Fish & Shellfish Immunology 41 (2014) 102–112

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Identification and characterisation of the IL-27 p28 subunits in fish: Cloning and comparative expression analysis of two p28 paralogues in Atlantic salmon *Salmo salar*

Mansourah Husain, Samuel A.M. Martin, Tiehui Wang*

Scottish Fish Immunology Research Centre, Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK

ARTICLE INFO

Article history:
Available online 26 June 2014

Keywords:
IL-27
p28
Atlantic salmon *Salmo salar*
Cloning
Expression

ABSTRACT

Interleukin (IL)-27 is an IL-6/IL-12 family member with pro-inflammatory and anti-inflammatory properties. It is a heterodimeric cytokine composed of an α-chain p28 and a β-chain Ebi3 (Epstein–Barr virus induce gene 3). The p28 subunit can also be secreted as a monomer and function as IL-30 that acts as an inhibitor of IL-27 signalling. At present, the p28 gene has only been described in mammals. Thus, for the first time outwith mammals, we have identified seven p28 molecules in six divergent teleost fish species, Atlantic salmon, two cichlids, two cyprinids and a yellowtail. The fish p28 molecules have higher similarities to mammalian p28 than other IL-6/12 family members. Critical residues involved in the interaction with Ebi3 and the receptor gp130 are highly conserved. The prediction that these are true orthologues is supported by phylogenetic and synteny analysis.

Two p28 paralogues (p28a and p28b) sharing 72% aa identity have been identified and characterised in Atlantic salmon. There are multiple upstream ATGs in the 5′-UTR and ATTTA motifs in the 3′-UTR of both cDNA sequences, suggesting regulation at the post-transcriptional and translational level. Both salmon p28 genes are highly expressed in immune relevant tissues, such as thymus, gills, spleen and head kidney. Conversely salmon Ebi3 is highly expressed in other organs, such as liver and caudal kidney. The expression of p28 but not Ebi3 was induced by PAMPs and recombinant cytokines in head kidney cells, and in spleen by Poly I:C challenge *in vivo*. The dissociation of the expression and modulation of p28 and Ebi3 suggest that both p28 and Ebi3 may be secreted alone or with other partners.

1. Introduction

In mammals, IL-27 is a heterodimeric cytokine of the IL-6/IL-12 family composed of an α-chain p28 and a β-chain Ebi3 (Epstein–Barr virus induced gene 3). p28 is a four-helix bundle cytokine and resembles other helical cytokines such as IL-6, cardiotrophin-1 (CT-1), CT-2, p35 (α-chain of IL-12), and p19 (α-chain of IL-23). In contrast, Ebi3, as with p40 (the β-chain of IL-12 and IL-23), has homology to soluble receptors, such as IL-6Rα, ciliary neurotrophic factor (CNTF) receptor, and cytokine-like factor 1 (CLF) [1–4]. IL-27 signals via a heterodimeric IL-27 receptor (IL-27R) consisting of IL-27Rα (also known as WSX-1 and TCCR) and glycoprotein 130 (gp130), and activates both the JAK/STAT1/3 and MAPK pathways. Although IL-27 is capable of binding to IL-27Rα in the absence of gp130, both subunits are required for signal transduction [5,6].

Mammalian IL-27 is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory properties. These different functions depend on the immunological context, the temporal regulation of IL-27 production, and cell-specific expression of components of the IL-27R. Human and mouse IL-27 is expressed by antigen-presenting cells (APCs), including monocyte/macrophages, dendritic cells (DC) and B cells in the early phase following antigen mediated activation [5]. IL-27 production has been noted during viral, bacterial, and parasitic infections [4]. In response to bacterial and viral pathogen associated molecular patterns (PAMPs), Toll-like receptors (TLR) 3, 4 and 9 on APCs are activated to induce IL-27 production. Additionally IL-27 subunits can be upregulated by host-derived inflammatory stimuli such as IL-1β, IFN-γ and by interaction of T cells and APCs through CD40/CD40L ligation [7,8].

In mammals, the gp130 subunit of IL-27R is expressed in virtually all tissues and is a common signal-transducing chain of other IL-6 family cytokines, including IL-6, IL-11, CNTF, and leukaemia inhibitory factor amongst others. In contrast, IL-27Rα expression is restricted to immune cells, such as natural killer (NK),

* Corresponding author. Tel.: +44 1224 272883; fax: +44 1224 272396.
E-mail address: t.h.wang@abdn.ac.uk (T. Wang).

http://dx.doi.org/10.1016/j.fsi.2014.06.024
1050-4648/

NKT, T, B and plasma cells, and cells of the myeloid lineage [1,3]. Thus human and mouse IL-27 can promote inflammatory responses by (1) initiation of the T helper (Th)1 response via T-bet and IL-12 receptor induction, (2) induction of IFN-γ synthesis by NK, CD4+ and CD8+ T cells, (3) constraint of the Treg cell population, and (4) induction in CD4+ T cells of the expression of IL-21 that supports follicular helper CD4 T cell differentiation and proliferation, and antibody production by B cells [9]. In terms of anti-inflammatory properties, IL-27 induces IL-10 production from various T cell subsets (Th1, Th2, Th17, Treg, and Tr1 cells). IL-10 is a potent anti-inflammatory cytokine that restrains inflammation in a variety of contexts [1,3,4]. In addition IL-27 is a suppressor of Th2 or Th17 responses by down-regulating key transcription factors GATA3 or RORγt expression, respectively [1,3].

Compared with other mammalian IL-12-related cytokines, such as IL-12 and IL-23, IL-27 differs in that its subunits are not covalently linked by a strong disulphide bond [2,9]. Additionally, IL-27p28 and Ebi3 can be differentially expressed in different cell types, implying that p28 and Ebi3 might associate with other factors to form novel cytokines or have biological functions of their own [10]. Early studies suggested that p28 is not secreted unless it is co-expressed with Ebi3. However, over-expression of mouse p28 revealed that it is also secreted as a monomer, IL-30, that acts as an inhibitor of IL-27 signalling [11]. Mouse p28 subunit also binds to CLF, a soluble cytokine receptor, to form a novel heterodimer that requires IL-6R for signalling and induces cytokine production by T and NK cells [12], and enhances B cell proliferation and plasma cell differentiation [13]. Interestingly, an engineered composite heterodimeric cytokine mouse p28/p40 is biologically active, suggesting that additional αβ pairing of the IL-6/IL-12 family may exist in nature [14]. So far, the p28 gene has only been described in mammals.

Genomic resources have led to the identification of a growing number of IL-6/IL-12 family members in teleost fish. These include IL-6 [15–18], IL-11 [19–21], CNTF like [22] and M17 [22,23]. Of the IL-12 subfamily, all of the known subunits (p19, p35, p40 and Ebi3), except p28 have been isolated in fish, with the presence of two and three isoforms of teleost p35 and p40 subunits, respectively [24–31]. In this report, we identified and characterised the p28 genes, for the first time outwith mammals, in six fish species. Two p28 loci have been identified and their transcripts cloned in Atlantic salmon *Salmo salar*, most likely as a result of the salmonid whole genome duplication that occurred approximately 88 Myr ago [32]. The expression of salmon IL-27 subunits p28a, p28b and Ebi3 was comparatively investigated and was found that Poly I:C, PHA, PMA and IL-1β are strong inducers of p28 expression in head kidney (HK) cells.

2. Materials and methods

2.1. Identification, cloning and sequence analysis of IL-27 p28

The Atlantic salmon whole genome shotgun sequence (WGS) was searched (TBLASTN [33]) using mammalian IL-27 p28/IL-30 protein sequences. A candidate contig (Acc. No. AGKD01172157) was identified and exons predicted. Primers (Table 1) were designed against the predicted exons and used for 5′ and 3′-RACE using thymus SMART cDNA as described previously [34,35]. Two overlapping RACE products were obtained, cloned and sequence analysed as described previously [35]. The resulting cDNA sequence matched the WGS contig, contained an open reading frame (ORF), and was designated as salmon p28a. Further analysis of salmon WGS data identified two non-overlapping WGS contigs (AGKD01075909 and AGKD01068023) that code for a second p28 (designated as p28b). Primers F1 and R1 (Table 1), designed against these WGS contigs were used to amplify a 1.4 kb cDNA from thymus cDNA and cloned as above. Using the salmon p28 sequence, other fish p28 molecules were identified *in silico*. The DNA and protein sequence analysis was as described previously [35,36]. Briefly, protein identification was carried out on http://www.expasy.org/tools/ [37] and global sequence comparison was performed using the MatGAT program [38]. The signal peptide and protein modular structure were predicted using the SignalP 3.0 program [39] and Jpred [40], respectively. Multiple sequence alignments were generated using CLUSTALW (version 1.82) [41] and shaded using BOXSHADE (version 3.21, http://www.ch.embnet.org/software/BOX_form.html). The synteny analysis of orthologs between fish and mammals was carried out using Genomicus [42]. Finally phylogenetic trees were created by the neighbour-joining method using the MEGA program (V5.2) [43] and were bootstrapped 10,000 times.

Table 1
Primers used for cloning and expression analysis.

Gene	Primer	Sequences (5′- to 3′)	Application
Atlantic salmon p28a	F1	GAGCAGTTGGATAACAAGCAGTTTG	3′-RACE
	F2	TCCCTCTCCACAGACTTCGACC	3′-RACE
	R1	GTGAGGTAGCGCTCCAGGTC	5′-RACE
	R2	CTCACATTGGAGCTCTTCACACA	5′-RACE
	F	GGTGGCAGGTTCTGTTTCTCCA	Real-time PCR
	R	TCAGACGCTCCCAGTCCTTCAT	Real-time PCR
Atlantic salmon p28b	F1	GAGCCATTACAGAGTTCCACAAGTAGC	PCR cloning
	R1	AGGCAAATAGGCATTGAAGTCAGT	PCR cloning
	F	CCAGATGGCTGTTTGGTGGTG	Real-time PCR
	R	TCAGACGCTCCCAGTCCTTCAT	Real-time PCR
Zebrafish p28	F	CTTACATTTTCTCTCTCTATCCTCCAG	PCR cloning
	R	CGCTCTGATCTCTGAGTTGCTG	PCR cloning
Atlantic salmon Ebi3	F	ACATCGCCACCTACAGTATGAAAGG	Real-time PCR
	R	GGGTCCGGCTTCACAATGT	Real-time PCR
Atlantic salmon EF-1α	F	CAAGGATATCCGTCGTGGCA	Real-time PCR
	R	ACAGCGAAACGACCAAGAGG	Real-time PCR

2.2. Comparative gene expression analysis by real-time RT-PCR

The primers (Table 1) for real-time-PCR were designed so that at least one primer crossed an intron, to ensure that genomic DNA could not be amplified under the PCR conditions used. The salmon Ebi3 primers was designed against a WGS contig (Acc. no. AGKD01090807). To directly compare the expression level of the two salmon p28 paralogues and Ebi3, a reference was constructed using equal mole amounts of PCR product from each gene, including the house keeping gene elongation factor-α (EF-1α). The relative expression level of each sample was normalised against the expression level of EF-1α.

2.3. Transcript expression of the two p28 paralogues and Ebi3 in vivo

Six healthy Atlantic salmon (average weight, 34 g) reared in a fresh water aquarium at 13 °C and fed with commercial pellets (EWOS) twice daily. Fish were killed by anaesthetic overdose followed by destruction of brain (schedule 1 method) and 16 tissues (tail fin, gills, thymus, brain, scales, skin, muscle, adipose tissue, liver, spleen, gonad, HK, caudal kidney, intestine, heart and blood) were collected. The blood cells were washed once with PBS and dissolved in TRI reagent (Sigma). 300 mg of adipose tissue, or 50 mg of other fresh tissues were directly homogenised in 1 ml of TRI reagent using a TissueLyzer II (Qiagen). The oily phase of the adipose tissue homogenate was discarded before