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Costimulatory Receptors in a Teleost Fish: Typical CD28, Elusive CTLA4[1]

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T cell activation requires both specific recognition of the peptide-MHC complex by the TCR and additional signals delivered by costimulatory receptors. We have identified rainbow trout sequences similar to CD28 (rbtCD28) and CTLA4 (rbtCTLA4). rbtCD28 and rbtCTLA4 are composed of an extracellular Ig-superfamily V domain, a transmembrane region, and a cytoplasmic tail. The presence of a conserved ligand binding site within the V domain of both molecules suggests that these receptors likely recognize the fish homologues of the B7 family. The mRNA expression pattern of rbtCD28 and rbtCTLA4 in naive trout is reminiscent to that reported in humans and mice, because rbtCTLA4 expression within trout leukocytes was quickly up-regulated following PHA stimulation and virus infection. The cytoplasmic tail of rbtCD28 possesses a typical motif that is conserved in mammalian costimulatory receptors for signaling purposes. A chimeric receptor made of the extracellular domain of human CD28 fused to the cytoplasmic tail of rbtCD28 promoted TCR-induced IL-2 production in a human T cell line, indicating that rbtCD28 is indeed a positive costimulator. The cytoplasmic tail of rbtCTLA4 lacked obvious signaling motifs and accordingly failed to signal when fused to the huCD28 extracellular domain. Interestingly, rbtCTLA4 and rbtCD28 are not positioned on the same chromosome and thus do not belong to a unique costimulatory cluster as in mammals. Finally, our results raise questions about the origin and evolution of positive and negative costimulation in vertebrate immune systems. ***The Journal of Immunology,*** **2006, 176: 4191–4200.**

T cell activation is initiated through complex cell-to-cell interactions. TCRs expressed on the surface of T cells first recognize antigenic peptides presented by MHC molecules on the surface of APCs. TCRs and other surface receptors and ligands stabilize the contact between T cells and APCs, which triggers signal transduction pathways resulting in T cell activation. According to the two-signal model (1, 2), T cell activation by Ag requires both specific recognition of the peptide by the TCR (signal 1) and additional signals delivered by other costimulatory receptors (signal 2). Among the known costimulatory receptors, CD28 and CTLA4 (also known as CD152), which are expressed on T cells and bind their corresponding ligands B7-1/B7-2 (CD80/CD86) on APCs, represent a well-studied system in mammals (3).

CD28 is a glycosylated homodimeric protein expressed on the surface of double-positive thymocytes, mature $CD4^+$ T cells, $CD11b^-CD8^+$ T cells, and $\gamma\delta$ $CD3^+$ T cells (4, 5). CD28 is expressed at higher levels on activated T cells than on resting cells (6). In mammals, CD28 binds to B7-1 and B7-2, which are expressed on the surface of APCs, and delivers a critical costimulatory signal to T lymphocytes. In the absence of CD28 ligation, TCR binding either induces apoptosis or anergy in T cells (5). Engagement of CD28 alone cannot activate T cells, even if synergetic effects are provided via stimulation by T cell mitogens or anti-CD3 treatment (7). A tyrosine-based motif in cytoplasmic tail of CD28 functions as a binding site for the p85 PI3K subunit when it is phosphorylated (8, 9). Other CD28 intracytoplasmic motifs, which associates with IL-2-inducible tyrosine kinase, lymphocyte-specific tyrosine kinase, and the adaptor growth-factor receptor bound protein-2 are responsible for signal transduction (10–12). The binding of growth-factor receptor bound protein-2 to CD28 also activates the GTPase RAS (13). CD28 signaling is also thought to contribute to the mobilization of lipid rafts to the immunological synapse (14), the region of contact between T cell and APC, which lowers the overall threshold of TCR engagement required for effective cytokine production and proliferation (15).

In contrast, CTLA4 is a powerful negative regulator of T cell activation and was first cloned via differential screening of a cytotoxic T cell cDNA library (16). CD28 and CTLA4 belong to the same family and share high sequence similarity to each other (16, 17). Both receptors interact with B7-1 and/or B7-2 but induce different signals: CD28 amplifies signaling triggered by the TCR-CD3 complex, whereas CTLA4 generates negative signals that inhibit T cell activation (18). CTLA4 has higher affinity for B7-1 and B7-2 compared with CD28, and its expression is also induced by TCR engagement (19). The mechanisms of CTLA4-mediated suppression likely involve both competition with CD28 for B7-1/B7-2 binding and potent inhibitory signals delivered by CTLA4 (20). The inhibitory signaling mechanisms triggered by CTLA4 are not clear. The CTLA4 cytoplasmic tail lacks typical ITIM motifs and shares

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with CD28 a p85 binding site (21). Thus, the extent of an immune response is likely controlled through the finely tuned expression of costimulatory receptors from the CD28/CTLA4 family on the surface of activated T cells (22, 23).

Very little is known about T cell responses and activation in bony fish. TCR-$\alpha\beta$ cDNAs (24–27) and polymorphic class IA and class IIA/B MHC molecules (28–30) have been reported in several species, suggesting that the fish TCR recognizes antigenic peptides presented by MHC molecules similar to that of mammals. T cell-mediated responses in fish are supported by several lines of evidence. Allograft rejection provided the first experimental indications suggesting that bony fish possess a functional T cell-mediated immune response (31, 32). In vitro assays for allospecific cytotoxicity have also been established using clonal and nonclonal catfish cell lines (33, 34) or clonal rainbow trout (35). Autologous cell-mediated specific lysis of virus-infected syngenic target cells has also been reported in cloned goldfish (36, 37). Finally, both public and private T cell-specific expansions have been observed in rainbow trout during secondary immune responses to viral hemorrhagic septicemia virus (VHSV),[3] a fish rhabdovirus, using TCR-β CDR3-length spectratyping (38). TCR-MHC-peptide interactions induce signaling events through CD3 in mammals and most likely in lower vertebrates because a complete set of *CD3* genes, including *TCR*ζ, *CD3*ϵ, and *CD3*$\gamma\delta$, has been found in Pufferfish and *Xenopus* (39, 40). CD8α has been identified in rainbow trout (41); however, the cytoplasmic tail lacks the consensus p56^{lck} motif, suggesting that TCR/CD3/CD8-mediated signaling events may be different in teleosts.

In this study, we have identified and initially characterized two members of the CD28 family in rainbow trout, rbtCD28 and rbtCTLA4. Their sequence features and expression patterns suggest that they are the likely homologues of mammalian CD28 and CTLA4. The potential costimulatory capacities of these fish receptors were investigated using a human T cell line expressing chimeric receptors composed of the extracellular domain of human CD28 (hCD28) fused to the cytoplasmic tail of rbtCD28 or rbtCTLA4. The chimeric hCD28-rbtCD28 receptor mediated enhanced TCR-induced IL-2 production, suggesting a costimulatory function for rbtCD28. In contrast, the divergent cytoplasmic tail from rbtCTLA4 did not mediate similar signaling activities. This study therefore provides the first characterization of costimulatory receptors in lower vertebrates.

Materials and Methods

Fish, tissue preparation, and leukocyte stimulation

Rainbow trout were raised in the Institut National de la Recherche Agronomique experimental fish facility at Jouy-en-Josas. Fish were sacrificed by overexposure to 2-phenoxyethanol diluted 1/1000. Relevant organs were removed aseptically. Leukocytes from the head kidney tissue were isolated by centrifugation through a Ficoll gradient. Gut intraepithelial lymphocytes were prepared by mechanical extraction and extensive washing using culture medium. For PHA polyclonal stimulation, leukocytes were prepared from a single fish and divided into several aliquots. Cells from one aliquot were used immediately for RNA preparation, whereas cells from the other aliquots were incubated for 4 days with PHA (Sigma-Aldrich). The attenuated variant (25–11) of VHSV strain 07-71 was used to infect fish via i.m. injection of 1–5 $\times$ 10^5 PFU/trout. This first mode of infection typically leads to a strong protection against VHSV. Four weeks later, fish received a second injection of the same virus (7.5 $\times$ 10^7 PFU/trout).

[3] Abbreviations used in this paper: VHSV, viral hemorrhagic septicemia virus; BAC, bacterial artificial chromosome; EST, expressed sequence tag; ORF, open reading frame.

RNA preparation and isolation of full-length cDNAs

Total RNA was extracted with TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions and treated with RNase free DNaseI (Invitrogen Life Technologies). The treated RNA was used to generate full-length cDNAs. 5′- and 3′-RACE were performed using the SMART RACE cDNA amplification kit (BD Clontech) with universal primers provided within the kit and gene-specific primers for rbtCD28/CTLA4. PCR products were purified with Sephacryl S-400 columns (Pharmacia Biotech) and then cloned using the TOPO T/A cloning kit (Invitrogen Life Technologies). Purified plasmids were subjected to automated sequencing with M13 forward and reverse primers.

Northern blot and real-time RT-PCR

Procedures for Northern blot analysis have been described previously (42). Briefly, RNA from different tissues was blotted on a nylon membrane. A rbtCD28 V region probe was amplified using CD28VF1 and CD28VR1 primers (Table I). The blot was hybridized with the CD28 probe, washed under high stringency (65°C), and exposed for 7 days for CD28, then stripped and reprobed with EFTu1 probe (16-h exposure).

First-strand cDNAs were synthesized using an oligo(dT) primer and Superscript reverse transcriptase (Invitrogen Life Technologies) with RNase-free DNAseI-treated RNA templates. Conventional PCR were performed using Promega *Taq* polymerase. Real-time RT-PCR were performed using the SYBR green reagent and ABI PRISM 7700 cycler (Applied Biosystems), according to the manufacturer's instructions. A standard curve was included in each plate for rbtCD28, rbtCTLA4, and rainbow trout β-actin genes. All reactions were performed in duplicate. Data analysis was performed as described in the ABI PRISM 7700 sequence detection bulletin no. 2 from Applied Biosystems. Oligonucleotides (Table I) used for real-time RT-PCR were designed from rainbow trout β-actin, rbtCD28, and CTLA4 using the Primer Express software.

Cloning of CD28 and CTLA4 introns

Primer pairs were designed based upon CD28 and CTLA4 cDNA sequences to amplify intron-containing regions (CD28gen1 and -2 for CD28 intron 1; CD28gen3 and -4 for CD28 intron 2; CD28gen5 and -6 for CD28 intron 3; CTLA4gen3 and -4 for CTLA4 intron 2; and CTLA4gen5 and -6 for CTLA4 intron 3). Amplification was performed with genomic DNA from rainbow trout liver. Primer sequences are indicated in Table I. PCR conditions consisted of 3 min at 94°C followed by 30 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 90 s with a final incubation at 72°C for 5 min. PCR products were purified using Sephacryl S-400 columns (Pharmacia Biotech), cloned into pCR2.1 plasmid (TOPO T/A cloning kit; Invitrogen Life Technologies), sequenced, and compared with trout CD28 and CTLA4 cDNAs to determine exon/intron boundaries.

Table I. *Primers*

Primer Name	Primer Sequence
ACTIN-2F	GGGAGAAGATGACCCAGATCATG
ACTIN-2R	GGTGGTACGGCCAGAGGC
CD28-1F	CTTGGTCCTGGTTGAAGCATT
CD28-1R	CCGTGACAACAAGACCATAGAT
CTLA4-1F	CCTACTCTATATAACAGAGGAACCAGG
CTLA4-1R	AGGATAGCAATGGCAGAGATTACTATT
CD28gen1	TGAACGTTTACTGGATACCCACGAT
CD28gen2	CAAGGTCTGAGGCTCGTCTGGTA
CD28gen3	AGCGCGCTGGAGTCTATACCTGT
CD28gen4	ACCATAGATGGTGGTGACCCAGA
CD28gen5	GTAGGCCCTAGAGTGCATGGTGTCC
CD28gen6	TGAGGATGTCCGATCTGAGGATGAC
CTLA4gen3	GGTCTCAGGGGTAACGACACTGACC
CTLA4gen4	ATCTCTTCTCTGGGCCTCTGGGGTA
CTLA4gen5	GAGGCCCAGAGAAGAGATGATGTAAC
CTLA4gen6	GGAAGTTCCCATAATCAAATCTTCCA
CD28-V-F1	TCTGTCAACGGCATTGCAT
CD28-V-R1	GCTCGTCTGGTACTTTCTCAA
CTLA4-V-F1	AGCTGTTCTGCTCCTACCA
CTLA4-V-R1	TCTGTTATATAGAGTAGGGTACCAT

Plasmids and cell lines

Human CD28 insert (*Sal*I-*Bam*HI) was excised from pHβApr-1-neo-CD28WT (43), and replaced with a truncated human CD28 sequence lacking the cytoplasmic tail (plasmid pHsCD28Δ30). The cytoplasmic tails of rbtCD28 or rbtCTLA4 were then inserted in-frame with the extracellular portion of human CD28 to generate chimeric proteins, where the human cytoplasmic tail was replaced by rainbow trout CD28 or CTLA4 cytoplasmic tails (plasmids phuCD28-rbtCD28 and phuCD28-rbtCTLA4, respectively). All constructs were entirely sequenced. CH7C17 Jurkat cells ($CD28^-$ $CTLA4^-$ $TCR\alpha\beta^+$ $CD3^+$) (44) (7×10^6) were electroporated with the constructs at 250 V, 960 μF, using a Gene Pulser apparatus (Bio-Rad). After 48 h, cells were distributed in 96-well culture plates in selective RPMI 1640 (4 mg/ml G418; Sigma-Aldrich). After 2 wk of selection, cells were stained (30 min, 4°C) with anti-human CD28-PE mAb (eBioscience) and analyzed by flow cytometry. Positive clones were identified based on surface expression of the human CD28 extracellular domain. Several clones were selected and subcloned. A T cell clone expressing full-length human CD28 receptor (A14) (44) was provided by Dr. F. Michel (Institut Pasteur, Paris, France) that served as a positive control.

Immunoblotting

T cell clones (10^8/ml) were incubated for 30 min at 4°C with anti-human CD28 (5 μg/ml; clone CD28.2; eBioscience) in complete RPMI 1640. Control cells were kept at 4°C in the same volume of complete RPMI 1640. Cells were washed, incubated with complete RPMI 1640 for 5 min at 37°C, and cross-linked for 2 min with goat anti-mouse IgG1 (5 μg/ml; eBioscience). After centrifugation, cells were lysed at 4°C for 1 h in lysis buffer (100 mM Tris-HCL (pH 7.4), 1.5 M NaCl, 5 mM EDTA, 1 mM Na-orthovanadate), in the presence of complete proteinase inhibitor mixture (Roche). Insoluble material was removed by centrifugation at 14,000 rpm for 1 h, and total proteins within the supernatant were then analyzed by SDS-PAGE using a 4–20% gradient gel (Invitrogen Life Technologies). Proteins were transferred to Immobilon (Amersham Biosciences) membranes, blocked for 30 min with 5% BSA in PBS-0.1% Tween 20 and incubated overnight either with anti-human phospho-ERK phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) mouse mAb or with anti-human ERK p44/42 MAPK Ab (Cell Signaling) at 4°C. After washing, the membranes were incubated with goat anti-mouse or anti-rabbit IgG peroxidase (PARIS), washed, stained with ECL reagent (Pierce), and exposed to x-ray film.

Analysis of IL-2 production

Jurkat CH7C17 transfectants were incubated in the presence or absence of anti-human CD28 mAb (5 μg/ml) (clone CD28.2; eBioscience) for 30 min at 4°C. After washing, 10^5 cells were deposited on flat-bottom, anti-human CD3 T cell activation plates (BD Biosciences). Goat anti-mouse IgG1 Ab (γ1 chain specific; Southern Biotechnology Associates) was added (5 μg/ml) to cross-link human CD28. As a control, 10^5 T cells were incubated in normal flat-bottom 96-well plates in 200 μl of complete RMPI 1640 (5% FCS) with goat anti-mouse IgG1 Ab (5 μg/ml). Supernatants from five replicate wells were collected after 24 h at 37°C. IL-2 was then quantified by ELISA using the Human IL-2 ELISA Kit II (BD Biosciences).

Genetic mapping and in situ hybridization

Genotyping was conducted on a doubled haploid background as described previously (45). Bacterial artificial chromosomes (BACs) positive for rbtCD28 and rbtCTLA4 were identified by screening the OSU-142 4.5× BAC library using PCR-generated probes specific for rbtCD28 (CD28 V-F1/R1 primers) and rbtCTLA4 (CTLA4 V-F1/R1 primers) on high-density filters. BAC clones were confirmed via direct sequencing and secondary PCR before usage. Genotyping was accomplished by SNaPshot (Applied Biosystems) analysis of the PCR products. Analyses were performed with GeneMapper 3.5 application software (Applied Biosystems). Genotype readouts were assigned to specific linkage groups as described previously (45) using Mapmaker 2.0 and EXP 3.0 software. Procedures for in situ hybridization on trout chromosomes have been previously described (42).

In silico sequence analysis

The Genetic Computer Group (Madison University, Madison, Wisconsin) package was used for alignment and sequence assembly. Multiple alignments were performed with ClustalW and edited using Boxshade software. Signal peptide and transmembrane hydrophobic regions were identified using Tmpred (⟨http://www.chnet.org⟩), and *N*-linked glycosylation sites were predicted by NetNGlyc 1.0 Server (⟨http://www.cbs.dtu.dk/services/NetNGlyc⟩). Similarity searches were performed using Blast programs at ⟨http://www.ncbi.nlm.nih.gov/⟩.

Results

Identification of rainbow trout CD28 and CTLA4

We used the murine CD28 sequence in conjunction with tBlastn to search for rainbow trout homologues in the salmonid expressed sequence tag (EST) databases at the National Center for Biotechnology Information. Several rainbow trout ESTs were retrieved, and a consensus was computed and then used to produce a conceptual translation of a partial open reading frame (ORF), which was aligned with several mammalian CD28 sequences. Primers were then designed from the rainbow trout consensus sequence for 5′- and 3′-RACE experiments. With this approach, several full-length cDNA clones were obtained from pronephric cDNA. The rainbow trout CD28 cDNA sequence was 941-bp long and contained a 687-bp long ORF, coding for 229 residues (Fig. 1*A*). A BlastP query with this amino acid sequence (GenBank accession no. AY789435) retrieved only CD28 sequences (E values between 10^{-9} and 10^{-2}, ~28% identity to mammalian CD28) from higher vertebrates,

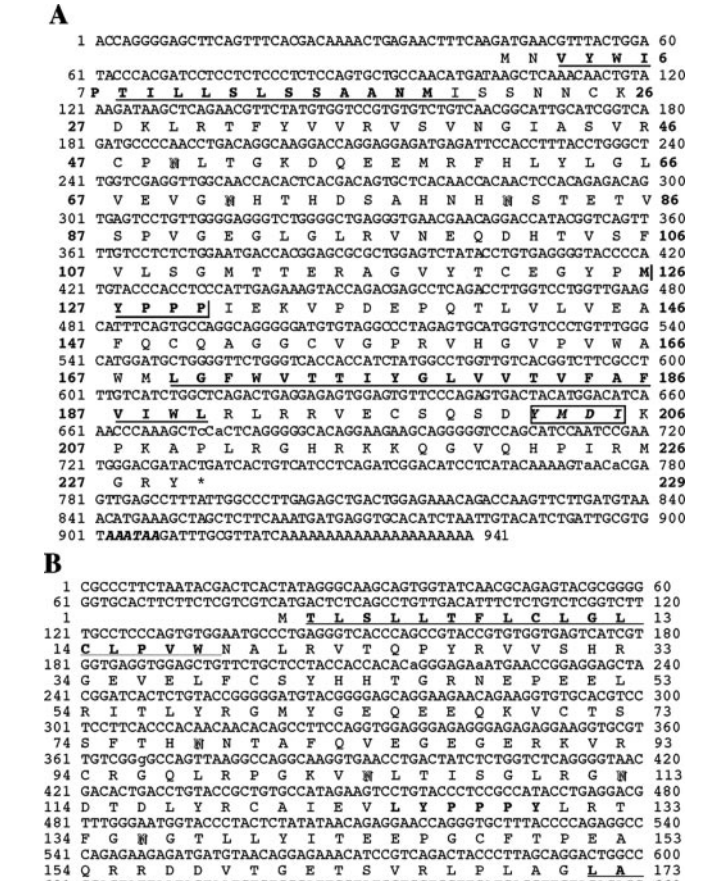

FIGURE 1. Sequence of rbtCD28 and rbtCTLA4 mRNAs. rbtCD28 (*A*) and rbtCTLA4 (*B*) cDNA and deduced amino acid sequence of the putative ORFs. ∗ indicates the STOP codon. Putative polyadenylation signal is bold italic. Putative B7-binding is bold and boxed, and potential Tyr-based motifs are bold, italic, and boxed. Signal peptide and transmembrane hydrophobic regions were identified using Tmpred (⟨www.chnet.org⟩), and are bold underlined. *N*-glycosylation sites predicted by NetNGlyc 1.0 Server (⟨http://www.cbs.dtu.dk/services/NetNGlyc/⟩) are bold-shadowed. These sequences have been submitted to GenBank (rbtCD28, AY789435; rbtCTLA4, AY789436).

suggesting that it likely corresponds to an ortholog of CD28. Sequences similar to rainbow trout CD28 were also found among ESTs from Atlantic salmon (ca369939; ca059267; ca064339; ca062851; ca053916; ca769496; ca057139).

In mammals, CTLA4, a receptor structurally related to CD28, delivers negative costimulatory signals and constitutes a powerful negative regulator of T cell responses. To search for such a costimulatory sequence in rainbow trout, the human CTLA4 sequence was compared with EST and other sequence databases available for salmonids using tBlastn. An Atlantic salmon EST similar to mammalian CTLA4 was identified (CA044588). This sequence was used to design primers for 5′- and 3′-RACE. Using PHA-stimulated pronephric lymphocyte mRNA as the template, a putative full-length cDNA for a CTLA4-like sequence was obtained (Fig. 1*B*). It included a 651-bp ORF, encoding a peptide of 217 aa residues. BlastP analysis with the deduced amino acid sequence (GenBank accession no. AY789436) showed it was most similar to CTLA4 from several mammalian sources (E values below 10^{-10}).

Considering the striking sequence similarity with mammalian CD28 and CTL4, the two putative proteins identified in rainbow trout were named rbtCD28 and rbtCTLA4, respectively.

rbtCD28 displays the general characteristics of CD28

Sequence analysis and secondary structure prediction of rbtCD28 identified a V-type Ig superfamily domain that is preceded by a hydrophobic N-terminal region (residues 2–20) consistent with a signal peptide (Fig. 1*A*). General characteristics of a CD28 Ig V-domain were observed in the predicted extracellular domain of rbtCD28, including the conservation of canonical Cys^{47} and Cys^{121} involved in the intra-V domain disulfide bridge and the absence of Trp residue in strand C. Three potential glycosylation sites were identified in rbtCD28, of which only two were conserved between fish and mammalian sequences. The MYPPPY motif, which is conserved from birds to mammals and essential for CD28 binding to B-7 ligands, is well conserved between trout (MYPPPI) and mammals (MYPPPY). In addition, Cys^{149}, which is required for CD28 dimerization is also conserved in trout and mammals. rbtCD28 possesses a second hydrophobic region (aa 169–190), which likely corresponds to the transmembrane region. The cytoplasmic tail of rbtCD28 sequence contains a ^{199}YMDI motif, which is similar to the YMNM PI3K binding sites of mammalian CD28 (Fig. 2*A*). However, the consensus SH3 PYAP motif of mammalian CD28 was not present in the fish sequence.

rbtCTLA4 has a B7 binding site, but lacks conserved tyrosine-based signaling motif

Similar to CD28 sequences, typical features of a Ig V-domain from the CD28/CTLA4 family were present in the predicted extracellular domain of rbtCTLA4 (Fig. 1*B*). The rbtCTLA4 sequence had two hydrophobic stretches (residues 2–18, and residues 171–192) representing a putative peptide signal and a transmembrane region, respectively. Four potential sites of *N*-linked glycosylation were identified in the sequence, including Asn^{105} and Asn^{136}, which are strictly conserved from chickens to mammals. Four Cys residues in the V domain (positions 39, 71, 94, and 120) were conserved in trout and mammals. Another Cys residue is present at position 148 that is likely involved in rbtCTLA4 dimerization. The ligand-binding motif M/LYPPPY was also present in the rbtCTLA4 sequence. Another striking feature in rbtCTLA4 is found in the G strand of the extracellular Ig superfamily V-domain, which contains a conserved GNGTXY motif that is also present in mammalian CTLA4 sequences (Fig. 2*B*). This sequence is reminiscent of a J segment, implying the presence of a diglycine β bulge. In contrast, the multiple tyrosine-based motifs that contain the PI3K binding site and regulate the subcellular distribution of mammalian CTLA4 were not present in the trout sequence. A unique tyrosine-based motif (YGNF) was found in the cytoplasmic tail of rainbow trout and Atlantic salmon, close to the C terminus of the protein that may play a similar role as the human Tyr^{218} Tyr-based motif (46).

rbtCD28 and rbtCTLA4 genes share the intron/exon structure of the CD28 family

The intron/exon structure of vertebrate genes is generally well conserved inside a gene family. To further ascertain that rbtCD28 and rbtCTLA4 sequences belong to the CD28 family, we compared

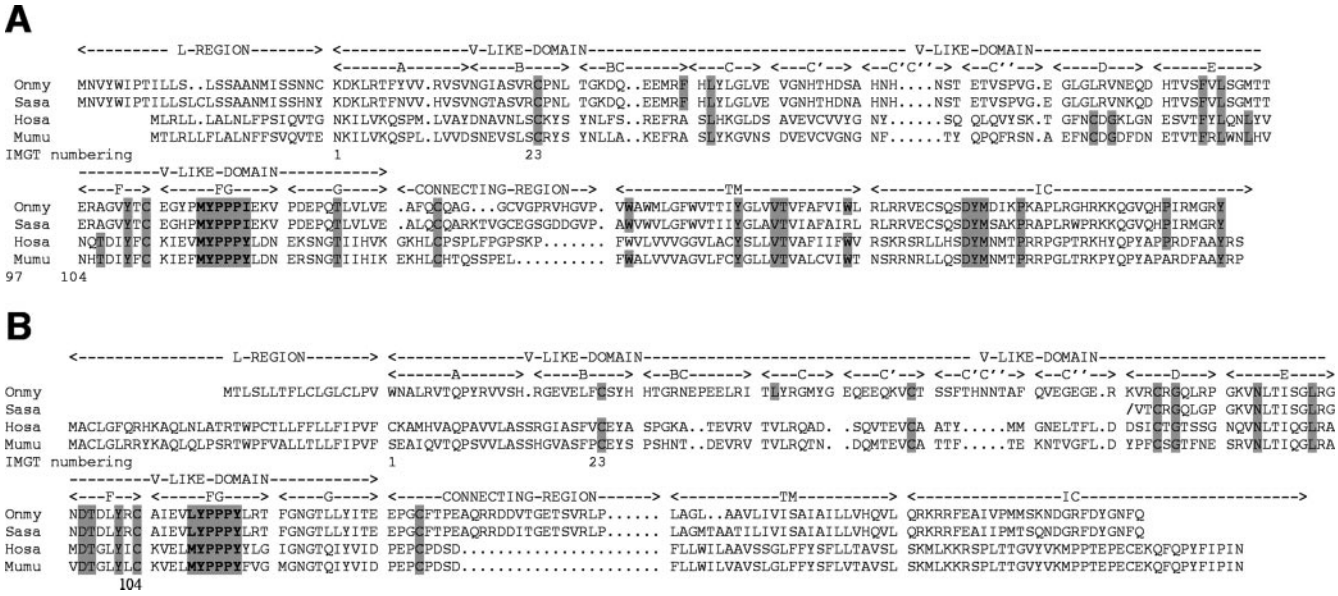

FIGURE 2. Multiple alignments of salmonid and mammalian CD28 (*A*) and CTLA4 (*B*) proteins. Salmonid sequences were aligned following the ImMunoGeneTics numbering for Ig superfamily domains (Onmy, *Oncorhynchus mykiss*; Sasa, *Salmo salar*) with human (Hosa) and mouse (Mumu) sequences. Residues of the ligand binding site are bold. Relevant conserved positions are shadowed.

their genomic structure to that of their mammalian counterparts. In mammals, *CD28* and *CTLA4* genes have four exons: the first exon encodes up to the signal peptide, the second exon codes for the Ig V-like domain, the third exon encodes the transmembrane and the first part of the cytoplasmic tail, whereas the end of the transcript is encoded by the fourth exon. We used this information to predict the intron positions in *rbtCD28* and *rbtCTLA4* genes. Specific primers were designed from cDNA sequences to amplify and clone genomic sequences corresponding to the putative intron regions. PCR products were cloned and sequenced, except for the first intron of *rbtCTLA4*, which could not be fully amplified. Introns were indeed located at the sites predicted from mammals (Fig. 3). Where the sequence was highly conserved, for example at the limit between exons 2 and 3, it was possible to verify that the intron was inserted in the same position in human and rainbow trout sequences. Most importantly, intron frame was also strictly conserved. These observations indicate that *rbtCD28* and *rbtCTLA4* genes have the same intron/exon structure as their mammalian counterparts, further substantiating the hypothesis they belong to the same gene family.

rbtCD28 and rbtCTLA4 mRNAs are primarily expressed in lymphoid tissues

In mammals, the expression pattern of CD28 and CTLA4 is strongly controlled by their respective positive and negative costimulatory functions. To gain further insight into the function of rbtCD28 and rbtCTLA4 genes, we investigated their mRNA expression patterns in different organs using real-time RT-PCR (Fig. 4*A*). rbtCD28 was only expressed in lymphoid tissues, especially in the thymus. This is reminiscent of the expression pattern described in mammals, where CD28 is predominantly expressed on T cells and developing lymphocytes (47). rbtCTLA4 was expressed at 3- to 10-fold less than rbtCD28 in the thymus, pronephros, and spleen, consistent with the expression of these genes in mammals. Interestingly, trout intraepithelial lymphocytes from the gut also expressed rbtCD28 and rbtCTLA4, respectively, at similar levels as found for pronephric lymphocytes. Expression of rbtCD28 or rbtCTLA4 was not detected in the trout liver, a nonlymphoid tissue. The pattern of rbtCD28 expression in different organs was also analyzed by Northern blot. This experiment confirmed that rbtCD28 was strongly expressed in the thymus, and at lower levels in other lymphoid organs (Fig. 4*B*). Two bands were observed at 1600 bp and 950 bp, respectively. The short transcript likely corresponds to the sequence identified using 5′ and 3′RACE, whereas the long transcript may result from an alternative polyadenylation site.

rbtCTLA4 is induced upon PHA stimulation and viral infection

Although CD28 is constitutively expressed at the surface of T cells in mammals, CTLA4 has been described as an activation-induced

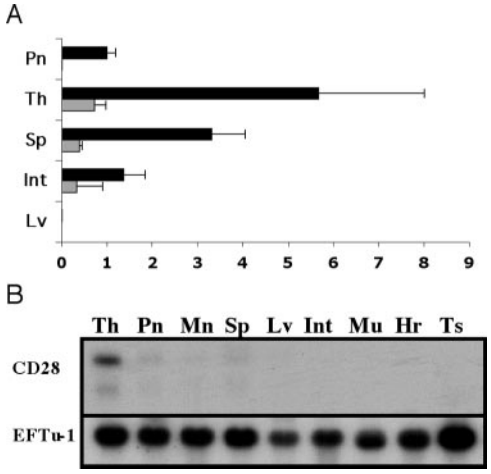

FIGURE 4. Expression pattern for rbtCD28 and rbtCTLA4. *A*, RNA samples were prepared from different tissues and subjected to reverse transcription. The amount of rbtCD28 or rbtCTLA4 cDNA was measured by real-time RT-PCR. Normalized values for rbtCD28 or rbtCTLA4 were determined by dividing the average rbtCD28 or rbtCTLA4 value by the average β-actin value. Then, normalized rbtCD28 (■) and rbtCTLA4 (□) values were subjected to a calibration relative to the pronephros level of rbtCD28 (i.e., 3,3 rbtCD28 copies for 100 β-actin copies). *B*, Northern blot analysis of rbtCD28 expression in thymus (Th), pronephros (Pn), mesonephros (Mn), liver (Lv), intestine (Int), muscle (μ), heart (Hr), and testis (Ts). The same blot was rehybridized with an EFTu-1 probe, as a control for the total amount of mRNA loaded in the gel.

gene. We therefore analyzed rbtCTLA4 expression after stimulation with a conventional T cell mitogen. Because lymphoid cell lines are not available for the rainbow trout model, we analyzed the relative kinetics of rbtCD28 and rbtCTLA4 in PHA-stimulated Ficoll-purified pronephric leukocytes. The pronephros is the bone-marrow equivalent in teleosts. We assessed mRNA expression by real-time RT-PCR throughout 4 days of PHA stimulation, which corresponds with the generation of blast cells in culture. We observed a striking modification of the rbtCD28:rbtCTLA4 mRNA ratio (Fig. 5*A*), corresponding to a rapid increase of rbtCTLA4 expression compared with that of rbtCD28 during the first hours following activation. Such a pattern was not observed in pronephric cells maintained in parallel without PHA stimulation (data not shown).

In mammals, the expression of CD28 and CTLA4 on the surface of activated T cells controls the activation, the extent, and the decay of the cellular immune response. We have previously shown that VHSV, a fish rhabdovirus, induced a strong T cell response in rainbow trout (38). To determine whether the expression of rbtCD28 and rbtCTLA4 are modified in vivo, we experimentally infected rainbow trout with an attenuated strain

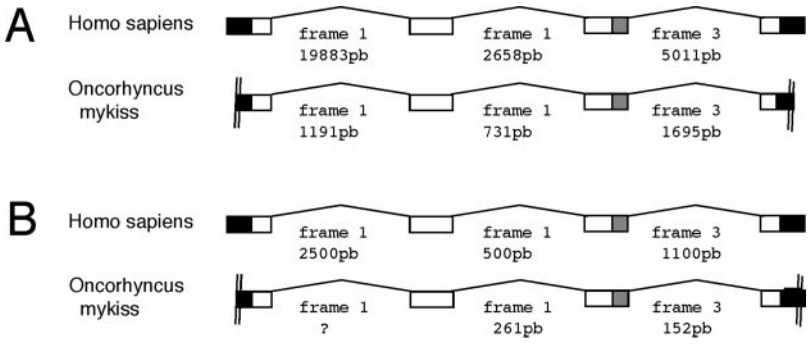

FIGURE 3. Intron/exon structure of rbtCD28 (*A*) and rbtCTLA4 (*B*) genes compared with their human counterpart. Human CD28 and CTLA4 structures were obtained from ⟨http://www.ensembl.org⟩ (ENSG00000178562 and ENSG00000163599). GenBank accession nos. for rbtCD28 and rbtCTLA4 introns are as follows: AY789437, AY789438, AY789439, AY789440, AY789441. The first intron for rbtCTLA4 intron was only partially sequenced to determine splice sites, and therefore its length was not determined.

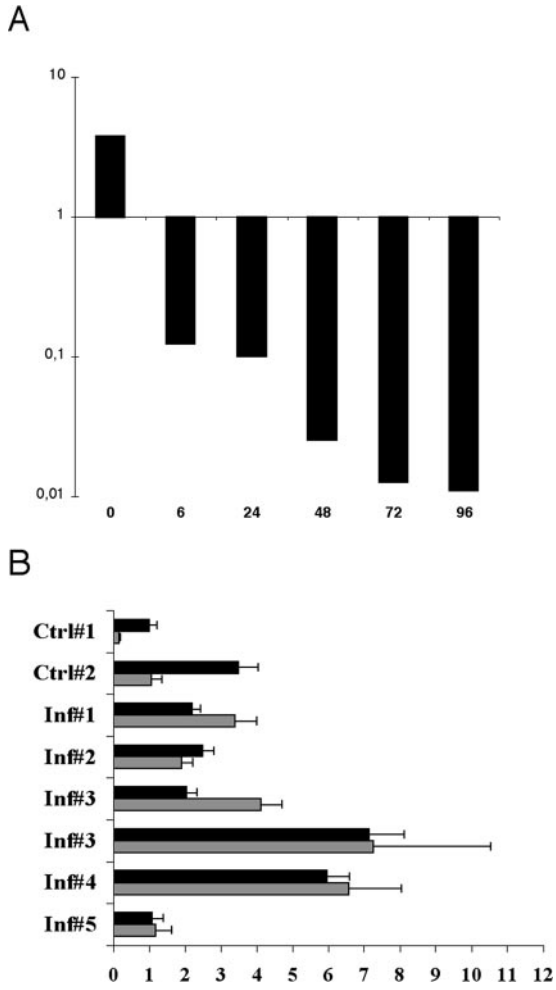

FIGURE 5. rbtCD28 and rbtCTLA4 mRNA expressions upon PHA and viral stimulation. *A*, RNA was prepared from fresh pronephros cells, and after incubation with PHA for 6, 24, 48, 72, and 96 h. The levels of rbtCD28 and rbtCTLA4 mRNAs expression were quantified by real-time RT-PCR, and the rbtCD28:rbtCTLA4 ratio was then calculated. *B*, The amount of rbtCD28 or rbtCTLA4 cDNA was measured from the spleen of VHSV-infected fish (Inf# 1–5) or from PBS-injected controls (Ctrl# 1 and 2) by real-time RT-PCR. The rbtCD28 or rbtCTLA4 normalized value was determined by dividing the average rbtCD28 or rbtCTLA4 value by the average β-actin value. Normalized rbtCD28 (■) and rbtCTLA4 (□) values were then subjected to a calibration relative to the level of rbtCD28 expressed in the first control fish.

of VHSV. Twenty-seven days postinfection, fish were infected again and sacrificed 2 wk later. We then used real-time RT-PCR to assess the expression of rbtCD28 and rbtCTLA4 mRNAs in splenic leukocytes 2 wk after secondary infection with VHSV. Although rbtCD28 expression was not significantly different in control and infected fish, rbtCTLA4 was clearly up-regulated in splenocytes of the VHSV-infected animals (Fig. 5*B*). Thus, rbtCTLA4 is strongly induced in splenocytes in the context of an antiviral T cell response in vivo.

The cytoplasmic tail of rbtCD28 triggers ERK phosphorylation

To gain insight about the capacity of rbtCD28 and rbtCTLA4 to deliver an activation signal, we produced human T cell clones expressing chimeric receptors composed of the extracellular and transmembrane regions of the human CD28 fused to the cytoplasmic tail of either rbtCD28 (huCD28/rbtCD28) or rbtCTLA4 (huCD28/rbtCTLA4). We stably transfected a human T cell line (Jurkat CH7C17) that is deficient for CD28 and CTLA4 (48) with different constructs, as described in Fig. 6*A*. For each construct, growing clones were expanded and tested by flow cytometry for the expression of the chimeric CD28 receptor. Typical results of expression of the chimeric CD28 receptor are shown in Fig. 6*B* for clones expressing either huCD28/rbtCD28 (2F5, 3B8) or huCD28/rbtCTLA4 (26H6) receptors. T cell clones expressing the full-length human CD28 (A14) and CD28 with a truncated cytoplasmic tail (Del30) were used as positive and negative control, respectively. Surface expression of the relevant receptors and TCR/CD3 was monitored for all T cell clones.

Previous reports showed that stimulation of huCD28 with Abs induced the phosphorylation of the MAPK ERKs (49, 50). We thus investigated the respective role of the rbtCD28 and rbtCTLA4 in mediating this signaling pathway. Western blot analysis of ERK phosphorylation showed that cross-linking CD28 clones 3B8 and 2F5 (huCD28/rbtCD28 receptors; Fig. 7) and clone A14 (full-length huCD28; Fig. 7) lead to ERK activation. In contrast, no clear effect on ERK phosphorylation could be detected in cells expressing huCD28/rbtCTLA4 (Fig. 7; clone 26H6). Several clones expressing chimeric receptors were tested and showed consistent results. These observations indicate that the rbtCD28 cytoplasmic tail is capable of inducing an activation-based signaling cascade, whereas the cytoplasmic tail of rbtCTLA4 did not show any such effect.

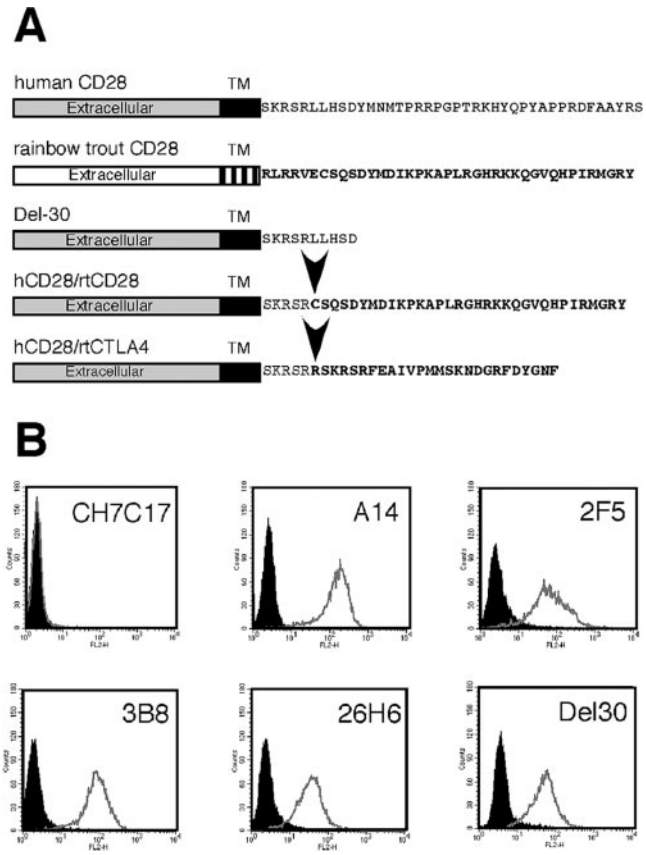

FIGURE 6. T cell clones expressing chimeric costimulatory receptors. *A*, Sequence schematic of proteins encoded by constructs for chimeric receptor expression. *B*, FACS analysis of Jurkat CH7C17 cells and transfectants expressing human CD28 (A14), huCD28/rbtCD28 receptor (3B8, 2F5), huCD28/rbtCTLA4 receptor (26H6), or a truncated CD28 (Del30). Cells were stained by direct immunofluorescence with an anti-human CD28-PE Ab.

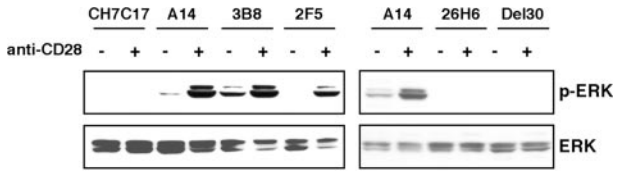

FIGURE 7. The cytoplasmic tail of rbtCD28 but not rbtCTLA4 mediates phosphorylation of ERK. Jurkat CH7C17 transfectants expressing huCD28 wild-type (A14), huCD28/rbtCD28 receptor (3B8, 2F5), huCD28/rbtCTLA4 receptor (26H6), or a truncated CD28 (Del30) were incubated with (+) or without (−) anti-human CD28 Ab at 4°C, washed, and stimulated for 2 min at 37°C with goat anti-mouse Ab. Lysates were analyzed by immunoblot with anti-phospho-ERK Ab (p-ERK). Immunoblots were reprobed with an anti-ERK Ab to show that similar amounts of proteins were loaded on the gel (ERK).

The cytoplasmic tail of rbtCD28 induces IL-2 production

To further confirm the signaling capacities of rbtCD28, we investigated whether chimeric clones were able to increase IL-2 production upon stimulation with anti-human CD3 and anti-human CD28 Abs. A significant induction of IL-2 production was observed when cells expressing full-length huCD28 or huCD28/rbtCD28 receptors were incubated in anti-CD3 Ab-coated plates (Fig. 8; clones A14 and 2F5, respectively). Furthermore, when T cell clones expressing either the full-length version of human CD28 or the chimeric huCD28/rbtCD28 receptor were subjected to double cross-linkage treatment (anti-human CD28 and anti-human CD3), IL-2 production was significantly increased, confirming that the cytoplasmic tail of rbtCD28 can deliver positive costimulatory signals.

Intriguingly, cells expressing huCD28/rbtCTLA4 did not induce significant production of IL-2 when incubated on the anti-CD3 plates (Fig. 8; clone 26H6) or upon double cross-linkage (anti-CD28 and anti-CD3).

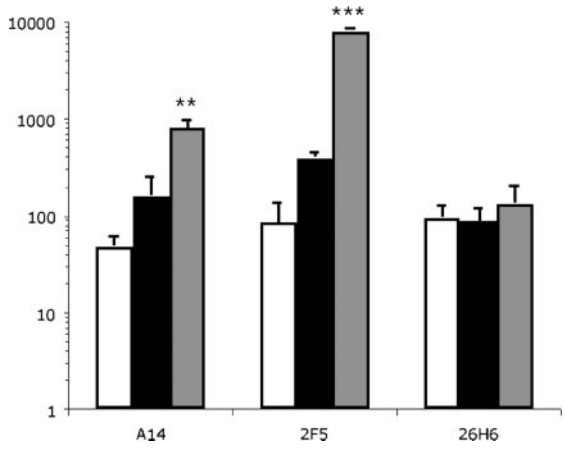

FIGURE 8. Combined triggering of CD3 and huCD28/rbtCD28 induces IL-2 production. Jurkat CH7C17 transfectants expressing huCD28 wild-type (A14), huCD28/rbtCD28 receptor (2F5), huCD28/rbtCTLA4 receptor (26H6) were incubated with (□) or without (■) anti-human CD28 Ab for 30 min at 4°C. After washing, cells were deposited on anti-CD3-coated plates, and goat anti-mouse IgG1 Ab was added to cross-link human CD28 or chimeric receptors. Cells incubated only with goat anti-mouse IgG1 Ab were used as a control (□). Supernatants were collected after 24 h at 37°C, and the IL-2 titer was determined by ELISA. IL-2 concentration is indicated in pg/10 ml. Asterisks indicate that the production of IL-2 by cells incubated using anti-human CD3-coated plates in the presence of anti-human CD28 Ab was significantly different from the production of IL-2 by cells solely plated on anti-human CD3-coated wells (two-tailed Mann-Whitney *U* test; **, $p < 0.05$, ***, $p < 0.01$).

rbtCD28 and rbtCTLA4 are localized on different chromosomes in trout

An interesting feature of the CD28 family is that *CD28*, *CTLA4*, and *ICOS* are closely linked (within 200 kbp) in humans, mice, and rats, forming a costimulatory cluster in these genomes. To determine whether this feature is shared among all vertebrates, we screened a 4.5× BAC library derived from clonal trout with probes corresponding to the rbtCD28 and rbtCTLA4 V regions. Twenty independent BAC clones were initially identified, of which 16 were positive for rbtCD28 and one was positive for rbtCTLA4 by secondary PCR screening. We then determined by in situ hybridization that three independent positive clones for rbtCD28 and one for rbtCTLA4, respectively, localized to the short arm of chromosome 7 (linkage group 12) and to the telomeric region of the long arm of chromosome 22 (linkage group 5) (Fig. 9). Genetic mapping using single nuclear polymorphism analysis within a double-haploid cross supported the linkage group assignment. Taken together, these results indicate that *rbtCD28* and *rbtCTLA* are located on different chromosomes, and therefore do not belong to a unique immunological

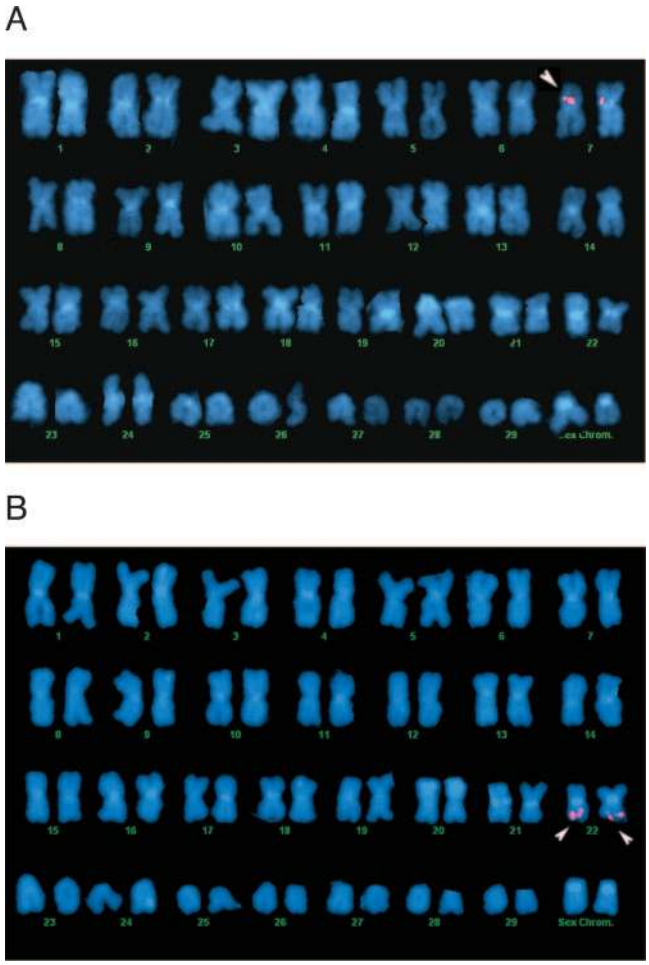

FIGURE 9. rbtCD28 and rbtCTLA4 are positioned on different chromosomes. rbtCD28 (*A*) and rbtCTLA4 (*B*) localization was visualized by in situ hybridization using rbtCD28 and rbtCTLA4-positive BAC clones as probes. rbtCD28 resides on the short arm of chromosome 7, whereas rbtCTLA4 is physically located on the telomeric region of the long arm of chromosome 22.

cluster, suggesting that they may have been subjected to different evolutionary constraints in fish compared with mammalian lineages.

Discussion

Recent progress in evolutionary immunology supports the notion that the main components and cell types of the adaptive immune system are conserved in gnathostomes (51, 52). Bony fish possess lymphocytes, which express typical Igs and TCRs that participate in Ag-specific responses, but costimulatory molecules have yet to be reported in teleosts. In this study, we describe rainbow trout genes that are homologous to the mammalian CD28 and CTLA4 costimulatory receptors, which deliver the second signal for T cell activation thereby contributing to the regulation of T cell responses in mammals.

Both rbtCD28 and rbtCTLA4 receptors encode an extracellular Ig V-domain containing a highly conserved B7 binding site, a transmembrane region, and a cytoplasmic tail. rbtCD28 and rbtCTLA4 sequences shared ≈25 and 35% similarity with their mammalian counterparts, respectively. The Ig superfamily V domain was well conserved for both, whereas the cytoplasmic tail was more divergent for rbtCTLA4 than for rbtCD28. In mammals, CD28 and CTLA4 bind the B7 ligands through a loop in the V domain that is conformationally constrained by the presence of three consecutive P residues. Similar motifs were present within the rbtCD28 (MYPPPI) and rbtCTLA4 sequences (LYPPPY), suggesting that these receptors would likely recognize similar ligand(s) as compared with their mammalian counterparts. In support of this likelihood, B7 family member orthologs are present among rainbow trout ESTs (J. D. Hansen and P. Boudinot, manuscript in preparation). Thus, receptors belonging to the B7 family are expressed in teleosts, and some may constitute the functional ligands for rbtCD28 and rbtCTLA4.

rbtCTLA4 displays several other structural features that are specific to mammalian CTLA4. An Asn residue at position 78 is strictly conserved in all CTLA4 sequences thus far examined including trout. In humans, the Asn^{78} glycan interacts with residues from the D/E/B β-strands of the V domain and is thought to be important for the structural integrity of the protein (53). In addition, a GNGTXY motif was also conserved in the G strand of both rbtCTLA4 and mammalian CTLA4 V domains that is reminiscent of a J segment. Interestingly, replacement mutagenesis demonstrated that this region was critical for higher affinity binding of human CTLA4 to its ligands compared with CD28 (54). These results suggest that rbtCTLA4 displays several structural characteristics that are specifically involved in mammalian CTLA4 functionality.

rbtCD28 and rbtCTLA4 genes display expression patterns that are quite similar to what has been reported in mammals. rbtCD28 and rbtCTLA4 transcripts are expressed in lymphoid tissues, and rbtCD28 mRNA is clearly expressed at quantitatively higher levels than rbtCTLA4 in naive lymphocytes. In mice and humans, CD28 is expressed on most T cell lineages and is up-regulated during initial T cell activation events, but upon ligation with its ligand (i.e., B7.1) on APCs, it is quickly down-regulated. Conversely, CTLA4 mRNA is expressed at low levels within T cells, but upon activation and CD28-B7 ligation, transcription is rapidly up-regulated. In trout a similar scenario emerged in that in vitro PHA stimulation quickly reversed the rbtCD28:rbtCTLA4 ratio in primary leukocyte cultures, indicating that rbtCD28 and rbtCTLA4 are differentially regulated during cell activation. Along the same line, rbtCTLA4 was also up-regulated in splenocytes during an in vivo immune response against VHSV, a rhabdovirus. rbtCD28 and rbtCTLA4 also share the intron/exon gene organization of the CD28 gene family. Thus, rbtCD28 and rbtCTLA4 possess expression patterns and genomic organizations that are similar to those of their mammalian counterparts, supporting the fact that rbtCD28 and CTLA4 are likely the orthologs of mammalian CD28 and CTLA4.

Comparison of the cytoplasmic tails of rbtCD28 and rbtCTLA4 with their mammalian counterparts suggests that only rbtCD28 may have signaling properties. In mammals, the short cytoplasmic tail of CD28 and CTLA4 share a YXXM motif, which binds the Src homology 2 domain of p85, an adaptor subunit of PI3K (55). The presence of this motif in both cytoplasmic tails indicates an overlapping signaling function for CD28 and CTLA4 (56). In contrast, no apparent motif is shared between the rbtCD28 and rbtCTLA4 cytoplasmic tails. Rainbow trout CD28 contains a DYMDI motif that likely corresponds to the mammalian motif DYMNM binding motif for PI3K (55) and Vav-1 (13, 57), suggesting that rbtCD28 and mammalian CD28 may have related costimulatory functions. Signaling properties of a chimeric receptor huCD28/rbtCD28 support this hypothesis. Indeed, stimulation of the chimeric receptor promoted vigorous TCR-induced IL-2 production, and induced the phosphorylation of the MAPK ERKs, a downstream effector of T cell activation. In contrast, the PYAP motif that binds ITK, TEK, and LCK in human and mouse CD28 (10, 11, 50) is absent in rbtCD28, suggesting that some CD28 signaling pathways may be restricted to mammals.

More surprising, however, was the absence of the DYVKM motif in the rbtCTLA4 cytoplasmic tail, which in mammalian CTLA4 binds PI3K, the clathrin adaptor protein complexes AP1 and AP2, and two phosphatases, SHP2 and PP2A (21, 58, 59). The absence of this signaling motif in rbtCTLA4 suggests that it may not have the same function as mammalian CTLA4. In fact, the cytoplasmic tail of rbtCTLA4 did not trigger the phosphorylation of ERK, nor did it modify the production of IL-2 upon anti-CD28 cross-linkage and anti-CD3 treatment. These observations neither demonstrated nor excluded that rbtCTLA4 negatively regulates immune responses, but indicated that rbtCTLA4 and rbtCD28 likely act through different signaling pathways. Conversely, it may simply be that the cytoplasmic tail of teleost CTLA4 interacts with different signaling components to control T cell activation, as suggested by the YGNF motif that conserved between rainbow trout and Atlantic salmon CTLA4.

Finally, it is interesting to consider the divergent nature of the cytoplasmic tail of rbtCTLA4 in light of its chromosomal localization. Although *CD28* and *CTLA4* belong to the same immunological cluster in humans (chromosome 2), mice (chromosome 1), and chickens (chromosome 7), *rbtCD28* and *rbtCTLA4* are localized to two different chromosomes, implying that they have been subjected to different evolutionary constraints. This is reminiscent of the genomic organization of MHC *class I* and *class II* genes, which are linked in all vertebrates but teleosts. Such a split of gene complexes onto different chromosomes in teleosts may have resulted from the whole genome duplication event that occurred in the fish lineage subsequent to its divergence from the other vertebrates (60).

This study shows that homologues of mammalian CD28 and CTLA4 are present in teleosts, implying that factors governing the "second signal" for T cell activation have been conserved during evolution. The emergence and diversification of this family of costimulatory receptors therefore occurred in the first vertebrates, predating the divergence between bony fish and tetrapods.

rbtCD28 appears to be a positive costimulatory receptor as found for CD28 in mammals. Additional studies are therefore needed to determine whether rbtCTLA4 down-regulates T cell activation in vivo during trout immune responses similar to its mammalian counterpart.

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Disclosures

The authors have no financial conflict of interest.

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