Developmental and Comparative Immunology xxx (2016) 1–7

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Molecular characterisation, evolution and expression analysis of g-type lysozymes in *Ciona intestinalis*

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ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form
18 September 2016
Accepted 19 September 2016
Available online xxx

Keywords:
Ascidian
Lysozymes g-type
Inflammation
LPS
Ciona intestinalis

ABSTRACT

Lysozyme is an important defense molecule of the innate immune system. Known for its bactericidal properties, lysozyme catalyzes the hydrolysis of b-(1,4)-glycosidic bonds between the N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of bacterial cell walls. In this study, the complete coding sequence of four g-type lysozymes were identified in *Ciona intestinalis*. Phylogenetic analysis and modelling supported the hypothesis of a close relationship with the vertebrate g-type lysozymes suggesting that the *C. intestinalis* g-type lysozyme genes (*Ci*Lys-g1, *Ci*lys-g2, *Ci*Lys-g3, *Ci*Lys-g4) share a common ancestor in the chordate lineage. Protein motif searches indicated that *C. intestinalis* g-type lysozymes contain a GEWL domain with a GXXQ signature, typical of goose lysozymes. Quantitative Real-Time PCR analysis results showed that transcripts are expressed in various tissues from *C. intestinalis*. In order to determine the involvement of *C. intestinalis* g-type lysozymes in immunity, their expression was analyzed in the pharynx, showing that transcripts were significantly up-regulated in response to a challenge with lipopolysaccharide (LPS). These data support the view that *Ci*Lys g-type are molecules with potential for immune defense system against bacterial infection.

1. Introduction

Lysozyme is a ubiquitous bacteriolytic enzyme produced by diverse groups of organisms, ranging from bacteria and bacteriophages to fungi, plants and animals (Jollès and Jollès, 1984). It catalyzes the hydrolysis of 1, 4-beta-linkages between N-acetyl-*d*-glucosamine (NAG) and N-acetylmuramic acid (NAM) in peptidoglycan heteropolymers of prokaryotic cell walls, leading to the breakdown of bacterial cells (Smirnow and Wislowska, 2001; Nilsen et al., 1999). As a result, lysozyme acts directly on Gram-positive bacteria, causing the lysis of their outermost peptidoglycan layer. Gram-negative bacteria, however, are not directly damaged by lysozyme as their outer membrane is significantly coated with lipopolysaccharide (LPS) moieties. Instead, the outer membranes of Gram-negative bacteria must first be disrupted by cationic antimicrobial peptides that expose the inner peptidoglycan layer of bacteria to lysozyme (Banks et al., 1986; Hancock and Scott, 2000; Ibrahim et al., 2002).

Based on differences in structural, catalytic and immunological characteristics, lysozymes are generally classified into six main types: chicken (c-type) (Hultmark, 1996), goose (g-type) (Prager and Jollès, 1996), invertebrate (i-type) (Jollès and Jollès, 1975), T4 phage (phage-type) (Fastrez, 1996), bacterial (Holtje, 1996), and plant (Beintema and Terwisscha van Scheltinga, 1996). The g-type lysozyme was initially identified as an antibacterial enzyme in egg whites from the Embden goose (Canfield and McMurry, 1967), but it was later found to exist in the egg whites of several other bird species (Prager et al., 1974). More recently, the g-type lysozyme has been identified in vertebrate species, including mammals (Nakano and Graf, 1991), fish (Mohanty and Sahoo, 2010), urochordates (Nilsen et al., 2003) and molluscs (Zhao et al., 2007; Zhang et al., 2012).

Lysozyme is a well-known antibacterial protein which is active against Gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus stearothermophilus* and *Clostridium tyrobutyricum* (Proctor and Cunningham, 1988). It is also known to act as an opsonin and as an activator of the complement system and circulating phagocytes (Jollès and Jollès, 1984; Grinde, 1989). In addition to these antibacterial functions, some lysozyme family members have been demonstrated to have antiviral (Ferrari et al., 1959; Lee-Huang et al., 1999), anti-inflammatory activities (Jollès and Jollès, 1984; Samarayanake et al., 1997; Ogundele, 1998;

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http://dx.doi.org/10.1016/j.dci.2016.09.010
0145-305X/

Zhang et al., 2008), to be involved in immune modulatory (Valisena et al., 1996; Rymuszka et al., 2005) and antitumor activities (Sava et al., 1989), thus, it is possible that lysozymes function as multipurpose defense factors.

Ascidians (subphylum: Tunicata) occupy a key phylogenetic position in chordate evolution and are considered the sister group of vertebrates (Zeng and Swalla, 2005; Delsuc et al., 2006; Tsagkogeorga et al., 2009). They are proto-chordates which possess an innate immune system, including inflammatory humoral and cellular responses. An inflammatory response induced by LPS injection in the body wall of *C. intestinalis* is a well-established model for the analysis of regulator and effector inducible host defense molecules of the innate immune system (Bonura et al., 2009; Parrinello et al., 2008, 2010; Vizzini et al., 2012, 2013; 2015a, 2015b; 2016). In the present paper, we report on the identification, characterization and expression of *C. intestinalis* g-type lysozymes. Phylogenetic analysis was conducted to determine their evolutionary relationships. Real-Time PCR analysis revealed that *Ci*Lys-g(1-4) are expressed in several type of tissue, and their transcription is up-regulated by LPS inoculation.

2. Materials and methods

2.1. Tunicates and LPS inoculation

Ascidians were collected from Sciacca Harbour (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with a marine invertebrate diet of coraliquid (Sera Heinsberg, Germany). LPS (Escherichia coli 055:B5, LPS, Sigma-Aldrich, Germany) solution was prepared in sterile sea water (12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0). LPS solution (100 μg LPS in 100 μl sea water per animal) was inoculated into the tunic matrix close to the pharynx wall at the median body region. Ascidians, both untreated (naive ascidians) and injected with MS (sham ascidians), were used as controls.

2.2. Total RNA extraction

Ascidian tissue fragments (200 mg) explanted at various times (from 1 to 72 h) were immediately soaked in RNAlater Tissue collection solution (Ambion, Austin, TX), and stored at −80 °C. Total RNA extraction was performed by using an RNAqueousTM-Midi Kit purification system (Ambion, Austin, TX).

2.3. Cloning and sequence analysis

A search conducted in the Ensembl genome browser identified the sequence: *Ci*Lys-g1 (ENSCING00000007365), *Ci*Lys-g2 (ENSCING00000024417), *Ci*Lys-g3 (ENSCING00000018223), *Ci*Lys-g4 (ENSCING00000007365). The sequence of the cDNA was obtained by using the GeneRacerTM kit (Invitrogen, USA). 5′-and 3′ RACE was conducted using the primers listed in Table 1. The overlapping RACE products were cloned into the pCR™IIvector (TA Cloning Kit, Invitrogen) and sequenced. They contained the complete coding regions.

2.4. Bioinformatic analysis

The full length *Ci*Lys-g(1-4) cDNA were analyzed using the ExPASy translation tool (http://web.expasy.org/translate/) to obtain their ORF region, leader and trailer sequences (UTR), and the nucleotide sequence was translated into a protein sequence. A BLAST (http://blast.ncbi.nlm.nih.gov/Blast) search was conducted to identify the known protein sequences that are homologous to *Ci*Lys-g(1-4). The Exon-Intron Graphic Maker (http://wormweb.org/exonintron) was used to create an image of the genomic organization of *Ci*Lys-g genes and for comparison. Physical and chemical parameters such as molecular mass, and theoretical isoelectric point were computed using the Prot-Param tool on ExPASy (http://www.expasy.org/tool/protparam/). The NCBI Conserved Domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to predict domain architecture and other conserved domains based on sequence homology. Functional motifs were determined by comparison on the Prosite database (http://prosite.expasy.org/scanprosite/). The putative cleavage site of the signal peptide was predicted by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). The subcellular location of lysozyme proteins was predicted using the MultiLoc tool (http://abi.inf.uni-tuebingen.de/Services/MultiLoc/). Multiple sequence alignment was conducted using CLC (Version 7.0.0). A secondary structure was evaluated using Polyview (http://polyview.cchmc.org). Different 3D structures were predicted using the I-TASSER program (http://zhanglab.ccmb.med.umich.edu/I-TASSER) and the structures were validated by Ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) to obtain the best structure among those predicted. Finally, a phylogenetic tree using Neighbor-joining method was constructed using MEGA 6.0 after 1000 bootstrap iterations.

Table 1
Primers used for cloning and expression.

Gene	Primer sequence (5′-3′)	Application
*Ci*Lys-g1	5′-TAACCCATACCCATGCCCGT-3′	RACE5′
	5′-GCTTGCCCAATCTCGTCTTT-3′	NESTED5′
	5′-TGACGGGGATTCGAACAGGA-3′	RACE3′
	5′-ATCTGGAAAGTGTGGAGGGC-3′	NESTED3′
*Ci*Lys-g2	5′-CTCCCAAACCCTTGCTGTTC-3′	RACE5′
	5′-AATTAGATCGGGCGGCCTTA-3′	NESTED5′
	5′-GCAATGGCGACTCGTTTCAA-3′	RACE3′
	5′-GCTGAACGAGAATGGCTACG-3′	NESTED3′
*Ci*Lys-g3	5′-TGCGACGACTCTAACACTTG-3′	RACE5′
	5′-CGTACATTGCTGACCCCAAA-3′	NESTED5′
	5′-AAAAGATCTGCGTGCGATGG-3′	RACE3′
	5′-AGGTTGATAAGCGGCACCAT-3′	NESTED3′
*Ci*Lys-g4	5′-TCCTGCCACCATTTTACCAC-3′	RACE5′
	5′-AGCCACTACATCGCTAGAGT-3′	NESTED5′
	5′-TTGCTTTTCCTGTTCGTCGG-3′	RACE3′
	5′-GACGATCGTTACCACACCAT-3′	NESTED3′
*Ci*Lys-g1	5′-AACTTTGTATGGACGCTGCTG-3′	Real-time PCR
	5′-GCCCCTGCACGACTTTCA-3′	Real-time PCR
*Ci*Lys-g2	5′-CACGGTGCCGACACAAAGT-3′	Real-time PCR
	5′-GCGCCTTGTAAAATGTGATCTC-3′	Real-time PCR
*Ci*Lys-g3	5′-GCAAGCCGCGAAAGCA-3′	Real-time PCR
	5′-TCACCAAGCCCGTCTTTGTC-3′	Real-time PCR
*Ci*Lys-g4	5′-CGGCGTAGCCATCGCTTA-3′	Real-time PCR
	5′-CGGTGGTGTCAGTGTTGTAGAT-3′	Real-time PCR
Actin	5′-TGATGTTGCCGCACTCGTA-3′	Real-time PCR
	5′-TCGACAATGGATCCGGT-3′	Real-time PCR

2.5. Real-Time PCR analysis

Tissue Differential expression of the *Ci*Lys-g(1-4) cDNAs was studied by Real-Time PCR using the Sybr-Green method and the specific sets of primers listed in Table 1. Real-Time PCR analysis was performed using the Applied Biosystems 7500 Real-Time PCR System. Tissue Differential expression was performed in a 25 μl PCR reaction containing 2 μl cDNA converted from 250 ng of total RNA, 300 nM forward and reverse primers, and 12.5 μl of Power Sybr-Green PCRMasterMix (Applied Biosystems).

The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95 °C, followed by a denaturing step at 95 °C for 15 s, and then annealing/extension was carried out

Please cite this article in press as: Di Falco, F., et al., Molecular characterisation, evolution and expression analysis of g-type lysozymes in *Ciona intestinalis*, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.09.010