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A novel type-1 cytokine receptor from fish involved in the Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal pathway

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Abstract

Type I cytokine receptors mediate the action of the members of the long chain cytokines canonically involved in numerous physiological function. Here we report a novel cytokine receptor termed Japanese flounder glycoprotein 130 homologue or JfGPH, exhibiting the unique type I cytokine receptor motifs i.e. having a cytokine binding domain (CBD) containing two pairs of conserved cysteine (C) residues, a WSXWS motif, three fibronectin domains all in the extracellular region. It is also composed of the Jak binding domains Box 1 and Box 2, and a STAT 3 binding motif (Box 3) in the cytoplasmic region suggesting its mediatory role for Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal pathway. The JfGPH cDNA is about 3 kb encoding 801 amino acid residues with a predicted molecular weight of 90 kDa and its gene has an 11-exon/10-intron architecture. While JfGPH shows significant homology with the members of type-1 cytokine receptor family including IL6ST (or gp130), IL31α (or GLMR), CSF3R (or GCSFR), LIFR, OSMR, IL12Rβ1 and LEPR, structural and phylogenetic analysis of its protein revealed that it is a novel and an ancestral cytokine receptor found in teleost. JfGPH gene is ubiquitously expressed in Japanese flounder tissues and in a natural embryo (HINAE) cell line showing its critical role in teleost physiological functions similar to gp130 in higher vertebrates. High expression of JfGPH transcripts in immune-related tissues and, in ovary and embryo-derived cell line suggest its role in immune responses, and reproduction/development, respectively. *In vitro* stimulation of spleen, kidney, peripheral blood leukocytes (PBLs) and HINAE revealed that JfGPH is down-regulated by polyinosinic:polycytidylic acid (poly I:C), an interferon (IFN) inducer, suggesting an apparent control of the JfGPH's expression during IFN-induced Jak/STAT signaling.

Keywords: Japanese flounder (*Paralichthys olivaceus*); Type-1 cytokine receptor; Novel; Poly I:C; Jak/STAT signal pathway; Immunity; Development

1. Introduction

Type-1 cytokine receptors are a group of related molecules that mediate the signaling action of class-1 helical cytokines, classified as such based on a shared modular architecture i.e. having a cytokine-binding domain (CBD), fibronectin type-III (Fn3) domains, and a signature WSXWS motif (Bazan, 1990; Taga and Kishimoto, 1997). The cytokines these receptors mediate, which include interleukin 6 signal transducer (IL6ST) or glycoprotein 130 (gp130), granulocyte colony-stimulating factor (CSF3), interleukin 6 (IL-6), interelukin 11 (IL-11), ciliary neurotrophic factor (CNTF), leukemia inhibiting factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC) likewise share a common tertiary structure i.e. composed of four bundles of α-helices and are involved in numerous physiological processes including immune regulation, host defense, reproduction, development, blood formation and energy metabolism (for review see Huising et al., 2006).

Type-1 cytokines and their cognate receptors form complexes via three interaction epitopes; a site I, located at the distal portion of the D helix (CBD), a site II, composed of residues in the A and C helices that allows for heterodimerization with other receptors and, a site III, a feature only shown in gp130 receptor family located at the N-terminal tip of the D helix- and Ig-like domain (as reviewed by Bravo and Heath, 2000). Upon binding of the cytokine ligand to its receptor, the Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal pathway is activated. Janus kinases or Jaks are brought to the

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Box 1 or Jak binding site, a proline rich motif, and in some receptors, to Box 2, a cluster of hydrophobic amino acids followed by positively charged amino acids. Jaks are then subsequently trans-phosphorylated and activated following binding. The activated Jaks then phosphorylate the receptor chain and STATs are recruited through the interaction of the Src homology 2 domain (SH2) with sites of receptor tyrosine phosphorylation such as STAT3 binding motif (Box 3). STATs then form dimers through the intermolecular association of the SH2 domains with the carboxyl sites of tyrosine phosphorylation (Ihle, 1996; Heinrich et al., 1998).

Fish class-1 helical cytokine orthologues have been reported including IL6, IL11, carp cytokine-like (M17), interleukin 12 (IL12), leptin, erythropoietin (EPO), prolactin (PRL) and growth hormone (GH) (Huising et al., 2006), and recently, the granulocyte colony-stimulating factors (CSF3s) from Japanese flounder, fugu and pufferfish (Santos et al., 2006). Some cognate receptors of these group of signal molecules have likewise been reported in fish particularly from *Tetraodon nigroviridis* genome although their orthology is not yet clear since assignment of names was done only up to the *in silico*-prediction level and the genes were compared only to human (Jaillon et al., 2004). These include growth hormone receptor (GHR), prolactin receptor (PRLR), erythropoietin receptor (EPOR), interleukin 12 receptor γ (IL2Rγ), interleukin 7 receptor A (IL7Rα), interleukin 12 receptor β/interleukin 4 receptor A (IL2Rβ/IL4RA), interleukin 21 receptor (IL21R), interleukin 12 p40 (IL12p40), ciliary neurotrophic factor (CNTFR), interleukin 11 receptor A (IL11Rα), interleukin 13 receptor A (IL13Rα), interleukin 6 receptor A (IL6Rα), thrombopoietin receptor, interleukin 12 receptor β2 (IL12Rβ2), glycoprotein 130 (gp130), leukemia inhibiting factor receptor (LIFR) and obese protein receptor (OBR) or leptin receptor (LEPR). Fish cytokine receptors that have been cloned and characterized so far include LIFR and PRLR of gold fish (Hanington and Belosevic, 2005; Tse et al., 2000) and the growth hormones of fugu, zebrafish, Southern catfish and Nile tilapia (Jiao et al., 2006). Information about the kinds of type-1 cytokine receptor molecules in fish and their function in cell signalling, e.g. Jak/STAT pathway, which is important in understanding fish physiological processes such as immunity, is very much lacking.

Here, we report a novel cytokine receptor gene that is structurally and phylogenetically related to the class-1 helical cytokine receptors. Because of its close similarity with gp130 compared with other cytokine receptors, we termed it as JfGPH, short for Japanese flounder gp130 homologue. JfGPH appears to be a critical fish receptor because it is ubiquitously expressed in tissues and in an embryo-derived cell line, and is apparently regulated during IFN-induced Jak/STAT signalling.

2. Materials and methods

2.1. Cell culture

Primary cultures of Japanese flounder peripheral blood leukocytes were prepared using Percoll gradient isolation and placed in Medium B containing RPMI, 5% fetal bovine serum (FBS), and 100 IU ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin. Kidney and spleen cells were prepared by slowly mash-filtering tissues using a sterile mesh net and then placed in Medium B. Japanese flounder-derived cell line Hirame Natural Embryo (HINAE) were grown in Leibovitz's L-15 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS) and 100 IU ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin (Gibco-BRL, Grand Island, NY).

2.2. Molecular cloning

The full-length JfGPH cDNA was determined following Santos et al. (2006). An expressed sequence tag (EST) clone (Accession no: AU050570) showing putative homology to mouse CSF3 receptor (CSF3R) was used as a probe to screen a Japanese flounder kidney cDNA library. 5′ SMART Random Amplification of cDNA Ends (RACE) PCR and PCR cloning was used to complete the cDNA fragment generated from the Japanese flounder cDNA library. On the other hand, the JfGPH gene was completed by "primer walking" using overlapping primers designed from the JfGPH cDNA (Table S1).

2.3. In silico analysis

The nucleotide sequence, translated amino acids, and average molecular weight were analyzed and determined using GENETYX 7.0.3 (GENETYX Corporation). SignalP (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 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. The ProDom Server, release 2005.1 (http://protein.toulouse.inra.fr/prodom/current/html/home.php/) was used to predict protein domains while Phobius (http://phobius.cgb.ki.se/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) servers were used to identify the transmembrane region. For phylogenetic analysis, we used the unweighted pair group method with arithmetic mean (UPGMA) algorithm implemented in the MEGA3 (http://www.megasoftware.net/index.html/) employing the Poisson method with 1000 bootstrap tests and with complete deletion of gap sites. The bootstrap consensus tree was shown.

2.4. Constitutive expression in tissues and cell lines

Regular RT- and semi-quantitative RT-PCR analysis was carried out following Santos et al. (2006). Primers used for amplification—JfGPH: Forward 5′-GTGCCACTGTGAGCTGGATCA-3′ ĭand Reverse 3′-CTCTGACTCCGATAGGGGCT-5′. For constitutive expression, total RNA was extracted from brain, eyes, gills, kidney, heart, intestine, peripheral blood leukocytes (PBLs), liver, muscle, ovary, skin, spleen, stomach from three (3) apparently healthy Japanese flounder. β-actin was used as a positive control. 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.

2.5. In vitro stimulation of tissues

Immunostimulation studies was carried out by first extracting total RNA from primary cultures of kidney, spleen and peripheral blood leukocytes (PBLs) from Japanese flounder treated with final concentration of 0.5 mg/ml LPS and poly I:C and sampled at 1, 3 and 6 h post-induction. Primers used for amplification—JfGPH: Forward 5′-GTGCCACTGTGAGCTGGATCA-3′ and Reverse 5′-CTCTGACTCCGATAGGGGCT-3′; Mx: Forward 5′-AACAGCCAAGGCAAAGATTG-3′ and Reverse 5′-AATGTCCAGCTCCTCCTTCA-3′ (Caipang et al., 2003). β-Actin was used as above. PCR conditions were: initial denaturation at 95 °C for 5 min, 27 cycles (95 °C: 30 s, 55 °C: 30 s, 72 °C: 1 min), and final elongation at 72 °C for 5 min.

2.6. In vitro stimulation of HINAE cell line

Total RNA was extracted from HINAE cell lines treated with final concentration of 0.5 mg/ml: poly I:C sampled at 1, 3 and

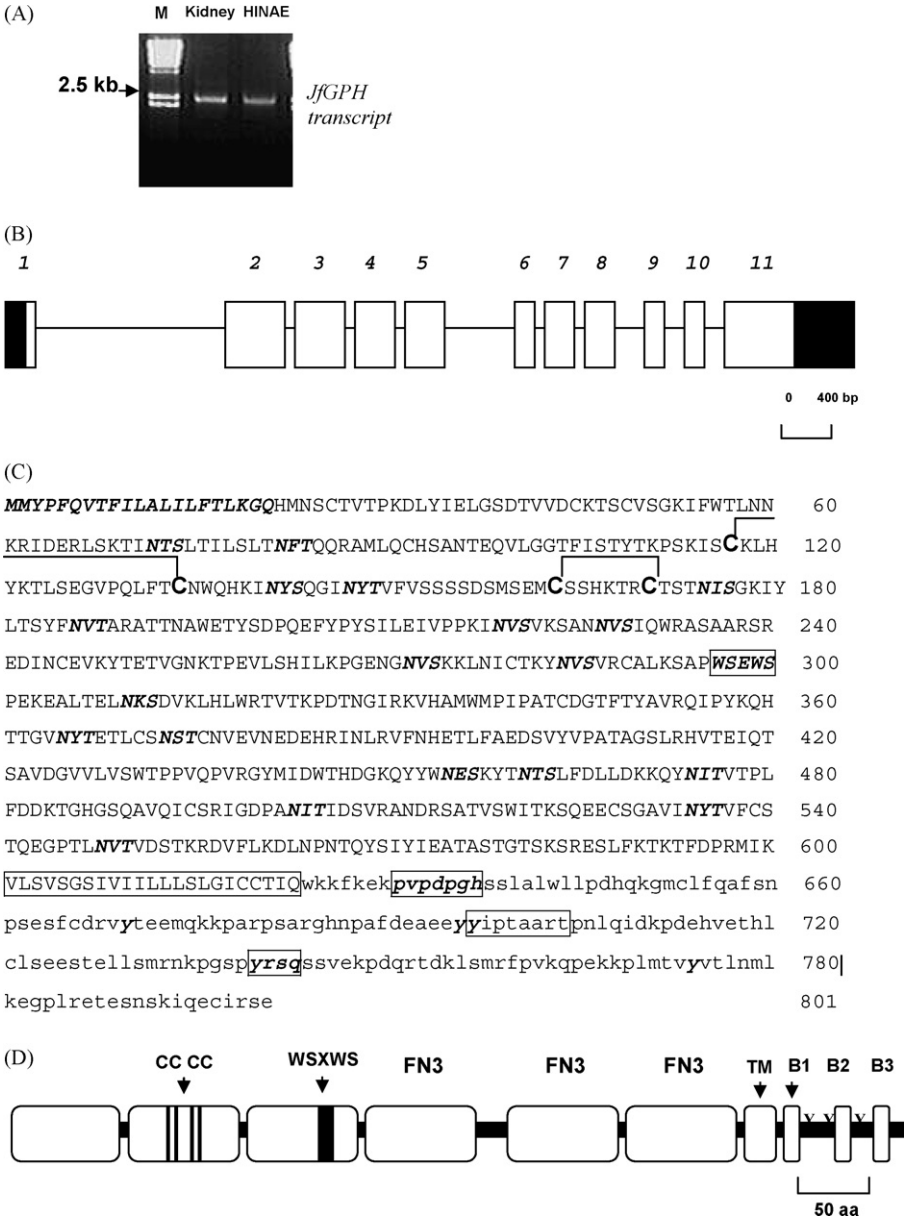


Fig. 1. Japanese flounder gp130 homologue (JfGPH) structure and transcript (Accession no. AB281273). (A) Gene organization showing 11 exons (boxes), 10 introns (line) and untranslated regions (black shade). (B) Amino acid sequence exhibiting the extracellular region (uppercase) signal peptide (uppercase bold), glycosylation sites (uppercase italics), the cytokine binding domain with the four conserved cysteine (C) residues (arial font bold with connecting line), WSXWS motif (uppercase bold boxed), transmembrane region (uppercase boxed), intracellular region (lowercase), Box 1 (lowercase bold boxed), Box 3 (lowercase italics boxed) and tyrosine (Y) residues (lowercase bold italics). (C) Schematic drawing of the JfGPH protein structure including position of regions in item B plus the 3 fibronectin III (FnIII) domains. (D) Partial JfGPH open reading frame (ORF) transcript. Primers F65 and R1295 (Table 1), which starts from bp 148 and bp 2401, respectively were used to produce the ∼2.2 kb amplicon that spans 93% of the ORF.

6 h post-induction. Primers for JfGPH, Mx and β-actin were the same as above. PCR conditions were: initial denaturation at 95 °C for 5 min, 27 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 gp130 homologue (JfGPH)

Screening of a Japanese flounder cDNA library using an EST fragment corresponding to a CSF3R as a probe yielded a partial cDNA fragment of about 2 kb that includes the polyA tail. Subsequent RACE PCR of the 5′ region (confirmed with RT-PCR using specific primers) completed the 3 kb cDNA fragment. RT-PCR likewise verified the existence of the full JfGPH transcript (Fig. 1A) BLAST analysis showed that it was most similar to gp130, thus it was named as Japanese flounder gp130 homologue or JfGPH (Table 1).

JfGPH cDNA is about 3 kb long and has an open reading frame (ORF) of 2406 bp (Fig. 1B, Fig. S1). It encodes for a protein with 801 amino acid residues having a predicted molecular weight of 90 kDa. *In silico* analysis revealed that the JfGPH protein has an extracellular region of 600 amino acids that includes a potential signal peptide of 21 amino acids, followed by a cytokine binding domain (CBD) consisting of two (2) pairs of conserved cysteine residues (aa 117 and 134; 163 and 170), a WSEWS motif, and a sequence with three (3) fibronectin III (FnIII) domains. This region also consists of 19 potential *N*-glycosylation sites. A transmembrane domain consisting of 23 amino acids was predicted at aa 601 to aa 623. The intracellular tail is composed of 178 amino acids with two (2) Jak binding domains, Box 1 (aa 631–636) and Box 2 (aa 696–703), and a Box 3 containing the canonical YXXQ motif (aa 40–439) for STAT 3 binding. It also contains 5 tyrosine (Y) residues (aa 670, 696, 697, 740 and 774), amino acids which are involved in the binding of signaling molecules with SH2 domains (Fig. 1C and D). These results indicate that JfGPH is a receptor molecule belonging to the type-1 cytokine receptor family and mediates a Jak/STAT signal cascade.

The JfGPH gene, on the other hand, is about 6 kb consisting of 11 exons and 10 introns (Fig. 1B and Fig. S1). Exons 1 to 8 encode for the extracellular region, Exon 9 for the transmembrane domain, and Exons 10 and 11 for the cytoplasmic domain that includes the Box 1, 2 and 3 motifs.

Since we supposed that JfGPH is a type-1 cytokine receptor, we next compared its identity with other members of the family (Table 1). As expected with these closely related receptors, JfGPH has significant and about equal identity with known orthologues of IL31Rα, IL6ST, CSF3R and OSMR, the nearest being is the chicken IL31Rα at 26%. Interestingly, however, JfGPH has significant identity with human and mouse LIFR but not with goldfish LIFR, with human but not mouse IL12Rβ1, and with chicken but not with human and mouse LEPR. It has no identity with human and mouse IL12Rβ2 orthologues, and fly Dome even though these receptors are significantly identical with the other receptors of the family. These homologies confirm the assignment of JfGPH to the type-1 cytokine receptor family but at the same time suggest the occurrence of positive selection among the group, and that JfGPH may be a distinct gene.

To know which receptor JfGPH is orthologous to in the absence of a clear homologue, we conducted phylogenetic and structural analysis (Fig. 2). The bootstrap concensus UPGMA tree, which is based on the assumption that the rate of change along the branches of the tree are constant and the distances are approximately ultrameric, showed significant placement of JfGPH outside and at the root of the IL6ST, IL31Rα, CSF3R, IL12Rβ2, LIFR and OSMR clades at 99% bootsrap values suggesting that JfGPH is a unique receptor gene that is novel and appears to be ancestral to some cytokine type-1 receptors. The same sequences were actually run in neighbor-joining (NJ) and maximum parsimony (MP) algorithms (data not shown). Both NJ and MP concensus trees showed that JfGPH is grouping with IL31Rα, but was not conclusive as the bootstrap values supporting the JfGPH node were very low, at only 35% and 17%, respectively. Since human, mouse and chicken IL31Rα and IL6ST are known to tandemly occur, we checked the fugu, pufferfish and zebrafish genomes to see whether such genes and gene arrangement occur in fish and perhaps link JfGPH to a fish "IL31Rα". The fish IL6ST was indeed present (pufferfish, AY374498; fugu, NEWSINFRUG00000140477; zebrafish ENSDARG00000030498, ENSDARG00000053908, ENSDARG00000053957) but a tandem "IL31Rα" was not, nor there was significant BLAST homology between JfGPH and the

Table 1
Comparative identity of Japanese flounder gp 130 homologue (JfGPH) with other type-1 cytokine receptor orthologues of different vertebrates

Gene	Species	JfGPH (%)
Interleukin 31receptor A (IL31Rα) or gp130-like monocyte receptor (GLMR)	Human	23
	Mouse	23
	Chicken	26
Interleukin 6 signal transducer (IL6ST) or glycoprotein 130 (gp130)	Human	21
	Mouse	22
	Chicken	22
	Pufferfish	22
Colony stimulating-factor 3 receptor (CSF3R) or granulocyte colony-stimulating factor receptor (GCSFR)	Human	23
	Mouse	20
	Frog	22
	Rainbow Trout	21
Domeless	Fly	–
Leukemia inhibitory factor receptor (LIFR)	Human	20
	Mouse	19
	Goldfish	–
Oncostatin M receptor (OSMR)	Human	19
	Mouse	20
Interleukin 12 receptor β1 (IL12Rβ1)	Human	19
	Mouse	–
Interleukin 12 receptor β2 (IL12Rβ2)	Human	–
	Mouse	–
Leptin receptor (LEPR)	Human	–
	Mouse	–
	Chicken	20

–: no significant identity.

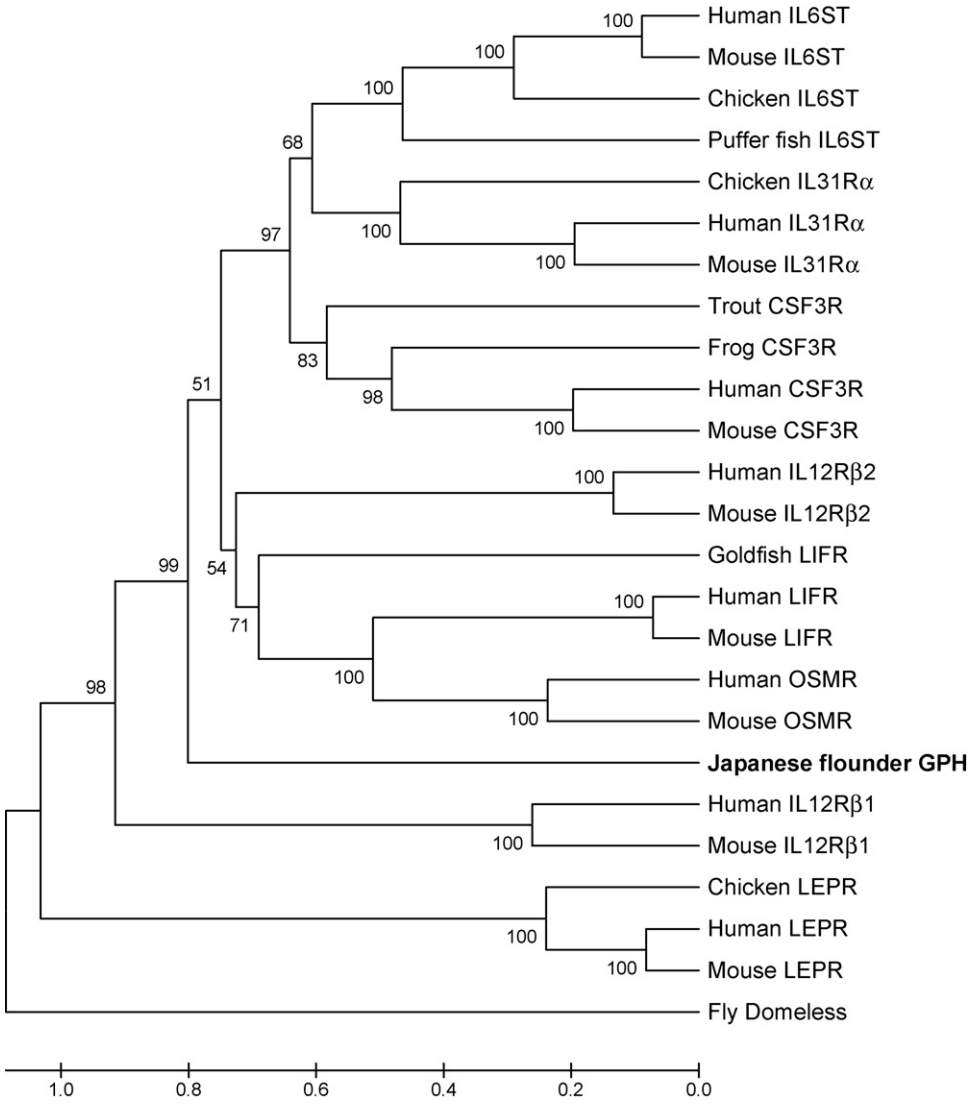


Fig. 2. UPGMA tree of type-1 cytokine receptor family including Japanese flounder gp130 homologue (JfGPH). Accession numbers: Human GLM-R (NP_620586); Mouse GLM-R (AF486621); Chicken GLM-R (XP_424732); Human IL6ST (AAI17405); Mouse IL6ST (AAH58679); Chicken IL6ST (NP_990202); Pufferfish IL6ST (AY374498); Human CSF3R (Q99062); Mouse CSF3R (AAI16636); Frog CSF3R (AAH77792); Trout CSF3R (CAE83614); Human LIFR (P42702); Mouse LIFR (P42703); Goldfish LIFR (AAU94362); Human OSMR (NP_003990); Mouse OSMR (NP_035149); Human IL12Rβ1 (P42701); Mouse IL12Rβ1 (NP_032379); Human IL12Rβ2 (P97378); Mouse IL12Rβ2 (NP_032380) Human LEPR (AAB09673); Mouse LEPR (AAH82551); Chicken LEPR (AAF31355) and Fly Domeless (AAN64333).

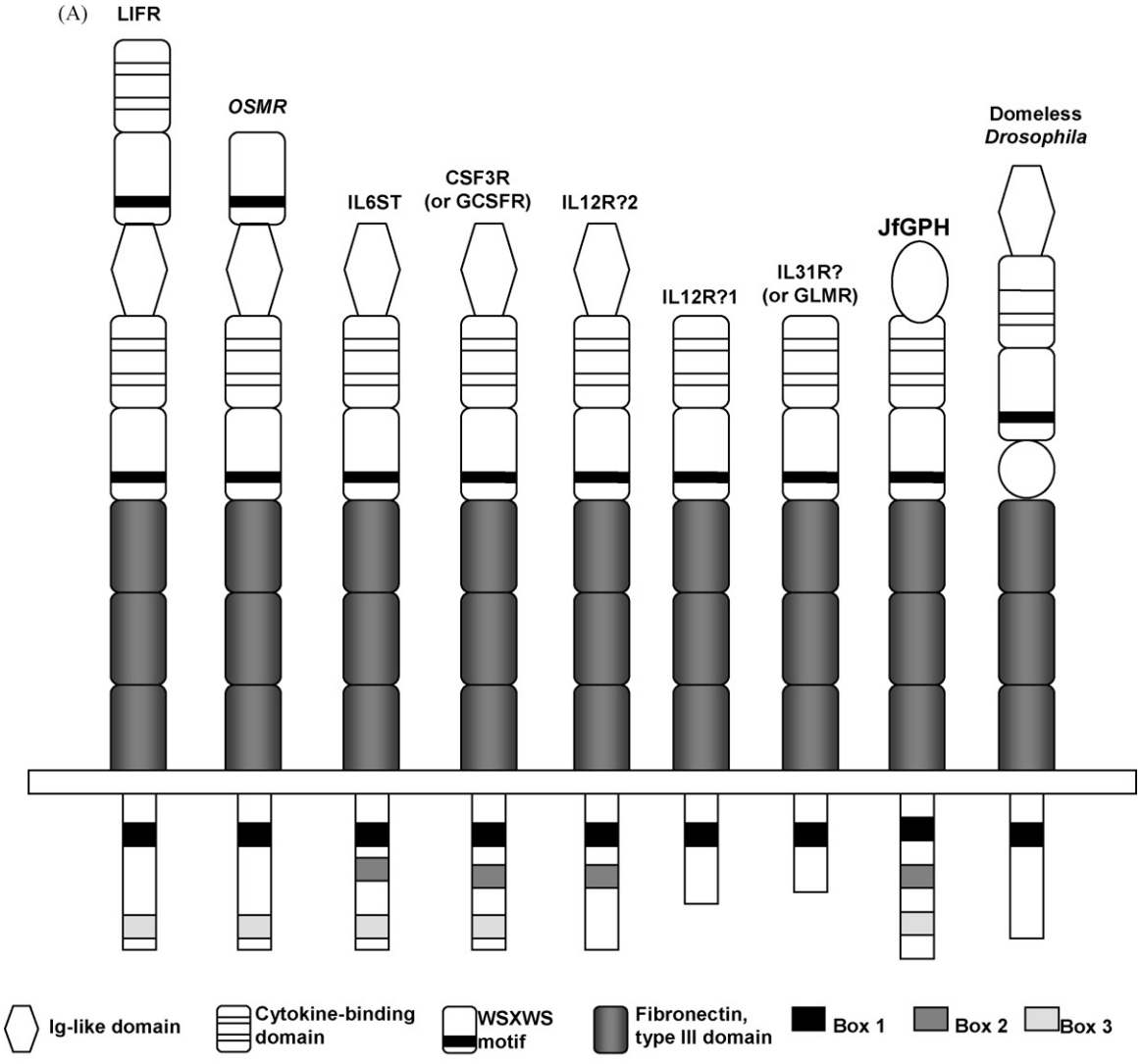
flanking regions of the fish IL6ST. We next compared the protein structures of the same receptors (Fig. 3A). JfGPH general protein architecture is very similar to other type-1 cytokine receptors except for the N-terminal region where there is a non-conserved stretch of 95 amino acids starting from the signal cleavage site to the first conserved cysteine (C) residue of the CBD (Fig. 3A and B). This fragment, which is suppose to correspond to the Ig-like domain of LIFR, OSMR, IL6ST, CSF3R and IL12Rβ2, does not exhibit any known protein domain as searched through ProDom. In addition, JfGPH clearly exhibits all the binding motifs (Boxes 1–3) similar only to IL6ST and CSF3R. The UPGMA tree result, the absence of a link to a possible fish "IL31Rα", the presence of an N-terminal region with unknown domain, and the cytoplasmic tail motifs lead us to conclude that JfGPH is indeed a novel type-1 cytokine receptor present in Japanese flounder.

3.2. Constitutive expression of JfGPH transcript

Semi quantitative RT-PCR analysis showed that JfGPH is expressed ubiquitously in different tissues/organs (Fig. 4). It is particularly expressed in high amounts in tissues involved in immune responses such as gills, intestine, kidney, blood, liver, skin, spleen, stomach and in reproduction such as ovary. JfGPH is also constitutively expressed in HINAE, whose origin is from embryo cells suggesting further its involvement in reproduction and/or development.

3.3. Regulation of JfGPH expression by interferon-inducing poly I:C

Stimulation of primary cultures of spleen, kidney and peripheral blood leukocytes (PBLs) and of embryonic HINAE cell lines



(B)

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Human IL6ST     ELLDPCGYISPESPVVQLHSNFTAVCVLKEKCMDYFHVNAN--YIVWKTNHFTIPKE--
Mouse IL6ST     Q..E.....Y..F....RG.....I.....A.LQHYY...S--.........AAV.R.--
Human CSF3R     ------..H..VSA.I.H.GDPI..S.II.QN.SHLDPEPQ----.L.RLGAE-LQPGGR
Mouse CSF3R     ------..H.EISP...R.GDPVL.S.TISPN.SKLDQQAK----.L.RLQDEP.QPGDR
Human IL31Rα    ------------------------------------------------------------
Mouse IL31Rα    ------------------------------------------------------------
JfGPH           -HMNS.T-VT.KDLYIE.G.DTVVD.--.TS.VSGK--------.F.TL.NKR.DER--
Fly Domeless    --ILDP.WVI.SKVEQLIGGD.NLS.T.N.DYFNGKSAEDCPVEKLYF.GGGRVYRDSK

Human IL6ST     QYTIINRTASSVTFTDIASLNIQLTCNILTFGQLEQNVYGITIISGLPPEKPKNLSC
Mouse IL6ST     .V.V....T.......VVLPSV.......S...I......V.ML..F..D..T..T.
Human CSF3R     .QRLSDG.QE.IITLPHLNHTQAFLSCC.NW.NSL.ILDQVELRA.Y..AI.H....
Mouse CSF3R     .HHLPDG.QE.LITLPHLNYTQAFLFCLVPWEDSV.LLDQAELHA.Y..AS.S....
Human IL31Rα    ----------------------------------------A..-A..E.I..
Mouse IL31Rα    ----------------------------------------VL.T..E.I..
JfGPH           ---LSKTINT.L.ILSLTNFTQ.RAMLQCHSANT..VLG.-.F..TYT--..SKI..
Fly Domeless    HIR.L.N.TILFSD.NAVEQENDYH.MCDEY-----VINKSKVYV.TR.LLVRDFN.
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Fig. 3. Comparison of class 1 helical cytokine receptors with significant identity with Japanese flounder gp130 homologue (JfGPH). (A) Schematic drawing of protein motifs and domains (similar to Fig. 2C) are shown. Figure with question mark corresponds to the 93 amino acids in the N-terminal region with no known domain. (B) Multiple sequence alignment of the Ig-like domain of IL6ST and CSF3R, and the sequences upstream of the first cysteine residue of IL31Rα and JfGPH minus the leader peptide. Conserved amino acids (dots), introduced gaps (dash) and the first conserved cysteine residue of the cytokine binding domain (boxed) are shown.

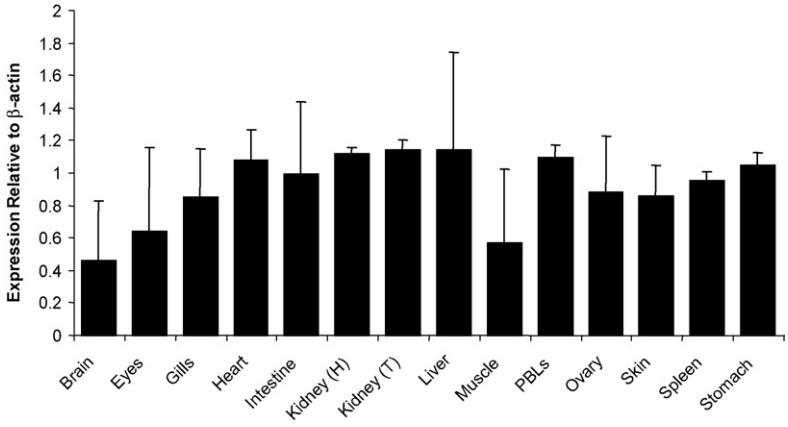

Fig. 4. Expression of Japanese flounder gp130 homologue (JfGPH) relative to β-actin in various tissues in Japanese flounder determined by semi-quantitative RT-PCR. Mean values (bars) of three samples plus standard deviation are shown.

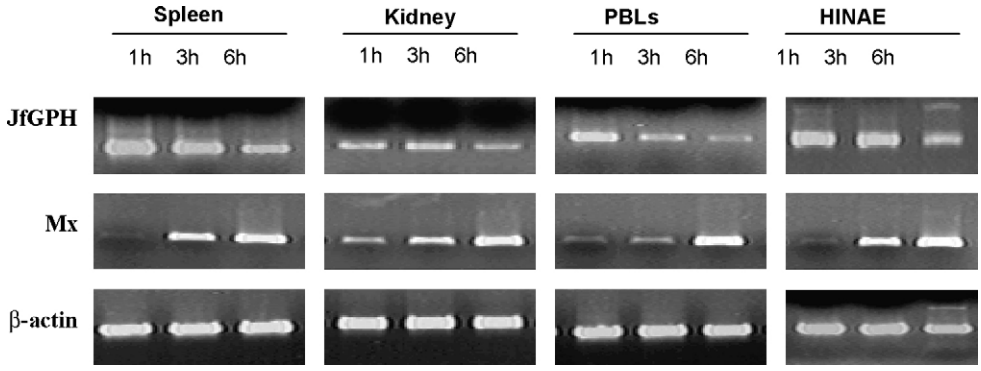

Fig. 5. Expression of Japanese flounder gp130 homologue (JfGPH) gene in response to polyinosinic:polycytidylic acid (poly I:C). JfGPH, Mx and β-actin transcript expression *in vitro* in Japanese flounder spleen, kidney, PBLs and HINAE at 1, 3 and 6 h post-stimulation with poly I:C.

revealed that JfGPH gene expression is down-regulated by poly I:C in a time-series manner *in vitro* (Fig. 5). Correct stimulation by poly I:C was confirmed by the up-regulation of Mx while the integrity of the RNA extraction and cDNA synthesis was verified through the expression of β-actin.

4. Discussion

Here we report a novel molecule that belongs to the type-1 cytokine receptor family found in fish. It exhibits the classic structural features of the family i.e. having a cytokine binding domain with the four (4) conserved cysteine residues, a WSEWS motif, three FNIII domains in its extracellular region, and 2 Janus kinase (Jak) binding motifs (Box 1 and Box 2), and a STAT3 binding domain (Box 3) in the intracellular tail. Subsequent structural and evolutionary analysis of the protein revealed that JfGPH is another long type-1 cytokine receptor, which does not exhibit an Ig-like domain in its N-terminal region unlike IL6ST and CSF3R, but instead possess a stretch of amino acids with no known domain or function. Such discovery is not surprising as a cytokine type-1 receptor in human has only been cloned and characterized just recently (Ghilardi et al., 2002; Diveu et al., 2003; Dreuw et al., 2004).

JfGPH is clearly involved in a Jak/STAT signal transduction pathway because of the conserved Box 1, Box 2-like and Box 3 motifs, and tyrosine (Y) residues in its cytoplasmic tail. Box 1 is a membrane-proximal, proline rich motif, which could associate the Janus kinase (Jak) family upon ligand binding while the hydrophobic Box 2 could also serve as a docking site for Jaks. Box 3 is established to be the binding site of STAT3 signal molecules. While it may be expected that fish do involve Jak/STAT signaling, JfGPH structure together with the identification of Japanese flounder Jak2 (EST accession no. AU091091), STAT1 (EST accession no. AU261169) and STAT3 (EST accession no. AU083083) ESTs (Table 2) reinforces this idea. The Jak/STAT pathway is responsible for numerous physiological responses including hematopoieis, immune responses and development in mammals (Hou et al., 2002; Ihle, 1996) and even in development and antiviral responses in drosophila (Chen et al., 2001; Agaisse and Perrimon, 2004; Costert et al., 2005). With the presence of the CBD and three (3) FN3 domains in the

Table 2
Identities of putative Japanese flounder Jak, STAT1 and STAT3 with known orthologues from other species

Japanese flounder (EST accession no.)	Zebrafish	Human
AU091091	62% (Jak2)	61% (Jak2)
AU261169	51% (STAT1)	37% (STAT1)
AU083083	83% (STAT3)	76% (STAT3)

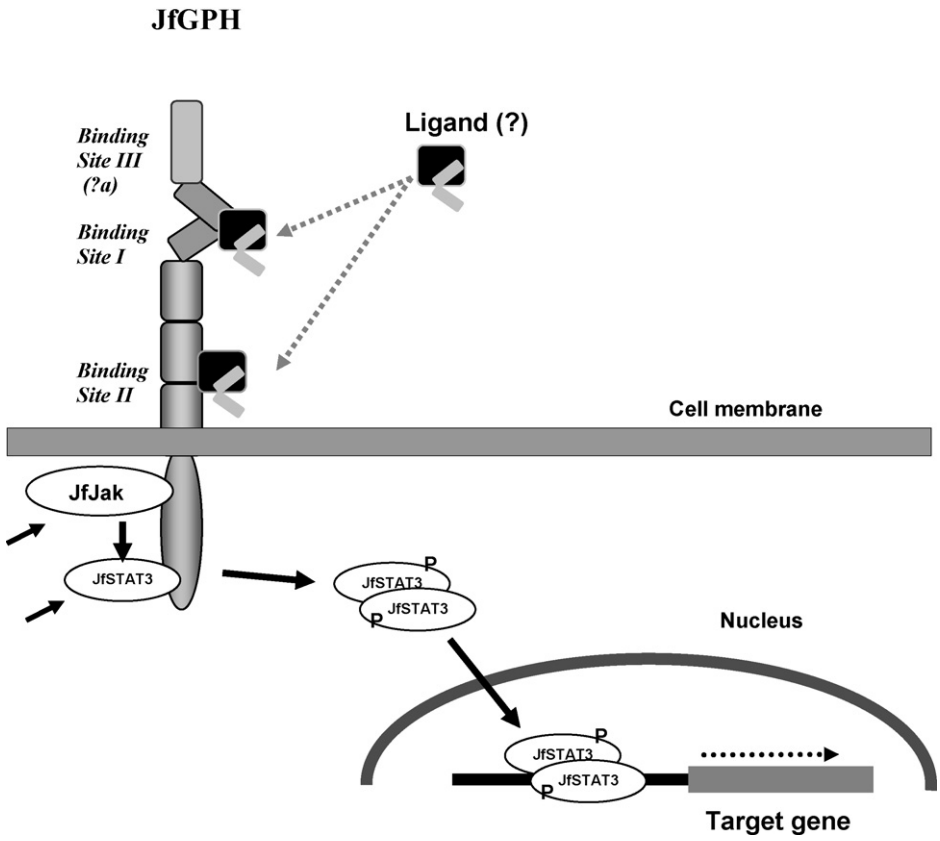

Fig. 6. Diagram of the putative Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal cascade in Japanese flounder mediated by Japanese flounder gp130 homologue (JfGPH). Japanese flounder Janus kinase (JfJak), Japanese flounder signal transducers and activators of transcription 3 (JfSTAT3) and ligand binding sites are shown. Specific ligand for JfGPH and Site III's putative function as a cytokine binding site are unclear (?).

extracellular region of JfGPH, it is likely that it utilizes two (site I and site II) of the three binding sites used by the long chain cytokines to bind to their receptors (Bravo and Heath, 2000). Hence, we speculate that a specific ligand for JfGPH binds to the receptor's binding site I or II or perhaps III and allows for the binding of JfJak to its Box 1 or Box 2. This in turn signals the recruitment of the JfSTAT3 in the Box 3 and undergoes phosphorylation. The phosphorylated STAT3s form dimers and translocates to the nucleus to effect target gene expression. We show a diagram of such putative signal cascade mediated by the JfGPH in Fig. 6. The ligand for JfGPH is not yet known and it is not clear whether JfGPH utilizes the peptide sequence that is parallel but not similar to the Ig-like domain for a site III interaction or whether JfGPH functions as a homodimer or a heterodimer similar to gp130. These unknowns warrant further study to better understand JfGPH function.

Interestingly, while JfGPH shows significant homology with all known IL31Rα, IL6ST, CSF3R, and OSMR genes, it does not have identity with every LIFR, IL12Rβ1, IL12Rβ2 and LEPR orthologues, in particular the goldfish LIFR, suggesting that JfGPH and perhaps the type-1 cytokine receptor family in lower vertebrates is undergoing a certain level of positive selection, which is not an unusual event for cytokine molecules (Huising et al., 2006; Jaillon et al., 2004; Santos et al., 2006). It is believed that, tandem gene duplication potentially expanded the type-1 cytokine and receptor genes in vertebrates as evidenced by their short physical distances. In addition, short type-1 cytokine and receptor genes may have occurred later in evolution than long type I cytokines and receptors accounting for the more developed acquired immunity in higher vertebrates (Boulay et al., 2003). The identification of a novel long type-1 cytokine receptor suggests that this group is also expanding at least in teleosts. Just recently, we have cloned a partial cDNA fragment, which surprisingly appears to have identities with chicken IL12Rβ, chicken OSMR, frog gp130 and frog PRLR (unpublished data). This variation could also be the reason why our BLAST search of existing fish genomic databases through *Ensembl* could not identify a homologous JfGPH gene.

The phylogenetic tree in this study resembles the tree generated for the group 2: gp130 family by Boulay et al. (2003), which also suggested that this same group likely represents the ancestral forms of the type-1 cytokine receptors. It is interesting to note that JfGPH appears to be a molecule ancestral for the type-1 cytokine receptors because of its cross similarity and basal phylogeny. However, it has no significant homology with the drosophila Domeless receptor, which is said to be the ancestral molecule for the mammalian cytokine receptor family (Chen et al., 2001). It is possible that JfGPH has significantly diverged from the Domeless receptor due to selection as mentioned previously.

High expression of specific JfGPH transcripts in kidney, spleen, kidney, PBLs liver, skin, stomach and gills suggest that it

is involved in immune responses. Moreover, JfGPH's significant expression in ovary and in a cell line whose origin is from embryo cells shows that it has a role in reproduction and development. This highlights the important role of JfGPH in the physiological processes in fish similar to gp130, a type-1 cytokine receptor well studied in higher vertebrate. Gp130 has been established to be a critical receptor molecule to an organism such that mutation or knockdown of the said gene has been fatal to the organism (Kishimoto et al., 1995).

The involvement of JfGPH in immunity and particularly in the Jak/STAT pathway is further confirmed through the down-regulation of its expression following poly I:C stimulation in tissues and in cell lines. poly I:C is a double stranded RNA known to induce IFN-α/β production. We speculate that the IFN-induced Jak/STAT pathway somehow inhibits the expression and thus the function of JfGPH-dependent Jak/STAT signaling presumably to regulate and balance the system. Actual mechanism though should be confirmed and fully explored. Down regulation of JfGPH by poly I:C is in contrast to the up-regulation of GPL, IL12Rβ2 and IL23R β1, receptors homologous to JfGPH, by IFN-γ treatment in monocytes and dendritic cells (Diveu et al., 2003; Parham et al., 2002). The difference in JfGPH expression as compared with other receptors further reflects its uniqueness at the transcriptional level.

It is important to identify the ligand(s) that could specifically bind to JfGPH and confirm the Jak/STAT signal cascade it would induce. Four (4) cytokines of the IL-6 cytokines from Japanese flounder namely *po*CSF3 (Santos et al., 2006), IL6 (Nam et al., 2007), IL11 type b (unpublished data) and an M17 homologue (submitted) have been cloned and are potential ligands. Identification of the JfGPH ligand could greatly help in understanding further the biological function of JfGPH and other related receptors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2007.02.018.

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