This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier’s archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright
Short communication

Teleostean IL11b exhibits complementing function to IL11a and expansive involvement in antibacterial and antiviral responses

Mudjekeewis D. Santos, Motoshige Yasuike, Hidehiro Kondo, Ikuo Hirono, Takashi Aoki *

Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Konan 4-5-7 Minato-ku, Tokyo 108-8477, Japan

Received 26 December 2007; received in revised form 4 February 2008; accepted 6 February 2008

Abstract

Interleukin 11 is a class-1 helical cytokine, having the four-helix bundle structure, possessing pleiotropic characteristics involved in physiological processes including blood production, bone formation and placentation. The interleukin 11 paralogues (IL11a and IL11b) have been identified in fish with only IL11a from carp and trout have been characterized and analyzed for its expression thus far. Here, we cloned and studied the structure and expression of IL11b in Japanese flounder (Paralichthys olivaceus), and compared this with the existing information on fish IL11 paralogues. Japanese flounder IL11b (poIL11b) cDNA is composed of 1536 bp encoding for 201 aa residues with a 23 aa leader peptide, three cysteine residues (C12, C183 and C198) and four potential N-linked glycosylation sites. poIL11b does not show constitutive expression in tissues of adult fish except for the very slight expression in kidney and spleen, and the very high expression in peripheral blood leukocytes (PBLs). poIL11b is transiently up-regulated by bacterial lipopolysaccharide (LPS) and increasingly stimulated by the IFN inducer poly I:C in kidney, spleen and peripheral blood leukocytes of adult fish in vitro. It is likewise slightly stimulated by Edwardsiella tarda infection but is highly expressed after hirame rhabdovirus (HIRRV) infection in kidney of juvenile fish. The stimulation studies suggest that poIL11b, aside from its role in bacterial infection, is well involved in antiviral responses. Moreover, poIL11b structure and expression pattern appears to be slightly distinct and opposite to IL11a, respectively, suggesting a complementation of function of the duplicate fish IL11 genes.

Keywords: Interleukin 11b; Gene duplication; Subfunction; Teleost; Immunity

1. Introduction

Interleukin 11 (IL11) is a pleiotropic cytokine that possesses many functions such as production of thrombocytes (thrombocytopoiesis), megakaryocytes (megakaryo-cytopoiesis) and other blood cells, bone formation and osteoblastosis, and in placental development (for reviews see Huising et al., 2006; Du and Williams, 1997). Because of its influence in hematopoiesis, particularly in megakaryocyte and thrombocyte development, it has been used to prevent thrombocyte/platelet loss following cancer therapy (Kurzrock, 2005) and is also being explored as a cure for neonatal thrombocytopenia (Ramasethu, 2004).

Mammalian IL11 is a single copy signal molecule that is expressed by and acts on various types of cells. It is composed of a high number of proline (P), leucine (L) and positively charged amino acids making it a basic molecule. It possesses a 4 α-helix bundle structure even in the absence of disulphide binding cysteine (C) residues, made possible by hydrophobic interactions (Czupryn et al., 1995). Because of the 4 α-helix configuration and the formation of a hexameric complex by IL11, IL11 receptor and gp130 during signal transduction (Heinrich et al., 2003), IL11 has been classified as a member of the class-1 helical cytokines that include interleukin 6 (IL6), ciliary neurotrophic factor (CNTF), leukemia inhibiting factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-2 (CT-2) and cardiotrophin-like cytokine (CLC) (Huising et al., 2006).

Teleost fish IL11 orthologue was first reported in trout (Wang et al., 2005). Subsequent cloning studies on carp coupled with in silico analysis of zebrafish (Danio rerio), tiger pufferfish (Takifugu rubripes) and green spotted pufferfish (Tetraodon nigroviridis) genomes allowed for the identification...
of a duplicated fish IL11 gene (IL11a and IL11b) (Husing et al., 2005). In both these reports, a partial expressed sequence tag (EST) fragment (AU090873) from Japanese flounder has been included in the phylogenetic analysis and was shown to cluster with fish IL11b. The IL11a and IL11b genes from carp, trout, zebrafish, tiger pufferfish and green-spotted pufferfish have been thoroughly characterized for its genomic and secondary protein structures. However, only IL11a from carp and trout has been so far investigated for its expression where it was found to be ubiquitously expressed in all tissues including brain, intestine, skin, muscle, liver, spleen, head kidney, kidney, thymus and gills except peripheral blood leukocytes (PBLs). IL11a expression has likewise been observed to be enhanced by lipopolysaccharide (LPS), bacteria (Aeromonas salmonicida MT423), concanavalin A, poly I:C and recombinant IL-1β, albeit at different levels, but significantly inhibited by cortisol (Wang et al., 2005; Husing et al., 2005). It is not known whether IL11b is functional and to what extent is its role in fish immunity in relation to a functional duplicate IL11a given that there is only a single IL11 protein in higher vertebrates.

Japanese flounder is one of the economically important culture species that is being impacted by diseases such as Edwardsiella tarda and hirame rhabdovirus (HIRRV) (Oh and Choi, 1998; Pluma, 1999). As such, it has been the subject of extensive studies on host–pathogen interactions and immunology at the molecular level.

Here, we report the cloning of the full Japanese flounder IL11b cDNA (poll11b) and the analysis of its expression constitutively and in response to LPS and poly I:C stimulation in vitro, and to E. tarda and HIRRV infection in vivo. poll11b amino acid structure is generally conserved compared to other vertebrate IL11 orthologues. However, IL11bs has two extra C residues compared with fish IL11as. Further, comparative analysis between the IL11 duplicates showed different and opposite expression patterns, constitutively and in response to immunostimulation. These suggest that there is complementation of function of the duplicate genes. Moreover, poll11b is significantly up-regulated by both bacterial and viral agents in vitro and in vivo exhibiting its involvement in a wide range of immunological responses.

2. Materials and methods

2.1. Molecular cloning

The full-length Japanese flounder IL11 type b cDNA was cloned by first selecting an EST clone (Accession no: AU090873) showing putative homology to published fish IL11. An anti-sense primer (5′-GTCCACCTGATGGATCCTCG-3′) was then designed from the upstream portion of this clone and, in partner with M13 reverse primer (5′-AGCGGA-TAACAAITTCACACAGG-3′) amplified a resulting ~200 bp fragment using a previously constructed AZipLox vector-based Japanese flounder cDNA library (GIBCO BRL/Life technologies) as template.

2.2. In silico analysis

The nucleotide sequence, translated amino acids, isoelectric points (pI) and average molecular weight were analyzed and determined using GENETYX 7.0.3 (GENETYX Corporation). SignalP (http://www.cbs.dtu.dk/services/SignalP/) and Net-NGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) servers were used to predict signal peptide cleavage and N-glycosylation sites, respectively. Identities were calculated using BLASTp (BLOSUM 62) implemented in BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and the complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters. Protein domain prediction was carried out using ProDom (http://prodom.prabi.fr/prodom/current/html/home.php) and 123D+ (http://123d.ncifcrf.gov/123D+/html) servers. For phylogenetic analysis, we used the neighbor joining (NJ) algorithm implemented in the MEGA3 (http://www.megasoftware.net/index.html/) employing the Poisson correction method with 1000 bootstrap re-sampling and with complete deletion of gap sites. The bootstrap consensus tree was shown.

2.3. Constitutive expression in tissues

For RT-PCR analysis of constitutive expression, total RNA was extracted from brain, eyes, gills, kidney, heart, intestine, peripheral blood leukocytes, liver, muscle, skin, spleen, stomach from three apparently healthy Japanese flounder. cDNA synthesis was done in each of the tissue samples. Resulting cDNA were amplified using the following poll11b primers (IL11F, 5′-CAGCCTTGCAGAGAGTGCT-3′ and IL11R, 5′-TGAACCTCCTGCGCCAGAG-3′). PCR conditions were: initial denaturation at 95 °C for 5 min, 30 cycles (95 °C: 30 s; 55 °C: 30 s; 72 °C: 1 min), and final elongation at 72 °C for 5 min. The PCR products were electroforeosed on a 1.0% agarose gel containing ethidium bromide. Resulting bands were photographed with a densitometer and were semi-quantitatively assessed for their relative expression following Santos et al. (2006) using the ImageJ software (Abramoff et al., 2004).

2.4. Expression in tissues after in vitro immunostimulation

Primary cultures of kidney and spleen were prepared by first dissecting out the tissues from about 3 kg fish and placed in a solution containing heparin, FBS, RPMI and penicillin/streptomycin. These were then mash-filtered using a sterile mesh net, suspended in 1 × phosphate buffer saline (PBS), centrifuged then re-suspended in a medium containing FBS, RPMI and penicillin/streptomycin. On the other hand, PBLs were isolated using Percoll solution, centrifuged then re-suspended in a medium similar to the kidney and primary cultures. 1 ml of each primary culture suspensions was equally placed in cell culture plates and then treated with immunostimulants accordingly. The following final concentration of 0.5 mg/ml LPS and poly I:C and with PBS were used and sampling was done at 1, 3 and 6 h post-induction. Total RNA was extracted from the treated and control cells for cDNA synthesis and subsequent RT-PCR anal-
2.5. Expression in tissues after infection in vivo

Apparently healthy juvenile fish samples weighing about 2 g each were used in the in vivo studies. Five fish individuals were placed in re-circulating aquarium tanks. For the E. tarda experiment, one tank containing 15 fish samples were infected with the bacteria (2.5 × 10^7 CFU/ml) by immersion while one untreated tank was used as a control. Kidney from five fish samples was sampled at 1 day, 3 days and 7 days post-infection and also from the control. For the HIRRV experiment, one tank containing 15 fish samples were infected with the bacteria (3.2 × 10^3 TCID50) and another 15 with PBS for the control. Kidney from five samples was taken at 1 day, 3 days and 7 days post-injection. For each of the sampling time points, kidney was pooled and then total RNA was extracted for cDNA synthesis and subsequent RT-PCR analysis. poIL11b, CSF3, Mx and β-actin gene primers (same as above) were used for the analysis. PCR conditions were: initial denaturation at 95 °C for 5 min, 26 cycles (95 °C: 30 s; 55 °C: 30 s; 72 °C: 1 min), and final elongation at 72 °C for 5 min.

3. Results

3.1. Japanese flounder IL11b

The complete Japanese flounder interleukin 11 type b (poIL11b) cDNA was composed of 1536 bp encoding for a putative protein of 201 aa residues (Fig. 1). The nucleotide sequence showed four mRNA destabilizing AUUUA motifs and a typical poly-adenylation signal "AATAAA". The polypeptide on the other hand had a predicted signal sequence of 23 aa cleavage of this peptide results to a mature poIL11b protein containing 178 aa with a predicted molecular mass of about 20kDa and predicted isoelectric point (pI) of 6.94. It was leucine (L) and serine (S) rich at 16% and 15%, respectively, had four C residues and four predicted N-linked glycosylation sites. Using the ProDom and 123D+ servers, poIL11b protein has been identified as homologous to the mammalian IL11 motif and belongs to the structural classification of proteins family of long-chain cytokines (A.26.1.1), respectively.

Alignment of fish IL11 orthologues and paralogues revealed some interesting results (Fig. 2). The poIL11b leader peptide of 23 aa was 3-less than the other fish IL11a and 1-less than mammalian IL11a. The other fish IL11a and 1-less than mammalian IL11a.
Fig. 2. Alignment of interleukin 11 orthologues and paralogues. Signal peptides and charged amino acids (aspartic acid, D; glutamic acid, E; lysine, K; arginine, R) and the polar histidine (H) are shaded in black while important functional residues are denoted by asterisks (*). /H9251-H9252 Helices A–D are shaded gray. Conserved cysteines are boxed while other conserved residues are represented by dots (...). Gaps (−) were introduced to maximize alignment. NI means no significant identity.

malian IL11s. Three C residues of poIL11b were shown to be part of the mature peptide. One C residue (C183) was well conserved in fish IL11s but the other two C residues (C12 and C198) were only conserved among IL11bs [poIL11b, tiger pufferfish (trIL11b) and green-spotted pufferfish (tnIL11b)] and not with IL11as (trIL11a, tnIL11a, ccIL11a, omIL11a, drIL11a.1 and drIL11a.2). It is important to note that there are two zebrafish drIL11s identified so far that are homologous to type a. The putative poIL11b helices (A–D) were mainly composed of charged residues aspartic acid (D), glutamic acid (E), lysine (K) and the polar histidine (H). Some residues that have been reported in mammals to be important for receptor binding were conserved in poIL11b including L28 and L34 in Helix A, L173 at the start of Helix B, L115 in Helix C, and tryptophan (W165), arginine (R168), L171 and L173 in Helix D. Another important residue which was conservatively replaced to phenylalanine (F) is the hydrophobic L170. The comparative identities among fish and mammalian IL11 orthologues as well as isoelectric points were also interesting (Fig. 2). Between poIL11b and the other fish IL11b, the identities were significantly conserved at more than 50%. poIL11b has limited identity compared to IL11a orthologues (24–29%), and no significant identity with mammalian IL11s. Teleost fish IL11bs were also neutral compared to the IL11as, which are basic including the mammalian IL11s.

With the complete poIL11b amino acid sequence and the limited identities especially between Japanese flounder poIL11b and two zebrafish IL11as, we ran a phylogenetic analysis with fugu IL6 as an outgroup. Our results confirmed the clustering between IL11as and IL11bs and hence the orthology of poIL11b. Moreover, we also show a, lineage-specific, secondary duplication for zebrafish IL11a (Fig. 3).

3.2. poIL11b tissue distribution

At 30 PCR cycles, Japanese flounder poIL11b was remarkably expressed in PBLs and slightly in kidney and spleen but not in brain, eyes, gills, heart, intestine, liver, muscle, skin and stomach (Fig. 4).

3.3. poIL11b expression is regulated by LPS and poly I:C treatment

We checked the expression of poIL11b vis-a-vis with the Japanese flounder granulocyte colony stimulating factor (poCSF3), Mx (poMx) and β-actin genes in primary cultures of kidney, PBLs and spleen of adult fish following LPS and poly I:C treatment (Fig. 5). Expectedly poIL11b, identified as a pro-inflammatory cytokine, was induced by LPS although not
comparable to poCSF3 in the three tissues studied, where the latter was highly induced. Surprisingly, however, it was highly stimulated by poly I:C in a time-series manner similar to poMx expression pattern, a gene that is well known to be highly induced by poly I:C and virus. Interestingly, poCSF3, was highly induced at 6 h post-stimulation by poly I:C in PBLs but not in kidney or spleen.

3.4. poIL11b involvement in E. tarda and HIRRV infection

At 26 cycles, Japanese flounder poIL11b was expressed, albeit faintly, in the kidney of juvenile Japanese flounder infected with E. tarda starting at day 3, in contrast to poCSF3 where there was visible expression starting at day 1 and moderate expression at day 3 and day 7. On the other hand, kidney of juvenile fish infected with HIRRV showed increasingly high amounts of poIL11b transcripts as compared to PBS-injected controls starting from day 1 until day 7 (Fig. 6). This result was confirmed by controls through poMx expression, where poly I:C is known to stimulate it, and by the constant expression of β-actin. Consequently, from this in vivo experiment, we noticed that poIL11b is not constitutively expressed in the kidney of juvenile Japanese flounder.
also act as a receptor for IL11s (Santos et al., 2007). Earlier studies reported that the helix A of mammalian IL11 particularly the P13, E16, L17, R25, L28, T31, R32, L34 and R39 residues has been implicated in gp130 binding site (Site II) critical for bioactivity while helix D particularly its R130, H153, D164, W165 and R168 residues has been reported as a primary receptor-binding site (Site I) (Du and Williams, 1997). In a more recent study, the R168, L171 and L173 residues in the helix D at the COOH-terminal have been found to be very important for binding of mouse IL11 to IL11 receptor, while the R111 and L115 residues were extremely crucial for binding to gp130. Furthermore, the W146 within the CD loop was determined to be critical for biological activity but not necessary for binding using A residue replacement experiment (Barton et al., 1999).

Despite the conserved IL11 structures, we were, however, able to spot some structural differences between fish IL11b and IL11a, which we think would have significant implication in their individual function. Firstly, there are two extra C residues conserved only among fish IL11b. These residues, particularly C183 and C198, could potentially form a di-sulphide bond in this area and influence the stability of the peptide particularly the COOH-terminal of the protein. This could consequently affect the bioactivity of the molecule as this region is the primary receptor-binding site of the protein. This could consequently affect the bioactivity of the molecule as this region is the primary receptor-binding site of the protein. IL11a, which we think would have significant implication in their individual function. Secondly, IL11b orthologues are neutral compared to the basic nature of IL11a and even mammalian IL11s, which is a result of the presence of lesser-charged amino acid residues in the helices and thus more neutral pH. IL11bs are therefore more cytokine-like in terms of pH and the fish IL11as including mammalian IL11s are unusually basic. Thirdly, the poIL11b mRNA is composed of four destabilizing AUUUA motifs (Akashi et al., 1994) in its 3' UTR region and based on the presence of one poly (A) signal could only produce a single transcript. The DNA sequence of the trout IL11a on the other hand, has several AUUUA motifs and four poly (A) signals that potentially could produce different sized transcripts with varying stability depending on which transcript is produced (Wang et al., 2005). Assuming that the largest of the potential trout IL11a transcripts is transcribed (since this is the one cloned in the study), such IL11a is far more unstable than the poIL11b, which has only four ATTTA motifs. Taken together, these structural data suggests that fish IL11a and IL11b may very well exhibit different functional attributes.

The difference between IL11a and IL11b becomes clearer when expression data from both published reports and this study is compared, and that the difference appears to be complementary. First, studies show that carp cIL11a and trout omIL11a is expressed significantly high in tissues except in PBLs (Wang et al., 2005; Huising et al., 2005). In contrast, we found that IL11b is highly expressed only in PBLs and very slightly in spleen and kidney. Second, in vitro studies showed that for LPS treatment, there is significant immediate expression of IL11b and is then dissipated afterwards while trout IL11a is persistently expressed at the duration of sampling. For poly I:C, a viral mimic (Fortier et al., 2004), the effect is more dramatic and increasing from 0 h to 6 h post-treatment, and approximates that of poMx expression. This is in contrast to the expression of rainbow trout IL11a where it was observed to be slightly up-regulated by poly I:C
at 3 h and 7 h sampling but then disappears at 24 h. Third, the results showed that E. tarda infection up-regulates poIL11b but not significantly, at least in the kidney of juvenile fish, unlike trout IL11a, which is up-regulated in the liver, head, kidney and spleen of trout, 1 day after challenged with bacterial Aeromonas salmonicida MT423. HIRRV infection, on the other hand, stimulated high expression of poIL11b at 1–7 days sampling, again mirroring the expression of the antiviral Mx.

The fish IL11 paralogues appears to have complementing function, a characteristic that is very similar to fish M17s and MSHs, also members of the IL6-cytokine subfamily (Hwang et al., 2007), that follows the Duplication–Degeneration–Complementation (DDC) model (Force et al., 1999). There is evidence to support, at least at the transcriptional level, of the partitioning of ancestral functions of these molecules. This model was initially supported by the analysis of the engrailed gene in zebrafish. Since then, a number of duplicate genes have been reported to follow such model including among others the protocadherin 15 and ß-crystallin genes in zebrafish (Seiler et al., 2004; Smith et al., 2006) and the pro-opiomelanocortin genes in green-spotted pufferfish (Souza et al., 2005).

Focusing on its immune function, poIL11b was indeed shown to be involved in both bacterial and antiviral responses based on its in vitro and in vivo expression in immune-related tissues that include the kidney, spleen and PBLs. Bacterial (LPS and E. tarda) and viral (poly I:C and HIRRV) mimics/agents have been shown to regulate numerous immune-related genes, including cytokines, during infection (Matsuyama et al., 2007; Yasuike et al., 2007). poIL11b is yet another cytokine molecule that responds to these agents and as such underscores its important role in host–pathogen interactions. This result adds to the growing information about fish IL11s. For example, trout omIL1a has been reported to be induced by a plasmid construct containing the glycoprotein gene of VHSV and a CMV promoter although the induction is significant only at day 3 in spleen and curiously only at day 10 in kidney (Jiménez et al., 2006). The non-expression of poIL11b, in juvenile Japanese flounder indicates that during development, it is functional only at the adult stage. A similar pattern has been observed with other genes related to hematopoiesis where they are expressed at specific stages in zebrafish development (Davison and Zon, 2004). Furthermore, the viral challenge experiment and poly I:C treatment in trout, suggests that IL11a is not very much involved in antiviral responses unlike the poIL11b. It is therefore likely that IL11b has more antiviral function as compared to antibacterial responses and such broad involvement in pathogen responses makes it a good vaccine adjuvant candidate as compared to its counterpart IL11as in fish.

It is interesting to note that the Japanese flounder IL6-cytokine poCSF3, which was immediately up-regulated by LPS in kidney, PBLs and spleen similar to what was reported previously (Santos et al., 2006), was strongly expressed only in PBLs and only at 6 h following stimulation by poly I:C. This suggests that poCSF3 is differentially regulated by bacterial and viral infection, similar to poIL11b, and that its function is tissue-specific. The reason for such kind of expression pattern is not yet clear and would have to be studied further.

In conclusion, whether the observed difference in structure and expression between IL11a and IL11b are linked remains to be confirmed. Also, complementation of function of both duplicates at the protein level within a species should be investigated further. Nevertheless, we can conclude that both IL11 paralogues in fish are ‘functional’ at the expression level and in a manner that is complementary to each other. This has implication to for example strategies on the development of vaccines against fish pathogens. Combinatorial use of these genes for therapy may prove to be more effective than used separately.

Acknowledgements

This study was supported in part by the Grants-in-Aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. Nature 431, 946–957.


