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

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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).

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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′

template.

2.5. Real-time PCR analysis

Tissue differential expression of the three cDNAs was studied by real-time PCR using the Sybr-Green method and 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 (5′TTTCAGGGACCCAAAAACGA3′) and reverse (5′GCCAG CTATAATGACATCCAAGGT3′) *Ci*TGF-β primers, 300 nM forward (5′-TGATGTTGCCGCACTCGTA-3′) and reverse (5′- TCGACAATGGAT CCGGT-3′) actin 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 denaturing step at 95 °C for 15 s, and then the annealing/extension was carried out at 60 °C for 45 s when the fluorescent signal was detected. Each set of samples was run three times and each plate contained quadruplicate cDNA samples and negative controls.

The specificity of amplification was tested with real-time PCR melting analysis. To obtain sample quantification, the $2^{-\Delta\Delta Ct}$ method was used and the relative changes in gene expression were analysed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). The amount of *Ci*TGF-β transcripts from different tissues was normalised to actin in order to compensate for variations in input RNA amounts. Relative *Ci*TGF-β expression was determined by dividing the normalised value of the target gene in each tissue by the normalised value obtained from the untreated tissue.

2.6. Pharynx preparation and histology

The tunic surface was cleaned and sterilised with ethyl alcohol and body wall fragments (200 mg, containing both tunic and pharynx tissue) were excised from the injection site at various times after the injection (1–72 h).

For *in situ* hybridisation studies, body wall fragments were fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid 15:5:1) for 24 h, paraffin embedded, and serially cut at 6 μm (Leica RM2035 microtome, Solms, Germany).

2.7. In situ hybridisation assay (ISH)

To examine tissue excised from the inflamed body wall, ISH was carried out with digoxigenin-11-UTP-labelled riboprobes (1 μg/ml final concentration). The *Ci*TGF-β probe was generated by PCR amplifying a cDNA fragment of 776 bp covering the 3′ region from nucleotide 1349 to nucleotide 2042.

The DNA fragments were cloned in the pCR4-TOPO vector (Invitrogen, USA). The digoxigenin-11-UTP-labelled riboprobes were carried out according to manufacturer's instructions (Roche Diagnostics). The re-hydrated histological sections were digested with proteinase K (10 μg/ml) in PBS for 5 min, washed with PBS-T, and treated for hybridisation with 50% formamide, 5X SSC (1X SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween 20, at 37 °C overnight. After exhaustive washing in PBS-T and 4XSSC (twice for 10 min), the sections were incubated for 1 h with anti-DIG-Fab-AP conjugate (Roche Diagnostics) diluted 1:500 and washed in PBS-T. Finally, the sections were incubated in the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma–Aldrich, Germany). Colour development was stopped after 30 min at room temperature.

2.8. Statistical methods

Multiple comparisons were performed with a one-way analysis of variance (ANOVA), and different groups were compared using Tukey's t-test. Standard deviations were calculated on four experiments. $P < 0.01$ was considered statistically significant.

3. Results

3.1. Sequence analysis of CiTGF-β in C. intestinalis

The *Ci*TGF-β full-length mRNA was isolated using a 5′ and 3′ RACE strategy. *Ci*TGF-β gene encodes for a unique transcript. Sequence analysis showed a 2136 bp full-length cDNA, with 5′ and 3′ untranslated regions of 53 bp and 767 bp, respectively (Fig. 1). The cDNA sequence analysis showed that the *Ci*TGF-β cDNA had an ORF of 1308 bp encodings 435 aa with a predicted molecular weight of 49 kDa. *In silico* analysis of the *Ci*TGF-β amino acid sequence using the Delta-Blast algorithm, Signal P 4.0 and SMART showed the presence of putative structural domains: a predict signal peptide (from the aa1 to 33), a 210 aa TGF-β pro-domain (from 42 to 251 aa) and a 99 aa mature peptide (337-435aa), a putative cleavage site, the cysteine residues and two hypothetical glycosylation sites (N81 and N153) (Fig. 1).

The *Ci*TGF-β-deduced amino acid sequences, examined in GeneBank through BLAST analysis, showed significant homologies with components of the TGF family. In particular, the highest similarity (S) and identity (I) percentages were found as follows: 39% S and 59% I with *M. Musculus* TGFβ 2 (NP_033393.3) and 40% S and 59% I with *H. sapiens* TGFβ 2 (NP_003229.1). *Ci*TGF-β gene was retrieved by a search in the Ensembl genome database: *Ci*TGF-β (ENSCING00000008565), localised on Chromosome 3: 6,256,534–6,269,539. *Ci*TGF-β genomic organisation and comparison with Human TGF-β genes were performed (Fig. 2). The *Ci*TGF-β gene contained 5 exons and four introns. To some degree, the number of exons is lower with respect to mammals' TGF genes and, in particular, a shorter introns length was shown (Fig. 2).

3.2. Phylogenetic analysis

By using the MEGA 6 program, *Ci*TGF-β was aligned with vertebrate and invertebrate members of TGF β family proteins and a phylogenetic tree was constructed using the Neighbor-Joining method. The tree shows three main clusters (Fig. 3). The first includes vertebrate TGF-β3 and TGF-β2 (*O. mykiss, D. rerio, B. taurus, R. norvegicus, X. tropicalis, M. musculus* connected with the second one including the protochordate *C. intestinalis,* and the hemichordates *S. kowalevskii and B. japonicum* TGF-β. The third cluster includes TGF-β1, *(M. musculus, H. sapiens)* and TGF-β of fish (*O. mykiss, S. salar, M. saxatilis, D. rerio).* Finally, some TGF-β of protostome invertebrate is located as an outgroup (*C. ariakensis* and *A. queenslandica)* (Fig. 3).

3.3. Alignment and structural analysis

In Fig. 4A, using the CLC workbench 6.4 alignment and secondary structure elements of *Ci*TGF-β and human TGF-β1, TGF-β2, TGF-β3, the amino acid positions conserved in functionally and structurally motif are shown. The conserved height cysteine residues (Cys338, Cys339, Cys367, Cys371, Cys400, Cys401, Cy429s, Cys431) that may form intra-molecular disulphide linkages are indicated with black asterisks. Arrows represent secondary structure elements: α – helices, β – strands and a putative cleavage site (RRRK) is indicated by a black triangle.

A structural homology analysis was carried out using the SWISS-

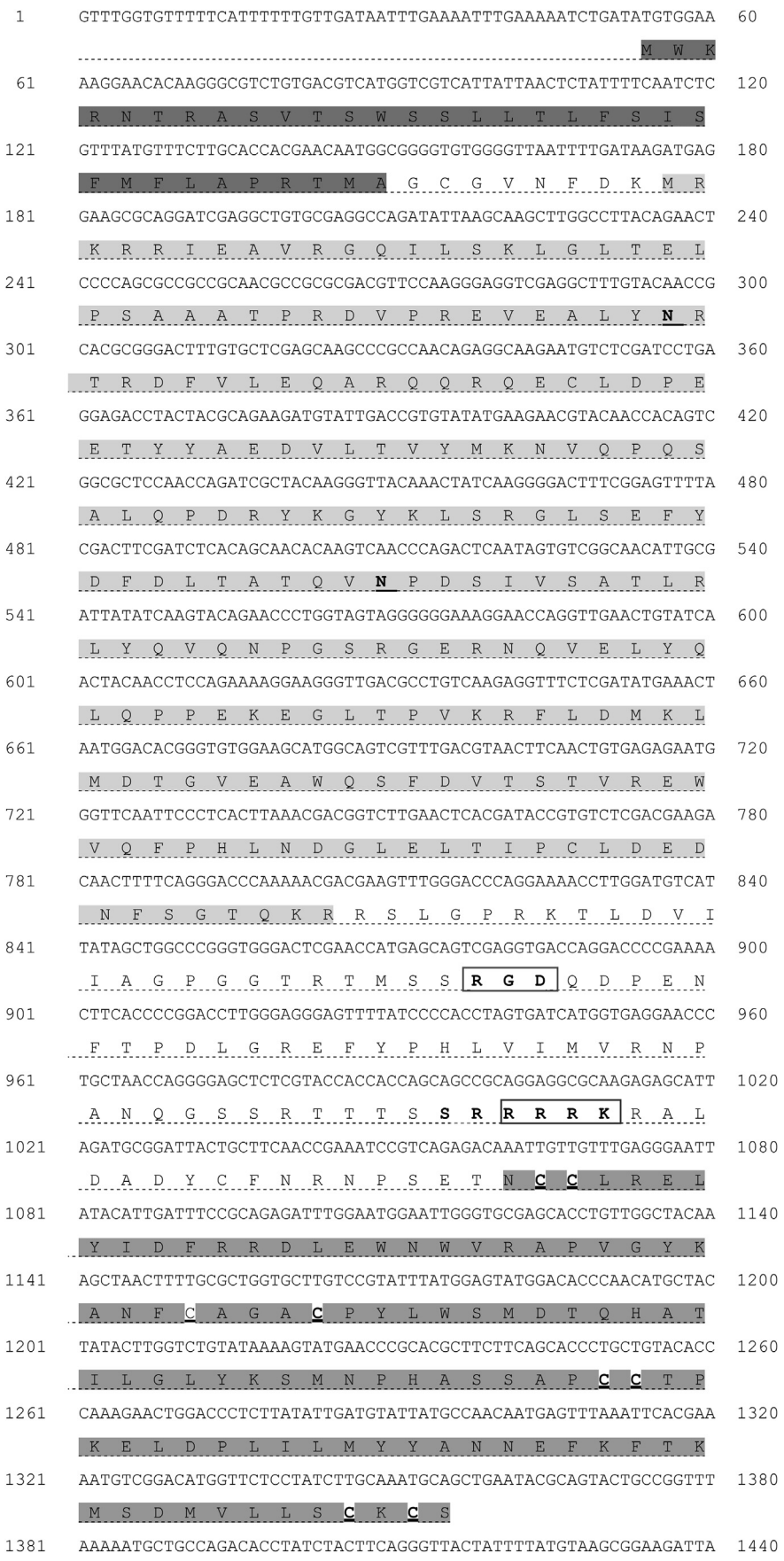

Fig. 1. *Ci*TGF-β nucleotide and aminoacidic sequence. Light grey shading signal peptide, mid-grey shading TGF-β pre-domain, dark grey shading mature peptide. The convertase cleavage site with the consensus sequence RRRK is boxed, N-glycosylation sites and height conserved cysteine residues are indicated in bold and underlined.

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1441  TCTGGCAACACTATAGAAATTTGAAAACAACAATCTATTGTGCCGCAACAATCTGCATTG  1500

1501  AATTAAATTATGACGTCAACAATGTGTTCCTTGCCCACCGCAGTGCGTCACAAAGGACTG  1560

1561  TTTATTTATAAATTTTCATTTTTTCTTTCTTTTTCCTCTGTACAAAAGATTACGTCACAC  1620

1621  ACGCTTACTGCGTGTTCAAGGACTTTTATAATTTCCTCATTGCCAGCAATTAAAATTTGC  1680

1681  TTAGAAATGCTGTTTCTGGTTAAAAACTACTAAATACACAACACATAACAGCATATACAA  1740

1741  CATATTCGTTATATCAAGTGAGTTTTGTTAACTACAACGTAAAATATACAGGAGCGTTCT  1800

1801  ATTCATTTTTTATGCTACTCGTTCAAATGTGTCTCAAACCGCTTTTACTGTGATAACAAC  1860

1861  AATAACATAATCATTTTTAAATTTCGGCAAATCGTCGTAAAATCTGGCAACGAAAATTTC  1920

1921  TCTTTGTTTTTGTTGTTGTCGATATATAATGTTACTGTTGATTTACCATTATGTTCTGAT  1980

1981  TTCCCATAAGTTGTGTCGTAAGCGATATAAACGACGAACATTCCAAAGACATGATTAAAC  2040

2041  TTTATCCAATTTTTAACTTAAACTCTTATTATGTTGCAGTTGTATTAAAAATACACAATT  2100

2101  GCCTCTCAAAAAAAAAAAAAAAAAA                                    2125
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Fig. 1. (*continued*).

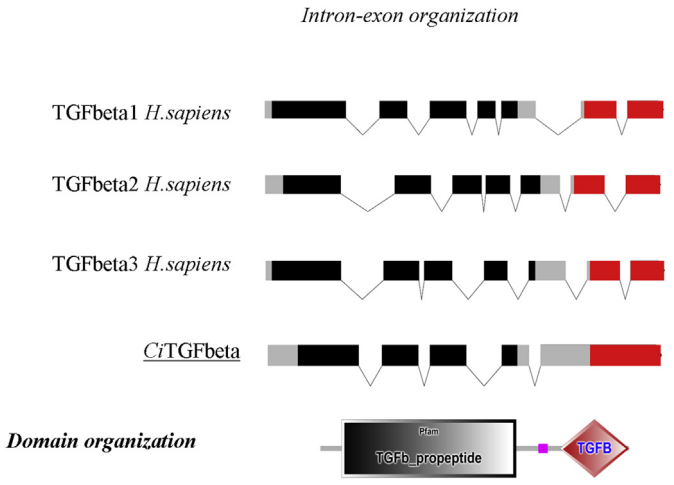

Fig. 2. Comparison of the intron/exon sizes between human TGF-β family genes (introns are reduced) and the *C. intestinalis* TGF-β genes (in grey). The thin line represents the introns and the open boxes indicate the exons of the respective genes.

MODEL program. The homology modelling process was performed on the basis of the known crystal structure of human TGF-β2 (4kxz.1.A). The global and per-residue model Qmean4 is equal to −2.40. Fig. 4B–D show the *Ci*TGF-β molecular model resulting from the super-imposing of the 337–435 residue sequence that corresponds to the mature peptide and shares 58.59% identity with the template.

3.4. *CiTGF-β gene expression is up-regulated by LPS*

Real-time PCR analysis of the inflamed ascidian pharynx showed enhanced *Ci*TGF-β mRNA levels as an effect of the LPS challenge (Fig. 5). To examine the time course of the response, four ascidians in three distinct experiments were examined at increasing post inoculation time points (1, 2, 4, 8, 12, 24, 48,72 h). At each time point, four sham ascidians were the controls (Fig. 5). The *Ci*TGF-β gene expression significantly boosted at 1–4 h, decreased at 4–24 h and increased again at 48–72 h. The response by sham ascidians indicates that the inoculation procedure did not significantly modulate the mRNA expression (Fig. 5).

3.5. *In situ hybridisation assay (ISH)*

Histological sections of the pharynx from ascidians were examined from naïve ascidians (Fig. 6A), at 1 h LPS post inoculation (Fig. 6B) and at 72 h LPS post inoculation (Fig. 6C). LPS induces *Ci*TGF-β gene up-regulation in comparison to the gene expression in naïve ascidians. Fig. 6D,E shows that the *Ci*TGF-β transcript signal is present in tightly packed hemocytes clusters within the vessel lumen. In Fig. 6D&E higher magnifications show *Ci*TGF-β expressing haemocytes, and reveal that cell that resemble granulocyte (GC) (Fig. 6D) and univacuolar refractile granulocytes (URGs) cells (Fig. 6E) were mainly involved. Controls with the sense strand probe were negative (Fig. 6A, insert).

4. Discussion

The inflammatory response triggered by bacterial infections involves the coordinated delivery of humoral and cellular components to the site of infection, and the involvement of receptors of the innate immune system such as toll-like receptors (TLRs) (Barton, 2008). This initial recognition of infection leads to the production of a variety of inflammatory mediators, including

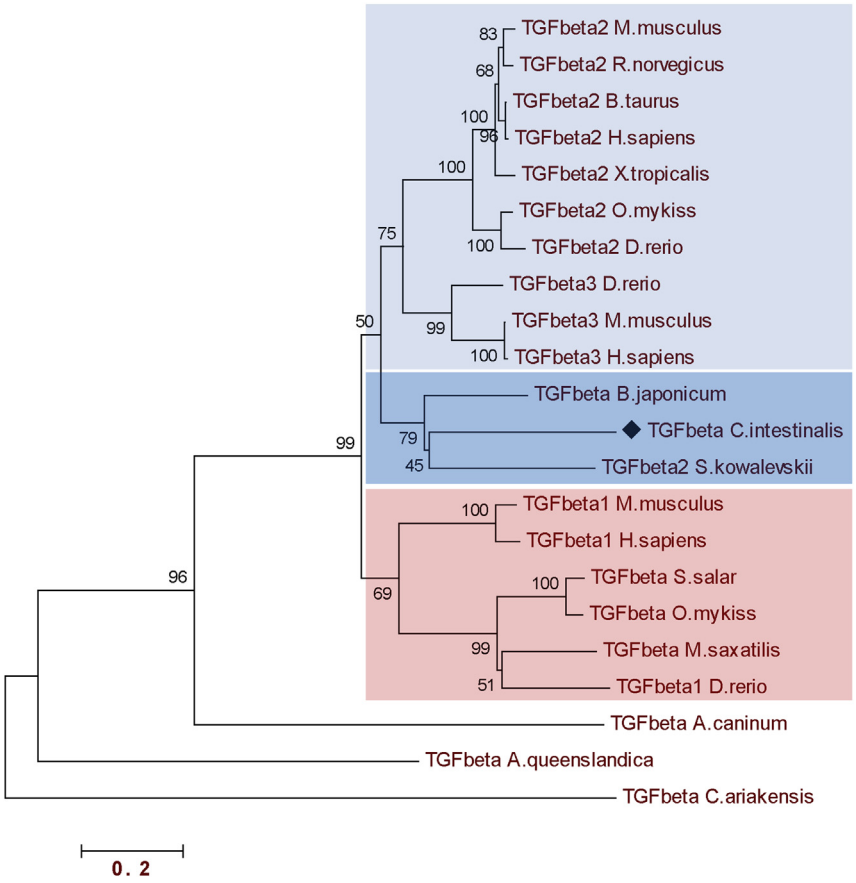

Fig. 3. Phylogenetic tree of vertebrate and invertebrate members of TGFβ family proteins: TGFβ-1, TGFβ-2, TGFβ-3, from vertebrate (*H. sapiens, M. musculus, X. tropicalis, D. rerio, O. mykiss, B. taurus, R. norvegicus, M. saxatilis, S. salar, X. laevis*) and invertebrate *(C. intestinalis, B. japonicum, S. kowalevskii,, C. ariakensis, A. caninum, A. queenslandica)*. The tree was constructed by the Neighbor-Joining method and bootstrap analysis. Bootstrap value indicates the number particular node occurrences in 1000 trees generated by bootstrapping the sequences. Bar indicates the number of amino acid residues substitutions for site.

cytokines, that play an important role in host defence via the induction of pro-inflammatory gene expression and promoting cell infiltration (Mesquita et al., 2009; Hartupee et al., 2007).

TGF-β are pleiotropic cytokines involved in development, tissue repair, remodelling and haematopoiesis. They increase the production and secretion of ECM proteins and protease inhibitors, play numerous roles in the function and development of the immune system (Li and Flavell, 2008) and show immunosuppressive and anti-inflammatory activities (Massaguè, 1990).

In this study, we identified and characterised the *C. intestinalis Ci*TGF-β gene located on chromosome 3, and showed its involvement in the inflammatory response modulation induced by LPS in the pharynx, which is the main hematopoietic organ in ascidians (Giacomelli et al., 2012).

The *C. intestinalis* inflammatory response to several irritants appears to comprise a set of complex reactions. The cellular reactions in the tunic involve hemocyte infiltration, haemocyte proliferation and epidermis activities, vacuolization and cell disruption, while cell products can contribute to form capsule components and/or cause a tunic wound (Parrinello, 1981; Parrinello et al., 1984a, 1984b). In this response the involvement of the pharynx, as the main immune-competent organ, has been disclosed by the lipopolysaccharide (LPS) challenge that up-regulates innate immunity genes in hemocytes (Parrinello et al., 2008; Vizzini et al., 2008, 2012, 2013a, 2013b, 2015a, 2015b).

Phylogenetic analysis of invertebrate and invertebrate TGF-β proteins supported a conserved evolution from a common TGF-β ancestral gene with protochordate, emichordate and vertebrate TGF β. In addition, *Ci*TGF-β is found to consist of 5 exons and 4 introns, differing from that of mammals (7 exons and 6 introns), homologous in the introns size, especially. These differences suggested that the size and exons of TGF genes varied with vertebrates. The invertebrate *Crassostrea virginica* gene reveals a single ancestrally common intron of around 3 Kb, of which the position was well conserved with the corresponding intron of both *C. intestinalis* and vertebrates (Lelong et al., 2007). This TGF-β gene organisation suggests an evolutionary model based on gene duplication and sequence diversification.

The TGF-β signalling process appears to be widely conserved in the animal kingdom since components of the pathway have characterised in protostome and deuterostome. *Ci*TGF-β is structurally related to members of the superfamily and is synthesised as a large pre-pro-protein composed of a hydrophobic signal peptide, an N-terminal pro-domain and a C-terminal active peptide, and also exhibits a cleavage site at a consensus site (RRRK) to generate a C-terminal domain. As with all members of the superfamily, the pro-domain shows a low degree of conservation for correct processing and secretion of the mature dimeric complex and C-terminal active peptide exhibits height cysteine residues at invariant positions, that are engaged in intramolecular disulphide bonds resulting in the adoption of a tri-dimensional structure with a cysteine knot motif and shared secondary structures with two alpha helix and seven β sheets.

In addition an RGD motif is present with a potential binding of

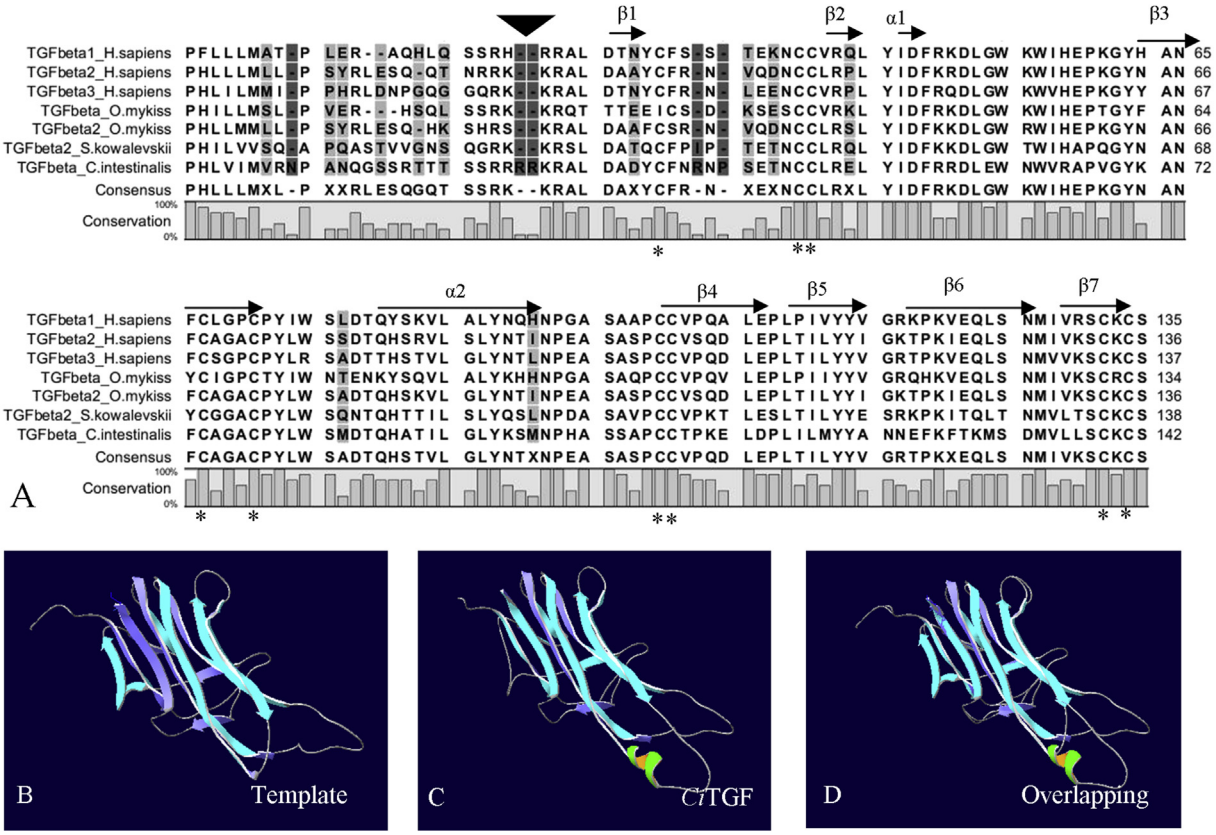

Fig. 4. (A) Alignment and secondary structure elements of *Ci*TGF-β and human TGF-β1, TGF-β2, TGF-β3. The conserved cysteines that may form intra-molecular disulphide linkages are indicated with black asterisks. Arrows represent secondary structure elements: α – helices, β – strands. (B) Homology model of *Ci*TGF-β (C) Used template structure of human TGFβ-2 (4kxz.1.A) (D) overlap of target/template structures.

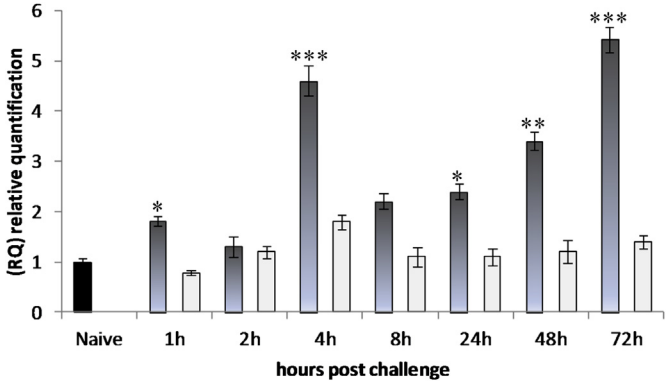

Fig. 5. Real-time PCR analysis. Time-course of *Ci*TGF-β gene expression in *C. intestinalis* pharynx after inoculation into the body wall of 100 μg bacterial lipopolysaccharide (LPS) in 100 μl marine solution (MS) (in grey), compared with the gene expression in ascidians injected with 100 μl MS (sham ascidian, in white). Values, plotted as mean ± SD, were inferred from four ascidians examined in three distinct experiments; each assay was performed in triplicate. Asterisks indicate significant differences, at each time point, between LPS and SW inoculation (post hoc Tukey *t*-test). *P < 0.05; **P < 0.01; ***P < 0.001; (n = 4).

integrins that can activate TGF-β1 present in LAP-β conformation. In mammals, upon binding, it induces adhesion-mediated cell forces that are translated into biochemical signals which can lead to liberation activation of TGF β from its latent complex (Munger et al., 1999).

The real-time PCR profile shows that *Ci*TGF-β is up-regulated at 4 h p.i. and at 72 h p.i., with a higher expression at 72 h p.i.

This double peak of *Ci*TGF-β mRNA production could be related to the potential function of TGF as a pro-inflammatory cytokine and anti-inflammatory molecule that at the end of the reaction helps to restore the homeostasis.

The *In situ* hybridisation assay, showing that the *Ci*TGF-β gene is expressed in tightly packed hemocyte clusters within the vessel lumen by inflammatory hemocytes (cell that resemble granulocytes and URGs) in the pharynx vessels, is intriguing. Indeed, a future research direction might be the possibility that this cell gathered in clusters could represent epithelia host stationary cells derived from the lymph nodules (Ermak, 1976). According to previous findings (Parrinello et al., 2008, 2010; Vizzini et al., 2008, 2012; Cammarata et al., 2008; Bonura et al., 2009, 2010), the LPS inoculation enhanced the population density of hemocytes expressing immune-related genes including *Ci*TGF-β. In invertebrates, TGF1 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).

In *Crossostea gigas* TGF was involved in the immune function by up-regulating the expression in hemocytes during infection by gram-negative bacterium.

In mammals TGF-β have a role in T cell differentiation during the immune response - in particular, Th17 and in IL-17 production (Lohr et al., 2006). All these findings suggest that *Ci*TGF-β may be involved in an acute inflammatory response in the elimination of the infectious agents and thereafter, also playing a crucial role in the resolution of inflammation, including the initiation of tissue repair.

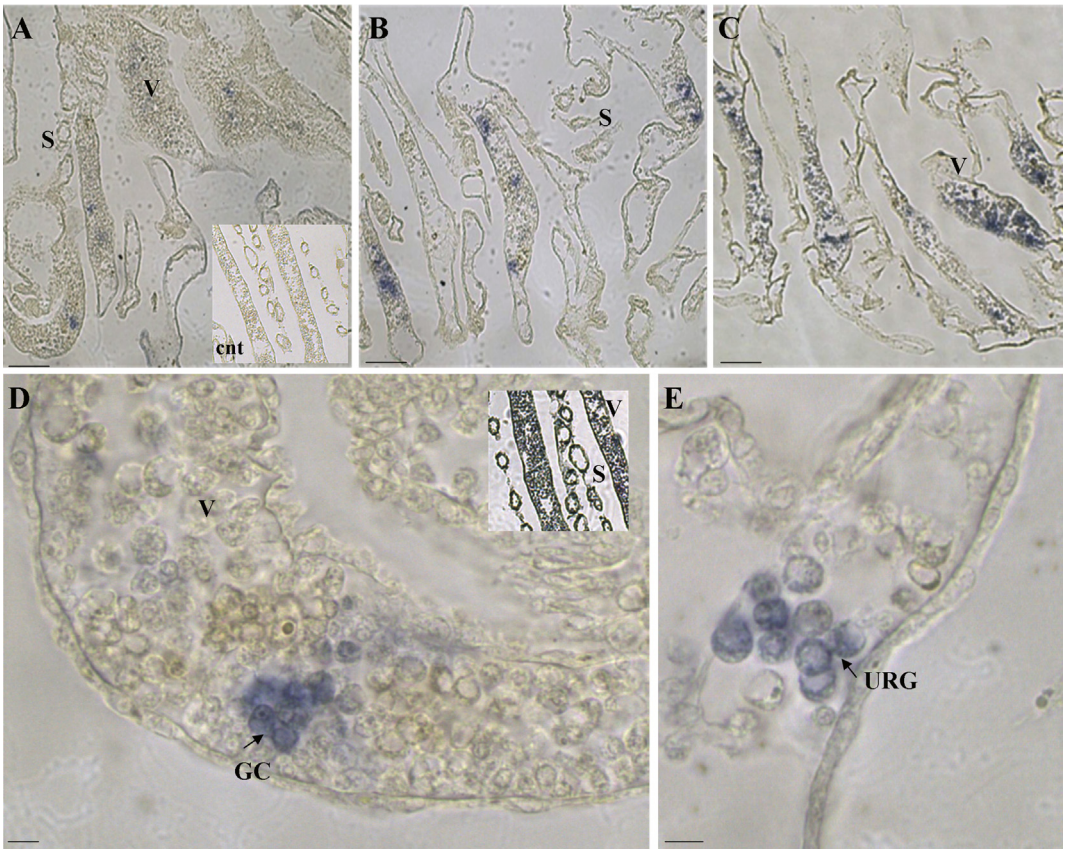

Fig. 6. Histological sections of *C. intestinalis* pharynx vessels. *In situ* hybridisation with the *Ci*TGF-β riboprobes. Naive ascidian (A), ascidian at 1 h (B) and 72 h (C) after LPS inoculation. Control with the sense strand (A inset). Bar size: A, B, C 50 μm, D, E 4 μm. URG (univacuolar refractile granulocyte), GC (granular cells). D (inset) showing Gomori stained section. Branchial basket stigmata (S); Vessel (V).

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