Developmental and Comparative Immunology 55 (2016) 102–110

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Transforming growth factor β (*Ci*TGF-β) gene expression is induced in the inflammatory reaction of *Ciona intestinalis*

Aiti Vizzini, Felicia Di Falco, Daniela Parrinello, Maria Antonietta Sanfratello, Matteo Cammarata*

Marine Immunobiology Laboratory, Department of Biological Chemical Pharmaceutical Science and Technology, University of Palermo, Via Archirafi 18, Palermo, Italy

ARTICLE INFO

Article history:
Received 5 July 2015
Received in revised form
15 October 2015
Accepted 15 October 2015
Available online 19 October 2015

Keywords:
Ascidian
Transforming growth factor
TGF
Hemocyte
Inflammation
LPS
Ciona intestinalis

ABSTRACT

Transforming growth factor (TGF-β) is a well-known component of a regulatory cytokines superfamily that has pleiotropic functions in a broad range of cell types and is involved, in vertebrates, in numerous physiological and pathological processes. In the current study, we report on *Ciona intestinalis* molecular characterisation and expression of a transforming growth factor β homologue (*Ci*TGF-β). The gene organisation, phylogenetic tree and modelling supported the close relationship with the mammalian TGF suggesting that the *C. intestinalis* TGF-β gene shares a common ancestor in the chordate lineages. Functionally, real-time PCR analysis showed that *Ci*TGF-β was transcriptionally upregulated in the inflammatory process induced by LPS inoculation, suggesting that is involved in the first phase and significant in the secondary phase of the inflammatory response in which cell differentiation occurs. *In situ* hybridisation assays revealed that the genes transcription was upregulated in the pharynx, the main organ of the ascidian immune system, and expressed by cluster of hemocytes inside the pharynx vessels. These data supported the view that *Ci*TGF-β is a potential molecule in immune defence systems against bacterial infection.

1. Introduction

Transforming growth factor TGF-β belongs to a family of regulatory cytokines that have pleiotropic functions in a broad range of cell lineages involved in numerous physiological and pathological processes such as embryogenesis, carcinogenesis, and the immune responses (Blobe et al., 2000; Wharton and Derynck, 2009; Li et al., 2006). TGF-βs are the prototype of the TGF-β superfamily (Massagué, 1990). In mammals, three members of the TGF-β family (TGF-β1, -β2 and -β3) have been identified, with TGF-β1 being the predominant form expressed in the immune system (Massagué, 1990; Lawrence, 1996; Clark and Coker, 1998). All the TGF-βs are synthesised as a precursor: the pre region contains a signal peptide, and pro-TGF-β is processed in the Golgi by a furin-like peptidase that removes the N terminus of the immature protein. A TGF-β homodimer called the latency-associated protein (LAP) is non-covalently associated with a homodimer of mature TGF-β. This latent complex can be secreted, or may associate with latent-TGF-β-binding protein (LTBP), which plays an important role in targeting TGF-β to the extracellular matrix. TGF-β cannot bind to its receptors in its latent form, but needs to be liberated from the constraints of LAP and LTBP by a TGF-β activator (TA) through LAP proteolysis or a conformational change (Annes et al., 2003). This unique activation step for TGF-β provides a means for this secreted molecule to integrate signals from multiple cell types to regulate cellular responses. Active TGF-β mediates its biological functions by binding to TGF-β type.

Genome comparative analysis of the TGF-β pathway genes in 33 species has shown that they are universally present in metazoans. The TGF-β pathway genes evolved rapidly to a high degree of complexity. In bilateria at least one type II receptor and multiple type I receptors could can be detected, and the ancestral bilaterian repertoire can be inferred as consisting of two type II receptors and three type I receptors.

In ascidians the ancestral bilaterian TGF receptor repertoire is expanded to three type II receptors: this is the first example of a bilaterian TGF- β receptor duplication, mapping to chordates which is propagated through vertebrates (Huminiecki et al., 2009).

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

http://dx.doi.org/10.1016/j.dci.2015.10.013
0145-305X/

The signalling output of TGF-β elicits diverse cellular responses that are primarily mediated through the actions of SMAD transcription factors (Massague, 1998; Massague and Gomis, 2006; Shi and Massague, 2003).

In ascidians, at least two R-SMADs (one TGF-b and one BMP), one Co-SMAD and one I-SMAD have been described (Huminiecki et al., 2009).

The TGF-β superfamily includes nearly 30 proteins in mammals. The founding member of this family is the product of the *Drosophila melanogaster* gene Mad and TGF-β/BMP-like proteins have been found in invertebrate species including *Caenorhabditis elegans, D. melanogaster* (Schmierer and Hill, 2007) and in the Pacific oyster *Crassostrea gigas* (Corporeau et al., 2011).

The presence of immune-reactive molecules to TGF-β 1 has been detected in immunocytes from molluscs *Planorbarius corneus, Viviparus ater, Viviparus contectus, Lymnaea stagnalis, Mytilus galloprovincialis*, in the insects *Calliphora vomitoria* and in the annelids *Eisenia foetida* (Franchini et al., 1996; Ottaviani et al., 2000). In invertebrates, TGF-β1 affects immunocyte and human monocyte migration in dose-correlated fashion and induces hemocytes to migrate in a chemotactic manner (Ottaviani et al., 1997a). In addition TGF provokes changes in the cellular shape and affects phagocytosis in a species-specific manner (Ottaviani et al., 1997b).

Ascidians (subphylum: Tunicata) occupy a key phylogenetic position in chordate evolution and are considered the sister group of vertebrates (Swalla et al., 2000; Zeng and Swalla, 2005; Delsuc et al., 2006; Tsagkogeorga et al., 2009). They are proto-chordates provided by an innate immune system, including inflammatory humoral and cellular responses. For this reason, they represent an intriguing model for studying the evolution of the innate immune system. In particular, the inflammatory reaction in the body wall of *Ciona intestinalis* is a well-established model for the analysis of inducible host defense molecules of the innate immune system: Type IX collagen-like (Vizzini et al., 2008), CAP-like (Bonura et al., 2010), MBL-like (Bonura et al., 2009), TNFα-like (Parrinello et al., 2008, 2010), galectin-like (Vizzini et al., 2012), peroxinectin (Vizzini et al., 2013b), Interleukin 17 (Vizzini et al., 2015a), and proPO-system (Cammarata et al., 2008; Trapani et al., 2015., Vizzini et al., 2015b).

The pharynx is the main hemopoietic organ in which circulating hemocyte populations are renewed, and it can be challenged by inoculating inflammatory agents and express immune related genes upregulated by lipopolysaccharide (LPS) such as MBL-like (Bonura et al., 2009), galectin-like (Vizzini et al., 2012), Interleukins 17 (Vizzini et al., 2015a). In the present paper, we report on the identification, characterisation and expression of the *C. intestinalis* TGF-β gene. Real time PCR analysis revealed that their transcription is up-regulated by LPS inoculation, while an *in situ* hybridisation assay revealed that they are expressed by hemocytes (granulocytes, URG) in the inflamed pharynx tissues.

2. Materials and methods

2.1. Tunicates and LPS inoculation

Ascidians were collected from Sciacca Harbour (Sicily, Italy), maintained in tanks with aerated seawater at 15 °C, and fed every second day with a marine invertebrate diet coraliquid (Sera Heinsberg, Germany). Although the systematic of this species has been reexamined (Brunetti et al., 2015) we used the *C. intestinalis* until definitive clarification of Mediterranean species had been established.

Lipopolysaccharide (*Escherichia coli* 055:B5, LPS, Sigma–Aldrich, Germany) solution was prepared in sterile seawater (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 seawater per animal) was inoculated into the tunic matrix close to the pharynx wall in the median body region. The pharynx is the initial part of the ascidian digestive tract; it consists of two epithelial monolayers perforated by rows of ciliated stigmata aligned dorso-ventrally and enclosed in a mesh of vessels (transversal and longitudinal bars) where hemolymph, rich in hemocytes, flows. The ciliated stigmata generate a water current that serves as respiration and supply of dissolved organic particles, including bacteria.

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 (Ambion, Austin, TX), and stored at −80 °C. Total RNA extraction was performed using an RNAqueousTM-Midi Kit purification system (Ambion, Austin, TX).

2.3. Cloning and sequences analysis

A search at NBCI (www.ncbi.nlm.nih.gov) identified the sequence: TGF-β (acc no. NM_001078370.1). The sequence of the *Ci*TGF-β cDNA was obtained by using the GeneRacerTM kit (Invitrogen, USA). 5′-and 3′ RACE was conducted using 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 region.

2.4. Phylogenetic and structural analysis

Similarity searches were performed using the BLAST algorithm (www.ncbi.nlm.nih.gov/blast/). Sequences were subjected to multiple alignments using CLC workbench 6.4. A phylogenetic tree was made by the Neighbor-Joining method (NJ) after 1000 bootstrap iterations by using MEGA 6 (4). The accession numbers are as follows: ABX90061.1 (*Amphimedon queenslandica* TGF-β), AAX36083.1 (*Ancylostoma caninum* TGF-β), NP_001106723.1 (*Bos taurus* TGF-β2), AEE90023.1 (*Branchiostoma japonicum* TGF-β), ACA96823.1 (*Crassostrea ariakensis* TGF-β), AAO60240.1 (*Danio rerio* TGF-β1), AAQ18012.1 (*D. rerio* TGF-β2), AAU14139.1 (*D. rerio* TGF-β3), AAA50405.1 (*Homo sapiens* TGF-β2), NP_000651.3 (*H. sapiens* TGF-β1), NP_003230.1 (*H. sapiens* TGF-β3), AAD46997.1 (*Morone saxatilis* TGF-β), NP_035707.1 (*Mus musculus* TGF-β1), EDL13059.1 (*M. musculus* TGF-β2), NP_033394.2 (*M. musculus* TGF-β3), CAA07707.1 (*Oncorhynchus mykiss* TGF-β), AHH92867.1 (*O. mykiss* TGF-β2), XP_006250510.1 (*Rattus norvegicus* TGF-β2), NP_001171727 (*Saccoglossus kowalevskii* TGF-β2), ABU53678.1 (*Salmo salar* TGF-β), XP_002936067.1 (*Xenopus tropicalis* TGF-β2).

The signal peptide and protein domain was predicted using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0) and SMART (Simple Modular Architecture Research Tool). The protein structural models were developed with SWISS-MODEL and the Swiss-PdbViewer (Guex and Peitsch, 1997; Arnold et al., 2006; Schwede et al., 2003) by using human TGFβ-2 (4kxz.1.A) as a

Table 1
Primers used for cloning.

Primer name	Sequence 5′-3′	Application
*Ci*TGF-β	5′ATCAAGGGGACTTTCGGAGT3′	RACE 3′
*Ci*TGF-β	5′GCCTGTCAAGAGGTTTCTCG3′	NESTED 3′
*Ci*TGF-β	5′TACGAGAGCTCCCCTGGTTA3′	RACE 5′
*Ci*TGF-β	5′TTTGGGTCCCTGAAAAGTTG3′	NESTED 5′