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Short communication

Cloning and expression analysis of an IL-6 homolog in rainbow trout (*Oncorhynchus mykiss*)

Dimitar B. Iliev [a], Barbara Castellana [b], Simon MacKenzie [c], Josep V. Planas [b], Frederick W. Goetz [a,*]

[a] *Great Lakes WATER Institute, University of Wisconsin Milwaukee, 600 E. Greenfield Avenue, Milwaukee, WI 53204, United States*
[b] *Departament de Fisiologia, Facultat de Biología, Universitat de Barcelona, Barcelona, Spain*
[c] *Unitat de Fisiologia Animal, Departament de Biologia Cellular, Fisiologia i d'Immunologia, Facultat de Ciencies, Universitat Autonoma de Barcelona, Bellaterra, Barcelona, Spain*

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Abstract

A partial cDNA with significant similarity to IL-6 was identified in rainbow trout. Rapid amplification of cDNA ends was used to obtain the full sequence of the trout IL-6 homolog which contains 1180 nucleotides. The transcript encodes a predicted protein of 219 amino acids and eight instability motifs in the 3′UTR. While the complete sequence of the trout IL-6 is poorly conserved, the protein contains a distinct IL-6/G-CSF/MGF family consensus pattern and predicted characteristic α-helical tertiary structure. However, like in fugu, trout IL-6 lacks a pair of cysteine residues, which in mammals are involved in formation of a disulphide bond. The expression of the IL-6 homolog in trout mononuclear phagocytes was highly up-regulated by LPS but not poly(I:C) as demonstrated by Northern analysis. Using RT-PCR the IL-6 expression was detected in trout spleen, gill, gastrointestinal tract, ovary and brain. The highest transcript levels were detected in the ovary suggesting that IL-6 may perform specific functions within this organ.

Keywords: Rainbow trout; Interleukin-6; Cytokine evolution; Cloning; Expression; Inflammation; Lipopolysaccharide; Monocytes/macrophages

1. Introduction

Interleukin-6 (IL-6) (Kishimoto and Hirano, 1988) belongs to a group of molecules that share tertiary structure formed by four antiparallel alpha helices and a common signaling receptor subunit (reviewed in Heinrich et al. (1998)). In addition to IL-6, this gene family also includes leukemia inhibitory factor (LIF), cilary neurotrophic factor (CNTF), oncostatin M (OncM), cardinotrophin-1 (CT-1) and interleukin-11 (IL-11).

IL-6 is produced by a diverse group of cells including T-lymphocytes, macrophages, fibroblasts, neurons, endothelial and glial cells. The pleiotropic effects of IL-6 are mediated by a 2-subunit receptor (Taga and Kishimoto, 1997) and include the regulation of diverse immune and neuro-endocrine processes. IL-6 has been implicated in the control of immunoglobulin production, lymphocyte and monocyte differentiation, chemokine secretion and migration of leukocytes towards sites of inflammation (Hirano, 1998; Inoue et al., 2005; Kaplanski et al., 2003). In addition, IL-6 is involved in the control of metabolism in adipose tissue (Chida et al., 2006; Kralisch et al., 2005), the development of the central nervous system (Taga and Fukuda, 2005), angiogenesis (Motro et al., 1990) as well as the function of gonads and the synthesis of steroids (Alpizar and Spicer, 1994; Van der Hoek et al., 1998).

Consistent with its diverse functions, the expression of IL-6 has been detected in various rat tissues with the highest levels in thymus, liver and ovary (Chung et al., 2000). The expression of IL-6 is inducible by proinflammatory mediators including TNF-

Abbreviations: IL-6, interleukin-6; LIF, leukemia inhibitory factor; CNTF, cilary neurotrophic factor; OncM, oncostatin M; CT-1, cardinotrophin-1; RACE, rapid amplification of cDNA ends; UTR, untranslated region; LPS, lipopolysaccharide; SSH, suppression subtractive hybridization; AP-1, activator protein-1; CREB, cAMP response element binding protein; C/EBP-β, CCAAT enhancer-binding protein-β; NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor alpha

* Corresponding author. Tel.: +1 414 382 1742; fax: +1 414 382 1705.
E-mail address: rick@uwm.edu (F.W. Goetz).

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α, IL-1β and lipopolysaccharide (LPS) (Ammit et al., 2002; Bergamaschi et al., 2006; Takezako et al., 2006) and is driven by activator protein (AP-1), cAMP response element binding protein (CREB), CCAAT enhancer-binding protein-β (C/EBP-β), and nuclear factor (NF-κB) (Ray et al., 1988, 1989, 1990).

Similar to other cytokines, the sequence of IL-6 is phylogenetically poorly conserved. However, recently, the conserved synteny within the IL-6 locus allowed for the isolation of the first piscine IL-6 homolog in fugu (Bird et al., 2005).

The current paper describes a trout IL-6 homolog which was identified in a suppression subtractive hybridization (SSH) cDNA library derived from LPS-stimulated mononuclear phagocytes. The IL-6/G-CSF/MGF family consensus pattern and the characteristic α-helical structure are present in the trout IL-6. In addition, trout IL-6 was highly inducible by LPS and tissue expression analysis indicates that the IL-6 mRNA levels are highest in unstimulated trout ovaries.

2. Materials and methods

2.1. Cell culture and stimulation

Rainbow trout mononuclear phagocytes were isolated, cultured and stimulated according to methods that have been previously described (Iliev et al., 2005). Briefly, head kidneys were homogenized using 200 μm nylon mesh in the presence of 10% fetal bovine serum in DMEM. The homogenates were plated on 60 mm poly-D-lysine coated cell culture dishes at 5×10^6 cells/dish and were kept at 15 °C and 5% CO_2. The cells were washed approximately 24 h after initial plating and supplied with fresh medium. The adherent cells were incubated for another 4 days before stimulation.

LPS from *E. coli* (serotype O26:B6, cat. no. L-8274, 2.2% protein) was purchased from Sigma–Aldrich. Poly(I:C) was purchased form InvivoGen.

To investigate the regulation of IL-6, the cells were either left untreated or were stimulated with 50 μg/ml of *E. coli* LPS or 10 μg/ml of poly(I:C) for 3, 6 and 24 h prior to sampling.

2.2. Cloning and sequencing trout IL-6

The partial IL-6 sequence was identified in a subtracted library generated with RNA from cells cultured as described above and stimulated with 10 μg/ml of *E. coli* LPS for 24 h. Briefly, mRNA from untreated and stimulated cells was obtained using the PolyAtract mRNA Isolation System (Promega, Madison, WI, USA). One microgram of mRNA from each the LPS-treated and the non-stimulated cells were subtracted against each other using the PCR Select Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The subtracted cDNA samples were cloned in TOPO pCR 2.1 (Invitrogen). The libraries were plated on LB agar plates and sequenced using BigDye Terminator (Applied Biosystems) on an ABI 3730 automated sequencer (Applied Biosystems). The trout IL-6 homolog was identified in a clone containing a 717 bp insert after translating blast (BLASTX) against the non-redundant GenBank protein database at: http://www.ncbi.nlm.nih.gov/BLAST/. The closest match for this sequence was fugu IL-6 (accession no. BAD18930) with 29%/58% identities/positives and an expected value of 3E − 06.

The full-length of trout IL-6 was obtained using rapid amplification of cDNA ends (RACE) with the SMART™ RACE cDNA Amplification Kit, the Advantage™ 2 PCR Kit (Clontech) and following manufacturer's instructions. For template, 1 μg of total RNA obtained from LPS-stimulated trout macrophages was used. The following gene specific IL-6 primers were used for the RACE—5′ (forward): TGAGGCACAAAGCTTCTC; 3′ (reverse): TCTTCAGCACGTTAAGGC. The 5′ and 3′ cDNA fragments obtained from RACE were cloned and sequenced as described above. The assembled full-length cDNA sequence was entered in the GenBank with accession number DQ866150.

2.3. Analysis of the expression of IL-6

The probe for the Northern analysis was labeled using PCR with a Biotin-N_4-dCTP Labeling Mix (Detector PCR DNA Biotinylation Kit, Kirkegaard and Perry Labs Inc.), the IL-6 specific primers shown above and cDNA from LPS-stimulated trout macrophages generated as described before (MacKenzie et al., 2006). The following PCR cycling protocol was used: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. The final step was an extension at 72 °C for 3 min. Total RNA from stimulated trout mononuclear phagocytes were isolated using Tri Reagent (Molecular Research Center Inc.) following the manufacturer's instructions. Total RNA (15 μg) was separated on a denaturing agarose gel and processed, as previously described (Iliev et al., 2005). Following hybridization and washing, the signal was visualized on a GeneGnome chemiluminescent imaging system (Syngene).

For the tissue expression analysis, total RNA was isolated from different trout tissues using Tri Reagent (Molecular Research Center Inc.) following the manufacturer's instructions. Total RNA (6 μg) was treated with 30 units of DNase I for 30 min at 37 °C and repurified using RNeasy MinElute Cleanup Kit (Qiagen). Repurified RNA (1 μg) was reverse transcribed with ImProm-II™ reverse transcriptase (Promega) according to the manufacturer's protocol. The cDNA was diluted (2×) with RNAse/DNAse free water and was used as a template in PCR reactions with primers (indicated below) for IL-6 (DQ866150) and acidic ribosomal phosphoprotein (AY685220):

- IL-6 forward: 5′-TTTCAGAAGCCCGTGGAAGAGA-3′;
- IL-6 reverse: 5′-TCTTTGACCAGCCCTATCAGCA-3′;
- ARP forward: 5′-TCCTTCGAAGCACTGCAAAGATGC-3′;
- ARP reverse: 5′-TGTGGAATGCAGACAGACAGGGAT-3′.

The PCR reactions were performed using *Taq* DNA Polymerase (Promega) with 3 mM MgCl, 150 nM primer concentration and 100 ng of reverse-transcribed tissue RNA. The cycling conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. The final step was an extension at 72 °C for 3 min. The reactions were run on 1.5% agarose gels stained with ethidium bromide.

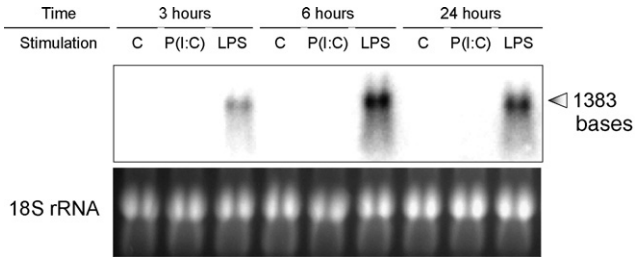

Fig. 1. LPS up-regulates the trout IL-6 homolog in mononuclear phagocytes. The cells were either left untreated (C) or were stimulated with 10 μg/ml of poly(I:C) (P(I:C)) or 50 μg/ml of *E. coli* LPS. Total RNA (15 μg) was run on a denaturing gel and the IL-6 expression was detected using chemiluminescent Northern blot analysis. The arrowhead shows the position of a marker band of the indicated size. Lower panel shows the ethidium bromide stained ribosomal RNA.

3. Results and discussion

A partial sequence of a trout IL-6 homolog was identified in a SSH library derived from LPS-stimulated trout mononuclear phagocytes. The full-length of trout IL-6 was obtained using RACE, and the size of the complete cDNA is 1180 nucleotides (DQ866150). However, an IL-6 probe was able to detect a transcript on a Northern blot which was approximately 1380 nucleotides long (Fig. 1). The larger size is most likely attributed to the additional poly-A tail which is, on average, 200 nucleotides.

The trout IL-6 mRNA contains a 660 nucleotide-long open reading frame, eight non-overlapping "ATTTA" instability motifs located in the 3′UTR and one "AATAAA" polyadenylation signal found eight nucleotides upstream of the poly A tail. The IL-6 protein sequence contains 219 amino acids (AA) and it is phylogenetically poorly conserved (Fig. 2). Trout IL-6 shares highest similarity with fugu IL-6 with 30% identical and 53% similar AA matches. The similarity between trout and different mammalian IL-6 homologs varies between 24–28% identical and 47–50% similar AA matches. Phylogenetic analysis further demonstrates that the trout IL-6 homolog is more closely related to IL-6 than to the other members of the family (Fig. 3). Despite the low sequence conservation, the general structure of trout IL-6 has been retained throughout the evolution of vertebrates. As with fugu, trout IL-6 features a signal peptide sequence and four conserved α-helices (Fig. 2). In addition, with the exception of a slightly shifted cysteine residue, the IL-6/G-CSF/MGF family consensus pattern is present in trout IL-6. The AA sequence alignment also shows that a pair of cysteine residues which participate in a disulphide bond and appear to be conserved within tetrapods, is not found in either trout (Fig. 2) or fugu IL-6 (Bird et al., 2005).

In mammals, the IL-6 promoter contains a set of transcription factor binding motifs recognized by AP-1, CREB, C/EBP-β and NF-κB (Ray et al., 1988, 1989, 1990). These factors render IL-6 inducible by cytokines, including IL-1, TNF-α and IL-10, as well as mitogenic stimuli, such as phytohemagglutinin (Ammit

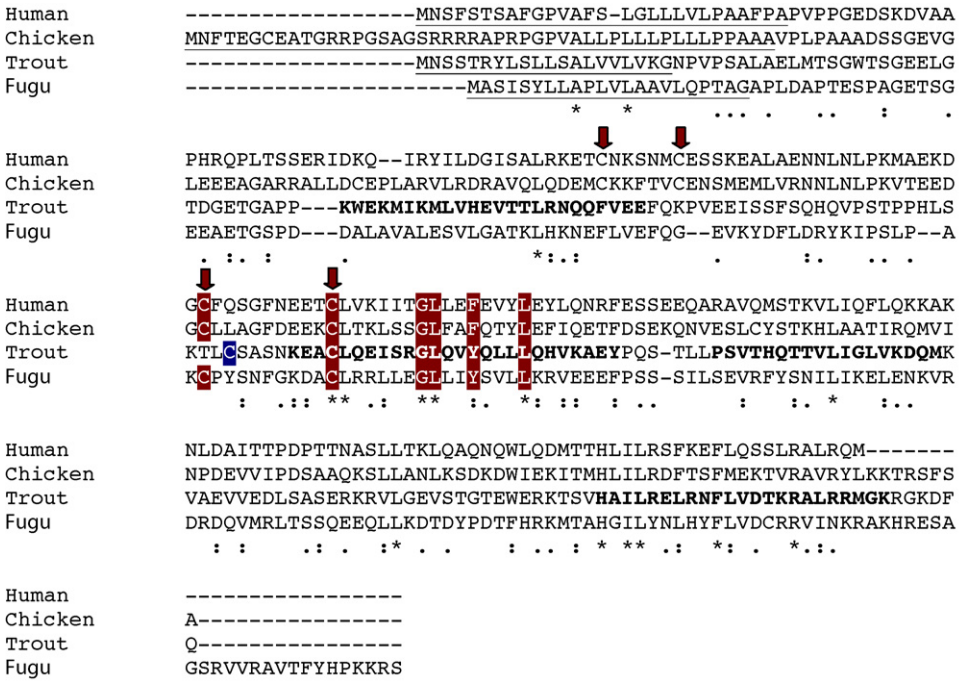

Fig. 2. Multiple alignment of IL-6. The predicted AA sequence of trout IL-6 were aligned with IL-6 homologs from other vertebrates using CLUSTALW. The AAs of the IL-6 consensus pattern (C-x(9)-C-x(6)-G-L-x(2)-[FY]-x(3)-L) are shaded. The positions of the cysteine residues that are known to form two disulfide bonds are indicated with arrows. Note that, although the position of the first cysteine of the IL-6 consensus pattern is not conserved in trout, a cysteine residue is situated in close proximity and may potentially be involved in the formation of the second disulfide bond. The first disulfide bond, which is not a part of the IL-6 consensus pattern, does not appear to be conserved in fish. The signal peptide of trout IL-6 was predicted to span AA 1–20 (underlined) using SignalP 3.0 V (Technical University of Denmark; http://www.cbs.dtu.dk/services/SignalP/). The sequences of the four conserved α-helices are marked in bold. Accession numbers: human, P05231; chicken, Q90YI0; trout, DQ866150; fugu, CAD67608.

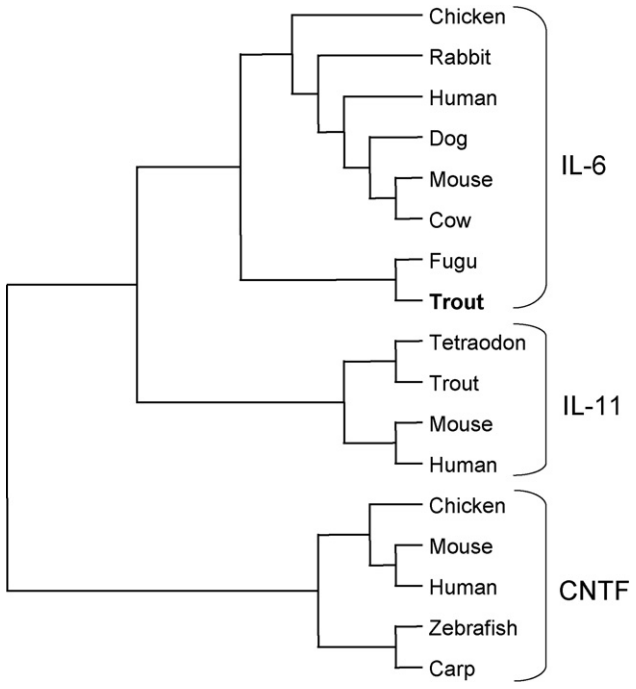

Fig. 3. Phylogenetic analysis of the IL-6 family. The protein sequences were aligned using CLUSTALW and further analyzed with PROTDIST and FITCH from the PHYLIP package. The Cladogram demonstrates that the analyzed trout sequence is most similar to IL-6. Accession numbers for IL-6: chicken, Q90YI0; rabbit, Q9MZR1; human, P05231; dog, P41323; mouse, AAA39301; cow, NP_776348; fugu, CAD67608 and trout, DQ866150. Accession numbers for IL-11: Tetraodon, AAR25699; trout, CAD59686; mouse, P47873; human, AAH12506. Accession numbers for CNTF: chicken, Q02011; mouse, P51642, human, P26441; zebrafish, XP_683402; carp, Q7ZT19.

et al., 2002; Bergamaschi et al., 2006; Bird et al., 2005; Robb et al., 2002; Takezako et al., 2006). Accordingly, we observed that trout IL-6 message was strongly and persistently (>24 h of stimulation) up-regulated by LPS whereas its induction by poly(I:C) was not detectable by Northern analysis (Fig. 1). In contrast, fugu IL-6 appears to be more responsive to stimulation with poly(I:C) as compared to LPS following in vivo challenges (Bird et al., 2005). This difference could be due to the presence of cell types in tissues (e.g., spleen) examined in fugu, that may be relatively more responsive to stimulation with poly(I:C) as compared to mononuclear phagocytes which were used in the current experiment. It could also be due to the stimulation of other factors that would subsequently up-regulate IL-6 in vivo that would not be present in vitro.

Interestingly, among the trout tissues examined in the present study, ovaries expressed the highest levels of the IL-6 message (Fig. 4). The ovaries sampled from the two females were at the same stage of development and were undergoing vitellogenesis. Similarly high levels of the IL-6 message were detected in the ovary of a third individual (data not shown). Within the ovary, IL-6 is produced by epithelium, follicular granulosa cells and, potentially, by residing leukocytes (Lidor et al., 1993; Ziltener et al., 1993). In fact, rat ovary was amongst the tissues that expressed the highest levels of IL-6 (Chung et al., 2000). Also, the expression of IL-6 in the rat ovary depended on the ovulatory cycle and was influenced by stimulation with IL-1β and

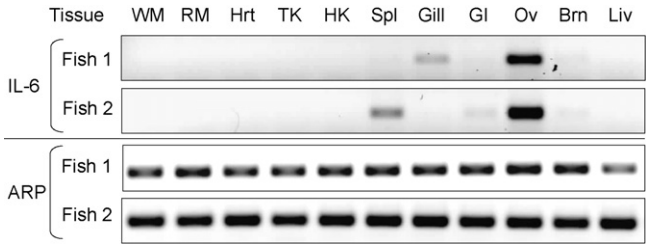

Fig. 4. Tissue expression analysis of trout IL-6. RT-PCR was performed on cDNA samples from two females. Amplification of acidic ribosomal protein (ARP) was used to confirm the cDNA integrity. WM, white muscle; RM, red muscle; Hrt, heart; TK, trunk kidney; HK, head kidney; Spl, spleen; GI, gastrointestinal tract; Ov, ovary; Brn, brain; Liv, liver.

prostaglandins (Chung et al., 2000). The production of ovarian steroids may be regulated by proinflammatory mediators, such as TNF-α and IL-1β (Brannstrom et al., 1993, 1995; Simon et al., 1994). In addition, it has been demonstrated that exogenous IL-6 may influence ovarian steroidogenesis and the proliferation of granulosa cells (Alpizar and Spicer, 1994; Van der Hoek et al., 1998).

The considerably higher expression of IL-6 in the trout ovary as compared to other tissues suggests that, like in mammals, trout IL-6 may be involved in ovarian function and it will be particularly interesting to further study the expression of IL-6 during the complete gonadal cycle and the factors that regulate it.

In addition to the ovary, the expression of trout IL-6 was detected in spleen, gill, gastrointestinal tract and brain but not in head kidney as reported for fugu (Bird et al., 2005). The levels of expression of trout IL-6 in the spleen, gills and GI tract were quite variable between the two individuals assayed which could be attributed to variations in the activation state and fluctuations in leukocyte populations within these tissues. On the other hand, the levels of IL-6 in the brain, albeit very low, were similar between the two individuals. This is not surprising since IL-6 is expressed in neurons and glial cells (Bergamaschi et al., 2006; Di Loreto et al., 2003) and, under non-pathological conditions, it may participate in the development and the function of the central nervous system (Taga and Fukuda, 2005).

In summary, similarly to other cytokines, the overall sequence of trout IL-6 is poorly conserved. Nevertheless, the key features of IL-6 appear to be phylogenetically well conserved within vertebrates. This includes a well-preserved IL-6/G-CSF/MGF family consensus pattern and retained positions of four α-helices. In addition, the data suggest that the control of expression and, possibly, the function of trout IL-6, may be similar to that of its mammalian homolog.

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