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Conservation of Structural and Functional Features in a Primordial CD80/86 Molecule from Rainbow Trout (Oncorhynchus mykiss), a Primitive Teleost Fish

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In mammals, interaction of CD28 with CD80 or CD86 molecules provides costimulatory signals for T cell activation that leads to increased IL-2 gene and protein expression by activated T cells. Thus far, CD80 and CD86 have been cloned and functionally characterized only in mammals and birds. To shed light into the evolution of CD80 and CD86, we have cloned and functionally characterized a rainbow trout (rt) molecule (rtCD80/86) that shows the highest degree of sequence conservation and phylogenetic relationship with CD80 and CD86 molecules. Moreover, its genomic organization was almost identical to that of human CD86. Rainbow trout possess one membrane-bound and two soluble CD80/86 transcripts, all of which are derived from the same rtCD80/86 gene. The membrane-bound form exhibited its highest degree of expression in lymphoid tissues, particularly on B cells. Incubation of trout leukocytes with LPS and bacteria leads to up-regulation of rtCD80/86 gene expression. Importantly, we show that trout and other teleost fish contain a single CD80/86 gene, thus suggesting that this gene may represent the ancestor from which CD80 and CD86 arose by gene duplication in more evolved species. To gain further insights into the function of rtCD80/86, we have identified and cloned trout IL-2 and have shown that recombinantly produced trout CD80/86 up-regulates the expression of IL-2 in trout blood leukocytes. Significantly, this finding indicates that the capacity to modulate IL-2 expression is a primordial function that has been conserved both in fish and mammalian CD80/CD86 molecules throughout 350 million years of evolution.


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2 The nucleotide sequences of rainbow trout CD80/86 and IL-2 presented in this article have been submitted to GenBank under accession numbers EU927451–EU927453, FJ467621–FJ467623, and FJ507781 (for CD80/86) and FJ571512 and FJ571513 (for IL-2).

3 Y.-A.Z. and J.-i.H. contributed equally to this work.

4 Address correspondence and reprint requests to Dr. J. Oriol Sunyer, Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, 413 Rosenthal Building, 3800 Spruce Street, Philadelphia, PA 19104. E-mail address: sunyer@vet.upenn.edu

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Beyond mammals and birds, IL-2 has only been cloned in Takifugu rubripes (fugu), a teleost fish species (21). Fugu IL-2 appears to have a secondary structure comprised of three helices instead of the four typically found in IL-2 from mammals and birds. An important finding that strengthened the identification of fugu IL-2 as a true ortholog of its mammalian counterpart was the conservation of synteny between the chromosomal areas containing the fugu and mammalian IL-2 genes (21). Although the biological roles of mammalian and avian IL-2 have been well characterized, virtually nothing is known about the function of fish IL-2. In this regard, the study on the fugu IL-2 molecule showed that its gene expression could be induced after the treatment of fish leukocytes with PHA. However, its inducibility through the action of a costimulatory molecule (like CD86 or CD80) remains to be investigated in fish.

Thus, the objective of this study was 2-fold. First, to establish the presence of a CD80 or CD86 ortholog in teleost fish; second, to evaluate whether the ability to induce IL-2 expression is conserved in the fish CD80 or CD86 molecule. The identification of CD28 in teleost fish provided the rationale for the existence of CD80- or CD86-like molecules in these species (22, 23). The aforementioned hypothesis is now supported in this study by the cloning of a rainbow trout (rt)5 gene (rtCD80/86) whose deduced primary sequence and overall structure appear to resemble those of both CD80 and CD86. We subsequently identified and cloned trout IL-2 with the goal of evaluating a putative involvement of trout CD80/86 in IL-2 expression. We found that recombinant rtCD80/86 induced the up-regulation of IL-2 expression on trout leukocytes.

Materials and Methods

Fish

Rainbow trout (Oncorhynchus mykiss) (50–100 g) obtained from Limestone Springs Fish Farm were maintained in aquarium tanks using a water recirculation system involving extensive biofiltration, UV sterilization units, and thermostatic temperature control. Water temperature was maintained continuously at 16–17°C. The fish were fed daily with commercial trout pellets, and feeding was terminated 48 h before sacrifice. Fish were acclimated to the laboratory conditions for at least 2 wk before being used in experiments.

Isolation of cDNAs for rtCD80/86 and IL-2

The amino acid sequences for human CD80 and CD86 (GenBank accession nos. NM_005191 and NM_175862, respectively) were used in TBLASTN-based searches to identify orthologous sequences within the teleost expressed sequence tag (EST) databases at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and The Institute for Genomic Research (TIGR; www.tigr.org/db.shtml) and the genome database at Ensembl (www.ensembl.org/index.html). ESTs displaying similarity to human CD80 and CD86 were retrieved from Atlantic salmon (Salmo salar) and other teleost species. Forward primers sCD86F1, -F2, and -F3 (see Table I) were designed based on the salmon EST sequences for 3′-RACE amplification of trout CD80/86 cDNA fragments. For 5′-RACE of trout CD80/86, cDNA produced from head kidney leukocytes was tail-dATP and terminal deoxynucleotidyl transferase (Promega); PCR was then performed with the gene-specific reverse primers sCD86-R1, -R2, and -R3, whose designs were based on the sequenced 3′-RACE amplification products of trout CD80/86. The obtained products were cloned and sequenced as described below.

The amino acid sequence for fugu IL-2 (GenBank accession no. NM_001037994) was used to search homologous sequences within salmonid EST databases at NCBI and TIGR. Candidate ESTs (TIGR: TC77909; NCBI: EG762969 and EG764237) encoding for putative IL-2 sequences were retrieved from Atlantic salmon. 5′-RACE was performed initially with the reverse primers sIL2-R1, -R2, and -R3, whose designs were based on the salmon EST sequences using the 5′-tailed cDNA from PHA-stimulated head kidney leukocytes as a template. Having isolated a 5′-end partial sequence, the 3′-RACE was performed using the gene-specific primers IL2-F4 and IL2-F5 (see Table I) based on the sequenced 5′-end cDNA of trout IL-2.

Identification of other B7 family members from teleost fish

Amino acid sequences for human B7-DC (NM_025239), B7-H1 (NM_014143), B7-H2 (NM_015259), B7-H3 (NM_001024736), and B7-H4 (NM_024662) were used in TBLASTN-based searches to identify orthologous sequences within teleost fish using the NCBI, TIGR, and Ensembl databases as described above.

Genomic analysis of the CD80/86 gene

Genomic DNA was isolated from the head kidney of rainbow trout with a blood and cell culture DNA mini kit (Qiagen) according to the manufacturer’s instructions. The trout genomic DNA was used as a PCR template. The rainbow trout CD80/86 gene was PCR-amplified using the Platinum Pf/S DNA polymerase (Invitrogen) with three specific primer sets as follows: 1) CD86-F2 and CD86ss-qR1; 2) CD86g-F2 and CD86g-R4; and 3) CD86m-qF3 and CD86m-R3 (where F is forward primer, R is reverse primer, g refers to genomic amplification, q refers to quantitative real-type PCR, and ss is short secretory isoform; see Table I). The PCR profile was as follows: 94°C for 3 min followed by 33 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 3 min and finally 68°C for 5 min. The PCR products were cloned and sequenced as described below.

Cloning and sequence analysis

The obtained PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen). Plasmid DNA was isolated from positive colonies using the QIAprep spin miniprep kit (Qiagen) and sequenced with an ABI 377 sequencer (Applied Biosystems) at the DNA Core Facility of the School of Veterinary Medicine, University of Pennsylvania (Philadelphia, PA). Comparing the obtained nucleotide and amino acid sequences for similarity with those in GenBank was performed using BLAST. Prediction of the open reading frame (ORF), the signal peptide, and the transmembrane helices was performed with the programs Translate, SignalP, and TMHMM, respectively, at the ExPasy proteomics server (ca.expasy.org/). Multiple sequence alignments were generated using ClustalW (version 1.81) and BioEdit (version 7.0.9.0), and phylogenetic trees were constructed from the ClustalW-generated alignments using the maximum parsimony methods within the PHYLIP program (version 3.68) and were bootstrapped 1000 times.

Expression and purification of recombinant trout CD80/86 in Escherichia coli

The pET-23b expression vector (Novagen) was modified by replacing the N-terminal T7 tag with a 6×His tag and used for constructing the expression plasmids. The DNA fragments encoding for the extracellular domain (aa 30–231) of rtCD80/86 (where the suffix “m” designates the membrane isoform) were amplified from a plasmid containing rtCD80/86us encoding the primers CD86(E)-F and CD86(X)-R (Table I) and the proofreading Platinum Pf/S DNA polymerase (Invitrogen). The resulting amplification was digested with EcoRI and XhoI and ligated to the modified pET23b vector, which was digested with the same restricted enzyme. The plasmid DNA was prepared and sequenced to confirm that the construct (p23bHis-rtCD80/86) encodes the extracellular domain of rtCD80/86 fused with an N-terminal 6×His tag for expression. As a negative control, an expression plasmid (p23bHis-rtIL-2D) containing an unrelated gene (encoding the constant domain (aa 347–845) of trout IgD (GenBank accession no. AY870261)) was constructed as described above for rtCD80/86.

The expression plasmids p23bHis-rtCD80/86 and p23bHis-rtIL-2D were subsequently transformed in BL21(DE3)-CodonPlus-RIL expression host competent cells (Strategen). Bacterial cells were cultured in Luria-Bertani broth. Recombinant proteins were expressed by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) and purified from inclusion bodies. Briefly, 10 ml of an overnight-saturated culture of bacteria transformed with the aforementioned constructs were inoculated into 1 liter of Luria-Bertani broth containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown at 37°C with shaking. The OD600 of this culture was allowed to reach 0.6 before induction with 0.4 mM IPTG and allowed to grow for an additional 4–6 h. Cells were harvested by centrifugation and resuspended in 25 ml of native lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, and 10 mM 2-ME (pH 8.0)). Cell suspensions

5 Abbreviations used in this paper: rt, rainbow trout (prefix); EST, expressed sequence tag; HPLC, high pressure liquid chromatography; Ig, immunoglobulin; IL, interleukin; MCP, monocyte chemotactic protein; MHC, major histocompatibility complex; NOD, neutrophil-destined, or NOD-LP-1, neutrophil inhibitory factor; PECAM-1, platelet endothelial cell adhesion molecule-1; RANTES, regulated on activation, normal T expressed and secreted; RT, reverse transcriptase; s, secretory isoform; ss, short secretory isoform; T, transmembrane; TLR, toll-like receptor; TTR, transthyretin; UTR, untranslated region.
were mechanically disrupted using an SLM Aminco French pressure cell press (SLM Instruments) and then centrifuged at 12,000 \( \times \) g for 20 min to pellet the inclusion bodies.

Inclusion bodies were solubilized with 25 ml of denaturing lysis buffer (50 mM NaH\(_2\)PO\(_4\), 10 mM Tris-HCl, 8 M urea, and 10 mM 2-ME (pH 8.0)). Recombinant proteins with a His tag were batch bound to a Ni-NTA resin by rocking at room temperature for 1 h. The Superflow resin (Qiagen) was washed three times with 30 ml of denaturing wash buffer (containing the same component as denaturing lysis buffer (pH 6.5)) before being packed into an empty column. The bound protein was eluted using a denaturing elution buffer (containing the same component as the denaturing lysis buffer (pH 5.8)). Fractions containing the recombinant protein samples were pooled and re-fol- ded by gel filtration using a Pharmacia Superdex 200 column (GE Healthcare). The concentration of the purified recombinant protein was determined by SDS-PAGE and Bradford assay (Bio-Rad). Endotoxin (LPS) concentration within the purified recombinant protein was tested at the Cell Center Services (Rad). Endotoxin (LPS) concentration within the purified recombinant protein sample was tested at the Cell Center Services (Rad).

### Table I. Primers used for gene cloning and expression

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<td>3′- or 5′-RACE</td>
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<td>AP</td>
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<td></td>
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IL-2

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β-Actin

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β-Actin

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CD80/86m

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CD80/86s

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IL-2

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\(^a\) V = A, C, or G.  
\(^b\) Salmon CD86.  
\(^c\) The restriction enzyme EcoRI recognition sequence is underlined.  
\(^d\) The restriction enzyme XhoI recognition sequence is underlined.  
\(^e\) Salmon Il2.  

**Isolation of PBLS and head kidney leukocytes (HKLs)**

Collection of trout leukocytes from blood and head kidney was done as previously reported by us (24, 25). Briefly, fish blood was obtained from the caudal vein and immediately diluted (1/5) with DMEM (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 U/ml heparin and then placed on ice. Trout head kidneys were removed aseptically and pressed through a 100-µm nylon mesh and suspended in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 U/ml heparin. The cell suspensions from head kidney or blood were desalting columns (GE Healthcare). As control fractions, we collected the fractions from the unrelated recombinant protein sample eluting at the same position (elution time) as recombinant trout CD80/86. Control fractions were treated identically as the fractions containing CD80/86 and, thus, were also concentrated and desalted into PBS (pH 7.5) with PD-10 desalting columns (GE Healthcare). Concentration of the purified recombinant protein was determined by SDS-PAGE and Bradford assay (Bio-Rad). Endotoxin (LPS) concentration within the purified recombinant protein samples and control samples was tested at the Cell Center Services facility at the University of Pennsylvania.
layered onto a 51/34% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 400 × g for 30 min. The band of leukocytes lying at the interface was collected and the cells were washed three times with DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μM heparin. Leukocytes were used for the experiments described in the sections below.

Rainbow trout IgM- B cells were isolated from PBLs using a MACS separator and the cell separation column LS (Miltenyi Biotec) with a biotinylated anti-trout IgM Ab (mAb 1.14). Ten million PBLs were used for the MACS isolation according to the manufacturer's instruction. The IgM+ cells in the flow-through were incubated at 17°C in DMEM for 2 h in a 24-well cell culture plate, and then the nonadherent IgM+ cells (in the medium) and the remaining adherent cells were collected separately for total RNA extraction.

Stimulation of PBLs and HKLs with LPS and Vibrio bacterin

The isolated PBLs or HKLs (5 × 10^6 cells per 100 μl of medium per well in a 96-well microplate (Nunc) were incubated at 17°C with 50 μg/ml LPS (E. coli 0111:B4; Invitrogen) or with formalin-treated Vibrio anguillarum/Vibrio ordalii bacterin (Vibrogen-2; Novartis Animal Health) at doses of 1/200 and 1/20 of the original bacterin in DMEM supplemented with 10% FBS in an incubator with 5% CO2. After 24 and 48 h of incubation, the stimulated cells were collected, washed with PBS and then subjected to total RNA extraction.

Induction of IL-2 expression by PHA, PMA, and rtCD80/86

PBLs (5 × 10^6 cells per 100 μl of medium per well in a 96-well microplate) were incubated at 17°C with PHA (0.1, 1.0, and 10 ng/ml; Sigma-Aldrich) or PMA (0.1, 10, and 100 ng/ml; Sigma-Aldrich) to analyze the ability of the aforementioned mitogens to induce IL-2 gene expression. In a different set of experiments, PBLs (5 × 10^6 cells per 100 μl medium per well in a 96-well microplate) were incubated at 17°C with recombinant rtCD80/86 at doses of 2 and 5 μg/ml in an incubator with 5% CO2. In parallel, PBLs were also incubated with equivalent volumes of control fractions or PBS as negative controls (see above paragraphs in this section under the heading Expression and purification of recombinant trout CD80/86 in Escherichia coli). To test the effect on the expression of IL-2 by the trace amounts of contaminating LPS present in the rtCD80/86 and control fractions, PBLs were also incubated with 0.1 and 10 μg/ml LPS (E. coli 0111:B4; Invitrogen). After 12 h of incubation, cells were collected and washed with PBS and then subjected to total RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from rainbow trout lymphoid and nonlymphoid tissues (whole blood, head kidney, trunk kidney, spleen, heart, gill, liver, brain, intestine, skin, and muscle), stimulated PBLs and HKLs, as well as sorted IgM+ cells and IgM- cells. Total RNA was extracted with the RNeasy mini kit (Qiagen). Isolated RNA was treated with an RNase-free DNase set (Qiagen) according to the manufacturer’s instruction. To normalize gene expression levels for each sample, equivalent amounts of the total RNA (100 ng) were used for cDNA synthesis with the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) in a 20-μl reaction volume. The synthesized cDNA (20 μl) was then diluted with 80 μl of RNase-free water and used as a template for regular RT-PCR or real-time PCR.

Quantitative real-time PCR (qPCR) and regular RT-PCR analyses of trout CD80/86 and IL-2 expression

qPCR was used to determine the abundance of trout CD80/86 and IL-2 transcripts in tissues and stimulated cells. The qPCRs were performed in duplicate and each contained 4 μl of a diluted cDNA template (4 ng of total RNA equivalents), 5 μl of Power SYBR Green PCR master mix (Applied Biosystems), and 150 nM forward and reverse primers (except for IL-2 primers, which were 250 nM; see Table I) in a 10-μl reaction volume. The amplification profile consisted of an initial denaturation step at 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min followed by elongation stage (95°C to 95°C in an ABI Prism 7500 sequence detection system (Applied Biosystems). Amplification efficiencies for all qPCR primer sets were analyzed following the protocol reported elsewhere (26). For all of the primer sets used in this study, the efficiencies were ≥90% as shown: 97% for CD86m-qF1 and CD86m-qR3 (R² > 0.998); 94% for CD86m-qF1 and CD86mss-qF1 (R² > 0.999); 96% for CD86m-qF1 and CD86mss-qR1 (R² > 0.999); 999% for IL-2b-qF and IL-2b-qR (R² > 0.986); 102% for TNEfa2-qF and TNEfa2-qR (R² > 0.992); 84% for IL-2F6 and IL-2R4 (R² > 0.998); and 93% for Bactin-qF and Bactin-qR (R² > 0.999) (where F is forward primer, R is reverse primer, and q refers to quantitative real-time PCR; see Table I). The specificity of the PCR amplification for all primer sets was verified from the dissociation curves. The relative expression levels for trout CD80/86 or IL-2 were determined using the trout β-actin gene as an internal reference (normalizer). The p values were calculated by Student’s t test.

RT-PCR was performed using Taq DNA polymerase (Invitrogen) with the primer sets for trout IL-2 (IL2-F4 and IL2-R4) and β-actin (Bactin-F and Bactin-R) (Table I). The amplification of the trout β-actin gene served as an internal reference for each sample. The PCR profile for IL-2 expression was as follows: 94°C for 3 min, 36 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 25 s followed by 72°C for 1 min. The PCR profile for β-actin was 94°C for 3 min and 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by 72°C for 1 min. The PCR products were separated on 1.6% agarose gels and visualized by staining with 100 μg/ml ethidium bromide solution (Invitrogen).

Results

Isolation and sequence analysis of trout CD80/86 cDNA

Using the amino acid sequences of human CD80 and CD86 to search salmonid EST databases, we found several Atlantic salmon EST sequences (NCBI: EG833543, DW580717, DR695874, DW580718; TIGR: TC111220) displaying 23–29% identity to either CD80 or CD86. The full-length cDNA for a rtCD80/86 ortholog was obtained by the RACE technique using primers based on the aforementioned salmon EST sequences and the cDNA from rainbow trout HKLs as a template. Using forward primers (Table I) for 3’-RACE, we obtained several partial cDNA products encoding for a molecule that showed roughly equal sequence identity to mammalian CD80 and CD86; thus, we decided to name this molecule rtCD80/86. Based on the consensus cDNA sequence of these rtCD80/86 fragments, 5’-RACE was performed using gene-specific reverse primers (Table I). Two full-length cDNA sequences were subsequently compiled, one of which was comprised of 1361 bp (the membrane isoform rtCD80/86m, GenBank accession no. FJ467621; Fig. 1A) while the other one was 940 bp (short secretory isoform (ss) rtCD80/86ss, GenBank accession no. FJ467623; Fig. 1B). The secretory isoform is probably generated by a differential use of the transcription termination signal that results in the exclusion of both transmembrane and cytoplasmic domains. The ORFs of the membrane (EU927451; 283 aa) and secretory (EU927452; 227 aa) isoforms of trout CD80/86 were confirmed by PCR with primer pairs CD86-F1/CD86m-R1 and CD86-F1/CD86ss-R1, respectively (Table I). Interestingly, among the PCR products amplified with primer pair CD86-F1/CD86m-R1, we obtained an additional secretory isoform (rtCD80/86ss, EU927453; 251 aa) slightly larger than the aforementioned rtCD80/86ss secretory product (Fig. 1B). This alternatively spliced variant is characterized by the loss of a large portion of exon 6, which encodes for the transmembrane (TM) domain. The absence of the highly hydrophobic segment represented by the TM domain in both secretory isoforms was further demonstrated by hydrophobicity plot analysis (data not shown), strongly suggesting that both isoforms are not membrane bound.

The deduced amino acid sequence of rtCD80/86m exhibits a typical structure for members of the Ig superfamily, including mammalian CD80 and CD86 (Fig. 1B). Thus, it consists of an extracellular Ig variable region-like (IgV) domain and an Ig constant region-like (IgC) domain followed by a TM domain and a cytoplasmic tail in which two potential sites for protein kinase C (PKC) phosphorylation at locations 255 (S) and 263 (T) were predicted. The extracellular regions of mammalian CD80 and CD86 are highly glycosylated (27, 28). Similarly, one and seven potential N-linked glycosylation sites (N-X-T/S) exist in the IgV and the
IgC domains of rtCD80/86m, respectively (Fig. 1; see supplemental Table I). Among the eight glycosylation sites, the only one located in the IgV domain (N100; Fig. 2A) is conserved between rtCD80/86m and mammalian/avian CD80. However, within the IgC domain of rtCD80/86m there are three glycosylation sites conserved in the mammalian CD80 (locations 161, 208, and 229) or CD86 (locations 156, 168, and 200) sequences.

A comparison of each domain of rtCD80/86m with the corresponding domains of human, mouse, and chicken CD80 and CD86 (Table II) revealed that the signal peptide, IgV, and IgC domains of rtCD80/86m showed the highest degree of homology with the corresponding domains of mouse CD86 with amino acid identities of 21, 25, and 21%, respectively. For the IgC domains, the highest percentage of identity is to chicken (30%) and mouse (28%) CD86, whereas the trout cytoplasmic domain showed a higher degree of identity to both mouse CD80 (27%) and human CD86 (27%). When making comparisons using the full-length sequences, we found that rtCD80/86m was slightly more similar to the known CD86 sequences (18–19%) than to those of CD80 (16–19%). Importantly, rtCD80/86m showed a higher degree of sequence identity to CD80 or CD86 members (16–19%) than to any of the other five members (B7-DC, B7-H1, B7-H2, B7-H3, and B7-H4) of the B7 family (11–16%, see supplemental Table I).

As shown in the multiple alignment (Fig. 2A), rtCD80/86m exhibits many structural features common to mammalian CD80 and CD86 molecules. The cysteine residues critical to form the interchain disulfide bonds within the IgV (C56 and C127) and IgC domains, respectively (Fig. 1; see supplemental Table I). A multiple alignment (Fig. 2A) reveals that the signal peptide, IgV, and IgC domains of rtCD80/86m showed the highest degree of homology with the corresponding domains of mouse CD86 with amino acid identities of 21, 25, and 21%, respectively. For the

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**FIGURE 1.** Sequences and domain structure of rainbow trout CD80/86 transcripts. A, Nucleotide and deduced amino acid sequences of rtCD80/86m (GenBank accession no. FJ467621). The start and stop codons are in boldface. The putative polyadenylation signals are in boldface and italics. The predicted signal peptide and TM hydrophobic region are in boldface and underlined. The potential N-glycosylation sites are in boldface and italics. The predicted signal peptide and TM hydrophobic region are in boldface and underlined. The potential N-glycosylation sites are in boldface and italics. The predicted signal peptide and TM hydrophobic region are in boldface and underlined. The potential N-glycosylation sites are in boldface and italics.

B, Domain structure of trout CD80/86 transcripts. The sequences have been submitted to GenBank (rtCD80/86m, EU927451; rtCD80/86ss, EU927452; rtCD80/86l, EU927453). All three trout CD80/86 transcripts consist of an IgV domain (IgV) followed by an IgC domain (Ig-C). The membrane-bound form, rtCD80/86m, has a TM domain and a cytoplasmic tail that contains two putative PKC phosphorylation sites. The small gray circles on the Ig domains indicate N-glycosylation sites. S - - S denotes interchain disulfide bonds. The circled P denotes putative PKC phosphorylation sites predicted with the NetPhosK 1.0 server.

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6 The online version of this article contains supplemental material.
FIGURE 2.  Comparison of trout CD80/86 with known CD80 and CD86 sequences.  A, Multiple sequence alignments of trout CD80/86m with mammalian and avian CD80 and CD86 molecules.  The identical and similar residues from the aligned sequences are shaded black and gray, respectively.  Dashes indicate gaps introduced to maintain maximal sequence alignment.  The secondary structure of the human CD80 is shown above the alignment.  Conserved cysteine residues within IgV and IgC domains are indicated by a dollar sign ($) below the alignment.  The amino acids from human CD80 participating in the binding with CD28/CTLA-4 are indicated by an asterisk (*) above the alignment (30, 53), whereas those from CD86 are indicated by an asterisk below the alignment (32).  The amino acids contributing to the CD80 (IgV domain) homodimer interface are indicated by a filled circle (●) above the alignment (29, 31).  Within the IgC domains, the amino acids contributing to CD80/CTLA-4 binding are indicated by a pound sign (#) (33, 54).  The conserved N-glycosylation sites (N-X-T/S) between trout CD80/86 and other CD80 or CD86 are indicated by an ampersand (&) above or below the alignment, whereas those conserved between trout CD80/86 and chicken CD80/CD86 are indicated by an “at” sign (@) above and below the alignment.  GenBank accession numbers of the sequences used in the multiple alignments are shown as follows: human (Hum) CD80, NM_005191; mouse (Mou) CD80, NM_009855; chicken (Chi) CD80, AM050146; Hum CD86, NM_175862; Mou CD86, NM_019388; Chi CD86, AM050135; trout (Tro) CD80/86, EU927451.  B, Hydrophobicity profiling of human CD80, CD86, and trout CD80/86 molecules. The profile shows a hydrophobic signal peptide and a TM region as well as a hydrophilic C terminus for all three molecules. The plot was generated using the Kyte-Doolittle method.
(C180 and C241) domains of mammalian B7 molecules are conserved in rtCD80/86m. Interestingly, the IgC domain of rtCD80/86m possesses four more cysteines (locations 166, 171, 230, and 262) than their mammalian counterparts. Similarly, the corresponding domain of chicken CD80 also contains four additional cysteines, two in the IgV domain (C57 and C145) and two in the IgC domain (C171 and C262), whereas the IgV domain of chicken CD86 contains only one additional cysteine (C149) (Fig. 2A).

In mammals, the IgV domains of CD80 and CD86 are responsible for binding to their receptors (CD28 and CTLA-4) (29). The binding occurs through the interaction of the CDR3-analogous loop (MYPPPY loop) from CTLA-4 and CD28, with the CD80/CD86 surface comprised primarily of the β-strands G, F, C, C’, and C” (29–32). The MYPPPY loops have previously been reported to be conserved in both the CD28 and the CTLA-4 of teleost fish (22, 23); thus, it would be conceivable that the residues involved in receptor binding in rtCD80/86m are similar to those of the mammalian CD80 and CD86 molecules. Confirming the previous hypothesis, we found that the residues Y72, Q74, D77, E126, Y155, G160, and Q165 are conserved in the IgV domain of rtCD80/86m. In addition, the residues H70, H221, and H238 are conserved in the IgC domain of rtCD80/86m.

**FIGURE 3.** Phylogenetic tree of vertebrate B7 family members. The tree was constructed in accordance with the maximum parsimony method of the PHYLIP program, using the amino acid sequences of the IgV and IgC domains. Percentage values shown for each node represent 1000 bootstrap replications. In the tree construction, GenBank accession numbers for the teleost fish B7 molecules reported in this paper are shown in supplemental Table II; GenBank accession numbers for known B7 family members are as follows: human (Hum; *Homo sapiens*) CD80, NM_005191; mouse (Mous; *Mus musculus*) CD80, NM_009855; rat (Rat; *Rattus norvegicus*) CD80, NM_012926; hamster (Ham; *Mesocricetus auratus*) CD80, AB085742; dog (Dog; *Canis lupus familiaris*) CD80, NM_001003147; sheep (Shee; *Ovis aries*) CD80, AY390555; pig (Pig; *Sus scrofa*) CD80, NM_214087; rabbit (Rabb; *Oryctolagus cuniculus*) CD80, D49843; chicken (Chic; *Gallus gallus*) CD80, AM050146; human CD86, NM_175862; mouse CD86, NM_019388; dog CD86, NM_001003146; sheep CD86, AY395982; pig CD86, AY826403; rabbit CD86, D49842; chicken CD86, AM050135; human B7-H1, NM_014143; mouse B7-H1, NM_021893; pig B7-H1, NM_001025221; chicken B7-H1, XM_424811; human B7-DC, NM_025239; mouse B7-DC, BC104138; chicken B7-DC (EST), CF251191; human B7-H2, NM_015790; chicken B7-H2, Y08823; human B7-H3, NM_025240; mouse B7-H3, NM_133983; rat B7-H3, XM_190319; chicken B7-H3, XM_413702; human B7-H4, NM_024626; mouse B7-H4, AY280973; chicken B7-H4, XM_416546. The fish species involved in this tree are rainbow trout (Trou; *O. mykiss*), Atlantic salmon (Sal; *S. salar*), medaka (Meda; *Oryzias latipes*), zebrafish (Zebf; *D. rerio*), stickleback (Stib; *G. aculeatus*), fugu (Fugu; *T. rubripes*), and tetraodon (Tetr; *Tetraodon nigroviridis*).

**Table II.** Protein homologies between trout CD80/86 and other vertebrate CD80 and CD86.

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<th>Protein</th>
<th>Species</th>
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<th>IgC</th>
<th>TM</th>
<th>CYTb</th>
<th>Overall</th>
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<td>18</td>
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<tr>
<td></td>
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<td>41</td>
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<td>14</td>
<td>33</td>
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a Percentages indicate amino acid identity. Protein sequences were aligned with ClustalW (Version 1.81). Signal cleavage sites were predicted with SignalP (version 3.0). The transmembrane domains were predicted with TMHMM (version 2.0). Boldface indicates the highest identity percentage between each domain of rtCD80/86m and the corresponding domains of tetrapod.

b Cytoplasmic domain.
c Percentage could not be determined because of the lack of sequence data.
and K138, which are critical for the interaction between mammalian CD80 and CTLA-4 (30), are conserved in rtCD80/86m. In contrast, only two similar residues (Y85 and S93) in rtCD80/86m show conservation with the corresponding area of mammalian CD86 (32), indicating that the contact interface between rtCD80/86m and its receptors is more similar to that of CD80 than to that of CD86.

Within the IgC domain, the motif SQD(P/N)(E/V)(S/T)(E/K)L(Y/F) between the -strands D and E (residues 212–220) have been suggested to contribute also to the binding of CD80 and CD86 molecules to their receptors CD28 and CTLA-4 (27). In rtCD80/86m, a conserved motif exists at the corresponding location except for the residues at locations 212, 213, and 218 (Fig. 2A). By site-mutagenesis studies, four residues (Q213, D214, E218, and L219) located in the aforementioned motif and another three (F157, P160, and I162) in -strand A were reported to participate in the contact interface between human CTLA-4 and CD80 (33). Among these residues, P160, D214, and L219 are conserved in rtCD80/86m.

In mammals, CD80 and CD86 adopt different oligomeric states on the cell surface; CD80 exists predominantly as a dimer whereas CD86 exists as a monomer (34). Roughly 13 aa within the IgV domain were revealed to contribute at the human CD80 homodimer binding interface (29, 31). Among these residues, eleven (V51, V62, L66, A67, G87, M91, I103, F104, I106, V113, and L115) are hydrophobic, whereas at the same locations most (9/13) of the residues of human and mouse CD86 are hydrophilic. Similar to mammalian CD80, many (7/13) of the residues in the corresponding areas of rtCD80/86 (V52, I61, L66, P67, L91, V104, and W115) and mouse CD80 (V52, P61, G87, L103, Y104, I113, and L115) are also hydrophobic. Furthermore, the only two hydrophilic residues (D105 and T107) at the dimer interface of human CD80 are also conserved in trout CD80/86 and mouse CD80.

Based on hydrophathic analysis (Fig. 2B), the hydrophathy plot of rtCD80/86m is very similar to that of human CD80 and CD86, showing a hydrophobic signal peptide and TM region, and a hydrophilic cytoplasmic region for all three molecules. Particularly in the extracellular IgV and IgC domains rtCD80/86m shows a nearly identical hydrophobicity profile with that of human CD86, whereas the hydrophobicity profile of the trout intracytoplasmic domain is more similar to that of human CD80.

**Identification of CD80/86 homologs from other teleost and phylogenetic analysis**

Taking advantage of available genome databases (i.e., Ensembl genome database) from several teleost fish, we used the trout CD80/86m sequence to search for homologous sequences from zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), and fugu (*T. rubripes*). Importantly, only one gene homologous to rtCD80/86m could be identified from each fish species (see supplemental Fig. 1 and supplemental Table II). None of the analyzed genomes appeared to contain distinct orthologs for CD80 and CD86 genes. It should be stressed that in rainbow trout and other teleost fish we found orthologs for all of the other members of the B7 family (B7-H1/DC, B7-H3, and B7-H4) except for B7-H2 (see Supplemental Table II), thus strongly suggesting that rtCD80/86m and the other teleost CD80/86m genes are not B7-H/DC members. The latter is further supported by the phylogenetic tree of Fig. 3, which shows that teleost fish B7-H1, B7-H3, and B7-H4 molecules group with their mammalian counterparts whereas teleost CD80/86 members form a
sister clade clustering together with the clade containing mammalian and avian CD80 and CD86.

**Genomic organization of trout CD80/86**

The genomic organization of the rtCD80/86 gene was determined by a PCR-based approach using rainbow trout genomic DNA as template. The full-length rtCD80/86 gene was comprised of 5,328 bp (GenBank accession no. FJ607781), which was obtained by assembling three genomic DNA fragments amplified by PCR (Fig. 4A). The exon/intron boundaries of the three transcripts derived from the rtCD80/86 gene were also analyzed (Fig. 4A). The full-length rtCD80/86 gene consists of eight exons and seven introns, including two large exons encoding for the IgV (303 bp) and IgC (279 bp) domains. Although the first four exons are common in all three transcripts, the 3’ regions of the two soluble transcripts have a different alternative splicing pattern (Fig. 4A). Thus, the rtCD80/86s transcript...
is translated differently due to a frame shift at the codon encoding the aa 225 located just after the TM domain (encoded by exon 6), whereas the exon 5 is spliced normally, like that of the membrane isoform (rtCD80/86m). In contrast, the rtCD80/86ss transcript is spliced at the intron 5 and ends abruptly at a stop codon.

Fig. 4B shows the comparison of the exon/intron organization between the rtCD80/86 gene and human genes of representative members of the B7 family. With regard to the exon number and domain organization, the trout CD80/86 gene is almost identical to that of the human CD86 gene, except for the signal peptide region. As can be observed, the gene organization of the IgV and IgC domains is conserved in all members of the B7 family.

Tissue distribution of trout CD80/86 transcripts

The gene expression of rtCD80/86m, rtCD80/86sl, and rtCD80/86ss transcripts was analyzed by qPCR in several rainbow trout tissues (Fig. 5A) and PBLs (Fig. 5B). The expression levels of the membrane isoform rtCD80/86m were the highest of the three isoforms in all of the lymphoid tissues analyzed (Fig. 5A). Within the lymphoid tissues, the strongest expression levels of the membrane isoform were detected in blood leukocytes. Interestingly, we found that within blood leukocytes, IgM/H11001 B lymphocytes showed their highest levels of expression when compared with the levels expressed by the adherent and nonadherent IgM/H11002 cell populations (Fig. 5B). Within the nonlymphoid tissues, the expression of rtCD80/86m was the highest in the gills and to a lesser degree in the liver, probably as a result of the large amount of blood leukocytes contained in these two tissues. With regard to the two secretory isoforms, they were both expressed at low levels in all tissues except for rtCD80/86ss, which showed the highest expression levels of all three isoforms in tissues with a high content of muscle cells, including the heart and muscle.

Regulation of rtCD80/86 gene expression by E. coli LPS and Vibrio bacterin

Regulation of rtCD80/86m, rtCD80/86sl, and rtCD80/86ss gene expression by LPS was studied in vitro using trout PBLs. After 12 h of incubation only the two secretory isoforms showed an increase, albeit minor, in their expression levels when compared with control values. Gene expression levels of all three isoforms increased moderately but in a significant manner, after 24 h of incubation (Fig. 6A). After 48 h, only the membrane isoform showed a significant increase in its expression levels. As a control of immune activation, we showed that IL-1β/H9252 and TNF-α/H9251 expression levels were significantly increased after 24 and 48 h of incubation. A similar gene expression trend was obtained with rtCD80/86m and one of the secretory isoforms (rtCD80/86ss) when incubating trout PBLs and HKLs with a Vibrio bacterin (Fig. 6B).

Cloning and expression analysis of trout IL-2

In mammals, a major effect of CD28 crosslinking by CD80 or CD86 is the induction of IL-2 expression mainly by activated T cells (2–4). To be able to study whether trout CD80/86 had a homologous role, we first had to identify and clone a trout IL-2 ortholog.

We initially identified two salmon EST sequences (GenBank accession nos. EG762969 and EG764237) encoding for a molecule with a high degree of sequence similarity to available IL-2 sequences (data not shown). Based on these EST sequences, reverse primers were designed to perform 5’-RACE using rainbow trout cDNA isolated from the head kidney as a template. This yielded a cDNA fragment that was similar to that of Fugu IL-2, the only teleost fish IL-2 molecule reported thus far (21). Subsequently, this
fragment was used to design gene-specific forward primers to perform 3'-RACE, which yielded a fragment corresponding to the 3'-end of trout IL-2. The compiled full-length cDNA sequence of trout IL-2 contained a 67-bp 5'-untranslated region (UTR), a 568-bp (IL-2A) or 76-bp (IL-2B) 3'-UTR, and a single ORF encoding a 142-aa IL-2 molecule. Interestingly, five copies of an mRNA instability motif (ATTTA) and a typical poly(A) signal (AATAAA) exists upstream of the poly(A) tail of IL-2A but not of IL-2B (sequences not shown; GenBank accession nos. FJ571512 and FJ571513 for IL-2A and IL-2B, respectively).

When compared with other known IL-2 amino acid sequences, trout IL-2 is predicted to have a 20-aa signal peptide (Fig. 7). The α-helix B portion of trout IL-2 exhibits the IL-2 family signature (T-E-[L/F]-X (2)-L-X-C-L-X (2)-E-L). Two cysteine residues located at positions 98 and 151 are conserved in all aligned mammalian, avian, and teleost IL-2 molecules, which are thought to be involved in disulfide bond formation. Furthermore, an additional pair of cysteines (positions 91 and 148) were found only in the avian and teleost IL-2 molecules. Two potential glycosylation sites (positions 65 and 140) were found in the trout IL-2 molecule.
were comparable, and very low (<0.08 ng/ml). We performed experiments using much higher dose of E. coli LPS (1,000 ng/ml) that failed to induce increases in IL-2 expression (data not shown), thus indicating that the minute amounts of LPS contained in the recombinant rtCD80/86 preparation did not have an influence on the obtained IL-2 expression levels. Similarly LPS also failed to induce IL-2 expression in fugu leukocytes (21).

Discussion

Thus far, CD80 and CD86 have only been cloned and functionally characterized in mammals and birds. To shed light into the evolutionary origins and primordial functions of CD80 and CD86, our studies focused on the search for CD80 and CD86 orthologs in teleost fish. Thus, in the present study we have cloned and functionally characterized a rainbow trout molecule (rtCD80/86) that shows a high degree of homology to CD80 and CD86 molecules. In addition, we have identified and cloned trout IL-2 and have shown that recombiantly produced trout CD80/86 induces the expression of IL-2 in trout blood leukocytes.

We initially identified salmon EST sequences with a high degree of homology to mammalian CD80 and CD86 molecules. Based on these sequences, primers were designed to perform 5’- and 3’- RACE using rainbow trout cDNA as a template. This led to the cloning of a trout cDNA whose deduced amino acid sequence showed comparable degrees of similarity with that of mammalian CD80 or CD86, and thus we named this trout molecule rtCD80/86. Three different transcripts for the rtCD80/86 gene were identified by PCR, a membrane form (rtCD80/86m), a short secretory isoform (rtCD80/86ss), and a long secretory isoform (rtCD80/86sl). The short secretory isoform is lacking the exons coding for both the TM and cytoplasmic domains, whereas the longer secretory isoform only lacks the TM domain. However, the latter isoform contains a frame shift at the C terminus, corresponding to the cytoplasmic tail of the membrane form. Comparable soluble forms of CD80 and CD86 molecules have also been described in several mammalian species (6–12).

Similar to mammalian CD80 and CD86 molecules, the trout membrane form is composed of two extracellular Ig-like domains, a hydrophobic TM region, and an intracellular cytoplasmic tail (Fig. 1A). Several lines of evidence strongly support the hypothesis that the rtCD80/86 gene represents an ortholog of mammalian and avian CD80 and CD86 genes: 1) the deduced amino acid sequence of rtCD80/86 showed higher degrees of similarity (16–19% identity) to the CD80 and CD86 sequences than to sequences of any of the other five members of the B7 family (see supplemental Table I); 2) trout orthologs of mammalian B7-DC/H1, B7-H3, and B7-H4 were identified (see supplemental Table II), thus excluding the possibility that rtCD80/86 could be an ortholog of any of the aforementioned B7 molecules; 3) the rtCD80/86 molecule showed a closer phylogenetic relationship to mammalian and avian CD80 and CD86 than to any of the other members of the B7 family (Fig. 3); 4) the genomic organization of the rtCD80/86 showed the highest degree of homology to that of human CD86 (Fig. 4B); 5) most of the key residues of mammalian CD80 and CD86 involved in the binding to their receptors (CD28 and CTLA4) are conserved in rtCD80/86 (Fig. 2A); and 6) the number of potential N-linked glycosylation sites (eight sites) in rtCD80/86 resembled that of mammalian CD80 and CD86 molecules (eight sites) and is more than that of any of the other members of the B7 family (4–7 sites) (see supplemental Table I).

We could only identify one CD80/86 gene in rainbow trout. To verify whether other teleost fish also contained a single CD80/86 gene, we searched for rtCD80/86 homologs in available teleost fish genome drafts and EST databases. Similar to what we found in rainbow trout, only one rtCD80/86 homolog could be identified for each of the teleost species analyzed, and thus none of the analyzed fish genomes contained distinct orthologs for the mammalian CD80 and CD86 genes. This suggests that teleost CD80/86 represents the ancestral gene from which CD80 and CD86 arose by gene duplication before the emergence of birds, because birds have already been reported to contain true orthologs of mammalian CD80 and CD86 genes (13, 14). During the writing of this article, a study was reported online that included a systematic in silico evolutionary analysis of B7 family members using available genome and EST databases from lower vertebrates (37). This study identified a CD80/86 gene in the genome of several teleost fish species that corresponds to the same CD80/86 gene we have identified in the analyzed fish genomes (see supplemental Table II). Similar to our findings, this study showed the presence of one single CD80/86 gene (termed B7R) in each of the analyzed fish genomes. In agreement with our conclusion and supported by elegant syntenic analyses, the aforementioned study concludes that fish B7R probably represents the ancestor from which CD80 and CD86 derived in more evolved species (37).

Tissue expression of rtCD80/86 was detected in all tissues analyzed. The membrane form (rtCD80/86m) was significantly more expressed in the lymphoid tissues, thus suggesting a larger involvement of this isoform in fish immunity. However, the two secretory forms were expressed at low levels both in lymphoid and nonlymphoid tissues, although the short secretory form rtCD80/86ss was predominantly expressed in the heart and muscle. Similarly as in rainbow trout, expression of membrane CD80 and CD86 in mammals is restricted to lymphoid cells including B cells, macrophages/monocytes, dendritic cells, and activated T cells and mainly play a role in immune modulation (13, 38). However, the expression of membrane forms of mouse and rat CD80 and/or CD86 molecules has also been shown in nonlymphoid tissues (i.e., heart, tongue, and liver), although to a much lesser degree than in lymphoid tissues (39, 40). Although CD80 and CD86 genes are not normally expressed in the human muscle cells, after activation with IL-4 or CD40 those genes can be induced in myoblasts (41).

Interestingly, we found that in blood leukocytes and HKLs, IgM+ B cells showed a significantly higher degree of rtCD80/86m expression when compared with the adherent and nonadherent populations of IgM+ leukocytes, suggesting an important role for the IgM+ B cell as an APC. The putative role of trout IgM+ B cells as APCs is perhaps related to the high phagocytic capacity that we have recently reported for teleost fish B cells (42), a hypothesis that will be examined in future studies.

In mammals, CD86 has been shown to be expressed constitutively at low levels and is rapidly up-regulated by a variety of stimuli including LPS, whereas CD80 is expressed only inducibly and later, after the activation of CD86 (1, 2, 43). In this regard rtCD80/86 appeared to behave more like a CD86 molecule, as we found that its gene expression was significantly up-regulated by E. coli LPS (Fig. 6A) and Vibrio bacterin (see Fig. 6B). The membrane form transcript (rtCD80/86m) showed the highest levels of up-regulation and, although the increases in rtCD80/86m expression were moderated, they were comparable to the up-regulation levels induced by LPS for mammalian CD86 (44, 45).

Data on the functional roles of CD80 and CD86 are restricted to mammalian species (1) and to a much lesser degree to birds (14). Perhaps the most important functional outcome resulting from the engagement of CD80 and/or CD86 with CD28 is the up-regulation of gene expression and protein synthesis of IL-2, mainly by Th cells (2–4). Thus, we resolved to determine whether this role in rtCD80/86 was conserved. To carry out such studies, we needed first to characterize trout IL-2, because its identification
had not thus far been reported. Therefore, we isolated cDNA encoding for trout IL-2 that represents the second IL-2 molecule to be cloned in a teleost fish. The primary amino acid sequence deduced from the trout IL-2 gene contains the key residues characterizing the IL-2 family signature (T-E-[L/F]-X (2)-L-X-C-L-X (2)-E-L) and the two cysteine residues involved in disulfide bond formation that are conserved in all known IL-2 molecules. The rainbow trout IL-2 showed the highest degree of sequence similarity to that of the recently reported fugu IL-2 molecule (21). The trout IL-2 transcript could be significantly up-regulated by PMA and PHA, both of which are also known to up-regulate the expression levels of IL-2 in mammals (15, 35). More importantly, IL-2 transcripts were able to be significantly up-regulated by recombinantly produced rtCD80/86 (Fig. 9). Our data show that the addition of recombinant rtCD80/86 alone to trout blood leukocytes provided a signal that was sufficient to induce an increase in the expression levels of IL-2. In this regard, IL-2 production in mammals normally requires two signals induced by the interaction of the TCR with the MHC/peptide complex (signal 1) and by the engagement of CD80 or CD86 with CD28, which provides a co-stimulatory signal (signal 2) (2). However, and in support of our results, it has been shown by several groups that engagement of CD80 alone (signal 2) by crosslinked anti-CD28 Abs is sufficient to induce stimulation of IL-2 gene expression in T cells, although to significantly lower levels than the simultaneous coengagement of CD80 and TCR (46–48). Moreover, other studies have also shown that up-regulation of IL-2 on T cells, or their activation, can happen independently of TCR engagement (49–51).

In this study the functional extracellular domain of rtCD80/86m was recombinantly produced in a prokaryotic expression system (E. coli). Similarly, a functional receptor binding domain of human CD86 (IgV-like domain) was also produced in E. coli (32, 52). This indicated that, similar to what we observed with rtCD80/86, the bacterially produced human molecule retains the conformational structure required to interact with its receptor and that glycosylation of the binding domain of CD86 does not play a role in binding to its receptor. In support of the latter, it has also been shown that soluble deglycosylated human CD80 produced recombinantly retains its ability to bind to both CD28 and CTLA-4 (36). Moreover, it has been reported that the potential N-linked glycosylation sites in human CD80 are localized to regions essentially opposite of the site for receptor interaction and are therefore unlikely to play a role in the interactions between CD80 with CD28/CTLA-4 molecules (33), a fact that further explains the observed binding of deglycosylated CD80 and CD86 products to their receptors.

It has been shown that CD80 but not CD86 forms homodimers on cell surfaces (34). Recombinantly produced soluble human CD80 maintains also its capacity to form dimers as well as higher m.w. oligomers, depending on its concentration in solution (5, 30). Similarly, our gel filtration studies showed that recombinant rtCD80/86 did not form monomers in solution; instead, it appeared to form tetramers. It has been shown that the residues at the dimer interface of human CD80 are primarily hydrophobic and, thus, critical for inducing the dimerization of CD80 (30), whereas in the same area most of the residues of CD86 are hydrophilic. Similar to mammalian CD80, many (seven of the 11 hydrophobic) residues in the corresponding area of rtCD80/86 are also hydrophobic, a fact that might explain the degree of oligomerization observed in recombinant rtCD80/86 (Fig. 8). In addition, the oligomeric state of recombinant rtCD80/86 may have contributed to is ability to efficiently crosslink its ligand on trout T cells, similar to the reported and above-mentioned ability of crosslinked anti-CD28 to induce IL-2 gene expression in T cells in a TCR-independent fashion (46, 47).

In conclusion, this study represents the first cloning and functional characterization of an ancestral CD80/86 ortholog in a poikilotherm species, as well as the cloning of the second IL-2 gene in a teleost fish. Significantly, we show that recombinantly produced rtCD80/86 up-regulates IL-2 expression levels in blood leukocytes, thus indicating that this capacity to modulate IL-2 expression is a primordial function that has been conserved in both fish and mammalian CD80/CD86 molecules throughout 350 million years of evolution.

Although teleost fish appear to have most of the cellular and molecular machinery necessary to elicit primary and secondary adaptive immune responses, it is clear that degree of such responses (e.g., specificity, affinity maturation, and duration) differ to a great extent between fish and mammals. It is likely that differences in the mechanisms of Ag uptake and presentation between fish and mammals account, at least in part, for some of the differences observed in their primary and secondary responses. For example, we do not know yet which cells are the main APCs in teleosts, as the presence of bona fide dendritic cells in these species remain to be demonstrated. Thus, the characterization of trout CD80/86 and IL-2 molecules presented in this article will allow us in the near future to determine further the mechanisms and cells involved in Ag presentation in this species. In turn, this will enable us to identify some of the cellular and molecular elements responsible for the differences seen between immune responses of fish and mammals. From a practical perspective, future studies on Ag presentation processes in fish will be instrumental for improving delivery strategies and adjuvant formulations with the goal of designing more efficacious fish vaccines.

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Disclosures

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