b) genes are differentially expressed in endostyle zones and challenged by LPS

Daniela Parrinello, Maria Antonietta Sanfratello, Aiti Vizzini, Nicolò Parrinello, Matteo Cammarata*

University of Palermo, Biological Chemical Pharmaceutical Sciences and Technologies, Via Archirafi, 18, Palermo, Italy

ARTICLE INFO

Article history:
Received 19 July 2014
Received in revised form
21 September 2014
Accepted 17 October 2014
Available online 8 November 2014

Keywords:
Ascidian
Galectin
Endostyle
Inflammation
Ciona intestinalis

ABSTRACT

Immunohistochemical and *in situ* hybridization assays were performed to answer the question whether the endostyle, that is the initial gastro-intestinal trait of *Ciona intestinalis* pharynx, is involved in galectin (*Ci*Lgals-a and *Ci*Lgals-b) production during the pharynx inflammatory response to LPS inoculation. Specific anti-*Ci*Lgal-a and anti-*Ci*Lgals-b antibodies, and oligonucleotide probes, that mark inflammatory hemocytes inside the pharynx vessels and vessel epithelium as shown by a previous paper, were assayed on endostyle histological sections. For the first time, we show that galectins are produced by endostyle zones, and both *Ci*Lgals-a and −b genes are upregulated by LPS. *Ci*Lgals-a and *Ci*Lgals-b are constitutively expressed in the endostyle zone 2 and 3, respectively, both genes are upregulated by LPS in the zone 2, and *Ci*Lgals-b in the zone 3 and 4. The antibody-reacting material contained in intracellular and extracellular large vesicles suggest an unexpected vesicle-dependent transporting mechanism of galectins not provided with signal peptide. Differential expression and gene upregulation in not-treated and LPS-treated specimens, support the role of endostyle galectins both in filter feeding and defense responses.

1. Introduction

Galectins form an evolutionarily conserved protein family characterized by homologous carbohydrate recognition domains (CRD) with a relatively high affinity to β-galactosides [1,2]. All known galectins are classified into three types in terms of molecular architecture, i.e. mono-CRD, bi-CRD and chimera types. In most animals, a complex galectin repertoire recognizes and cross-links glycan groups of glycoconjugates of cells and extracellular matrix [1–4]. Ligands can be cell membrane-bound, secretory molecules or bacterial lipopolysaccharides, so variations in glycan binding preference suggest galectin diversity in recognition. In mammals, at least 15 galectin subtypes have been identified [1] and several galectins have been found in birds, frogs, and many invertebrate species, including ascidians and cephalochordates [5–8]. Galectins lack of signal sequence and glycosilation, it is generally considered that they escape the ER/Golgi apparatus and are released extracellularly to be involved in cellular communication, self not-self recognition, host pathogen interaction, inflammation, development, differentiation [6,9,10] and malignant transformation [11].

Ascidians represent a key group in chordate phylogenesis, and are retained the sister group of vertebrates [12–15]. In *Ciona intestinalis*, two bi-CRD galectins (*Ci*Lgals-a and *Ci*Lgals-b) have been identified. According to Houzelstein et al. [5], the *Ci*Lgals-a exhibits the F4-CRD-linker-F3-CRD gene arrangement typical of the amphioxus and vertebrate Bi-CRD Lgals genes, the *Ci*Lgal-b presents a specific F4-CRD-linker-F4-CRD gene organization. Sequence homology and gene organization suggest a tandem duplication of an F4-CRD gene organization that would have given rise to the ancestral F4-CRD-linker-F3-CRD found in chordates. In a recent paper we reported that these *Ci*Lgals are inducible by LPS inoculation [16]. Real time PCR profiling, *in situ* hybridization and immunohistochemistry methods disclosed that both genes are promptly (within 4 h) upregulated in pharynx vessel epithelium and hemocytes. Finally, the homology modeling process showed that the N-CRD and C-CRD of *Ci*Lgals-a as well as the *Ci*Lgals-b domains, are suitable for binding to β-galactosides, and can be superimposed to human C-CRDs and N-CRDs showing a common structural model [16].

Although the pharynx has been defined as the main ascidian immune-competent organ [17], it is the initial part of the ascidian digestive tract. The branchial basket, consists of two epithelial monolayers perforated by rows of ciliated stigmata aligned dorso-ventrally and enclosed in a mesh of vessels where the hemolymph

* Corresponding author. Tel.: +39 09123891805.
E-mail address: matteo.cammarata@unipa.it (M. Cammarata).

http://dx.doi.org/10.1016/j.fsi.2014.10.026
1050-4648/

flow [18–20]. The endostyle is a pharyngeal organ that functions in internal filter feeding of urochordates, cephalochordates and larval cyclostomata; it is retained a key structure in the evolution of chordates [21,22]. It has the form of a trough-shaped structure in the ventral wall of the pharynx, histologically divided in eight functional units called "zones" that extend antero-posteriorly, and are numbered bilaterally from midventral to dorsolateral [18,23–25]. The endostyle extends to the esophagus and produces the mucus, a complex of mucoproteins and mucopolysaccharides. The resulting mucus net, produced by the cells of the zone 1–4, is an elongated mesh consisting of transverse and longitudinal filaments that form an extremely high porosity that can capture particles as small as bacteria that adhere to the mucus components [21,24,26]. The bottom of the groove (zone 1) is lined with a longitudinal row of very long cilia that move the mucus to the sides of the endostyle and then outward by lateral cilia. At first, to check for the galectins immune role, observations were focused on the pharynx vasculature and hemocytes [16]. To answer the question whether *C. intestinalis* endostyle is also involved in immune response and whether galectins are expressed in this gastrointestinal tract, histological sections were examined with the anti-*Ci*L-gal-a and anti-*Ci*nLgals-b antibodies, and oligonucleotide probes specific for each of the galectin subtypes. For the first time, we show that galectins are produced by endostyle zones, and genes are upregulated following LPS inoculation. In addition, differences in gene expression between not-treated and LPS-treated ascidians, suggest the endostyle galectins involvement both in filter feeding and internal defense. Surprisingly, the antibody-reacting material was contained within intracellular and extracellular vesicles indicating a galectin vesicle-linked transport mechanism.

2. Materials and methods

2.1. Animals and pharynx tissue preparation

Ascidians, from Termini Imerese marinas (Italy), were maintained in aerated sea water at 15 °C and fed every second day with a marine invertebrate diet (Coraliquid, Sera Heinsberg, Germany). Before any treatment the tunic surface was cleaned and sterilized with ethyl alcohol. As previously described [27], 100 μg LPS (*Escherichia coli* 055:B5, LPS, Sigma–Aldrich, Germany) in 100 μl sterile marine solution (12 mM $CaCl_2 \cdot 6H_2O$, 11 mM KCl, 26 mM $MgCl_2 \cdot 6H_2O$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0) *per* specimen, were inoculated into the body middle region, just under the tunic, to allow the LPS spreading into the pharynx tissue. At 4 h post inoculation (p.i.) the pharynx region containing the endostyle was excised. Ascidians, either untreated (naïve) or injected with 100 μl marine solution (sham ascidians), were used as a control. Samples from 10 specimens for each treatment or naïve animals, were examined.

The experiments were 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).

2.2. Histological methods

For histological studies, serial sections of paraffin-embedded pharynx, opportunely excised to contain the endostyle, were alternatively examined by *in situ* hybridization and immunohistochemistry under a Leica DMRE microscope. To display the endostyle histological organization in naïve ascidians, sections were stained with Mallory's trichrome stain [28].

2.3. In situ hybridization

The ISH method has been previously reported [16,27]. In brief, histological sections were treated with *Ci*Lgals-a and *Ci*Lgals-b digoxigenin-11-UTPlabeled riboprobes (1 mg/ml final concentration) (Roche Diagnostics) as reported. 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 *Ci*Lgals-b cDNA [16]. After digestion with proteinase K (10 mg/ml) in PBS-T (NaCl 0.1 M; KCl 0.02 M; KH_2PO_4 0.01 M; Na_2HPO_4 0.06 M, pH 7.4 containing 0.1% Tween 20) for 5 min, sections washed with PBS-T 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. Sense probe preparations were as controls.

2.4. Antibody specificity and immunohistochemical assays

In a previous study [16], specific antibodies versus *Ci*Lgals-a and *Ci*Lgals-b galectin subtypes were prepared and assayed in pharynx histological sections. As previously shown, the antibodies were selectively purified, and tested for their specificity. In brief, antibodies were raised in rabbit against peptides selected from the deduced mature *Ci*Lgals-a and *Ci*Lgals-b sequences, and provided of immunogenic properties (Sigma-Genosys, UK). *Ci*Lgals-a DTGIEIPKPAVDTL-C and *Ci*Lgals-b MFRTQRKLNRPAI-C peptides were synthesized, antiserum were produced by Sigma-Genosys, and stored at −80 °C until to be used. The rabbit serum antibody titer (1:25,000) was recorded by ELISA. Immunoglobulins were isolated from the rabbit serum by affinity chromatography through a Protein G-Sepharose column (GE Healthcare Biosciences) eluted with 0.1 M glycine-HCl pH 2.8, then an antigen peptide coupled to CNBR-activated Sepharose 4B (GE Healthcare Biosciences) column was used [16]. The antibody titer of pooled and PBS-dialyzed fractions was checked by ELISA on plates (Nunc, Denmark) coated with the peptide used as an antigen (10 μg/well). Then, the wells were incubated in blocking solution, with: 1. Anti-*Ci*Lgals-a and anti-*Ci*Lgals-b antisera diluted (1:1000–1:50,000); 2. Pre-immune rabbit serum (1:50–1:200); 3. Purified antibody fraction (1:1000–1:50,000). The antibody–peptide reaction 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). Histological sections were incubated with 3% BSA in phosphate buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBS-T) for 2 h at r.t., and then (overnight at 4 °C) with primary antibody (0.0170 μg/μl anti-*Ci*Lgals-a or 0.026 μg/μl anti-*Ci*Lgals-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.

3. Results

3.1. Expression of galectins by the endostyle zones of naïve and sham ascidians

A typical endostyle organization is displayed in Fig. 1(a) that shows a histological transverse section treated with Mallory's

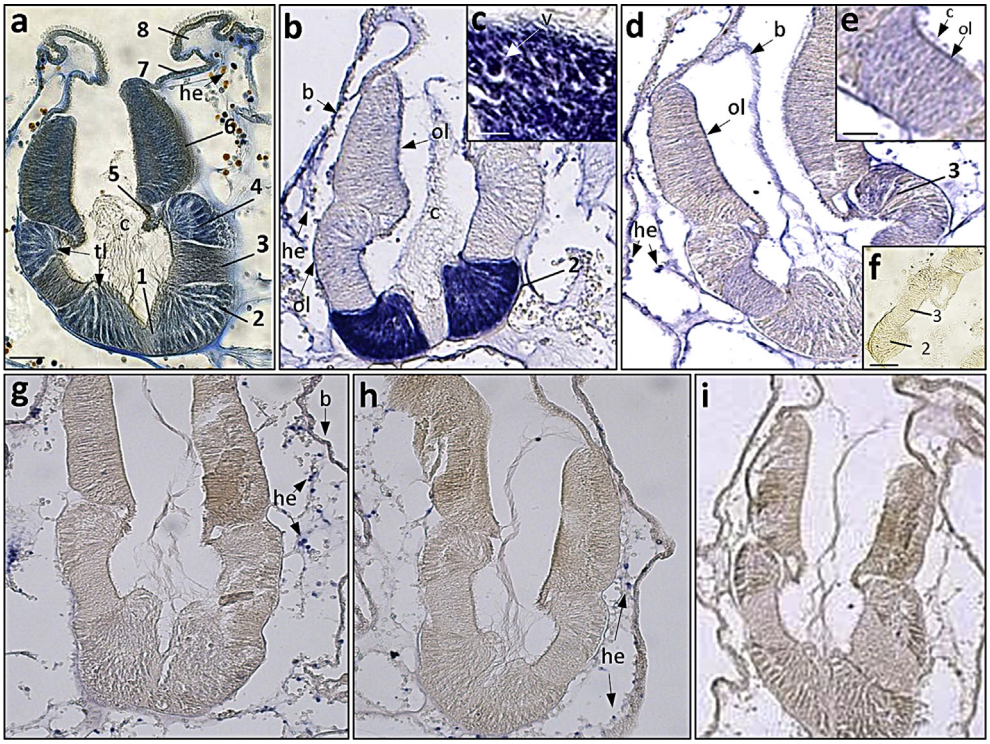

Fig. 1. Immunohistochemistry with anti-*Ci*Lgals-a and anti-*Ci*Lgals-b antibodies and *in situ* hibridization assays on transverse sections of the *Ciona intestinalis* endostyle from naïve and sham ascidians. a: Mallory's staining, the eight zones of the endostyle are indicated by corresponding numbers, the cells of the zone 1 bear very long cilia; hemocytes are scattered in the surrounding tissues. b: Treatment with anti-*Ci*Lgals-a antibody. c: Magnification of zone 2, seen in "b", showing numerous positive vesicles. d: Treatment with anti-*Ci*Lgals-b antibody, an outer layer and zone 3 are marked by anti-*Ci*Lgals-b antibody. e: Magnification of zone 3 shows tiny positive granules inside the narrow elongated cells, and positive material layered at the base of the cilia. f: Treatment with the secondary antibody. g: *In situ* hybridization with *Ci*Lgals-a riboprobe that shows marked hemocytes, only. h: *In situ* hybridization with *Ci*Lgals-b riboprobe, that shows marked hemocytes, only. i: *In situ* hybridization with sense riboprobes, where marked hemocytes cannot be seen. The numbers from 1 to 8 indicate the distinct endostyle zones. c: cilia; b: pharynx bar; tl: tuft-like area; v: vesicle; he: hemocytes; ol: outer galectin layer. Bar in a,b,d,g,h,i = 40 μm; bar in c = 20 μm, bar in e = 15 μm, bar in f = 100 μm.

thricrome stain. According to previous light and electron microscopy observations [25,29–31], eight zones run parallel to one another in longitudinal orientation. Zone 1 is the median region forming the bottom of the groove, where epithelial cells are provided with very long cilia. Zone 2, 4, 5, 6, 7 and 8 consist of two symmetrically rows forming the lateral walls of the endostyle. As shown in Fig. 1a, the zone 2 is formed by elongated columnar cells arranged in a fan-shaped pattern, the proximal side of this zone is covered by a layer of cubical cells. The cells stand more or less parallel with each other, their apices crowded together in a narrow zone, and form a tuft-like structure. The zone 3 presents markedly narrow (1–3 μm in thickness) ciliated cells. The median zone 4, resembles the histological organization of the ventral zone 2. Hemocytes are scattered in the surrounding pharynx tissues (Fig. 1a). Since galectin production was mainly disclosed in the zones 2, 3 and 4, details of the zones 1 and 5–8 are not reported. In the untreated or sham (inoculated with marine solution) ascidians, the immunohistochemistry reaction with the anti-*Ci*Lgals-a antibody strongly marked the cells of zone 2 (Fig. 1b). When sections from a more distal cutting plane were examined, numerous extracellular large vesicles (3.5–8.5 μm in diameter) full of antibody reacting material were seen at the apical area of the cells (Fig. 1c). A weak staining indicated a diffuse sharing of the *Ci*Lgals-a galectin scattered throughout the zones 3–6. The anti-*Ci*Lgals-b antibody mainly marked the zone 3 (Fig. 1d) where positive, finely granular material was scattered in the cytoplasm of the narrow elongated cells (Fig. 1e), while antibody-reacting material was scattered throughout the cells of the zones 2 and 4–6.

In addition, both antibodies reacted with some traits of extracellular material that form an outer layer strictly adhering to the endostyle surfaces (both proximal and distal), clearly visible at the base of the cilia lining the zone 3 (Fig. 1e). According to a previous paper [16], both antibodies also reacted with the epithelium lining the pharynx bars where positive hemocytes were scattered (Fig. 1b,d). In each of the 20 specimens examined, either naïve or sham, the riboprobe hybridization signal of *Ci*Lgals-a or *Ci*Lgals-b was not found in any endostyle zone (Fig. 1g,h), whereas, according to a previous paper [16] marked hemocytes adhering to the pharynx bars could be seen. Controls performed with the secondary antibody or the sense strand riboprobes were always negative (Fig. 1f and i).

3.2. Expression of galectins by endostyle zones following LPS inoculation

In the zone 2, the anti-*Ci*Lgals-a antibody showed the positive dense material contained in the cytoplasm of the cells which run parallel to one another (Fig. 2a). In the enlarged Fig. 2l, the antibody reaction displays the cytoplasmic and vesicular localization of the *Ci*Lgals-a. As shown by Fig. 2d the *Ci*Lgals-b was produced by the cells of the zone 2, and more strongly by the zone 4, while positive tiny granular material was scattered throughout the cells of zones 3 and 6 (Fig. 2d). Following the LPS inoculation, a more evident *Ci*Lgals-b content in the layer of extracellular material that lined the endostyle could be seen (Fig. 2d,m). The cytoplasmic and vesicular localization of the *Ci*Lgals-b in the columnar cells of the zones 2 and 4 is shown by magnified views (Fig. 2m,o,p). Similar pictures were seen for *Ci*Lgals-a. *In situ* hybridization assays, carried out on serial sections, revealed that the LPS inoculation challenged the cells of the zones 2, 3 and 4, and the genes were upregulated. An intense

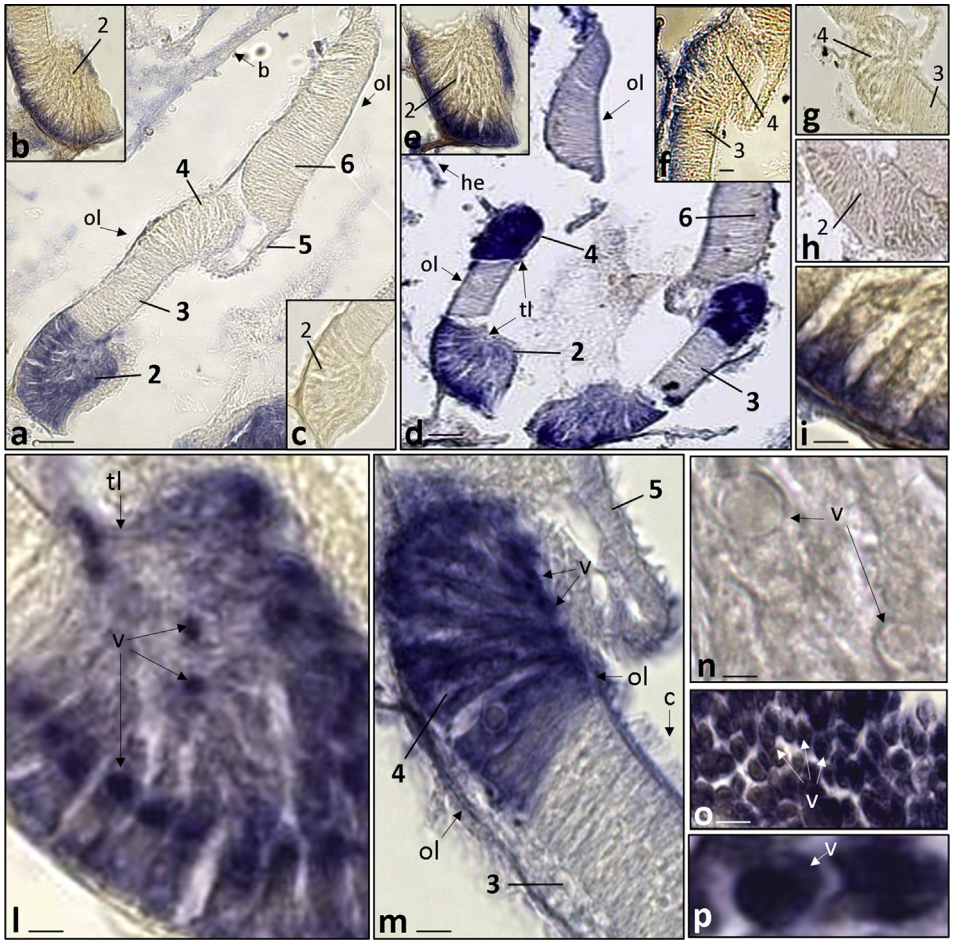

Fig. 2. Immunohistochemistry with anti-*Ci*Lgals-a and anti-*Ci*Lgals-b antibodies and *in situ* hybridization assays on transverse sections of the *Ciona intestinalis* endostyle following LPS inoculation. a: Treatment with anti-*Ci*Lgals-a antibody; the zone 2 is intensely marked, traits of outer material layer are slightly marked. b: *In situ* hybridization with *Ci*Lgals-a riboprobe, the basal area of the zone 2 cells is intensely marked. c: Zone 2 treated with the secondary antibody. d: Zone 2 and 4 treated with anti-*Ci*Lgals-b antibody; traits of positiver material lining the cells are positive, while tiny granules inside the cells of zones 3 and 6 are slightly positive. e: Zones 2 treated with *Ci*Lgals-b riboprobe. f: *In situ* hybridization with *Ci*Lgals-b riboprobe intensely marks the basal area of the zones 3 and 4 cells. g: Zones 3 and 4 treated with the secondary antibody. h: Zones 2 treated with the sense riboprobe. i: Magnification of the zone 4 treated with the *Ci*Lgals-b riboprobe. l: Magnification of the zone 2 treated with anti-*Ci*Lgals-a antibody. m: Magnification of zone 4 treated with anti-*Ci*Lgals-b antibody. n: Vesicles in the zone 4 treated with secondary antibody o: Vesicles in the zone 4 after treatment with the anti-*Ci*Lgals-b antibody. p: Magnification of vesicles seen in "o". The numbers from 2 to 6 indicate the distinct zones. c: cilia; ol: outer galectin layer; v: vesicle; t-l: tuft-like area, he: hemocytes, b: pharynx bar. Bar in a,b,c,e,g = 20 μm; bar in d,h = 25 μm; bar in f,o = 10 μm; bar in i: 7 μm; bar in l: 5 μm; bar in m: 4 μm; bar in n,p: 3.5 μm.

*Ci*Lgals-a riboprobe signal was found in the basal area of the zone 2 columnar cells (Fig. 2b). A Figure magnification better shows the *Ci*Lgals-a riboprobe localization at the basal area of the columnar cells of the zone 2 (Fig. 2i). A similar localization of the *Ci*Lgals-b riboprobe signal marked the cells of the zone 4 (Fig. 2f), and at the basal area of the narrow elongated cells of the zone 3 (Fig. 2f) where a lesser amount of protein had been recognized by the antibody (Fig. 2d). Although, in the examined sections, the antibodies did not mark the cubical cell layer that cover the proximal side of the zone 2, *in situ* hybridization following LPS challenge disclosed that they contained both *Ci*Lgals-a and *Ci*Lgals-b mRNAs (Fig. 2b, e). Fig. 2g and h show the features of the controls treated with secondary antibody and sense probe preparations, respectively.

3.3. Galectin containing vesicles

As shown by magnified views of sections from ascidians inoculated with LPS, the anti-*Ci*Lgals-b antibody marked material that filled large (about 3.4–8.5 μm in diameter) rounded vesicles located in the zones 2 (Fig. 2o,p) and 4 (Fig. 2m) at the middle part of every columnar cell and at the columnar apices that form the tuft-like structure. Similarly to what observed in naïve and sham ascidians (Fig. 1c), the anti-*Ci*Lgals-a antibody disclosed galectin-containing vesicles in the zone 2 (Fig. 2l). In both zones, vesicles found at the cellular apices crowded together to form a tuft-like structure (Fig. 2l), while more superficial sections disclosed numerous vesicles that could have been spread outside the cells from their apices (Fig. 1c). A control section treated with secondary antibody showed unstained vesicles which filled cells of the zone 4 (Fig. 2n), and a similar vesicle distribution was found in control sections of the zone 2.

4. Discussion

Although it is generally noted that galectin subtypes display cell and tissue-specific distribution, some of them are expressed in many tissues and cell types [32]. Anyhow, tissue localization and specific gene expression may contribute in elucidating their roles. Nothing is known about the galectins localization in the ascidian digestive tract, and the question arises as to whether the two already known ascidian galectins (*Ci*Lgals-a and -b), components of the *C. intestinalis* inflammatory response [16], are expressed by the

endostyle that is the initial digestive trait. According to previous reports [24,25], eight endostylar zones have been distinguished. Cells of zones 1, 3 and 5 are supporting elements involved in catching and transporting food. The zones 2, 4 and 6, are retained the main protein secreting glandular regions, and the zones 7 and 8 are thyroid-equivalent elements that incorporates iodine in iodoproteins [33]. By using specific antibodies and riboprobes, we show that galectins are expressed by endostylar zones and the immune-related *Ci*Lgals-a and *Ci*Lgals-b genes can be upregulated by a bacterial component, i.e. LPS. The cells of the zone 2 and 3 constitutively produce the *Ci*Lgals-a and *Ci*Lgals-b, respectively, being the density of the *Ci*Lgals-a protein in the columnar cells of zone 2 higher than that of the *Ci*Lgals-b in the narrow cells of the zone 3. Both cell types can be activated by LPS inoculation, the *Ci*Lgals-a and -b genes upregulated, and the riboprobe signals mainly localized at the basal area of the cells where the nucleus lays as shown by fine structure studies [25,26,29,34]. It is of interest the upregulation of the *Ci*Lgals-b gene in the zone 4 following LPS inoculation, where taller epithelial cells intensely produced the galectin. Although antibodies did not disclose galectins in the cubical cell layer that covers the proximal side of the zone 2, the *Ci*Lgals-b riboprobe marked these cells suggesting gene upregulation. An increased expression of *Ci*Lgals-b protein can also be indicated by the sharing of protein throughout the zone 3 and 5–6, as well as by the more dense outer material marked by the antibody at the endostyle surfaces where cilia are distributed.

The differential galectin expression levels between endostyle zones and the rest of the pharynx components, remains to be elucidated. In a previous paper, immunohistochemistry and *in situ* hybridization assays showed that both galectins were expressed by inflamed vessel epithelium and hemocytes inside the vessel lumen. In addition, real time PCR analysis of the whole pharynx disclosed a differential galectin genes modulation following LPS inoculation [16]. However, in that case the *Ci*Lgals-a appeared to be more expressed than *Ci*Lgals-b, whereas immunohistochemistry and *in situ* hybridization, show that in naïve and sham ascidians the endostyle *Ci*Lgals-a was overexpressed (zone 2) and the *Ci*Lgals-b was underexpressed. Although, the LPS inoculation challenged both genes, the *Ci*Lgals-b gene was upregulated and the protein produced both in zone 2 and 4 suggesting a differential zone expression and its immune role. The possibility exists that a different modulation could be imputed to methodological constrictions due to the whole pharynx tissue used for PCR analysis, whereas variation in ascidians collection and acclimation cannot be excluded [21]. A marked physiological differential regulation of galectin genes is an outstanding feature of mammalian galectins even of those widely expressed [32]. In this respect a distinct expression pattern can be normally quite but rises to high levels when cells are activated. Although our findings do not allow any speculation on the regulatory mechanisms involved, they disclose the endostyle contribution to the inflammatory response. In a previous paper [35] we reported that *C. intestinalis* galectin-like molecules, overproduced and released into the hemolymph following LPS inoculation, had opsonic properties. In *C. intestinalis*, the LPS locally inoculated permeates the pharynx and stimulates the expression of several immune-related factors [17,27,36–39] including TNFα-like [40,41] which could have a role in the regulatory network.

In addition, according to the constitutive galectin presence in the zones 2 (*Ci*Lgals-a) and 3 (*Ci*Lgals-b), they may also be involved in food collection. They may be released into the endostyle groove where mucus is secreted and food particles collected. In the extracellular space, galectins could absorb β-galacoside-containing microorganisms and form ligand lattices that would be moved to the digestive trait [42]. In this respect, BiCRD galectin-ligand binding can leads to oligomeration, forming trimers or pentamers more effective in ligand binding influenced by microenvironmental conditions [3,43,44]. The *Ci*Lgals-a and *Ci*Lgals-b oligomerization has previously been reported, delineating different galectin functions [16,35]. Although, we were not able to ascertain and distinguish the *Ci*Lgals-a and *Ci*Lgals-b functions, it is presumable that both are components of the secreted mucus. In this respect the zones 2–4 are producer of mucus, and the *Ci*Lgals could be coupled with the mucus production, while the modulation by LPS suggest both microorganisms collection for feeding and a defense role related to feeding [22,26,45]. The mucus net contains mucopolysaccharides and mucoproteins (composed of a peptide core surrounded by a stretch of polysaccharides) [26], therefore the possibility exists that galectins could be released in association with them. Accordingly, elongated mesh of mucus filaments containing galectins may form an outer layer strictly adhering to the endostyle surfaces, mainly in the ciliated traits. In mammals, galectins are expressed by epithelia of the digestive system [46–49], and the mucus layer coating the gastrointestinal tract is the front line of innate host defense. Mucus production and galectin genes expression could be regulated either through transcriptional or epigenetic modulation by bioactive factors including LPS and cytokines [21]. A striking common feature of both galectins, is the modulation under different physiological conditions, suggesting that the expression of galectin subtypes is finely tuned and possibly coordinate.

In mammalian cells, due to the lack of signal peptide, galectins are cytosolic and not compartmentalized within classical secretory compartments, and there is evidence of a mechanism distinct from the classical vesicle mediated exocytosis [32,50]. Although our findings cannot elucidate the transporting and releasing mechanism, we found galectins both scattered in the cytoplasm and contained within intra- and extra-cytoplasmic large vesicles. In this respect, the columnary cells of the zone 2 and 4 show large vesicles placed in the supranuclear cytoplasmic area, whilst vesicles were lined up toward the apical region (tuft-like) of the cells, where they were numerous. The vesicle size and cytoplasmic pattern cannot be related to the secretory granules reported by fine structure studies [25,29,34]. A membrane around the stained material that filled the vesicles could not clearly be showed by the used methods. However, a vesicular structure is indicated by magnification of antibody-treated and control sections. Subsequent fine structure studies could disclose a vesicle based transport in the absence of peptide signal. When released from the apical region of the cells, stressing factors, including infection and inflammation, can induce a vesicle destruction with release of their content into the outer space [50,51]. In conclusion, this study clearly shows the two galectin subtypes localization in the *C. intestinalis* filter feeding pharynx endostyle, the genes upregulation by LPS, the protein production that seems to be due to a vesicle-linked release. Although ligands of the endostyle galectins remain unknown, the present paper contributes to a better understanding of functions and defense role of glycan-binding molecules in the ascidian digestive tract.

Acknowledgments

This work was supported by a research grant from the Italian Ministry of Education (PRIN 2006 and 2010-2011 to N. Parrinello grant number: 20044057000), co-funded by the University of Palermo. We thank M. Guarcello for collecting ascidians for maintenance of the aquaria.

References

[1] Rapoport EM, Kurmyshkina OV, Bovin NV. Mammalian galectins: structure, carbohydrate specificity, and functions. Biochem Mosc 2008;73:393–405.

[2] Vasta GR, Ahmed H. Animal lectins: a functional view. Boca Raton, FL: CRC Press (Taylor & Francis Group); 2009 [QUANTE].
[3] Di Lella S, Sundblad V, Cerliani JP, Guardia CM, Estrin DA, Vasta GR, et al. When galectins recognize glycans: from biochemistry to physiology and back again. Biochemistry 2011;50:7842–57.
[4] Vasta GR, Ahmed H, Nita-Lazar M, Banerjee A, Pasek M, Shridhar S, et al. Galectins as self/non-self recognition receptors in innate and adaptive immunity: an unresolved paradox. Front Immunol 2012;3:1–14.
[5] Houzelstein D, Goncalves IR, Fadden AJ, Sidhu SS, Cooper DNW, Drickamer K, et al. Phylogenetic analysis of the vertebrate galectin family. Mol Biol Evol 2004;21:1177–87.
[6] Shoji H, Nishi N, Hirashima M, Nakamura T. Characterization of the xenopus galectin family. Three structurally different types as in mammals and regulated expression during embryogenesis. J Biol Chem 2003;278:12285–93.
[7] Yu Y, Yuan S, Yi Y, Huang H, Feng K, Pan M, et al. Molecular and biochemical characterization of galectin from amphioxus: primitive galectin of chordates participated in the infection processes. Glycobiology 2007;17:774–83.
[8] Ballarin L, Cammarata M, Franchi N, Parrinello N. Routes in innate immunity evolution: galectins and rhamnose-binding lectins in ascidians. Mar Proteins Peptides Biological Activities Appl 2013;9:184–205.
[9] Rabinovich GA, Rubinstein N, Toscano MA. Role of galectins in inflammatory and immunomodulatory processes. Biochim Biophys Acta 2002b;1572:274–84.
[10] Poirier F, Timmons PM, Chan CT, Guenet JL, Rigby PW. Expression of the L14 lectin during mouse embryogenesis suggests multiple roles during pre- and post-implantation development. Development 1992;115:143–55.
[11] Demetter P, Nagy N, Martin B, Mathieu A, Dumont P, Decaestecker C, et al. The galectin family and digestive disease. J Pathol 2008;215:1–12.
[12] Swalla BJ, Cameron CB, Corley LS, Garey JR. Urochordates are monophyletic within the deuterostomes. Rev Syst Biol 2000;49:52–64.
[13] Zeng L, Swalla BJ. Molecular phylogeny of the protochordates: chordate evolution. Can J Zool 2005;83:24–33.
[14] Delsuc F, Brinkmann H, Chourrout D, Philippe H. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 2006;439:965–8.
[15] Tsagkogeorga G, Turon X, Hopcroft RR, Tilak MK, Feldstein T, Shenkar N, et al. An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. BMC Evol Biol 2009;9:187.
[16] Vizzini A, Parrinello D, Sanfratello MA, Salerno G, Cammarata M, Parrinello N. Inducible galectins are expressed in the inflamed pharynx of the ascidian *Ciona intestinalis*. Fish Shellfish Immunol 2012;32:101–9.
[17] Giacomelli S, Melillo D, Lambris JD, Pinto MR. Immune competence of the *Ciona intestinalis* pharynx: complement system-mediated activity. Fish Shellfish Immunol 2012;33:946–52.
[18] Burighel P, Cloney RA. Urochordata: ascidiacea. In: Harrison FW, Ruppert EE, editors. Microscopic anatomy of invertebrates. Hemichordata, chaetognata, and the invertebrate chordates, vol. 15. New York: Wiley-Liss; 1997. p. 221–347.
[19] Ciona Millar R. Liverpool: The University Press of Liverpool; 1953.
[20] Pestarino M, Fiala-Medioni A, Ravera F. Ultrastructure of the branchial wall of a lower chordate: the ascidian *Ciona intestinalis*. J Morphol 1988;197:269–76.
[21] Petersen JK. Ascidian suspension feeding. J Exp Mar Biol Ecol 2007;342:127–37.
[22] Bone Q, Carre C, Chang P. Tunicate feeding filters. Mar Biol Ass U K 2003;83:907–19.
[23] Fujita H, Sawano F. Fine structural localization of endogenous peroxidase in the endostyle of ascidians, *Ciona intestinalis*. A part of phylogenetic studies of the thyroid gland. Arch Histol Jpn 1979;42:319–26.
[24] Olsson R. Endostyle and endostylar secretions. A comparative histochemical study. Acta Zool Stockh 1963;44:299–328.
[25] Thorpe A, Thorndyke MC, Barrington EJ. Ultrastructural and histochemical features of the endostyle of the ascidian *Ciona intestinalis* with special reference to the distribution of bound iodine. Gen Comp Endocrinol 1972;19:559–71.
[26] Flood PF, Fiala-Medioni A. Ultrastructure and histochemistry of the food trapping mucous in benthic filter feeders (ascidians). Acta Zool Stockh 1981;62:53–65.
[27] Vizzini A, Pergolizzi M, Vazzana M, Salerno G, Di Sano C, Macaluso P, et al. FACIT collagen (1alpha-chain) is expressed by hemocytes and epidermis during the inflammatory response of the ascidian *Ciona intestinalis*. Dev Comp Immunol 2008;32:682–92.
[28] Bancroft JD, Gamble M. Theory and practice of histological techniques. 6th ed. Philadelphia, PA: Churchill Livingstone Elsevier; 2008.
[29] Fujita H, Namba H. Fine structure and its functional properties of the endostyle of ascidians, *Ciona intestinalis*. A part of phylogenetic studies of the thyroid gland. Z Zellforsch 1971;121:455–69.
[30] Barrington EJW. The distribution and significance of organically bound iodine in the ascidian *Ciona intestinalis* Linnaeus. J Mar Biol Assoc U K 1957;36:1–16.
[31] Barrington EJW. The localization of organically bound iodine in the endostyle of Amphioxus. J Mar Biol Assoc U K 1958;37:117–26.
[32] Cooper DNW. Galectinomics: finding themes in complexity. Biochim Biophys Acta 2002;1572:209–31.
[33] Ogasawara M, Di Lauro R, Satoh N. Ascidian homologs of mammalian thyroid peroxidase genes are expressed in the thyroid-equivalent region of the endostyle. J Exp Zool 1999a;285:158–69.
[34] Aros B, Virágh S. Fine structure of the pharynx and endostyle of an ascidian (*Ciona intestinalis*). Acta Biol Acad Sci Hung 1969;20:281–97.
[35] Parrinello N, Arizza V, Cammarata M, Giaramita FT, Pergolizzi M, Vazzana M, et al. Inducible lectins with galectin properties and human IL1alpha epitopes opsonize yeast during the inflammatory response of the ascidian *Ciona intestinalis*. Cell Tissue Res 2007;329:379–90.
[36] Vizzini A, Parrinello D, Sanfratello MA, Mangano V, Parrinello N, Cammarata M. *Ciona intestinalis* peroxinectin is a novel component of the peroxidase–cyclooxygenase gene superfamily upregulated by LPS. Dev Comp Immunol 2013;41:59–67.
[37] Vizzini A, Bonura A, Parrinello D, Sanfratello MA, Longo V, Colombo P. LPS Challenge regulates gene expression and tissue localization of a *Ciona intestinalis* gene through an alternative polyadenylation mechanism. PLoS One 2013;8(4):e63235.
[38] Bonura A, Vizzini A, Salerno G, Parrinello N, Longo V, Colombo P. Isolation and expression of a novel MBL-like collectin cDNA enhanced by LPS injection in the body wall of the ascidian *Ciona intestinalis*. Mol Immunol 2009;46:2389–94.
[39] Bonura A, Vizzini A, Salerno G, Parrinello D, Parrinello N, Longo V, et al. Cloning and expression of a novel component of the CAP superfamily enhanced in the inflammatory response to LPS of the ascidian *Ciona intestinalis*. Cell Tissue Res 2010;342:411–21.
[40] Parrinello N, Vizzini A, Salerno G, Sanfratello MA, Cammarata M, Arizza V, et al. Inflamed adult pharynx tissues and swimming larva of *Ciona intestinalis* share CiTNF alpha-producing cells. Cell Tissue Res 2010;341:299–311.
[41] Parrinello N, Vizzini A, Arizza V, Salerno G, Parrinello D, Cammarata M, et al. Enhanced expression of a cloned and sequenced *Ciona intestinalis* TNFalpha-like (CiTNF alpha) gene during the LPS-induced inflammatory response. Cell Tissue Res 2008;334:305–17.
[42] Brewer CF, Miceli MC, Baum LG. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. Curr Opin Struct Biol 2002;12:616–23.
[43] Surolia A, Swaminathan CP, Ramkumar R, Podder SK. Unusual structural stability and ligand induced alterations in oligomerization of a galectin. FEBS Lett 1997;409(3):417–20.
[44] Dam TK, Brewer CF. Effects of clustered epitopes in multivalent ligand-receptor interactions. Biochemistry 2008;47:8470–6.
[45] Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA. Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. Cell Mol Life Sci 2007;64:1679–700.
[46] Oka T, Murakami S, Arata Y, Hirabayashi J, Kasai K, Wada Y, et al. Identification and cloning of rat galectin-2: expression is predominantly in epithelial cells of the stomach. Arch Biochem Biophys 1999;361:195–201.
[47] Kaltner H, Seyrek K, Heck A, Sinowatz F, Gabius HJ. Galectin-1 and galectin-3 in fetal development of bovine respiratory and digestive tracts. Comparison of cell type-specific expression profiles and subcellular localization. Cell Tissue Res 2002;307:35–46.
[48] Nio J, Kon Y, Iwanaga T. Differential cellular expression of galectin family mRNAsin the epithelial cells of the mouse digestive tract. J Histochem Cytochem 2005;53:1323–34.
[49] Wasano K, Hirakawa Y. Rat intestinal galactoside-binding lectin L-36 functions as a structural protein in the superficial squamous cells of the esophageal epithelium. Cell Tissue Res 1995;281:77–83.
[50] Nickel W, Seedorf M. Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. Annu Rev Cell Dev Biol 2008;24:287–308.
[51] Hughes RC. Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim Biophys Acta 1999;1473:172–85.