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Identification of a Novel IL-1 Cytokine Family Member in Teleost Fish

Tiehui Wang,* Steve Bird,* Antonis Koussounadis,† Jason W. Holland,* Allison Carrington,* Jun Zou,* and Christopher J. Secombes2*  

A novel IL-1 family member (nIL-1F) has been discovered in fish, adding a further member to this cytokine family. The unique gene organization of nIL-1F, together with its location in the genome and low homology to known family members, suggests that this molecule is not homologous to known IL-1F. Nevertheless, it contains a predicted C-terminal β-trefoil structure, an IL-1F signature region within the final exon, a potential IL-1 converting enzyme cut site, and its expression level is clearly increased following infection, or stimulation of macrophages with LPS or IL-1β. A thrombin cut site is also present and may have functional relevance. The C-terminal recombinant protein antagonized the effects of rainbow trout rIL-1β on inflammatory gene expression in a trout macrophage cell line, suggesting it is an IL-1β antagonist. Modeling studies confirmed that nIL-1F has the potential to bind to the trout IL-1RI receptor protein, and may be a novel IL-1 receptor antagonist. The Journal of Immunology, 2009, 183: 962–974.

The IL-1 family (IL-1F) of cytokines is characterized by their common secondary structure of an all-β fold, the β-trefoil, which they have in common with another cytokine family, the fibroblast growth factors (1, 2). To date, 11 members of this family are known, with IL-1F1 (IL-1α), 2 (IL-1β), 4 (IL-18), 6, 8, 9, and 11 (IL-33) all having agonist activity, and they generally promote inflammatory and adaptive immune responses (3). In several cases these agonist IL-1F proteins are produced as inactive precursors that require cleavage by IL-1 converting enzyme (ICE/caspase 1) to generate the biologically active mature protein, as seen with IL-1F1, 4, and 11 (only in vitro in the latter case (3)). The proteins signal via a number of receptors, namely IL-1RI for IL-1F1 and 2, IL-18R for IL-1F4, IL-1Rrp2 for IL-1F6, 8, and 9, and ST2 for IL-1F11 (5, 6). In each case an accessory protein (AcP) is needed to join the ligand-receptor complex to allow a signal to be transduced, and the IL-1RAcP is used by IL-1R1, IL-1Rrp2, and ST2, whereas IL-18R uses the IL-18Apc (3, 6, 7). Signaling, in common with the TLRs, requires recruitment of the adaptor molecule MyD88, which allows activation of the IL-1R-associated kinase (IRAK) (8) and ultimately activation of transcription factors such as NF-κB and MAPK-regulated transcription factors, leading to IL-1F-regulated gene transcription in the target cells (1).

Each of the pathways mentioned above has natural inhibitors that can down-regulate the elicited responses. In some cases the inhibitor is an IL-1F that can act as a receptor antagonist, as with IL-1F3 (IL-1ra) for IL-1RI and IL-1F5 for IL-1Rrp2. IL-1F7, which is another IL-1F that requires processing by ICE (9), is also an antagonist, in that it helps to reduce IL-1F4 activity by interacting with the IL-18 binding protein to form a complex that inhibits receptor signaling (10, 11). Additionally, soluble receptors can be produced to prevent binding to the signaling receptor, as with IL-1RII and soluble ST2, which in the latter case is a splice variant of the signaling receptor transcript (12). The last member of the IL-1F, IL-1F10, may also be involved in some form of regulation of IL-1F activity, and it binds to the soluble form of the IL-1RI, although the function of this is unknown. What is clear from these complex inhibitory pathways is that IL-1F actions need to be tightly regulated, and in humans many disease states are known to be associated with their actions if their activity is not appropriately controlled (3).

Most of the IL-1F are found on human chromosome 2 (q13–21) (13, 14), in the order IL-1F1, 2, 7, 9, 6, 8, 5, 10, 3, and this suggests that these genes have arisen by tandem gene duplication, with this region of chromosome 2 considered a “hotspot for IL-1 gene duplication” (15). The remaining IL-1F, IL-1F4 and IL-1F11, are found on chromosomes 11 and 9, respectively. Analysis of the genomic structure of the genes shows that they all possess introns that lie in similar positions within the proteins they encode, again indicating they have arisen from a common ancestor. Since the control of inflammatory events is likely to be ancient in origin, it seems likely that some IL-1F members will be universally present in vertebrates, although the possibility that independent duplication events may have given rise to unique IL-1F in particular vertebrate groups also exists.

Studies aimed at elucidating the cytokine network in fish have made large advances in recent years (16, 17). One of the earliest cytokines to be cloned in fish was IL-1β (18), partly due to a relatively high homology compared with known...
mammalian genes, and partly due to the high transcript level of this molecule. Since then, IL-1β has been cloned in many fish species, including cartilaginous fish (19), and the bioactivity of the recombinant protein has been established (20–22). In the search for other IL-1F genes, it was quickly discovered that several species of fish possess two IL-1F molecules (23, 24), thought to be a consequence of further genome duplication events in particular fish lineages. Additionally, alleles of IL-1F have been described, the receptor for IL-1F11, ST2, has been cloned recently, IL-18 (IL-1F4) has been discovered in fish, from in silico analysis of sequenced fish genomes and expressed sequence tag databases (26, 27). While other IL-1F have not yet been identified due to retroposition events within intron 3 (25). Moreover, IL-18 (IL-1F4) has been discovered in fish, from in silico analysis of sequenced fish genomes and expressed sequence tag databases (26, 27). While other IL-1F have not yet been described, the receptor for IL-1F1, ST2, has been cloned (28) and may suggest at least one other IL-1F has still to be discovered. In this paper we describe a novel IL-1F (nIL-1F) in fish, discovered by us during studies to identify immune genes involved in host defense against bacterial infection in rainbow trout, where the gene expression profile of bacterially challenged fish was surveyed by means of suppression subtraction hybridization and sequence analysis (29). This nIL-1F appears to have no clear homology to any IL-1F described to date, and it resides in the genome in a unique location. Functional and expression analysis of this molecule are also presented.

Materials and Methods

Identification of nIL-1F in trout

Rainbow trout suppressive subtractive hybridization (SSH) libraries from bacterially challenged fish were constructed as described previously (29). Analysis of SSH clones from a gill SSH library revealed a short sequence with limited homology to IL-1 family members. 3′- and 5′-RACE was conducted using SMART cDNA prepared from gills of Aeromonas salmonicida- (a Gram-negative fish bacterial pathogen) infected fish, as described previously (29). 3′-RACE using forward primers F1 and F2 (Table I) resulted in a 0.7-kb product that when sequenced contained the C terminus and the 3′-untranslated region (UTR). Additional primers R1 and R2 (Table I) were designed in the 3′-UTR and used for 5′-RACE. The resulting 1.5-kb product was cloned and sequenced and found to contain the 5′-UTR and complete coding region. The nucleotide sequences generated were assembled and analyzed with the AlignIR program (LI-COR). A sequence similarity search was performed using FASTA (30) and basic local alignment search tool (BLAST) (31). Direct comparison between two sequences was performed using the MatGAT program (32). Multiple sequence alignments were generated using CLUSTAL W (version 1.7) (33). Phylogenetic analysis was also performed on the predicted full-length amino acid sequences, with the known IL-1 family molecules, using the neighbor-joining method (34). The tree was drawn using CLUSTAL X version 1.81 and TreeView version 1.6.1 (35), and confidence limits were added (36).

Gene organization and promoter analysis of nIL-1F

A rainbow trout genomic library constructed with a GEM-11 was PCR screened with nIL-1F-specific primers at the 5′ (gF1 and gR1) and 3′ ends (gF2 and gR2) of cDNA (Table I), as described previously (37). Two overlapping positive clones were plaque purified and their DNA was prepared using a Wizard Prep DNA purification system (Promega). After an initial restriction enzyme analysis with SacI, XbaI, and XhoI (all from Promega), the digestions were subcloned into pGEM-7zF(+) and sequenced. Three contigs were identified that contained the full-length cDNA, as well as the 5′ and 3′ flanking regions. The large introns III and VII, −11 and 6 kb, respectively, as defined by PCR from genomic DNA, were not sequenced completely. The cDNA sequence was aligned to the genomic sequence, and the intron/exon boundaries were identified using the SIM4 program (38). The gene organization data of human IL-1F members, including exon/ intron sizes, intron phase, and coding regions, was extracted from the Ensembl database (www.ensembl.org/Homo_sapiens/exonview) for comparison to this fish nIL-1F. The 5′ flanking region sequence was next analyzed using the program Signal Scan (39). To get more insight into the regulation of gene expression of nIL-1F, a comparative

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Table I. Primers used for cloning and expression

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*a Note that TNF-α primers can amplify both trout TNF-α1 and TNF-α2.*
promoter analysis was conducted using the Possum program (http://zlab.bu.edu/~mfrith/possum/), which predicts cis elements in DNA sequences using the standard method of position-specific scoring matrices. The first 1-kb sequences (including the first exon) of both the nIL-1F and IL-1β genes from rainbow trout were analyzed for potential transcription factor binding sites.

Table II. Identity (percentage, top right)/similarity (percentage, bottom left) of the nIL-1F to other members of the IL-1 family from trout and humans

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Genome analysis of the IL-1 family in Tetraodon nigroviridis

Since the positions of IL-1F in the genome of mammals is well known, we undertook a comparison of the location of both the novel IL-1F and IL-1β in the genome of a fish species for which the genome had been sequenced, the pufferfish T. nigroviridis. The Tetraodon IL-1β and nIL-1F sequences were found using the Tetraodon genome, and evidence of conservation of synteny between the human and Tetraodon genomes was investigated. The Tetraodon genome was searched by BLAST analysis (31) using amino acid sequences for trout IL-1β and nIL-1F. Tetraodon homologs for IL-1β and nIL-1F were found on chromosome 12 and on chromosome 5, respectively.

Subsequently, the DNA sequence around these two genes was retrieved for further analysis using various sequence software programs. Using GENSEARCH (41), possible coding regions within the DNA sequence were predicted, and the amino acid sequences were analyzed using BLAST (31) and FASTA (30). Regions of chromosome 2 (the location of most IL-1F in mammals) and X (where some of the neighboring genes around nIL-1F are found) in the human genome were used to look for synteny to the regions of the Tetraodon genome analyzed.

Modeling

TROUT nIL-1F was modeled using the β-trefoil modeling method, as described previously (42). Briefly, an object-oriented database containing representative β-trefoil protein structures was queried using BLAST (31) to identify a template structure with the highest sequence similarity to the target, and then to identify segments in the database to model loops that differed in length from the template. Three criteria were applied in loop modeling. First, the templates had the correct number of residues for the target loop region. Second, the fragments had endpoints that geometrically matched the residues in the template structure where the loop was inserted. Third, only candidate fragments from the same β-trefoil loop category were considered for subsequent fitting. Structural information from the β-trefoil database was also used in side-chain modeling. The final three-dimensional model was built using MODELLER (43). The generated model was assessed for its stereochemical properties with PROCHECK (44). Molecular visualizations were prepared with the program DeepView version 3.7 (45).

Expression of IL-1 family members in vivo

A comparative expression analysis of the known trout IL-1β genes, IL-1β1 and IL-1β2, and the nIL-1F was undertaken by real-time PCR as described previously (46, 47) to assess levels of expression in vivo. Briefly, six healthy rainbow trout (average, 130 g/fish) were anesthetized with 2,2,2-tribromoethanol (A. E. Ellis (Fisheries Research Services Marine Laboratory, Aberdeen, Scotland, U.K.). The bacteria were spread onto a tryptic soy agar (Fluka Chemika) plate and incubated for 2 days at 22°C. Then, the bacteria were injected i.p. with bacteria (0.5 ml/fish). Fish in the control group were injected with PBS 6 h earlier, which was defined as 1. A fold change was also calculated as the average expression level in the bacterial challenged fish divided by that of the PBS-injected control fish at the same time point.

Modulation of the expression of trout IL-1 family members in vivo by bacterial Yersinia ruckeri infection

A pathogenic strain (MT3072) of the Gram-negative fish pathogen Y. ruckeri, the causative agent of enteric redmouth disease, was supplied by Prof. A. E. Ellis (Fisheries Research Services Marine Laboratory, Aberdeen, Scotland, U.K.). The bacteria were spread onto a tryptic soy agar (Fluka Biomerieux) plate and incubated for 2 days at 22°C. Then, the bacteria were scraped off and suspended in PBS (pH 7.2), washed three times with PBS, and resuspended in PBS to a concentration of 2 × 10^6 CFU/ml. Rainbow trout (~100 g), were put into two tanks (30 fish/tank) 2 wk before challenge. The water temperature throughout the experiment was controlled at 15 ± 1°C and the fish were fed with commercial trout pellets (EWOS) twice a day. The waste water was sterilized by ozonation. Fish from one tank were injected i.p. with bacteria (0.5 ml/fish). Fish in the second tank were injected i.p. with PBS (0.5 ml/fish) as control. Six fish from each treatment group were sampled at 6, 24, 48, and 72 h postinjection, when the spleen was collected and used for total RNA preparation using TRIzol (Invitrogen). The expression of trout IL-1 family members was quantified by real-time PCR as above. The relative expression of each member was normalized to the average level of IL-1β in the fish injected with PBS 6 h earlier, which was defined as 1. A fold change was also calculated as the average expression level in the bacterial challenged fish divided by that of the PBS-injected control fish at the same time point.

Expression of IL-1 family members during the modulation of the expression of trout IL-1 family members in vivo

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FIGURE 2. Phylogenetic tree showing the relationship between the full-length trout nIL-1F amino acid sequence, with other known vertebrate IL-1 family members. This tree was constructed using the CLUSTAL X and TreeView packages and was bootstrapped 10,000 times. Bootstrapping values <75 are shown (● ●). The GenBank accession numbers of the IL-1β genes are: cow, M53589; horse, D42147; human, M15330; mouse, M15131; pig, M86725; dolphin, BAA87947; dog, DQ251036; rabbit, B26295; platypus, AJ245728; possum, AF017539; catshark, A247101; leopard shark, AB074142; cod, AJ535730; goldfish IL-1β1, A249136; goldfish IL-1β2, AJ129137; halibut, BAB86882; icifish, CAD92853; salmon, AA360642; seabream, AJ269472; seabream, A277166; red seabream, AY257219; carp IL-1β1, AB010701; carp IL-1β2-1, AJ401030; carp IL-1β2-2, J401031; chicken, Y15006; xenopus, AJ101497; haddock, AJ550166; trout IL-1β1, AJ223954; trout IL-1β2, AJ243925; turbot, A295836; Tetraodon, AJ574910; Chinese perch, AY647430; cobia, AY641829; zebrasaffish, AY340959. The GenBank accession numbers of the IL-1α genes are: cow, X12497; dog, AF047011; dolphin, AB028215; horse, U92480; human, M15329; mouse, X01450; pig, M86730; rabbit, X02852. The GenBank accession numbers of the IL-1F3 genes are: cow, BC134577; dog, AY020662; dolphin, AB032868; horse, D83714; human, AY199608; mouse, AK076269; rabbit, M57526; pig, Q29056. The GenBank accession numbers of the human IL-1 family member genes are: IL1F5, AF186094; IL1F6, AF201831; IL1F7, AF201832; IL1F8, BC101831; IL1F9, AF200492; IL1F10, AF334755. The GenBank accession numbers of the mouse IL-1 family member genes are: IL1F5, AF200495; IL1F6, AF200493; IL1F8, AK009787; IL1F9, AK081783; IL1F10, AL732430. The GenBank accession numbers of the IL-18 genes are: rabbit, BIA384; dog, Q9XS50; human, Q6FGY3; horse, BIA8B; cow, B2LS56; pig, A19073; mouse, Q5BS85; chicken, Q6T44; trout, Q7OP11; Fugu, Q7OT31. The GenBank accession numbers of the IL-33 genes are: human, O95760; mouse, Q5BYYYYZ. The GenBank accession numbers of the nIL-1F genes are: trout, AJ555869; Tetraodon, FM207486.

Expression and modulation of trout IL-1 family members in the RTS-11 cell line by LPS, rIL-1β, and poly(I:C)

The mononuclear cell line RTS-11 (48) was routinely grown in L-15 medium supplemented with 30% FCS at 20°C. RTS-11 cells (3 × 10^5) were seeded in 25-cm² culture flasks in 5 ml medium (L-15 plus 0.5% FCS) and
cultured overnight before any treatments or RNA preparation. The cell culture supernatant was replaced with the same medium (0.5% FCS) with or without *Escherichia coli* LPS (Sigma-Aldrich, 25 μg/ml), poly(I:C) (Sigma-Aldrich, 50 μg/ml), or rIL-1 (30 ng/ml). These concentrations were chosen based on our previous studies (49) and were deemed optimal. The treatments were terminated by dissolving the cells in TRIzol (Invitrogen) at 1–24 h poststimulation and total RNA was prepared. The expression of trout IL-1F members was quantified by real-time PCR as above. The relative expression of each member was normalized to the average level in the control samples at 1 h, which was defined as 1.

Northern blot analysis

Total RNA isolated from RTS-11 cells stimulated with LPS for 4 h, or from control unstimulated cells, was also used for Northern blot analysis, performed as described previously (49). In each experiment, 10–25 μg of total RNA per lane was transferred from a 1.1% formaldehyde-MOPS agarose gel to nylon membranes by capillary action and hybridized overnight at 65°C with a 32P-labeled cDNA probe purified from a trout nIL-1F PCR fragment amplified using F and R primers (Table I). Following stringent washing, membranes were put into an x-ray cassette with intensifying screens and film (Kodak) and exposed for 2 days.

Expression of recombinant trout nIL-1F and bioactivity analysis

Although it is not known whether the trout nIL-1F is produced as a precursor that is subsequently cleaved, it seems likely that this is the case based on our modeling analysis. Thus, the C-terminal region downstream of the two possible cut sites (see Fig. 1) has 12 predicted -strand regions that typify the known mature IL-1F peptides. The recombinant protein produced was designed to contain the 12 predicted -strands (i.e., to begin upstream of the first -strand) and to start immediately downstream of the possible thrombin cut site. The C-terminal region chosen contained 197 aa and started at Gly169. Briefly, the trout nIL-1F sequence was amplified using primers (forward primer; GGTTGTCGTGACGGGAGCTCTT; reverse primer, TTTATGGATGACAAAGAAGAATGAC) and cloned to pTriEx-6 vector (Novagen) that has the option to express the cloned protein in *E. coli* as well as in eukaryotic cells. To facilitate purification, an N-terminal Strep-tag II and a C-terminal His tag from the vector were incorporated, and thus the recombinant nIL-1F had an N-terminal MASWSHPQFEKGALEGPS and a C-terminal GSSAHHHHHHHHH.
A sequence-confirmed plasmid was used to transform BL21 Star (DE3)-competent cells (Invitrogen), and protein expression was induced by isopropyl β-D-thiogalactoside as described previously (46). The recombinant nIL-1F was expressed at high yield as inclusion bodies. Thus, purification under native condition was not feasible. The nIL-1F was lysed in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 6 M guanidine hydrochloride, 15 mM 2-ME, and 15 mM imidazole, and purified using His GraviTrap columns (GE Healthcare Life Sciences). After binding and extensive washing with lysis buffer, the denatured nIL-1F was eluted in lysis buffer containing 500 mM imidazole. The purified denatured nIL-1F was refolded in a refolding buffer containing 50 mM Tris-HCl (pH 7.0), 0.5 M arginine, 0.5% Triton X-100, and 50 mM imidazole, and checked for purity on a NuPAGE Novex Bis Tris MiniGel (Invitrogen) under reducing conditions. When up to 20 μg/ml nIL-1F was added to RTS-11 cells for 4.5 h, the up-regulation of known LPS-responsive genes (IL-1β, nIL-1F, TNF-α, and cyclooxygenase (COX)-2) genes was not detectable, suggesting that LPS contamination was negligible.

A second nIL-1F of 175 aa, starting from Met191 (i.e., still upstream of the predicted 12 β-strands), was also produced in Chinese hamster ovary (CHO) cells to confirm whether native produced and potentially glycosylated protein also has the same activity as the E. coli-produced nIL-1F. The coding region was cloned using primers nIL-1F-r (F and R) (Table I) and directly cloned to pcDNA 6.2/GW/D-TOPO (Invitrogen), which allows production of the protein fused with a V5 tag from the vector for detection and bioactivity analysis. A sequence-confirmed plasmid was linearized using ScaI and used to transform CHO cells by electroporation using a Multiporator (Eppendorf). The transformed cells were selected using 5 μg/ml blasticidin (Invitrogen). The cell culture supernatant was removed and replaced with 1/20 volume of PBS and stored at −80°C. After freezing-thawing three times, the cell lysate was centrifuged at (12,000 rpm, 15 min) and the supernatant was used for Western blot and bioactivity analysis.

**Bioactivity analysis**

To assess the potential agonistic or antagonistic activity of the nIL-1F, the purified rIL-1F produced in E. coli, or the clarified lysate from CHO cells, was added to RTS-11 cells and the effect on expression of a number of genes involved in inflammatory responses (IL-1β, IL-8, TNF-α, TGF-β, COX-2), and on nIL-1F itself, was analyzed in the presence or absence of costimulation with rIL-1β (20), a potent proinflammatory molecule. It was anticipated that the nIL-1F may influence IL-1β activity since it had highest homology to trout IL-1β and the modeling studies identified human IL-1ra as the most similar structure, and that it was potentially able to bind to the IL-1R (see Results). In the case of single stimulations, analysis was performed 4.5 h poststimulation with nIL-1F or 4 h with IL-1β (10 ng/ml). In the costimulation experiments, the nIL-1F was added for 0.5 h before subsequent addition of the rIL-1β for a further 4 h to assess whether the nIL-1F could antagonize the rIL-1β-induced effects. While the optimal stimulatory concentration range for trout rIL-1β is well established, this was unknown for the nIL-1F, and so a range of concentrations (from 0.5 to 4 μg/ml) of the E. coli-produced nIL-1F or dilutions of the lysates from CHO cells (from 0.4 to 250 μl/ml) were used in these experiments. After treatment, the cells were lysed in TRIzol and gene expression was detected using a Multiporator (Eppendorf). The transformed cells were selected using 5 μg/ml blasticidin (Invitrogen). The cell culture supernatant was removed and replaced with 1/20 volume of PBS and stored at −80°C. After freezing-thawing three times, the cell lysate was centrifuged at (12,000 rpm, 15 min) and the supernatant was used for Western blot and bioactivity analysis.

**FIGURE 5.** Genome location of nIL-1F relative to other IL-1F. Comparative gene location maps between chromosome 2 in human and 12 in Tetraodon containing the IL-1β homolog and chromosome X in human and 5 in Tetraodon containing the telost nIL-1F homolog.

**FIGURE 6.** Multiple sequence alignment of human and mouse IL-1ra, trout IL-1β, and trout nIL-1F mature peptides. Identical residues and the 12 β-strands of the human structure are indicated.
by real-time PCR (E. coli-produced nIL-1F) or visualized on ethidium bromide-stained agarose gels, as described previously (25), for CHO cell-produced nIL-1F. The experiment was performed on three independent flasks of cells for each treatment.

Statistical analysis
Real-time quantitative PCR measurements were analyzed using the non-parametric Mann-Whitney $U$ test within the SPSS package 15.0, with $p < 0.05$ between treatment groups and control groups considered significant.

Results

Cloning and sequence analysis
The trout nIL-1F was cloned (EMBL accession no. AJ555869; www.ebi.ac.uk/embl/) and the full-length transcript shown to be 1736 bp, consisting of a 165-bp 5' UTR, an 1098-bp open reading frame encoding a protein of 365 aa, and a 473-bp 3' UTR (Fig. 1).

Within the 3' UTR a repeat (4) of ttttataccacca was present from position 1318, and a polyadenylation signal was present 17 bp upstream of a 20-bp poly(A) tail. Analysis of the predicted translation revealed a protein of 41.2 kDa, with a theoretical isoelectric point (pI) of 5.67. No signal peptide was apparent, in common with other IL-1F, but it contained a potential ICE cut site (LEXD) at aa positions 126–129, and a thrombin cut site (RGR) at aa positions 166–168. Interestingly, the latter divided the protein into an N-terminal acidic domain (pI of 4.67) of 18.6 kDa and a C-terminal basic domain (pI of 8.75) of 22.6 kDa. No signal peptide or transmembrane domain was detectable within the sequence, but two potential glycosylation sites (NXT) were found at aa positions 271–273 and 341–343 (Fig. 1).

A position-specific iterated (PSI)-BLAST search (20 iterations) vs nonredundant protein sequence databases revealed that nIL-1F is similar to IL-1\$\beta\$, IL-1ra, and other vertebrate IL-1F cytokines (the expect value $< 10^{-30}$), confirming that it is an IL-1F family member. The nIL-1F sequence contains a PROSITE (50) IL-1 signature (PS00253) in positions 327–347 that is modified in two residues. Other piscine IL-1F molecules, such as trout IL-1\$\beta\$, also contain the IL-1 signature with differences in the same positions.

The obtained sequences were used for homology and phylogenetic tree analyses and revealed that the trout nIL-1F, together with the Tetraodon nIL-1F in the latter case, had little relatedness to known IL-1F. Thus, the trout nIL-1F showed only 15–20% identity and 27–34% similarity with other known IL-1F in trout, and 13–19% identity and 20–33% similarity to the known human IL-1F (Table II), although the highest identity/similarity was to trout IL-1\$\beta\$, followed by mammalian IL-1\$\beta\$. In phylogenetic tree analysis (Fig. 2), the nIL-1F formed a separate and distinct clade, with no clear relationship with other known IL-1F.

A sequence-structure compatibility search using mGenTHR EADER (51) predicted the $\beta$-trefoil fold (52) and showed highest upstream of a 20-bp poly(A) tail. Analysis of the predicted translation revealed a protein of 41.2 kDa, with a theoretical isoelectric point (pI) of 5.67. No signal peptide was apparent, in common with other IL-1F, but it contained a potential ICE cut site (LEXD) at aa positions 126–129, and a thrombin cut site (RGR) at aa positions 166–168. Interestingly, the latter divided the protein into an N-terminal acidic domain (pI of 4.67) of 18.6 kDa and a C-terminal basic domain (pI of 8.75) of 22.6 kDa. No signal peptide or transmembrane domain was detectable within the sequence, but two potential glycosylation sites (NXT) were found at aa positions 271–273 and 341–343 (Fig. 1).

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A sequence-structure compatibility search using mGenTHR EADER (51) predicted the $\beta$-trefoil fold (52) and showed highest
similarity to human IL-1 and IL-1ra structures \( (p < 0.0001)\). These IL-1F family members are structurally classified in the “cytokine” \((50,353)\) and 2.80.10.50 superfamilies according to SCOP (53) and CATH classification (54), respectively. Other members of this structural superfamily include fibroblast growth factors. A BLAST search of the ProDom database of protein domain families (55) identified the PD002536 (IL-1, the expect value \(0.0001\)) and the PD932791 (IL-1F7, the expect value \(0.9\)) domains as the most similar. A search of Pfam database (56), a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families, identified the IL-1/18 domain (the expect value \(10^{-5}\)), supporting further that nIL-1F is indeed an IL-1F family member.

Gene organization and promoter analysis

The nIL-1F gene was \(\sim 22\ Kb\) in size (EMBL accession nos. FM207658, FM207659, and FM207660; www.ebi.ac.uk/emb1/). Analysis of the gene organization of nIL-1F revealed that it contained 8 exons and 7 introns and was a unique organization relative to other IL-1F (Fig. 3). The 5’-UTR was located in the first exon, together with the first 12 bp of the open reading frame, which finished in exon 8. The predicted ICE cut site was located over exons 4 and 5, while the thrombin cut site was wholly located in exon 5. Intron phase was also examined, and as with most of the other IL-1F, the trout nIL-1F has the typical 1,1,0 arrangement for the last three introns, with the IL-1 family signature located in the final exon after the phase 0 intron.

Analysis of potential transcription factor binding sites on the 5’-flanking region of the nIL-1F gene identified many sites, including those for CHOP:C/EBP, C/EBP (NF-IL-6), AP-1, AP-2, AP-4, NF-Y, Gfi-1, and ER (Fig. 4). In comparison to the trout IL-1\(\beta\) promoter region (40), most of these sites were also identifiable in the 5’-flanking region of the IL-1\(\beta\) gene, as well as a TATA box in both. The major differences were the lack of NF-\(\kappa\)B and Sp1 sites in the nIL-1F promoter and presence of the ER site.

Genome analysis

A search of the Tetraodon genome found the nIL-1F gene on chromosome 5 (Fig. 5), with FGIF and PIR downstream and ACES2 upstream. These neighboring genes were sought in the human genome, where they were also found to be colocated on chromosome X, in the same order and orientation but with the BMX gene (the gene for bone marrow tyrosine kinase, a TEC family kinase) in between rather than a nIL-1F. A broader look at the gene synteny between these two locations found a number of other genes conserved in this region, including GRPR, NHS, AP1S2, and ZRSR2 (Fig. 5). In contrast, analysis of the gene locus containing the fish IL-1\(\beta\) gene in Tetraodon found the gene on chromosome 12, with no further IL-1F members obvious and without any clear gene
synteny with the exception that CKAP2L was an immediate neighbor and is adjacent to the cluster of nine IL-1F genes seen on human chromosome 2 (Fig. 5).

**Modeling**

Since nIL-1F shows low similarity to other known IL-1F, a specialized protein modeling method for modeling proteins that adopt the β-trefoil fold was used (42). PSI-BLAST search within the Protein Data Bank (PDB) identified the human IL-1ra (PDB code: 1IRA, chain X) as the most similar structure. Sequences of relevant IL-1F were aligned with the C-terminal region of the trout nIL-1F (starting at Asn207) using CLUSTAL (33) (Fig. 6), and in accordance with observations in other IL-1F cytokines, the trout nIL-1F had higher sequence similarity within the 12 predicted β-strand regions than in loops connecting them, where nine regions of insertions and deletions were identified (Table III). The protein models of nIL-1F (Fig. 7) show the characteristic 12 β-strands of the β-trefoil structure folding into three similar β-β-β-loop-β trefoil subunits. The overall structure has a 3-fold pseudosymmetry and consists of a six-stranded barrel that is capped on its one side by a triangular hairpin triplet (52).

To simulate whether a potential interaction of the trout nIL-1F with the known trout IL-1R1 could occur, a theoretical model of the trout IL-1R receptor was retrieved from PDB (PDB code: 1OU1) (57). Similar to the human IL-1R, the trout receptor has three extracellular Ig-like domains. Domains I and II are intricately connected, forming an extensive interface, while domain III is linked via a flexible linker that allows rotation of domain III upon ligand binding. The trout nIL-1F/IL-1R complex was simulated by structural superposition of the trout nIL-1F and IL-1R models onto the human complex (PDB code: 1IRA). Analysis of the complex revealed a number of regions that may be of importance in binding of nIL-1F to the receptor as outlined below.

Within the receptor binding site A, the trout nIL-1F possesses three exposed arginines (Arg<sup>229–231</sup>) in the loop connecting β-strands 2 and 3. Their positively charged side chains are located proximal to an acidic, positively charged region of the receptor domain I (Asp<sup>25–28</sup>) located in the loop between strands b1 and c1. The close complementarity of the positive and negative sites suggests that they might interact directly with each other. Other charged surface residues, such as receptor Arg<sup>115</sup> located at the loop between strands b2 and c2 and Glu<sup>132</sup> of loop 10–11 of nIL-1F, may be involved in additional interactions. Other regions of potential interaction include nIL-1F loop 3–4, which is situated in the cleft between receptor domains I and II, potentially interacting with strand a2 of the receptor, and loop 1–2, which is situated proximal to receptor strands a2 and b2. It is therefore likely that as in the human complex, nIL-1F forms extensive interactions in site A with its receptor.

In humans, the largest conformational differences in IL-1ra upon receptor binding occur in site B loop 4–5 (58). This loop is shorter than that of the agonist by six residues and interacts with receptor loop f3–g3. In nIL-1F, loop 4–5 is longer than in trout IL-1β by three residues, suggesting that it interacts with the receptor domain III. The length of loop 7–8 is identical in the human agonist and antagonist molecules, but they differ in their sequence composition and structural conformation, and only the agonist interacts with the receptor loop c3–d3 (59). The trout nIL-1F and IL-1β loops 7–8 are longer by four residues than their human homologs. Despite the low confidence in the long loop regions of the protein models, it is likely that, as in the human antagonist/receptor complex, nIL-1F loop 7–8 and receptor loop c3–d3 are too far apart to interact.

Additional regions may be important in conferring the inactive receptor conformation, such as the receptor loop b3–c3. This is longer by six amino acids compared with the human receptor and is potentially interacting with residues from strand 12 and the barrel rim.

**Expression analysis**

The trout nIL-1F was found to be broadly expressed across a wide range of tissues (Fig. 8) and was relatively highly expressed in lymphoid tissues such as the gills, spleen, and head kidney. In comparison to constitutive expression of the two IL-1β isoforms present in trout, nIL-1F expression was noticeably higher in liver, spleen, head kidney, intestine, and brain. Following infection of fish with a common Gram-negative bacterial pathogen, Y. ruckeri, the expression level of nIL-1F in the spleen was studied. nIL-1F increased significantly (p < 0.05)
FIGURE 11. Northern blot analysis of the nIL-1F and IL-1β transcripts. Total RNA samples from control (unstimulated) and LPS-stimulated RTS-11 cells were separated using 1.1% formaldehyde-MOPS agarose gel transferred to nylon membranes and hybridized in three independent reactions with 32P-labeled cDNA probes for nIL-1F, IL-1β, and β-actin.

by 24 h postinfection, whether determined as relative expression (Fig. 9A) or fold change (Fig. 9B), and it remained high for the duration of the experiment (72 h). In comparison to the two IL-1β isoforms known in trout, which showed rapid increases in expression within 6 h of infection, the increase in nIL-1F was delayed and did not increase by the same magnitude but remained high when the IL-1β levels were beginning to decline at 72 h postinfection.

Attempts to determine cell types able to express the trout nIL-1F focused on available cell lines. nIL-1F was expressed in RTS-11 cells, a trout macrophage cell line. When these cells were stimulated with two proinflammatory stimuli, LPS or trout rIL-1β, nIL-1F was found to increase (p < 0.05) in expression level with similar kinetics to IL-1β but did not increase to the same degree, as found in vivo (Fig. 10). Northern blot analysis also showed the induction of the expression of nIL-1F and IL-1β in RTS-11 cells by LPS (Fig. 11). Only a single hybridizing band was detected, with the expected size from the nIL-1F cDNA sequence of ~2 kb, indicating that no major splice variants exist.

Recombinant protein production and bioactivity testing

The recombinant C-terminal protein, containing 197 aa and starting at Gly160, was successfully produced in E. coli and purified (Fig. 12A). The rnIL-1F was tested in terms of whether it had agonist or antagonist activity (Fig. 12B) on the expression of a number of proinflammatory genes, as determined by real-time PCR. For the former, addition of rnIL-1F to RTS-11 cells was found to have no effect on the expression of IL-1β, IL-8, COX-2, and TNF-α. It also did not affect its own expression or that of TGF-β. In contrast, addition of rIL-1β to these cells had a marked and significant effect on proinflammatory gene expression, and also increased nIL-1F expression, although again TGF-β expression was unaffected. When the cells were pre-treated with rnIL-1F before addition of rIL-1β, the up-regulation of gene expression induced by rIL-1β was inhibited (Fig. 12B). As the concentration of rnIL-1F was diluted out (from 4 to 0.5 μg/ml), keeping the amount of rIL-1β constant, the inhibitory effect on rIL-1β-induced proinflammatory gene expression and nIL-1F gene expression was progressively lost, showing that nIL-1F antagonized IL-1β activity. No effect on TGF-β expression was seen, suggesting that this was not a generalized effect on transcript expression. A second recombinant C-terminal protein produced in CHO cells, of 175 aa and beginning at Met191, showed essentially similar results (data not shown), indicating that the potential glycosylation sites in the C terminus were not essential for bioactivity, and that the loss of a further 22 aa also had little impact.

FIGURE 12. Production and bioactivity of recombinant nIL-1F. A. Induction of expression and purification of recombinant nIL-1F. Samples were mixed with LDS loading buffer and run on a NuPAGE Novex Bis Tris MiniGel (4–12%) (Invitrogen) under reducing conditions. Lane 1, Protein marker (New England Biolabs); lanes 2 and 3, lysates from uninduced and isopropyl β-D-thiogalactoside-induced bacteria transformed with pTriEx6/nIL-1F (theoretical molecular mass, 26.15 kDa); lane 4, purified and refolded nIL-1F. B. Antagonizing rIL-1β action with recombinant nIL-1F. Different amounts of recombinant nIL-1F (0.5–4 μg/ml) were added to RTS-11 cells half an hour before the addition of rIL-1β (10 ng/ml) for 4 h. The gene expression of a housekeeping gene (EF-1α), a rIL-1β-unresponsive gene (TGF-β1), as well as known IL-1β responsive genes (IL-1β, IL-8, TNF-α, COX-2) and nIL-1F itself, was detected by real-time RT-PCR. The gene expression level at each treatment was first normalized to that of EF-1α and then expressed as the percentage of the rIL-1β only group (100%). The results represent the average ± SEM of three samples from individual flasks. *, p < 0.05 of the mL-1F plus rIL-1β treatment groups relative to that of rIL-1β only treatment.
Discussion

The present paper describes the identification of a novel IL-1 family member present in teleost fish. The unique gene organization, genomic location, and low homology to known family members suggest this molecule is not homologous to known IL-1F. However, it bears the hallmarks of other members in having a predicted C-terminal β-trefoil structure, containing an IL-1F signature region within the final exon, an upstream potential ICE cut site that may allow processing of the precursor molecule, and a gene organization where the last three introns are in phase 1,1,0 in common with other IL-1F (although 2,1,0 is also seen in some IL-1F) (13). Additionally, expression of the gene is clearly increased following infection, or stimulation of macrophages with proinflammatory stimuli such as LPS or IL-1β. However, the recombinant protein does not appear to induce proinflammatory gene expression and in fact inhibits the effects of rIL-1β in in vitro studies, suggesting it may be a novel fish IL-1β antagonist.

Many of the IL-1F are produced as precursor molecules that are cleaved to release a biologically active mature peptide (3), so while formal proof that this occurs with the nIL-1F is still to be obtained, it would not be surprising for an IL-1F to undergo such processing. Several members are known to be cleaved by ICE, as with IL-1F1, 4, 7, and 11. While the ICE cut site is highly conserved during evolution in IL-1F4 (27), in IL-1F1 it is not obviously present outside of mammals (60). However, a number of other enzymes can also cleave the IL-1β precursor, and it is quite possible these are of more importance in lower vertebrates, where evidence of IL-1β processing is apparent (61, 62). In the case of the nIL-1F further possibilities also exist for processing, with a thrombin cut site present, that interestingly divides the protein into an N-terminal acidic domain and a C-terminal basic domain, and would give a mature peptide with a shorter N-terminal tail beyond the predicted first β-sheet of the β-trefoil. Processing of the precursor is associated with secretion in some IL-1F (e.g., IL-1F2 and IL-1F4), while others remain intracellular or membrane bound, as seen with IL-1F1 (3).

The produced C-terminal recombinant protein was demonstrated to have biological activity, in that it could antagonize the effects of trout rIL-1β. These studies could not distinguish between whether this effect was due to receptor antagonism or to some signaling interference brought about after binding to a different receptor. However, modeling of the nIL-1F and the trout IL-1RII does hint at the possibility that the nIL-1F could be a receptor antagonist. Thus, comparison of the human IL-1ra/IL-1R complex and the modeled trout nIL-1F/IL-1R complex revealed that potential interactions between the two molecules and the receptor involved the same loops. However, different types of bonds are possibly formed as a consequence of the lack of sequence similarity between human IL-1ra and the trout nIL-1F. This is particularly evident in receptor site A. For instance, the trout nIL-1F loop 10–11 is shorter by two residues compared with humans and may form fewer interactions in that region. Moreover in site B, modeling studies suggest that the receptor loop c3–d3, which is important in human IL-1β/IL-1R binding, in trout is located far away from loop 7–8 in a similar way to the human IL-1ra/IL-1R complex. Another region of interaction in receptor binding site B of human IL-1β/IL-1R and IL-1ra/IL-1R complexes is between the loop connecting strands 4 and 5 of IL-1β and IL-1ra and the loop between strands f3 and g3 of the receptor (2, 59). In humans, the length of the loop 4–5 in the antagonist is shorter by six residues compared with agonist molecules, and upon binding it confers a tilt by 20° to domain III of the receptor compared with the agonist/receptor complex. The structural conformation of domain III is critical in determining agonist/antagonist activity by inducing binding of the accessory protein and activation (58). The predicted nIL-1F loop 4–5 is longer than the corresponding loop of trout IL-1β, possibly resulting in alternative conformations of domain III in each complex. It is possible that nIL-1F binding confers an “inactive” orientation to domain III of the receptor. It is therefore likely that, as in the human complex, the orientation of domain III relative to domains I and II determines its agonist/antagonist activity by inducing binding of the accessory protein. However, if this is the case, then in trout the inactive conformation of the receptor is achieved by different antagonist-receptor interactions than in the human complex.

The trout nIL-1F is constituatively expressed at relatively high levels in a number of immune sites, including the gills, spleen, and head kidney, suggesting it is immune relevant. Additionally, it is highly inducible and within a day of bacterial infection increases some 10- to 12-fold. Curiously, relative to the expression levels of IL-1β isoforms in trout, the induction is slower to occur but remains higher for a longer period, perhaps supporting the contention that it is involved in the down-regulation of IL-1β-induced inflammatory responses. A macrophage cell line was shown to express this gene, and good induction was seen following stimulation with LPS or rIL-1β. Analysis for regulatory elements within the 5’ flanking region revealed a number of potentially important transcription factors involved in controlling the expression of this gene. However, in contrast to the IL-1β promoter (trout and mammals) (40) and IL-1-ra promoter (63, 64), no NF-κB sites were discovered, consistent with the rather slow induction seen, with NF-κB being a rapidly activated transcription factor induced via pattern recognition receptor recognition of pathogen-derived molecules (8). IL-1ra exists as secreted (sIL-1ra) and intracellular (iIL-1ra) molecules, driven by two independent promoters, with alternative splicing of the exon containing the signal peptide generating the two forms (65). The sIL-1ra promoter also requires PU.1 and GABP for LPS responsiveness (66), whereas the icIL-1ra promoter requires NF-IL-6 in addition to NF-κB (64), with AP-1 driving constitutive expression (66). Multiple NF-IL-6 and AP-1 sites were identifiable in the nIL-1F promoter region, although in contrast to the icIL-1ra promoter there was a TATA box, also present in the sIL-1ra promoter (65), suggesting conventional transcriptional initiation of the nIL-1F gene.

The genome analysis revealed that the IL-1β locus in fish lacks any other IL-1F, in distinct contrast to the situation in mammals. Thus the IL-1ra gene (IL-1F3), together with many others, is either lacking completely from fish or is in some other region of the genome yet to be discovered. However preliminary data here indicates that perhaps a functional homolog of an IL-1ra exists in fish, that together with IL-1RII (67) could potentially control IL-1β function. Nevertheless, many curious features of nIL-1F exist, and its regulation at the gene and protein levels and the means by which it exerts its biological activity require future investigation.

Disclosures

The authors have no financial conflicts of interest.

References


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