Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β

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Abstract

In this paper the cloning of interleukin−1β (IL-1β) from the fish *Dicentrarchus labrax* (sea bass) is described. Using degenerate primers designed from known IL−1β sequences, a cDNA fragment was amplified by PCR and elongated by 3' and 5' RACE to give the full-length coding sequence for sea bass IL-1. The cDNA is 1292 bp, lacks a putative ICE cut site, and codes for a deduced peptide of 29.4 kDa with a pI of 5.1. Sequence analysis showed highest amino acid similarity with rainbow trout (62%), Xenopus (46%), and carp (45.5%) IL−1β sequences. Expression studies show that sea bass IL−1β can be upregulated by bacterial lipopolysaccharide both *in vitro* and *in vivo* in leucocytes from blood, head-kidney, spleen, gills and liver, whereas the IL−1β transcript was not detectable in thymus and gut-associated lymphoid tissue. Northern blot analysis with head-kidney leucocyte RNA showed a main LPS-upregulated band at 1.3 kb, and two minor bands at 0.9 and 3.0 kb, respectively. Phylogenetic comparisons with IL−1β from other vertebrates is presented.

Introduction

Teleost fish preceded mammals by millions of years, and studies on their immune system will give valuable insights into the evolution of defence mechanisms within vertebrates and may have relevance for disease control in fish farming, where losses due to infectious disease are of major economic importance. Whilst major advances in some areas of fish immunology have been seen in recent years (Iwama & Nakanishi, 1997; Secombes & Pilström, 2000), studies of cytokine genes are meagre, in common with most nonmammalian vertebrates. One exception is interleukin−1β (IL-1), a common name for two diverse proteins, IL−1α and IL-1β, that represent the first members of a growing family of regulatory and inflammatory cytokines. The many biological activities of IL−1β have been extensively reviewed (Dinarello, 1997). Along with the IL−1 receptor antagonist (IL-1ra) and IL-18, IL−1β plays a pivotal role in the regulation of acute inflammation. Both IL−1α and IL−1β are produced as 31 kDa precursor molecules, they share about 23% homology in their peptide sequence and, in common with other IL−1β and fibroblast growth factor family members, have a -trefoil structure of 12 - strands (Nicola, 1994).

In fish, it has been known for many years that channel catfish macrophages and carp epithelial and macrophage cell lines can produce conditioned media containing IL−1β bioactivity (Clem et al., 1985; Siegel et al., 1986), and more recently this observation was extended to carp macrophages and neutrophils (Verburg van Kemenade et al., 1995; Weyts et al., 1997). In these studies the similarity in structure between fish and human IL−1β was confirmed by experiments where antisera to human IL−1β could inhibit the biological activity of fish IL-1. More recently, IL−1β has been cloned and sequenced in rainbow trout (Zou et al., 1999a), plaice (Secombes et al., 1999) and carp (Engelsma et al., 1999; Fujiki et al., 2000). Trout macrophages have been shown to be responsive to recombinant trout IL−1β (rIL-1β) (Zou et al., 2000a) and carp rIL−1β has been shown to have adjuvant properties (Yin & Kwang, 2000).

Recently, a second IL−1β gene has been found in trout, displaying 82% peptide sequence homology with the already reported IL−1β (Pleguenzuelos et al., 2000).

The sea bass *Dicentrarchus labrax* is present in the Mediterranean and Black Seas and the European Atlantic Ocean, and is a major aquacultured seawater fish species. In this paper the homology cloning of IL−1β from this species is described, and studies of the modulation of IL−1β gene expression in response to *in vivo* and *in vitro* stimulation with bacterial lipopolysaccharide are presented. The phylogenetic comparison of this important cytokine with other vertebrate species is discussed.

Materials and Methods

Fish and cells

Sea bass *Dicentrarchus labrax*, weighing 300–500 g, were grown and maintained in a local aquaculture fish farm (La Rosa, Orbetello, Italy). For in vivo stimulation, fish were kept in 6 m3 plastic tanks for 24 h. Prior to handling, fish were anaesthetised with 1 mg ml1 tricaine methanesulphonate (Sigma), and bu#ers and solutions used in handling fish cells were brought to 355 mOsm kg−1β with 2 M NaCl. All organs were immediately removed and disrupted by teasing through cell strainers (100 m) in cold HBSS. Leucocytes were obtained from blood, head-kidney, spleen, thymus, liver and intestine as previously described (Scapigliati et al., 1995, 1996, 2000a). Briefly, the resultant cell suspensions were resuspended at 1108 cells ml1, washed with HBSS at 680g, and then layered over a discontinuous density Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) diluted in HBSS to yield densities of 1.02 and 1.07 g cm3 (Romano et al., 1997). Peripheral blood leucocytes (PBL) were obtained from heparinised blood. Whole blood (2 ml) from individual fish was washed twice in HBSS-heparin, diluted in 8 ml of the same solution and loaded over Percoll gradients as described above. After centrifugation (30 min at 840g) at 4 C, cells at the interface between the two densities were collected and washed twice (10 min at 680g) at 4 C in RPMI 1640 medium (Gibco).

IL−1β cloning and sequencing

Total RNA was isolated from LPS-stimulated cells using Tripure (Boehringer, Mannheim, DE) following the manufacturers instructions and suspended in diethyl pyrocarbonate treated water. Poly(A) mRNA was purified using an Oligotex mRNA kit (Qiagen). cDNA was synthesised from 2 g of mRNA by MMLV reverse transcriptase (Gibco) at 42 C for 50 min with oligo-dT primer (Gibco) and used as a template for PCR reactions. Initially, PCR was performed using

degenerate forward (F) and reverse (R) primers designed to conserved regions of all known IL−1β sequences, to allow amplification of the sea bass IL−1β gene (F13 and R4, Table 1). All reaction mixtures were overlaid with mineral oil. The cycling protocol was one cycle of 94 C for 5 min, 35 cycles of 94 C for 45 s, 48 C for 45 s and 72 C for 45 s, followed by one cycle of 72 C for 10 min. PCR reactions were conducted using The Minicycler model PTC-150-16 (MJ Research, U.S.A.). PCR products (20 l) were visualised on 2% (w/v) agarose gels containing ethidium bromide (10 ng l1), using 123 bp ladder (Sigma) as size markers. Controls for the presence of DNA contamination were performed by PCR using the RNA samples as template. DNA amplified by PCR was inserted into the pGEM-T Easy vector (Promega). Following transfection into competent Escherichia coli cells, recombinants were identified through red-white colour section when grown on MacConkey agar (Sigma). Plasmid DNA from at least three independent clones was recovered using an alkaline lysis based method (Birnboim, 1983), and sequenced using an ABI 377 Automated Sequencer (Applied Biosystems). Sequences generated were analysed for similarity with other known sequences using the FASTA (Pearson & Lipman, 1988) and BLAST (Altschul et al., 1990) suite of programs. Direct comparison between cDNA sequences was performed using the GAP program (Needleman & Wunsch, 1970), within the Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package (version 10·0, 1999) and multiple sequence alignments generated using CLUSTAL W (version 1·74) (Thompson et al., 1994). Having isolated a partial sea bass IL−1β sequence, the 5 and 3 ends were obtained by rapid amplification of cDNA ends (RACE)-PCR, using gene-specific primers (Table 1). Poly(A) mRNA was purified using an Oligotex mRNA kit (Qiagen). cDNA was synthesised from 2 g of mRNA by MMLV reverse transcriptase (Gibco) at 42 C for 50 min with oligo-dT primer (Gibco) and used as a template for PCR reactions. In 3 RACE-PCR, cDNA was transcribed from poly(A) mRNA using an oligo-dT adaptor primer (Table 1). PCR was performed initially with the sea bass IL−1β F1 primer and the adaptor

primer, followed by a semi-nested PCR using a second sea bass IL-1-specific forward primer (F2) and the adaptor primer. In 5 RACE-PCR, cDNA was transcribed from poly(A) mRNA using an oligo-dT primer (Gibco), treated with E. coli Rnase H (Promega), purified using a PCR Purification Kit (Qiagen), and tailed with poly(C) at the 5 end with terminal deoxynucleotidyl transferase (TdT, Promega). PCR was performed initially with the sea bass IL-1-specific primer R1 (Table 1) and the oligo-dG primer, and then semi-nested with a second sea bass IL-1-specific reverse primer (R2) and oligo-dG. Sequencing and similarity searches were as described before. In addition, an unrooted phylogenetic tree was constructed by the 'neighbourjoining' method using CLUSTAL W and PHYLIP packages (Felsenstein, 1985; Saitou & Nei, 1987), on full-length amino acid sequences. For the alignments the gap opening penalty was 10 and the gap extension penalty was 0·05, and the data were bootstrapped 10 000 times. The sea bass IL−1β sequence was also analysed for the presence of a signal peptide using SignalP software (Nielsen et al., 1997) and for hydrophobicity (Kyte & Doolittle, 1982).

Sea bass IL−1β expression after *in vitro* and *in vivo* stimulation

For in vitro stimulation, washed leucocytes obtained as above were counted in trypan blue to assess cell viability, resuspended at 3106 cells ml1 in RPMI medium and maintained in culture with or without 5 g ml1 of E. coli serotype 055:B5 lipopolysaccharide (LPS, Sigma) for 4 h at 25 C. At the end of these periods, total RNA was isolated from approximately 5107 cells for each sample using Tripure Isolation Reagent (Boehringer, Mannheim) following the manufacturers procedure, and used for cDNA synthesis after checking its quality with Ready-To-Go PCR beads (Amersham, U.K.). For RT-PCR analysis sea bass-specific IL−1β primers PseabassIL−1β F and Pseabass IL−1β R (Table 1) were used with a cycling protocol of 94 C for 5 s, 16 or 36 cycles at 90 C for 45 s, 60 C for 45 s, 72 C for 45 s, one cycle at 72 C for 10 s, giving a product size of 238 bp. cDNA encoding for -actin was amplified and employed as

a positive control for RT-PCR, as it is highly conserved and expressed constitutively in the examined tissues (Laing et al., 1999). The -actin primers gave the expected product size of 540 bp.

For in vivo administration, 100 g LPS in 500 l of PBS was injected intraperitoneally into each fish, and 24 h later RNA was isolated from gill, head-kidney, spleen, PBL, liver, thymus and gut for RT-PCR as described above.

Northern blotting

RNA isolated from head-kidney leucocytes was separated on a 1·2% denaturing formaldehyde agarose gel. Twenty g of total RNA from control and LPS-stimulated cells was loaded, and electrophoresed at 30 V o/n in MOPS bu#er (Sigma). RNA was transferred to a Nylon Hybond N (Amersham) membrane by capillary blotting using 20SSC bu#er (Sigma). The RNA was fixed to the membrane by exposure to UV light. Blots were prehybridised at 65 C in 6SSC, 0·5% SDS, 5Denhardt's (Sigma) solution containing 100 g ml1 salmon sperm DNA for 2 h. A probe for IL−1β mRNA was prepared by purifying the 238 bp fragment obtained by PCR amplification (as described above) from an agarose gel, with a QIAquick Gel Extraction Kit (Qiagen, Germany). This fragment was subsequently labelled with 32P using the Prime-a-Gene Labelling System (Promega). Unincorporated nucleotides were removed by passage through Sephadex G-50 resin. The blots were hybridised at 65ºC o/n with the labelled probe using the same solution for prehybridisation. The filter was then washed in 2SSC+0·1% SDS at 65ºC for 15 min. This washing step was followed by two more 15 min washes at 65 C in 0·5SSC+0·1% SDS, and a final rinse in 0·1SSC+0·1% SDS. The membrane was put into an X-ray cassette with film (Kodak) and left for 3 days at 70ºC prior to development. To confirm equal loading of the RNA samples and to allow densitometric analysis, the probe was removed from the membrane using 0·1% SDS, at 95ºC for 15 min and the membrane reprobed using a sea bass -

actin cDNA probe. The actin probe was isolated from a gel and labelled as described above.

Hybridisation and washing conditions for the actin blots were exactly as for the IL−1β probe.

Densitometric analysis was performed on the autoradiographs using the Kodak Digital Science

ID System. Results were normalised with the corresponding actin control.

Results

cDNA cloning

Total RNA was extracted from LPS-stimulated head-kidney leucocytes, and its quality was checked by PCR with -actin primers. Initial PCR was performed using degenerate primers F13 and R4 (Table 1), which amplified a product of 201 bp (Fig. 1). The obtained product was sequenced and on analysis was found to have high homology with other fish and vertebrate IL−1β genes (not shown). To extend the sea bass sequence, semi-nested 3-RACE PCR was performed with specific primers F1 and F2 (Table 1), and a product of 635 bp was obtained that contained the 3-end of the gene, with an in-frame stop codon, 3 UTR containing six RNA instability motifs (ATTTA) and a polyadenylated tail (Fig. 2). Subsequently, 5 RACE-PCR was performed using sea bass specific primers R1 and R2 (Table 1), and a sequence of 661 bp was amplified (Fig. 1). The three cDNA products gave a contiguous sequence of 1292 bp that contained the full-length open-reading frame, with four potential glycosylation sites and the IL−1β family signature (Fig. 2). Nucleotide and amino acid homology of sea bass IL−1β with other known IL−1β sequences is shown in Table 2. At the nucleotide level the best scores were with IL−1β of trout (64%), carp (49·5%) and chicken (48%), whereas similarities of the deduced peptide sequence were 63% for trout, 46% for Xenopus and 45·5% for carp. Multiple alignment of these amino acid sequences revealed highest conservation of residues known to be involved in the secondary structure of IL-1, and showed a lack of aspartic acid residues in the region where ICE cuts the mammalian IL−1β precursor (Fig. 3). The deduced peptide has a MW of 29·4 kDa, and an isoelectric point of 5·1. The sea bass IL−1β peptide hydrophobicity profile showed that the sequence had several regions of high hydrophobicity (Fig. 4), and lacked a hydrophobic signal peptide, as determined by SignalP analysis. The EMBL accession number of the obtained sequence is AJ269472.

A phylogenetic tree was constructed using the CLUSTAL W and PHYLIP packages, and the results are shown in Fig. 5. The sea bass sequence branches with trout, with carp and then Xenopus/chicken as nearest neighbours, and mammals are more distant neighbours.

IL−1β expression analysis

Expression analysis was performed by RT-PCR using sea bass specific IL−1β primers (see Table 1) and RNA extracted from cells stimulated in vitro with LPS, or after in vivo administration of LPS (Figs 6 and 7, respectively). These experiments were performed initially using 35 cycles for PCR but the quantity of product obtained was almost the same in samples with and without LPS. Therefore, the number of cycles employed in PCR for DNA amplification was reduced to 16, the minimal number to have clear bands in agarose electrophoresis, to allow semi-quantitative analysis of the results.

For in vitro experiments, stimulation for 4 h with LPS (Sigma) at 5 g ml1 was used, because this concentration has been shown previously to be stimulatory for trout leucocytes (Zou et al., 1999a). Serum was not included in the cell culture medium to keep background levels of IL−1β expression to a minimum (Zou et al., 2000b). RT-PCR analysis of RNA extracted from LPSstimulated cells of gills, liver, head-kidney, PBL and spleen is shown in Fig. 5 (upper panel). A clear amplified product of the expected size (238 bp) was seen in both unstimulated and LPS-stimulated cells from gill, head-kidney and spleen. In the gill and head-kidney samples the IL−1β product was clearly induced by detectable in stimulated samples, being very weak in liver. A higher MW amplification product at c. 346 bp was also observed in LPS-treated gills, headkidney and PBL, the samples with the highest transcript level. When cloned and sequenced this band was found to be IL−1β but containing an intron of 108 bp. On one occasion a third faint product of approximately 320 bp was also seen, in the LPS-stimulated gill samples, but was not cloned and sequenced.

As a control, RT-PCR analysis was performed with the same samples using primers for -actin (Fig. 6, lower panel), and in all samples a band was readily detectable with transcript levels between pairs of unstimulated/stimulated samples being approximately equal. To check for genomic DNA contamination within RNA samples, RT-PCR experiments using sea bass specific primers were performed with RNA, before cDNA synthesis, and no amplified products were detected.

RT-PCR analysis of RNA extracted from gill, head-kidney, spleen, PL, liver, thymus and gut, after i.p. injection of LPS, is shown in Fig. 7 (upper panel). A clear amplified product of the expected size (238 bp) was seen in LPSstimulated cells from the gill, head-kidney, spleen and PBL, with the larger 346 bp product being present in the stimulated head-kidney and PBL samples. A weaker product was present in stimulated liver samples, whereas thymus and intestine samples were negative. All control tissues were also negative (not shown for thymus and intestine). As a control, RT-PCR analysis was performed on the same samples using primers for -actin (lower panel), and in all cases a product of the correct size was amplified.

Northern blot analysis is shown in Fig. 8. Three inducible transcripts were clearly detected in LPS-stimulated leucocytes from head-kidney; one dominant band at 1·3 kb, representing the previously described IL−1β transcript, plus two weaker transcripts at approximately 3 kb and 900 bp. The 1·3 kb transcript was also seen in RNA from control cells (Fig. 8a) but was clearly at a lower level of expression relative to the LPS stimulated cells (Fig. 8b). After normalisation of the data, using the -actin transcript level as an internal standard (not shown), densitometric analysis showed that stimulation with LPS induced a 2·2-fold increase in expression of the 1·3 kb transcript.

Discussion

IL−1β plays a pivotal role in regulating immune responses, and its recent discovery in teleost fish dates cytokine evolution at c. 350 My ago (Secombes et al., 1996, 1999; Scapigliati et al., 2000b). One strategy to isolate IL−1β in fish employs degenerate primers designed to amplify evolutionarily-conserved regions in the IL−1β molecule. Using this approach, cDNA from LPS-stimulated cells likely to secrete IL−1β has been used in the search for fish IL-1, and was successful for the cloning of IL−1β from rainbow trout (Zou et al., 1999a), the first non-mammalian sequence obtained. Taking advantage of observed sequence similarities, this study used sets of degenerate primers to isolate and sequence sea bass IL−1β from LPS-stimulated head-kidney leucocytes. An initial product of c. 201 bp was amplified, and when sequenced it showed high homologies with fish and mammalian IL-1. Further studies employed RACEPCR to obtain the 3- and 5-ends. The full-length sea bass IL−1β cDNA consisted of 1292 bp (Fig. 2), and its homology with other known IL−1β coding regions is summarised in Table 2. The best peptide similarity was with rainbow trout (63%) followed by Xenopus and carp (46% and 45·5%, respectively). When translated, this cDNA gave a peptide of 261 amino acids and had a deduced MW of 29·4 kDa. In mammals, IL−1β is produced as an inactive precursor that must be cleaved intracellularly by IL−1β converting enzyme (ICE) (Thornberry et al., 1992). In contrast to this, fish IL−1β precursors cloned to date lack a clear ICE cut site (Zou et al., 1999a; Fujiki et al., 2000), and this also appears to be the case for the analysed sea bass cDNA sequence. IL−1β bioactivity in fish is found in fractionated supernatants of 70 kDa and 15 kDa in catfish (Ellsaesser & Clem, 1994) and 22 kDa and 15 kDa in carp (Weyts et al., 1997). Based on the fish cDNA sequences, the full-length IL−1β precursor in trout (Zou et al., 1999b) and in carp (Fujiki et al., 2000) is predicted to be 28 kDa, and the predicted molecular mass of sea bass IL−1β appears quite similar to these species. Analysis of the sea bass IL−1β sequence

with SignalP software revealed the absence of a signal peptide, common to other known IL-1s, indicating the molecule is not secreted in a conventional manner. Nevertheless, a relatively large number of hydrophobic regions were present in the molecule, which may be useful for future studies on the interactions of sea bass IL−1β with its receptor (Scapigliati et al., 1989). A phylogenetic tree was constructed, based on the peptide similarity among known species, and sea bass IL−1β was located in the 'piscine' group being closer to trout than carp. This is an interesting observation, as salmonids (trout) are ancient teleosts and preceded percychthydae (sea bass and carp) in evolution. One di#erence between the trout and carp sequences relates to the organisation of the two genes. In trout, a relatively small gene consisting of six exons is present. Carp possess a more 'mammalian-like' gene with seven exons (Engelsma et al., 1999). Consequently, determining the gene structure of sea bass IL−1β will be valuable for the phylogenetic analysis of IL−1β evolution in vertebrates, and these studies are in progress.

Analysis of IL−1β expression in sea bass were performed using in vivo and in vitro approaches and, similar to the situation in trout, LPS was shown to upregulate the sea bass gene. From the in vivo experiments it was clear that IL−1β can be upregulated quite e$ciently, because in unstimulated fish no expression was detectable. However, the in vitro experiments showed expression of IL−1β in unstimulated samples from spleen, head-kidney and gills, although the expression was clearly upregulated by LPS. The discrepancies observed between the two di#erent experiments could be related to a low level of induction during the isolation and culture of the cells (Zou et al., 2000b), because adherence to plastic culture dishes is known to have this e#ect in mammals (Dinarello, 1991). In both these experiments a larger band at c. 346 bp was also seen to increase following LPS stimulation, in cell types where transcript levels of IL−1β were highest. This product was sequenced and found to contain an intron, and is likely to represent an incompletely spliced transcript as found in trout and carp IL−1β genes (Zou et al., 1996b; Engelsma et al., 2000), because no genomic DNA contamination of the samples

was detectable. It has been suggested that such transcripts may have a role in post-translational regulation of the mature, fully spliced transcript (Jarrous et al., 1994). It was clearly expressed at a lower level relative to the mature transcript, and this may explain why it was mainly detected in the stimulated samples. It was not universally present in tissues that expressed IL−1β after injection of LPS but again this could relate to relative transcript level, as in LPS-stimulated samples lacking the 346 bp product the -actin levels were typically low. By Northern blot analysis three transcripts were detected, a main one at 1·3 kb upregulated by LPS, a minor one at 0·9 kb, and a faint band at c. 3 kb. This pattern was similar to that observed by Northern blotting in LPS-stimulated head-kidney leucocytes from rainbow trout (Zou et al., 1996b). Whilst it is not clear if one of the additional transcripts could represent a second IL−1β gene, as recently discovered in trout (Pleguenzuelos et al., 2000), in fact in this species the additional transcripts appear to represent related genes, or transcripts varying in their untranslated regions. Future studies will be addressed towards the production of the sea bass recombinant IL−1β molecule, thus allowing 'in vitro' cell biology studies to elucidate the evolution of the many activities related to IL−1β in vertebrates.

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Table 1. Oligonucleotide primers used for the isolation and study of seabass IL-1.

Name	Sequence (5' to 3')	Information
Forward (F) 13	GGGAAAGAATCTRTACCTGTCYTG	Designed against multiple alignment of known interleukin-1β (IL-1β) sequences
Reverse (R) 4	GTGCTGATGAACCAGT	
Sea bass IL-1β -F1	ATCTGGAGGTGGACAAAGC	Designed against sea bass IL-1 β products
Sea bass IL-1β-F2	CCGGTTTGAACATCAGCACC	
Sea bass IL-1β-R1	GCTGATGTTCAAACCGGAGTC	
Sea bass IL-1β-R2	CGCTGTCCGAGGTGATGTT	
Oligo-dG	GGGGGGIGGGIIGGGIIG	Used for RACE-PCR
Oligo(dT)-adapter	CTCGAGATCGATGCGGCCGCT17	
Adaptor primer	CTCGAGATCGATGCGGCCGC	
Pseabass IL-1β-F	CACAAGGATGGAGAGGAGCC	Used for tissue expression
Pseabass IL-1β-R	GATGTTGAAGGCTCGGTGG	
Actin-F	ATCGTGGGGCGCCCCAGGCACA	
Actin-R	CTCCTTAATGTCACGCACGATTTC	Used for control expression

(R=A/G, Y=C/T, I=inosine)

Table 2. Nucleotide and amino acid percentage homologies of sea bass IL−1β with other known IL−1β genes

| | Amino acid | | Nucleotide |
	Similarity	Identity	identity
Human	38.8	29.6	45.2
Mouse	38.5	30.1	44.4
Chicken	44.2	33.9	48.3
Xenopus	46.0	34.6	44.0
Trout	62.7	56.2	64.0
Carp	45.5	35.5	49.5

Figure Legends

Figure 1. Position of primers used to isolate the sea bass IL−1β gene sequence and obtained products.

Figure 2. Compiled full length sea bass IL−1β sequence. Features in bold include the start and stop codon, potential glycosylation sites and RNA instability motifs (ATTTA) in the 3 untranslated region. The polyadenylation signal (AATAAA) is underlined, and the IL−1β family signature is boxed.

Figure 3. Alignment of the predicted sea bass IL−1β translation with other known IL-1s. Identical (*) and similar (: or .) residues identified by CLUSTAL are indicated. Position of the 12 beta sheets of the known human mature peptide crystal structure are indicated below the alignment. The ICE cut site (aspartic acid residue) in mammals is indicated with an arrow.

Figure 4. Kyte–Doolittle hydrophobicity plot of sea bass IL-1β. Positive values denote hydrophobic residues, negative values represent hydrophilic residues. Note the lack of a hydrophobic signal peptide.

Figure 5. Unrooted phylogenetic tree showing the relationship between the full-length sea bass IL−1β amino acid sequence with other representative IL−1β sequences in different vertebrate groups. The tree was constructed by the 'neighbour-joining' method using the CLUSTAL and PHYLIP packages, and was bootstrapped 10 000 times.

Figure 6. RT-PCR analysis of sea bass IL−1β (upper panel) and -actin (lower panel) expression of cells cultured in vitro without (−) or with (+) LPS. Cells were isolated from (a) gill, (b) liver, (c) head-kidney, (d) blood leucocytes and (e) spleen. Molecular markers were included between the gill and liver samples, and between the head kidney and blood leucocytes samples.

Figure 7. RT-PCR analysis of sea bass IL−1β (upper panel) and -actin (lower panel) expression of cells from control fish (−) and fish injected intraperitoneally with LPS 24 h earlier (+). Cells were isolated from (a) gill, (b) head-kidney, (c) spleen, (d) blood leucocytes, (e) liver, (f) thymus and (g) gut. Only stimulated samples are shown for thymus and gut.

Figure 8. Northern blot analysis of IL−1β expression in sea bass head-kidney leucocytes after in vitro culture without (a) or with (b) LPS.

Figure 1
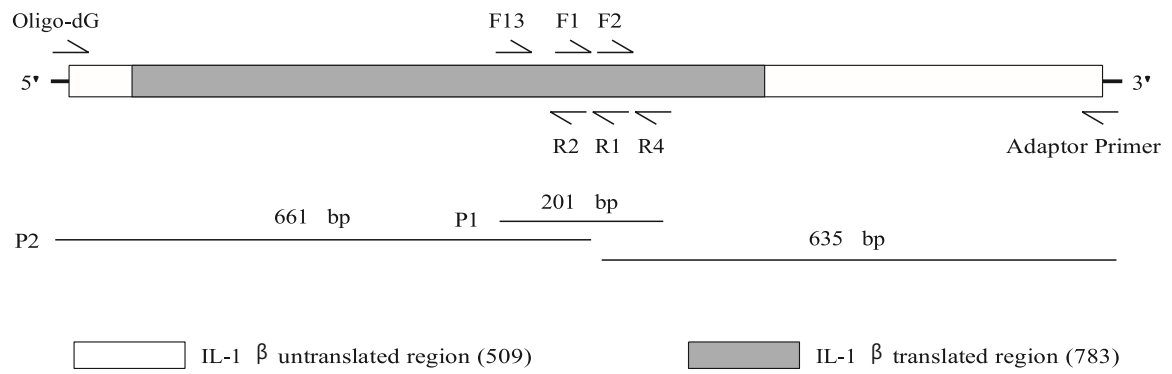
Figure 2

```
     ATCAACATTGACAGAGCAACTGTTGGGATCTTAACCAAACACACTAAAGCAACTTCTAAT
1    ---------+---------+---------+---------+---------+---------+    60

     CTACTGACCTTAACAGAAACATGGAATCTGAGATGAAATGCAACATGAGCGAGATGTGGA
61   ---------+---------+---------+---------+---------+---------+    120
                            M  E  S  E  M  K  C  N  M  S  E  M  W  R

     GATCCAAGATGCCCCAGGGACTGGACTTGGAGATTACCCACCACCCACTGACAATGAGGC
121  ---------+---------+---------+---------+---------+---------+    180
      S  K  M  P  Q  G  L  D  L  E  I  T  H  H  P  L  T  M  R  R

     GCGTGGTCAACCTCATCATTGCCATGGAGAGACTGAAGGGCTTCAGTTCAGAGACACTGA
181  ---------+---------+---------+---------+---------+---------+    240
      V  V  N  L  I  I  A  M  E  R  L  K  G  F  S  S  E  T  L  M

     TGAGCACTGAGTTCAGAGATGAAAACCTGCTAAACATCATGCTGGAGAGCATAGTGGAAG
241  ---------+---------+---------+---------+---------+---------+    300
      S  T  E  F  R  D  E  N  L  L  N  I  M  L  E  S  I  V  E  E

     AGAAAATTGTGTTTGAGCGCGGAACAACTCCAACAGCGCAGTACAGCAAGCGACGCGAGG
301  ---------+---------+---------+---------+---------+---------+    360
      K  I  V  F  E  R  G  T  T  P  T  A  Q  Y  S  K  R  R  E  V

     TCCAATGCAGCGTGACCGACAGCGAAAAGAGGAGCTTAGTTCTGGTCCCGAACAGCATGG
361  ---------+---------+---------+---------+---------+---------+    420
      Q  C  S  V  T  D  S  E  K  R  S  L  V  L  V  P  N  S  M  E

     AGCTCCACGCAGTGATGCTGCAGGGAGGCTCGGACAGATGCAAAGTCCAACTGAACATGT
421  ---------+---------+---------+---------+---------+---------+    480
      L  H  A  V  M  L  Q  G  G  S  D  R  C  K  V  Q  L  N  M  S

     CAACCTACTTGGACCGTACACCCAGTGCTGAGGCTCAGACTGTGGCTCTGGGCATCAAGG
481  ---------+---------+---------+---------+---------+---------+    540
      T  Y  L  D  R  T  P  S  A  E  A  Q  T  V  A  L  G  I  K  G

     GCACAAATTACTACCTGTCCTGCCACAAGGATGGAGAGGAGCCAACCTTGCATCTGGAGG
541  ---------+---------+---------+---------+---------+---------+    600
      T  N  Y  Y  L  S  C  H  K  D  G  E  E  P  T  L  H  L  E  V

     TGGTGGACAAAGCCAGTCTGGCGAACATCACCTCGGACAGCGACATGGTGCGATTTCTCT
601  ---------+---------+---------+---------+---------+---------+    660
      V  D  K  A  S  L  A  N  I  T  S  D  S  D  M  V  R  F  L  F

     TCTACAAACAGGACTCCGGTTTGAACATCAGCACCCTGACGTCTGTCCCCTTCAGCAACT
661  ---------+---------+---------+---------+---------+---------+    720
      Y  K  Q  D  S  G  L  N  I  S  T [L  T  S  V  P  F  S  N  W]

     GGTACATCAGCACAGCAGAGGAGAACAACCGGCCGGTGCAGATGTGCCAGGAGAGTGCCA
721  ---------+---------+---------+---------+---------+---------+    780
     [Y  I  S  T  A  E  E  N  N  R  P  V] Q  M  C  Q  E  S  A  R

     GACGCCACCGAGCCTTCAACATCGACAACCTGAAAGTGGATCCAACAACTGAGGATCAAG
781  ---------+---------+---------+---------+---------+---------+    840
      R  H  R  A  F  N  I  D  N  L  K  V  D  P  T  T  E  D  Q  V

     TTTGTCCCCTTTTGAATGGACAGTAAAATACACTGCACTTCAGATATTTTGTATTGTGTT
841  ---------+---------+---------+---------+---------+---------+    900
      C  P  L  L  N  G  Q  *

     CAAAAAAATCAGCAACAAAGATATCTATTTTTGTTTTAAATCACTACCACTGTTGACTAG
901  ---------+---------+---------+---------+---------+---------+    960

     AACGACTTCTGCAAGTTTTACAGATTGTTTATTTACTGTATGTACCAAGTACAAAAAGTA
961  ---------+---------+---------+---------+---------+---------+    1020

     AGATGCTTAATGTATTGACATTGTCAAGTATCACCACAAGGTGGCATTATTGTGCTGCCT
1021 ---------+---------+---------+---------+---------+---------+    1080

     GGTGAGCACCTTTTGTATTTATTGTGCTGAATGACATGAAACGCTATTATTGCTTTTATC
1081 ---------+---------+---------+---------+---------+---------+    1140

     TATTAATTTATTTTATTTTAATTATTTATTAACTAATTAATATAATATTTAATATATATG
1141 ---------+---------+---------+---------+---------+---------+    1200

     TATCAATTTTGAAAACTGCTTTCTAACAATTATTTAAATGTGCAATGAATCTTTAATAAA
1201 ---------+---------+---------+---------+---------+---------+    1260

     GCCTTTAATTTTCAGTAAAAAAAAAAAAAAAAA
1261 ---------+---------+---------+--    1292
```

Figure 3

```
Seabass                                                     MESEMKCNMSEM------WRSKMPQGLDL
Trout                                                       MDFESNYSLIKNTSESAAWSSKLPQGLDL
Carp        MACHEYVHQLDLSEAFETDSAI--YSDSADSDELDCPDPQSMSCQCDMH-------DIKL
Xenopus     MALVPDLSSIPMEGYSGDDEMFYSDSPSGMKDDMGDAAQWQSSTSHCSLDI---HVQITH
Chicken     MAFVPDLDVLESSSLSEETFYG—PSCLCLQKKPRLDSEHTTV-----------DVQVFT
Human       MAEVPKLASEMMAYYSGNEDDL--FFEADGPKQMKCSFQDLDLCPLDG------GIQLRI
                                                                       :


Seabass     EITHHPLTMRRVVNLIIAMERLKGFSSETLMSTEFRDENLLNI--MLESIVEEKIVFERG
Trout       EVSHHPITMRHIANLIIAMERLKGGEGVT-MGTEFKDKDLLNF—LLESAVEEHIVLELE
Carp        ELSSHPHSMRQVVNIIIAVERLKHIKNMSSG--KFCDEELLGF--ILENVIEERLVKPL-
Xenopus     GKGSL-HSFRKAVVLVVAVEKLKRGKE-----RFFGDEDLLLL--DSIFVEEEIGFSQAK
Chicken     TVRKGRGARSFRRAAVLVVAMTKL----LRRPRSRDFADSDS--ALLEEVFEPVTFQRLE
Human       SDHHYSKGFRQAASVVVAMDKLRKMLV--PCPQTFQENDLSTF--FPFIFEEEPIFFDTW
                              ::.:     :                 :           . *   .


Seabass     TT-----PTAQ--YSKRREVQCSVTDSEKRSLVLVPNS----MELHAVMLQGGSDRCKVQ
Trout       SAPP-ASRRAAG-FSSTSQYECSVTDSENKCWVLMNEA----MELHAMMLQGGSSYHKVH
Carp        -------NETPIYSKTSLTLQCTICDKYKKTMVQSNKLSDEPLHLKAVTLSAGAMQYKVQ
Xenopus     ETY----ASAST-YRYQRATTCRIKDTSNKCFVMQKFHEN--AQLVALQLQGANIQREEK
Chicken     SSY----AGAPA-FRYTRSQSFDIFDINQKCFVLESP-----TQLVALHLQGPSSSQKVR
Human       DNE-AYVHDAP-----VRSLNCTLRDSQQKSLVMSGP-----YELKALHLQGQDMEQQVV
Secondary                 ↑:          : *   ::   *         .* *: *..       :
Structure                     RSLNCTLRD   KSLVMS         YELKALHL          VV


Seabass     LNMSTYLDRTPSA----EAQTVALGIKGTNYYLSCHKDGE---EPTLHL-EVVDKASLAN
Trout       LNLSSYVTPVPIET---EARPVALGIKGSNLYLSCSKSGG---RPTLHLEEVADKDQLKS
Carp        FSMSTFVSSATQK----EAQPVCLGISNSNLYLACTQLDG--SSPVLILKEASGSVNTIK
Xenopus     VSMAFYATQPHQGG---SKRPVALGLAGKNLYLSCRATEDGQDSPKLYLEEISNIKDVKG
Chicken     LNIALYRPRGPRGSAGTGQMPVALGIKGYKLYMSCVMSGT---EPTLQLEEADVMRDIDS
Human       FSMSFVQGEESN-----DKIPVALGLKEKNLYLSCVLKDD---KPTLQLESVDPKNYPKK
Secondary   ..::               .*.**:   :  *::*        * * * .
Structure   FSMSF            DKIPVALGLK  NLYLSCVLK      PTLQLESVD


Seabass     ITSDSDMVRFLFYKQDSGL---NISTLTSVPFSNWYISTAEE--NNRPVQMCQESAR-RH
Trout       ISQQSDMVRFLFYRRNTGV---DISTLESASFRNWFISTDMQQDYTKPVDMCQKAAPNRL
Carp        AGDPN--DSLLFFRKETGT---RYNTFESVKYPGWFISTAFD--DWEKVEMNQMPTT-RT
Xenopus     EDL----NRFIFMKSQDGLNETSTNSFESVAFPGWYISTSQR--ENELVQMVHQKNQEAI
Chicken     VEL----TRFIFYRLDSPTE--GTTRFESAAFPGWFICTSLQ--PRQPVGITNQPDQVNI
Human       KME----KRFVFNKIEIN----NKLEFESAQFPNWYISTSQA--ENMPVFLGGTKGGQDI
Secondary          ::*  : .          : *. :  .*:*.*         * :
Structure          FVFNKIEI      KLEFESA   WYISTS       NMPVFLG        I


Seabass     RAFNIDNLKVDPTTEDQVCPLLNGQ
Trout       TTFTIQRHN----------------
Carp        TNFTLEDQKRI--------------
Xenopus     KDFNLFSVI----------------
Chicken     ATYKLSGR-----------------
Human       TDFTMQFVSS---------------
Secondary   :.:
Structure   TDFTMQFVS
```
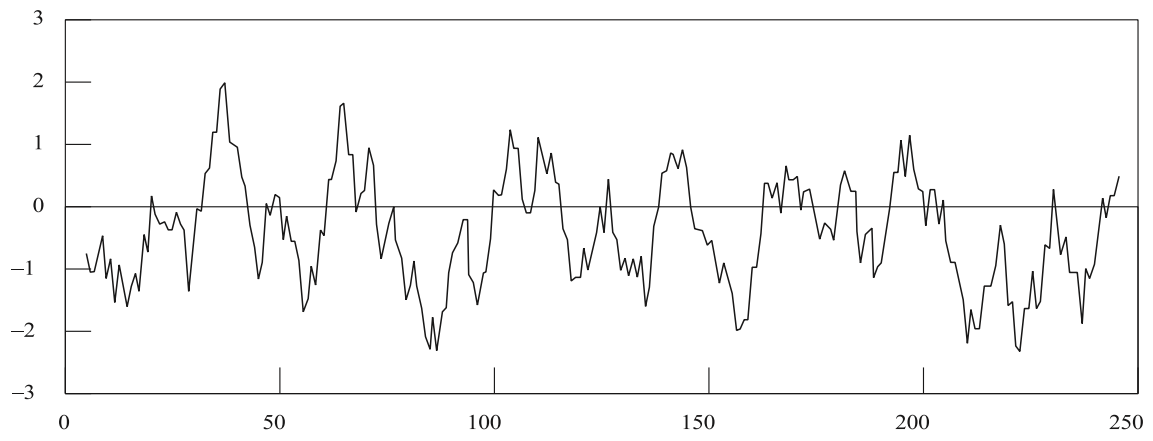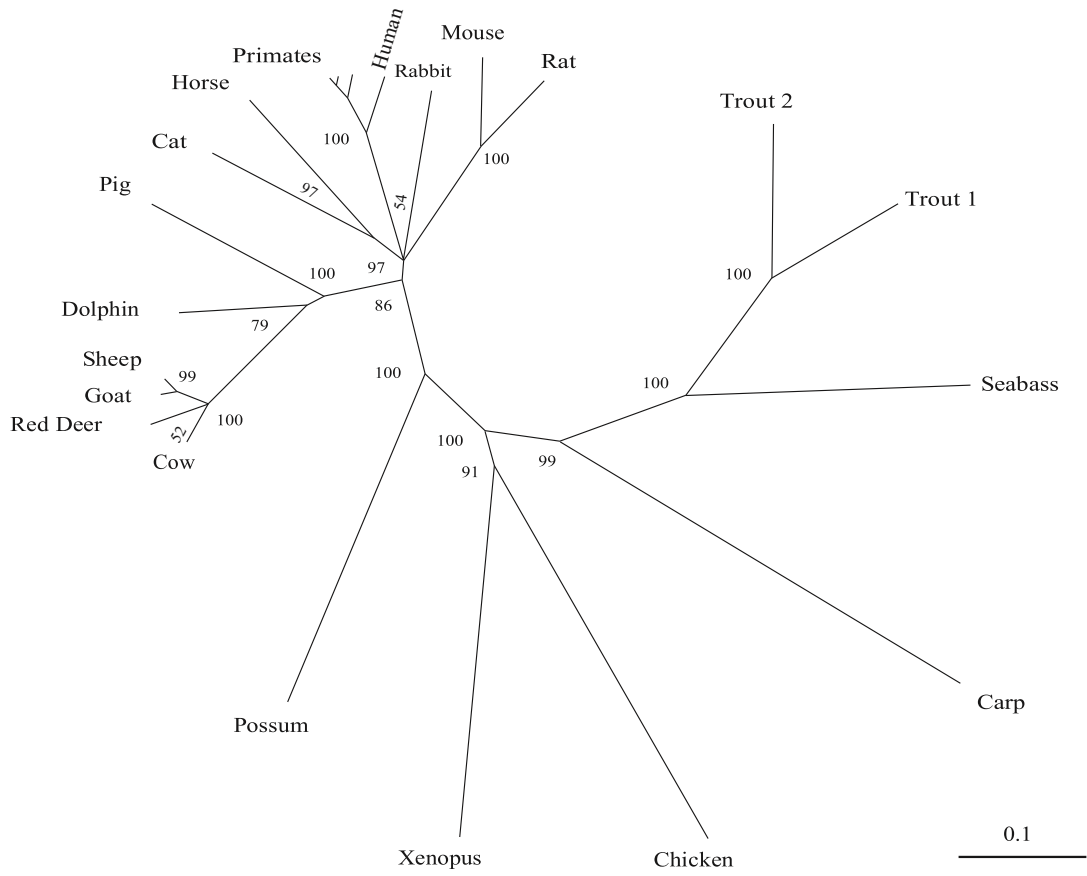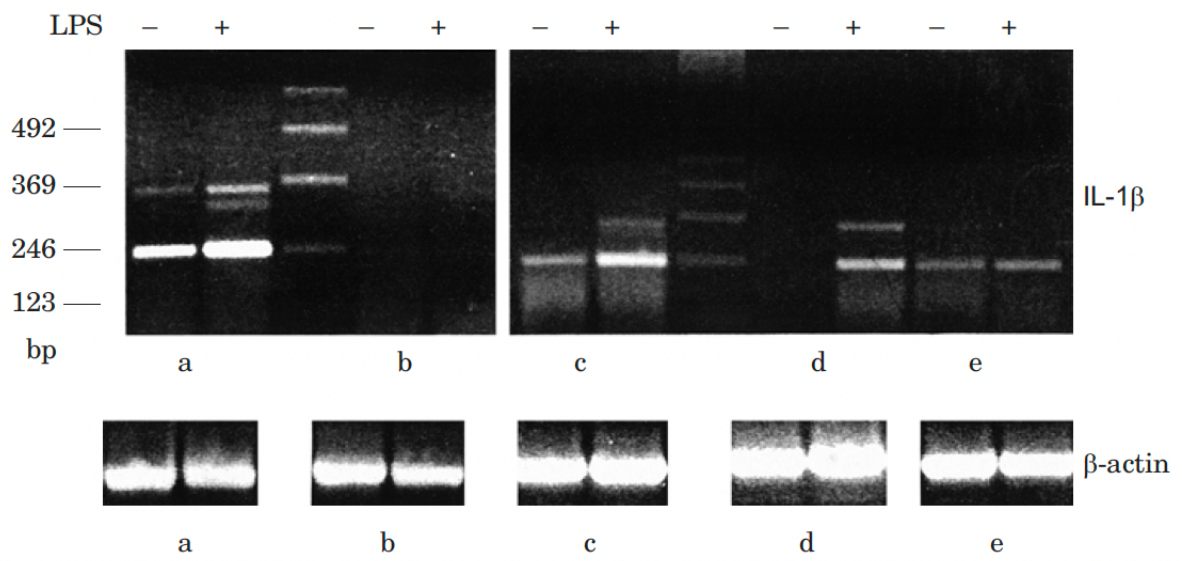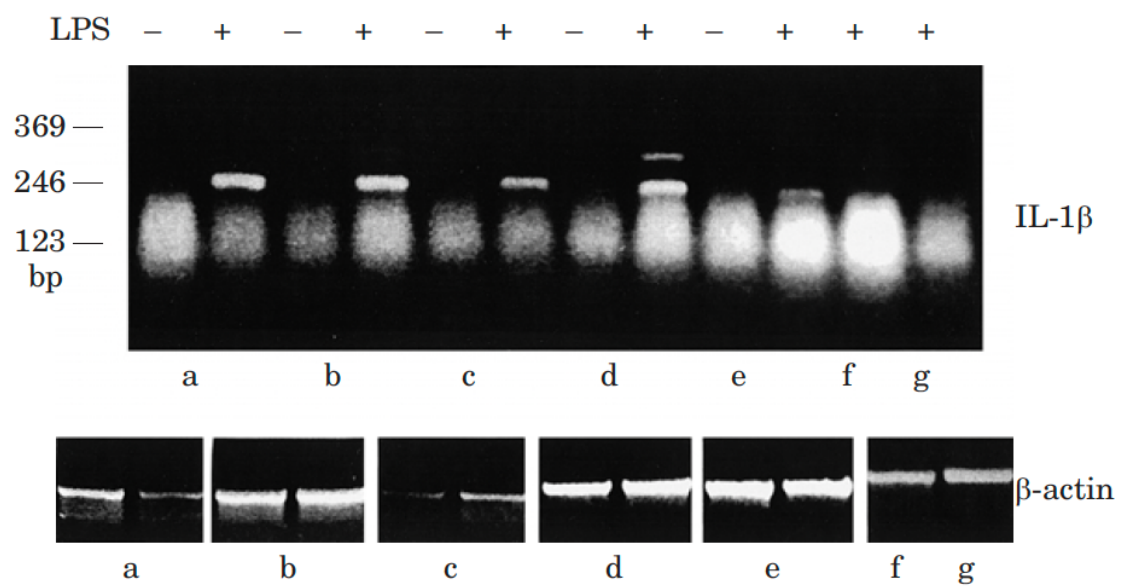
Figure 4

Figure 5

Figure 6

Figure 7

Figure 8