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