Fish & Shellfish Immunology 63 (2017) 157–164

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Morula cells as key hemocytes of the lectin pathway of complement activation in the colonial tunicate *Botryllus schlosseri*

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ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
2 February 2017
Accepted 5 February 2017
Available online 8 February 2017

Keywords:
Lectin pathway
Complement system
Cytotoxic cell
Invertebrates
Tunicates
Comparative immunobiology

ABSTRACT

The complement system is deeply rooted in the evolution of humoral mechanism of innate immunity. In addition to the alternative pathway of complement activation, lectins and associated serine proteases exert important roles in the recognition of non-self and activation of the effectors.

In the colonial tunicate *Botryllus schlosseri*, we identified, characterized and studied the expression of three orthologues of genes involved in the lectin pathway of complement activation of vertebrates, i.e., genes for a mannose-binding lectin (MBL), a ficolin and a mannose-associated serine protease 1 (MASP1). All the genes are transcribed by hemocytes, and specifically by morula cells, the same immunocytes responsible for the transcription of C3 and Bf orthologues. The transcription levels of MASP1 and ficolin orthologues are not affected by zymosan challenge, indicating a constitutive expression of complement system associated serine proteases, whereas the MBL orthologue is up-regulated after 15 min of zymosan exposure. Collectively, our data suggest the presence of a complete lectin activation pathway in *Botryllus*.

1. Introduction

One of the main goal in comparative immunology is the description of the molecular basis of the discrimination of self from non-self in different taxa, unraveling their evolution and the modifications that led to a growing complexity in the ability to recognize and eliminate foreign cells/materials.

Among the various effector mechanisms involved in immune responses, the complement system is one of the most ancient, deeply-rooted and important for its ability to orchestrate different cells and factors of both innate and adaptive immunity [1]. Once the complement system is activated, a cascade of reactions that involves proteolysis and polymerization occurs, known as complement activation pathway, resulting in the cleavage of the third complement component (C3) to give C3a and C3b. In vertebrates the proteolysis of C3 is followed by the activation of the lytic pathway during which the membrane-attack complex is formed.

There are three known complement activation pathways: the alternative, the classical and the lectin pathway. The alternative pathway starts with the auto proteolysis of C3 and the activation of a specific serine protease called factor B. The classical pathway is activated when the collectin C1q recognizes and binds antibody–antigen complexes. The lectin pathway is initiated by the binding of collectins (e.g. mannose-binding lectins (MBLs)) or ficolin (FCNs) to carbohydrates on the surfaces of pathogens with the subsequent activation of associated enzymes that are known as MBL-associated serine proteases (MASPs) [2,3].

Collectins are lectins bearing a collagen domain, involved in the recognition of pathogens. They seem to exert a pivotal role in the coordination of complement-related molecules, especially in the transition from innate to adaptive immunity since the collectin C1q is able to recognize the antibody-antigen complex [4]. MBLs are a group of collectins, structurally defined by the presence of a C-type carbohydrate recognizing domain (CRD) and a collagen domain connected by an alpha-helical, coiled-coil, neck region. In humans, MBL exists in several oligomeric forms such as trimers, tetramers and pentamers [5]. Ficolins bear a N-terminal collagenous domain and a C-terminal fibrinogen-like domain (FReD) recognizing *N*-acetyl glucosamine [6]. Three human ficolin genes have been identified: *fcn1*, *fcn2* and *fcn3*, encoding ficolin-1 (M-ficolin), ficolin-2 (L-ficolin) and ficolin-3 (H-ficolin), respectively [7]. Ficolins exhibit differences in tissue expression and ligand specificity, suggesting individual roles of each ficolin [8–10].

As regards the serine proteases of the lectin pathway, three different MASPs have been described in mammals: MASP1 [11], MASP2 [12] and MASP3 [5]. The structures of these enzymes

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http://dx.doi.org/10.1016/j.fsi.2017.02.003
1050-4648/

resemble C1r and C1s of the classical pathway, with which they form the MASP/C1r/C1s subfamily of serine proteases [2,13]. The five members of the MASP/C1r/C1s subfamily share the following six domains: C1r/C1s/Uegf/bone morphogenetic protein 1 (CUB) 1, epidermal growth factor (EGF)-like, CUB2, complement control protein (CCP) 1, CCP2 and serine protease domain. MASP-1 and MASP-3 are generated from the same gene (*masp1*) by alternative splicing mechanisms. The gene has ten exons that encode the CUB1–EGF–CUB2–CCP1–CCP2 domains common to both MASP-1 and MASP-3. These domains constitute the heavy chain of the active forms of MASP-1 and MASP-3. Conversely, the exons codifying the serine protease domain, which constitutes the light chain of the active forms, are different between MASP-1 and MASP-3 [14]. MASP-2 is generated from the *masp2* gene.

Upon the binding of MBLs or ficolins to carbohydrates to the surface of pathogens, the proenzyme form of MASPs is cleaved between the CCP2 and the serine protease domains, resulting in the generation of the active form [2]. The function of mammalian MASP3 is not well known, but MASP2 is the enzyme that, like C1s in the classical pathway, cleaves the complement components C4 and C2 to form the C3 convertase C4bC2a which, in Vertebrates, is common to both the lectin and classical activation pathways. By contrast, MASP1 is essential for the activation of the lectin pathway [15] and it is able to cleave C3 both directly [5,16] and indirectly through the activation of factor D [17], resulting in the activation of the alternative pathway [18].

As regards the lectin pathway of the complement system of the invertebrate deuterostome, very few data are available. *Strongylocentrotus purpuratus* genome analyses revealed sequences with similarity to a mannose-binding protein (MBP), but no MASP/C1r/C1s models were found [19].

Tunicates are invertebrate chordates and their phylogenetic position of vertebrate sister group renders them interesting models for the study of the evolution of the defense strategies within chordates.

In the solitary ascidian *C. intestinalis* two MBL has been described [14,20] but no clear evidences of the existence of a serine protease with characteristics of MASP as ever found [21]. By contrast, in another solitary ascidian, *Halocynthia roretzi*, two MASPs have been recorded [22] as well as in *Clavelina picta* [23] and *Boltenia villosa* [24]. In the compound ascidian *Botryllus schlosseri* we have recently identified and described C3 and factor B, components of the complement alternative pathway, and demonstrated the importance of the complement system in the opsonization and clearance of foreign microbial cells [26]. As regard lectin pathway first evidences of transcripts for MBL and MASP came from transcriptome and genome screening [25] where putative ESTs were detected.

In the present work, we identified some of the genes involved in the lectin pathway, analyzed their structures and those of the putative proteins resulting from *in silico* translation, and studied their transcription under different experimental conditions. All the reported data agree with an evolutionary scenario where lectins are fundamental molecules for the non-self recognition of invertebrates and suggest the existence of a *bona fide* lectin pathway of complement activation also in *B. schlosseri*.

2. Materials and methods

2.1. Animals and zymosan treatment

Large colonies of *B. schlosseri* were collected in the southern Lagoon of Venice, near Chioggia, acclimated for 5 days in aerated aquaria filled with filtered seawater (FSW), at the constant temperature of 19 °C, and fed with Liquifry marine (Liquifry Co. Dorking, UK). Hemolymph was collected as previously described [27]. Briefly 4 different colonies were used in this experiment: after puncturing the colonial tunic vasculature of each colony with a fine tungsten needle, hemolymph was collected with a glass micropipette, equally distributed in 6 vials and centrifuged for 10 min at 800×*g*. Pellets were resuspended in FSW, with or without zymosan (1 mg/ml) to obtain a concentration of 10^6 cells/ml. Out of 6 cell batches, two were resuspended in FSW, used as controls (0 and 60 min) whereas the other four batches were incubated for 15, 30, 45 and 60 min with zymosan. After the treatments, cells were processed for RNA extraction as described below.

2.2. RNA extraction and reverse transcription

Total RNA was isolated from the hemocytes of *B. schlosseri* with the SV Total RNA Isolation System (Promega); its purity was determined by the $A_{260/280}$ and $A_{260/230}$ ratio. RNA integrity was determined by visualization of rRNAs in ethidium bromide-stained agarose gels (1.5%). The first strand of cDNA was reverse-transcribed from 1 μg of total RNA at 42 °C for 1 h in a 20 μl reaction mixture containing 1 μl of ImPromII Reverse Transcriptase (Promega) and 0.5 μg oligo (dT)-Anchor primer or random primers (Promega).

2.3. Amplification, cloning and sequencing

Amplification and cloning of transcripts for *B. schlosseri* MBL (BsMBL), *B. schlosseri* ficolin (BsFCN) and *B. schlosseri* MASP1 (BsMASP1) was achieved with specific primers designed on the sequences found in our EST collection [28] and in the database of the *B. schlosseri* Genome Project (http://genepyramid.stanford.edu/botryllusgenome/). In both cases the obtained EST sequences contained the 5′ terminal untranslated region (UTR) and the entire coding region. The 3′ rapid amplification of the cDNA ends (RACE) was performed using the 2nd Generation of the 5′/3′ RACE Kit (Roche). In order to obtain the 3′ sequences of BsMBL, BsFCN and BsMASP1 cDNA, five specific primers, BsMBLF1, BsMBLF2, BsFCN1, BsMASPF1 and BsMASPF2 (Table 1), were designed for direct and nested PCR with anchor reverse primer according to the manufacturer's instruction (Roche). To study the location of BsMBL, BsFCN and BsBMASP mRNAs through *in-situ* hybridization (ISH), tree specific primers (BsMBLR, BsFCNR and BsMASPR1) were designed and used with BsMBLF1, BsFCNF and BsMASPF1, respectively. The primer pair BsMBLF2-BsMBLR, BsNCF2-BsNCR2 and BsMASPF3-BsMASPR2 (Table 1), were used to perform the relative RealTime-PCR (rRT-PCR).

PCR reactions were carried out in a 25 μl reaction mixture

Table 1

Primer name	Primer 5′-3′
BsMBLF1	AAGGGGGACCAAGGCGAGT
BsMBLF2 rt	CAACACGGGTCTGGCTTAGC
BsMBLR rt	GCAGTGGAACCGTCGTCCC
BsMASPF1	CCAAACTTCCCCGAGCCTTAC
BsMASPF2 rt	GCACTCCACGCATCAATAGGT
BsMASPR1 rt	CGTTGACGCAGAAGTGGTTG
BsMASPF3	GGTTTCAATCTGACCTGTCCGT
BsMASPR2	GGTGATTGTTCCGTTTGTTGGC
BsFCNF	GCAATGGTGCGATAATACGG
BsFCNR	CCTCGGGAATGATAACTTGG
BsFCNF2 rt	GCAAGGAGATCATTGGTTGG
BsFCNR2 rt	ATCTCCGTAGGTTCCGTTCC
BsACTF rt	ACTGGGACGACATGGAGAAG
BsACTR rt	GCTTCTGTGAGGAGGACAGG
Oligo dT Anchor	GCAGTGGTAACAACGCAGAG (-dT)20VN