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J Immunol 2005; 175:2427-2437; doi: 10.4049/jimmunol.175.4.2427 http://www.jimmunol.org/content/175/4/2427

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Characterization of a C3a Receptor in Rainbow Trout and \textit{Xenopus}: The First Identification of C3a Receptors in Nonmammalian Species\textsuperscript{1}

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Virtually nothing is known about the structure, function, and evolutionary origins of the C3aR in nonmammalian species. Because C3aR and C5aR are thought to have arisen from the same common ancestor, the recent characterization of a C5aR in teleost fish implied the presence of a C3aR in this animal group. In this study we report the cloning of a trout cDNA encoding a 364-aa molecule (TC3aR) that shows a high degree of sequence homology and a strong phylogenetic relationship with mammalian C3aRs. Northern blotting demonstrated that TC3aR was expressed primarily in blood leukocytes. Flow cytometric analysis and immunofluorescence microscopy showed that Abs raised against TC3aR stained to a high degree all blood B lymphocytes and, to a lesser extent, all granulocytes. More importantly, these Abs inhibited trout C3a-mediated intracellular calcium mobilization in trout leukocytes. A fascinating structural feature of TC3aR is the lack of a significant portion of the second extracellular loop (ECL2). In all C3aR molecules characterized to date, the ECL2 is exceptionally large when compared with the same region of C5aR. However, the exact function of the extra portion of ECL2 is unknown. The lack of this segment in TC3aR suggests that the extra piece of ECL2 was not necessary for the interaction of the ancestral C3aR with its ligand. Our findings represent the first C3aR characterized in nonmammalian species and support the hypothesis that if C3aR and C5aR diverged from a common ancestor, this event occurred before the emergence of teleost fish. \textit{The Journal of Immunology}, 2005, 175: 2427–2437.

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\textsuperscript{1}This work was supported by National Science Foundation Grant MCB-0417078, the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education and Extension Service, Grant 2004-01599; a contract from the European Community (QLSR-2001-002211); and Grant AGL2000-0349 from the Ministerio de Ciencia y Tecnologia.

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worth noting that there appears to be very little sequence homology of this loop among species. Interestingly, studies of guinea pig C3aR have identified two alternatively spliced receptors, lacking 34 residues of the large ECL2. No functional differences could be found in the expressed guinea pig spliced C3aR products (36).

Little is known about the evolution of C3aR and C5aR. However, anaphylatoxin activity has been demonstrated in primitive invertebrate species, where C3a-like peptides have been shown to induce hemocyte chemotaxis in tunicates, thereby implying the presence of a C3a-like receptor in these animals (37, 38). A recent report in trout showed for the first time in teleosts the presence of three C3a molecules generated from three trout C3 isoforms (C3-1, C3-2, and C3-4). Each of the three C3a isoforms stimulates to a significant degree the respiratory burst of trout head kidney leukocytes, suggesting that teleost fish contain a C3aR (39). Recombinant trout C5a has been produced and shown to play a prominent role in inducing leukocyte chemotaxis (40, 41) and respiratory burst (40). Thereafter, a C5aR was identified in rainbow trout, representing the first cloned (42, 43) and functionally characterized anaphylatoxin receptor in a nonmammalian species (42). It is well known that C3aR and C5aR share a high degree of homology, to the extent that it has been hypothesized that both receptors represent the duplication products of a single ancestral receptor. Thus, the presence of a bona fide C5aR in teleosts is relevant because it may be indicative of the existence of C3aR in these animal species. Thus, this study was initiated to explore the above-mentioned hypothesis with the idea of finding a homologue of C3aR in rainbow trout. In this study we report the characterization of a trout molecule whose primary structure appears to be highly similar to that of mammalian C3aRs (MC3aRs). However, this trout C3a-like receptor (TC3aR) was found to lack a significant portion of the large extracellular loop between TM4 and TM5 characteristic of MC3aRs, which is substituted instead by a much smaller loop. We also demonstrate the ability of this trout receptor to interact with trout C3a.

Materials and Methods

Fish

Rainbow trout (100–200 g) were obtained from Limestone Springs Fish Farm. Fish 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 12–14°C.

cDNA cloning of TC3aR and sequence phylogenetic analysis

Trout cDNA was generated from trout liver as previously described (42) using an Oligotex Direct mRNA kit (Qiagen), according to the manufacturer’s recommendations. mRNA (2.0 μg) was reverse transcribed to negative strand cDNA with oligo(dT) (0.05 μg/μl) and 40 U of SuperScript reverse transcriptase II (Invitrogen Life Technologies) for 1 h at 42°C. A primer set was designed on the basis of a 729-bp established sequence tag (EST; GenBank accession no. CA373689) from rainbow trout (Oncorhynchus mykiss) that was found to be homologous in sequence to mammalian chucks mykiss (EST; GenBank accession no. CA373689) from rainbow trout (42). This fragment was also used as a probe for Northern and Southern blotting.

PCR products were cloned into a TOPO zero blunt vector (Invitrogen Life Technologies). This fragment was also used as a probe for Northern and Southern blotting.

Consensus sequences were generated from comparisons of repeated amplifications from trout liver mRNA using SeqMan and MegAlign (DNA Star) software. The full-length cDNA of TC3aR was obtained by performing 5’ and 3’ RACE using SMART cDNA prepared from liver tissues, as described by Wang et al. (44). 3’ RACE was performed using a primer corresponding to the 3’ untranslated region (5’-CAGCACGAGCAACACGCACTA-3’) based on our initial TC3aR EST yield a 1463-bp fragment. When sequenced, the 3’ end was found to possess a stop codon, a 3’-untranslated region, and a poly(A) tail. 5’ RACE was performed using a primer corresponding to the 5’ untranslated region (5’-CAGCACGAGCAACACGCACTA-3’) and yielded an additional 13 bp located 5’ to the original EST. Sequence alignments and the phylogenetic tree were generated using the Clustal X software package (45). TM regions in TC3aR were predicted using TMPred software (46).

Northern blotting

TRIzol reagent (Invitrogen Life Technologies) was used to obtain the total RNA from various tissues and leukocytes of rainbow trout. RNA was quantified, and 10 μg/ml was size fractionated on agarose, ethanol precipitated and transferred to nylon-supported nitrocellulose membranes (Bio-Rad) by capillary blotting. The blot was then exposed to UV cross-linking to fix the RNA to the membrane. The 713-bp cDNA corresponding to our initial TC3aR EST was gel purified and radiolabeled with [32P]dCTP using the Ready-To-Go labeling system (Amersham Biosciences) and was purified using ProQuant G-50 microcolumns (Amersham Biosciences). The probe was prehybridized in Express hybridization solution (BD Clontech) at 68°C for 30 min in a hybridization oven (Problot 6; Labnet). The blot was hybridized at 68°C for 60 min in Express hybridization solution with 1–2 × 106 cpm of labeled probe/ml. After hybridization, the blot was rinsed three times in 2 × SSC with 0.1% SDS for 30 min at room temperature, then washed in 1 × SSC with 0.1% SDS with continuous agitation at 50°C for 40 min. After washing, the blot was exposed to x-ray film (Kodak X-OMAT; Eastman Kodak) in an autoradiography cassette (Fisher Scientific). The expression of TC3aR RNA was normalized for equal loading and transfer to 28S RNA.

Southern blotting

Genomic DNA (10 μg) isolation and blotting procedures have been previously described (42). For Southern blotting, the 713-bp cDNA fragment corresponding to our initial EST was generated by PCR. The gel-purified PCR product was then randomly labeled (Amersham Biosciences) with [32P]dCTP and used as a probe (65°C) under stringent conditions (0.25 × SSC/0.25% SDS, 64°C final wash). The blot was exposed to film for 6 days.

Generation of Abs against TC3aR

A 20-aa peptide corresponding to the N-terminal region of TC3aR (EHYGFNFSENYYTESYGEFDCT) was synthesized by Biosynthesis. Matrix-assisted laser desorption mass spectrometry was used to determine the purity of the peptide. The synthesized peptide was coupled to keyhole limpet hemocyanin by the glutaraldehyde method and was used to raise Abs in rabbits (Biosynthesis). Abs were purified by ELISA. The Ig fraction of the antiserum was first purified using a HiTrap protein G column according to the instructions of the manufacturer (Amersham Biosciences). Thereafter, specific Abs against the TC3aR peptide were purified by affinity chromatography using the synthetic peptide coupled to cyanogen bromide-activated Sepharose (Amersham Biosciences).

Isolation of PBLs and head kidney leukocytes (HKLs)

PBLs were isolated as previously described (42). Briefly, blood was collected from trout through the caudal vessel using a heparinized syringe and a 21-gauge needle. After extraction, the blood was immediately diluted 1:5 with EEM (American Type Culture Collection) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μl/ml heparin, then placed on ice. The blood cell suspension was then washed in PBS and centrifuged at 400 × g for 30 min. The packed cells were washed with HBSS or kept in EEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. HKLs were isolated as previously reported (39).

Flow cytometric analysis

Flow cytometric analysis of PBLs and HKLs was performed using conditions previously described (42). Briefly, 1 × 106 cells were suspended in 1 × PBS/2% FCS and incubated for 30 min at room temperature with affinity-purified rabbit anti-TC3aR, alone or in combination with anti-trout systems. Consensus sequences were generated from comparisons of repeated amplifications from trout liver mRNA using SeqMan and MegAlign (DNA Star) software. The full-length cDNA of TC3aR was obtained by performing 5’ and 3’ RACE using SMART cDNA prepared from liver tissues, as described by Wang et al. (44). 3’ RACE was performed using a forward primer (5’-GGGACAACATGATTICTCAG-3’) based on our initial TC3aR EST yield a 1463-bp fragment. When sequenced, the 3’ end was found to possess a stop codon, a 3’-untranslated region, and a poly(A) tail. 5’ RACE was performed using a primer corresponding to the 5’ untranslated region (5’-CAGCACGAGCAACACGCACTA-3’) and yielded an additional 13 bp located 5’ to the original EST. Sequence alignments and the phylogenetic tree were generated using the Clustal X software package (45). TM regions in TC3aR were predicted using TMPred software (46).
IgM (mAb 1.14 provided by Dr. G. Warr, Medical University of South Carolina, Charleston, SC) or anti-trout thrombocyte (mAb28.D7) mAbs. As a negative control, cells were incubated with Abs purified from preimmunized rabbit serum or control mouse IgG. After two washes in PBS/2% FCS, cells were stained with either FITC-conjugated anti-rabbit IgG or allophycocyanin-conjugated anti-mouse IgG. Cells were washed two more times, and cell cytometric analysis was performed using a standard FACScan (BD Biosciences). For each sample, 20,000 individual cells were analyzed, and the resulting data were analyzed using the program CellQuest (BD Biosciences).

**Indirect immunofluorescence microscopy**

Indirect immunofluorescence microscopy was performed using cells stained with anti-TC3aR in a similar manner as described for cell cytometric assays, with an additional step of incubation with Hoechst stain.

**FIGURE 1.** Amino acid comparisons of TC3aR and XC3aR with MC3aR sequences. Putative TMs are overlined. Extracellular and intracellular domains are indicated by upward and downward arch symbols, respectively. Residues important for C3aR function are shown in bold and include the following: Tyr200 (trout numbering) and the PKC recognition domain in the trout sequence (FKSQRA). The serine/threonine C-terminal phosphorylation sites in trout and Xenopus are also in bold (see Results and Discussion). Predicted N-glycosylation sites are circled. Asterisks denote identities in all sequences, and gaps are denoted by dashes. Colons indicate strongly similar amino acids, whereas single dots infer weakly similar residues. The N and C termini are indicated by lines linked to TM1 and the C-terminal valine of the trout sequence, respectively. The peptide sequence used to generate polyclonal Abs (at the N terminus of the trout sequence) is boxed.
Generation of TC3a and C3adesArg

TC3a was generated as previously described (39) from C3-1, the most active and abundant TC3 isoform (47). Briefly, trout C3-1, Bf/C2, and factor D molecules were purified from trout serum as described previously (47, 48). To produce the C3a anaphylatoxin, purified TC3-1 (5 mg), trout factor B/C2 (250 μg), and trout factor D (30 μg) were incubated for 30 min at room temperature in the presence of 5 mM Mg2+ in PBS. To purify the C3a fragment, the total reaction mixture (0.8 ml) was passed through a Superdex 200 gel filtration column (Amersham Biosciences) equilibrated with PBS, pH 7.4. The purity of the C3a was determined by SDS-PAGE and N-terminal sequencing. In mammals, the C-terminal Arg of C3a is shown between TM4 and TM5. Numbers in parentheses indicate the amount of residues within the ECL2.

Measurement of intracellular calcium \([Ca^{2+}]_i\), mobilization by confocal microscopy

Trout leukocytes (HKLs) were incubated with the acetoxymethyl ester of the fluorescent calcium indicator fluo-3 (5 μM) for 15 min. Cells were then washed twice with Tyrode’s solution and left on coverslips for cell attachment and de-esterification of fluo-3 for at least 30 min before experiments were begun. Fluorescence was measured using a confocal microscope (Leica SP2; A0BS) with the excitation beam at 488 nm attenuated to 2.5%. Fluorescence emission was collected between 500 and 650 nm. Experiments were begun by flushing the cells with control Tyrode’s solution to remove debris and nonsticking cells. Baseline fluorescence was recorded in the control solution for at least 10 min before C3a (5 nM) was added alone or in combination with anti-TC3aR IgG or the control preimmune IgG. To determine the time course of the action of C3a with or without Ab, fluorescence was measured in 10–15 randomly selected cells every 30 s over a 15-min period. The fluorescence intensity was expressed as a percentage of the baseline fluorescence (control) before addition of C3a with or without Ab, and the peak fluorescence was measured after addition of C3a alone, C3a (5 nM) with the control preimmune IgG (40 nM), or C3a with anti-TC3aR IgG (40 nM). For statistical evaluation of the results, values from each animal were averaged, giving one value for each condition per animal. Statistical analysis was performed on the average values from each animal, with \(n\) representing the number of animals. Student’s \(t\) test for unpaired samples was used, and differences were considered statistically significant at \(p < 0.05\).

Results

Isolation and sequence analysis of TC3aR cDNA

We originally identified a 729-bp EST from rainbow trout that showed a high degree of homology to mammalian C3aR. Using 5′ and 3′ RACE, we obtained a 1476-bp product (TC3aR) that included an initiation site, an open reading frame encoding for 364 aa, and a 3′ polyadenylation signal. When aligned with C3aR from human, mouse, rat, and guinea pig, a significant degree of homology was observed throughout the molecule (Fig. 1), with one exception: a region spanning ~137 aa in MC3aRs was noticeably absent in TC3aR. Upon additional analysis, it was shown that this portion of sequence corresponded to a large segment of the ECL2 of mammalian C3aR (represented in Fig. 2). C3aR in mammals is the only rhodopsin receptor known to have such a large ECL2 (49, 50). In the alignment of Fig. 1, we also included a sequence from Xenopus tropicalis (a diploid frog) highly homologous to TC3aR and MC3aR. Like TC3aR, the X. tropicalis molecule (XC3aR) lacked a large portion of the ECL2 (113 residues), although this region contained 24 more residues than the ECL2 of TC3aR. The XC3aR sequence was found after a multiple alignment analysis of TC3aR and MC3aRs sequences with available X. tropicalis genomic scaffolds located at the Ensembl Genome Browser web site of the Wellcome Trust Sanger Institute (www.ensembl.org/). More excitingly, in silico analysis of the X. tropicalis scaffold containing the XC3aR gene showed that XC3aR and MC3aR genes were located in syntenic regions, further supporting designation of the trout and X. tropicalis genes as C3aRs (Fig. 3). We were unable to find the corresponding syntenic region in the genome of zebrafish, although we did find a molecule highly homologous to TC3aR and XC3aR in a different region of the zebrafish genome (data not shown). This lack of synteny in regions of the genome between the zebrafish and X. tropicalis (or mammals) has been observed for other genes (i.e., genes within the MHC region (51)). It should be stressed that exhaustive in silico analysis of the genomes of X. tropicalis or zebrafish did not yield a C3aR-like molecule with an extra large ECL2 similar in size to that of mammals. Similarly, analysis of the trout or salmon ESTs (~244,837 ESTs) deposited at the National Center for Biotechnology Information or at the Institute for Genomic Research did not yield either a C3aR-like molecule with an extra large ECL2. All the above results combined suggest that C3aR in fish and amphibians lacks the additional extra piece of sequence that is uniquely present in the ECL2 from all MC3aR molecules.

Hydropathy analysis confirmed that TC3aR and XC3aR did possess seven-transmembrane domains normally associated with C3aR (and all rhodopsin receptors) in higher vertebrates (Fig. 4). As stated above and shown in Figs. 1 and 2, there was a noticeable difference in the amount of residues of ECL2 between TM4 and TM5 of TC3aR and XC3aR compared with the same region of MC3aR molecules. In humans, the entire ECL2 contains 175 aa (50), whereas in TC3aR and XC3aR, the area spanning the ECL2 is significantly shorter, comprising 36 and 62 aa, respectively (Fig. 2). It is worth noting that MC3aR is fully functional even after deletion of 65% of the residues of ECL2 (52). In fact, mutagenesis studies have shown that the crucial residue (Tyr174 in humans) required for the interaction of C3aR with C3a is located at the beginning of the ECL2 (53). Significantly, this critical residue is conserved in the ECL2 of TC3aR and XC3aR. When aligned with other MC3aRs, this tyrosine is located two amino acids downstream from Cys172 (human numbering). In TC3aR and XC3aR, the
corresponding tyrosine is also two residues from Cys198 (trout numbering). It should be noted that mammalian and trout C5aR also possess a homologous tyrosine, but in both cases this residue is located four amino acids downstream from their respective cysteines.

To find meaningful percentages of amino acid identities between TC3aR and the mammalian and *Xenopus* C3aR sequences, the sequence alignments were performed excluding the extra 137 residues of the ECL2 from MC3aRs or excluding the extra 26 residues of the ECL2 from XC3aR lacking in TC3aR. The analysis showed that TC3aR presented a significantly greater degree of homology to C3aR than to C5aR molecules or other members of the rhodopsin gene family. Thus, TC3aR showed 40% identity to XC3aR, 38.3% identity to murine C3aR, 34.0% identity to human orphan receptor ChemR23, 32% identity to human formyl peptide receptor 1, 29.8% identity to TC5aR, and 25.3% identity to murine C5aR.

Further analysis of the structure of TC3aR and XC3aR indicated that similar to MC3aRs and MC5aRs, these sequences possessed a serine/threonine-rich C terminus in which these residues may represent phosphorylation sites that become modified as a result of ligand stimulation (36). Conservation of post-translational modifications, including N-linked glycosylation, was also observed. To date, all characterized MC3aRs have been found to possess three to eight glycosylation sites, in contrast with the one or two contained in C5aR molecules. It is interesting that one to four (depending on the species) of these glycosylation sites are localized in the ECL2 (36). TC3aR and XC3aR were both found to possess four potential glycosylation sites (Fig. 1). Despite their considerably shorter ECL2, both TC3aR and XC3aR still contained two N-linked glycosylation sites within that region. This observation is significant, because it indicates a higher degree of conservation of TC3aR to C3aR rather than to MC5aR, in which the ECL2 is devoid of glycosylation.

In our previous characterization of TC5aR, we found that TMs among C5aR molecules were more highly conserved than their intra/extracellular regions (42). This does not appear to be the case with TC3aR. Although TM2 and TM3 showed the highest degree of conservation among all C5aR TMs (61.5 and 68.2%, respectively), there also existed a high degree of conservation of all three C3aR intracellular domains (ICD), with sequence identity values ranging from 46.7% (ICD3) to 85.7% (ICD1). The extracellular domains, especially the N terminus and ECL2 regions, remained the least conserved of the receptor, with <6% sequence identity.

It has been hypothesized that C3aR serves as a putative substrate for protein kinase C; in all MC3aRs, this motif has been found to be conserved as XKSXXKX (36). Although TC3aR does not possess this exact motif, it contains a sequence signature that is consistent with protein kinase C (PKC) recognition (FKSQRA) (54), which is also located in IC3. PKC recognition domains analogous to those mentioned above in mammalian and trout C3aRs were absent in the IC3 of all cloned C5aR, including TC5aR.

In Fig. 5, a phylogenetic tree was constructed using TC3aR, XC3aR, MC3aR, C5aR, TC5aR, along with other mammalian rhodopsin receptors. Both trout and *Xenopus* C3aR molecules clustered with the MC3aR molecules (Fig. 5). The tree composite also suggests that TC3aR is the most ancestral of all C3aR molecules.

**Northern blot analysis of TC3aR**

Total RNA was obtained from a variety of trout tissues and leucocytes and was separated by formaldehyde agarose gel electrophoresis. After transfer to nylon membranes, TC3aR RNA expression was detected using a 713-bp P12 labeled probe, corresponding
to our original TC3aR EST. In all samples, no more than one band was observed, which was estimated to be ~2.4 kb (Fig. 6). In mammals, C3aR mRNA has been detected at sizes ranging from 2.1 kb (human and mouse) (17, 22) to 3 kb (guinea pig) (36). Normalization of the TC3aR signal with 28S rRNA indicated that expression was strongest in blood leukocytes. A significant level of TC3aR message was found in the rest of the samples tested within a relatively short period of exposure (1 day). It is difficult to state whether this expression was due to unavoidable blood contamination of the sampled organs or was the true expression of TC3aR in these tissues. In this regard, the expression of TC3aR in gills (an organ rich in blood leukocytes) was found to be comparable to that of PBLs (data not shown).

Southern blot analysis
As was the case for Northern blot analysis, the 713-bp probe was labeled with 32P and used as a probe for Southern blotting. This probe was used because analysis of genomic DNA by PCR indicated that no introns were present within this fragment. Each of the three restriction enzymes yielded two or three different digestion products (Fig. 7). Fish 2 and 3 showed additional bands in the blot, providing evidence of allelic variation in these animals. Taking into account that no restriction sites exist within the probe for the enzymes used in the digestion, the Southern blot data appear to suggest that two TC3aR genes exist in the trout genome. The later was almost expected due to the quasi-tetraploid nature of rainbow trout. It should be pointed out, however, that screening of trout liver and head kidney libraries by RT-PCR or colony blotting using the 713-bp EST fragment failed to yield other variants or isoforms of TC3aR. Moreover, the Northern blot analysis detected only a single band. Significantly, multiple sequence alignment analysis of our TC3aR sequence with all ESTs comprised at the Institute for Genomic Research and National Center for Biotechnology Information Unigene EST indexes (>155,000 trout ESTs) did not yield any EST significantly similar in primary and secondary structures to TC3aR. Although not definitive, these facts suggest that only one of the two TC3aR genes is expressed.

Binding of anti-TC3aR to PBLs
Polyclonal Abs were generated against a 20-aa peptide corresponding to a portion of the putative N-terminal extracellular region of TC3aR (boxed residues in Fig. 1). Anti-peptide specific Abs were affinity purified using a column to which the peptide had been coupled. When used for flow cytometric analysis, ~83% of all PBLs were stained with the Ab, as indicated in the shift of fluorescence shown in the histogram in Fig. 8A. Incubation of the Ab preparation with a molar excess of the TC3aR peptide inhibited >90% of the Ab staining of PBLs, providing additional evidence of the specificity of the Ab (data not shown). As shown in Fig. 8, staining was localized to two distinct cell populations, designated R1 and R2. The cells in R1 (~51% of the PBLs), displayed low forward and side scatters and showed the strongest staining. The R2 population (~25% of the PBLs) exhibited the highest forward and side scatters (composed of granulocytes in trout), although they stained to a lesser degree compared with the R1 population. Granulocytes in mammals have also been shown to express C3aR (35, 55). The R3 population (~17% of the PBLs) represented the negative cells, because these cells displayed the same fluorescence intensity as those stained with the preimmune polyclonal rabbit IgG (Fig. 8D). Costaining analysis using the anti-TC3aR and an anti-trout thrombocyte mAb showed that >95% of the TC3aR-negative cells (shown in R3, Fig. 8D) were, in fact, thrombocytes (data not shown). As expected, the anti-TC3aR did not stain trout erythrocytes (data not shown). The fact that the anti-TC3aR did not stain thrombocytes and erythrocytes supports the specificity of the anti-TC3aR and is in agreement with the lack of staining of thrombocytes and RBCs in humans when using anti-human C3aR (55). Costaining analysis using the anti-TC3aR in combination with a mAb specific for trout IgM (mAbs 1.14) that stains B lymphocytes in trout (56, 57) showed the presence of TC3aR in all B cells (Fig. 8F). This pattern was displayed in all fish analyzed (n = 8). The double-positive cells (representing ~36% of the PBLs) displayed the same low forward and side scatter properties of the R1 population in Fig. 8 (data not shown). This was expected, because B cells are small agranular cells. It should be noted that this double-positive population showed some variability among different individuals, ranging from ~29 to 55% of the PBLs analyzed.

Trout HKLs displayed a very similar staining pattern, in which B cells showed the highest binding to anti-C3aR, and ~95% of granulocytes stained, although once again, to a lesser extent (data not shown).
Immunofluorescence (IF) microscopy

IF microscopy using the affinity-purified Abs indicated that TC3aR is expressed on the cell surface of trout granulocytes and lymphocyte-like cells of PBLs (Fig. 9) and HKLs (data not shown), and that the staining pattern was punctuated and patchy (Fig. 9). In agreement with the flow cytometric results, it could be observed that in lymphocytes the patchy areas were generally more abundant than in granulocytes (Fig. 9). A similar scattered pattern of C3aR staining has been shown on human PMN (55) and astrocytes (34, 58).

Inhibition of C3a-mediated intracellular calcium [Ca^{2+}]

In mammals, it is well known that C3a induces increases in [Ca^{2+}] in a variety of cells (59–62). We determined first whether TC3a could have a similar effect on trout leukocytes, then we investigated the ability of the anti-TC3aR to inhibit potential C3a-mediated increases in [Ca^{2+}]. To evaluate the [Ca^{2+}],-mobilizing capacity of TC3a, its effect on fluo-3 fluorescence was measured, as shown in Fig. 10. Because analysis of [Ca^{2+}], using confocal microscopy have never been performed with trout leukocytes, we first optimized the experimental conditions to obtain stable baseline fluorescence measurements over time. This led to measurements in which the average baseline fluorescence in control cells increased only by 1.5 ± 2.1% over a 10-min period (85 cells from six trout). The middle panel of Fig. 10A shows one example of fluorescence from control cells after 10-min exposure to control Tyrode’s buffer. Most of the cells showed no or very little fluorescence. In the right panel of Fig. 10A, the increase in cell fluorescence induced by exposure to TC3a (5 nM) is clearly shown. As observed in that figure, a majority of the cells were stimulated (turned to green) by C3a, representing 68 ± 4% of the cells examined (59 cells from a total of three fish). The left panel in

**FIGURE 5.** Phylogenetic tree of TC3aR and other related members of the rhodopsin gene family. Amino acid alignments were performed using Clustal X, which was used to generate an unrooted neighbor-joining tree. Numbers on the branches indicate total recovery from 1000 bootstrap replications. The phylogenetic tree was created using alignments performed with the entire mammalian C3aR sequences. Accession numbers used to construct the tree are as follows: TC5aR, AY438032; human C5aR, NP_001727; guinea pig C5aR, O70129; rabbit C5aR, Q9TUE1; rat C5aR, NP_446071; mouse C5aR, AAB97774; dog C5aR, P30992; human C5L2, BAA95414; mouse C5L2, BAC35303; TC3aR, AJ616902; human C3aR, AAH020742; guinea pig C3aR, O88680; rat C3aR, O55197; mouse C3aR, AAH03728; mouse Dez, NP_032179; human ChemR23, CAA75112; human formyl peptide receptor 1 (FPR1), P21462; human FPR1, NM_002029; mouse FPR1, NP_038549; and mouse FPR1, NP_032068.

**FIGURE 6.** Tissue-specific expression of TC3aR by Northern blot. Ten micrograms of total RNA from the indicated tissues of rainbow trout was electrophoresed, blotted onto a nitrocellulose membrane, and hybridized with a DNA probe corresponding to a 713-bp fragment of TC3aR. Ethidium bromide-stained 28S is shown as a loading control.

**FIGURE 7.** Southern blot analysis. Genomic DNA from four individual rainbow trout was digested with EcoRI, EcoRV, and HindIII. The blot was then hybridized with a DNA probe corresponding to a 713-bp fragment of TC3aR. Values on the right of the blot indicate kilobases.
FIG. 10. A shows a transmission (brightfield) image of the microscope field with the cells selected for fluorescence analysis. Fig. 10B shows the time course of the increase in fluo-3 fluorescence after addition of 5 nM C3a. To verify that the effect of C3a was specific, the action of the desarginated form of C3a (C3adesArg) on [Ca^{2+}]_i mobilization was also analyzed. In contrast to C3a, C3adesArg did not increase [Ca^{2+}]_i in trout leukocytes, suggesting that the stimulatory effect was a direct effect of C3a (Fig. 10C). This result is in agreement with the situation in mammals, in which C3adesArg does not have an influence on [Ca^{2+}]_i mobilization (3, 63).

To verify that the cloned trout receptor (TC3aR) has the capacity to interact with the C3a ligand, we investigated whether the anti-TC3aR could block the C3a-mediated increase in [Ca^{2+}]_i, that is shown in Fig. 10C. To this end, cells were exposed to 5 nM C3a in the presence or the absence of an 8-fold excess of the anti-TC3aR IgG. Preimmune IgG was used as a control. Fig. 10C shows that the anti-TC3aR almost completely abolished the stimulatory effect of C3a in inducing increases in [Ca^{2+}]_i, whereas preimmune IgG had no effect. Thus, the inhibitory action of the anti-TC3aR in C3a-mediated increases in [Ca^{2+}]_i, supports the idea that TC3aR is a bona fide C3aR.

Discussion

Our current knowledge of the structure and function of C3aR molecules comes from the study of MC3aRs, with nothing being known about the evolutionary origins of this important proinflammatory molecule. Thus, up to this point, no C3aRs have been identified in nonmammalian species. The present study was therefore undertaken to identify a homologous receptor in an evolutionarily old vertebrate species, with the goal of better understanding the important structural elements and functions that have been conserved throughout the evolution of this receptor.

Recent studies by us have shown that teleost fish contain C3a and C5a anaphylatoxins that play important roles in chemotaxis and respiratory burst processes, implying the presence of anaphylatoxin receptors in these species. We (42) and others (43) have recently reported the characterization of a bona fide TC5aR in rainbow trout. These findings suggested that the duplication event giving rise to C5aR and C3aR from a common ancestor might have occurred before the emergence of teleost fish.

In this study we have characterized a 364-residue molecule in rainbow trout that is highly homologous to all known MC3aRs. Several lines of evidence indicate that the trout molecule reported in this study represents a true C3aR. 1) the overall primary and secondary structures of TC3aR has the capacity to interact with the C3a ligand, we investigated whether the anti-TC3aR could block the C3a-mediated increase in [Ca^{2+}]_i, that is shown in Fig. 10C. To this end, cells were exposed to 5 nM C3a in the presence or the absence of an 8-fold excess of the anti-TC3aR IgG. Preimmune IgG was used as a control. Fig. 10C shows that the anti-TC3aR almost completely abolished the stimulatory effect of C3a in inducing increases in [Ca^{2+}]_i, whereas preimmune IgG had no effect. Thus, the inhibitory action of the anti-TC3aR in C3a-mediated increases in [Ca^{2+}]_i, supports the idea that TC3aR is a bona fide C3aR.
A significant feature of TC3aR was the lack of 137 residues of the mammalian ECL2 region, which is unusually large in MC3aR molecules. MC3aR is the only member of the rhodopsin family of seven-transmembrane, G protein-coupled receptors with an unusually large ECL2. It is striking, however, that the only residue in the ECL2 that appears to be pivotal for the interaction of C3a with C3aR (Tyr\(^{174}\)) in humans (53) is positioned at the beginning of the loop, and it is conserved in the trout and Xenopus C3aR sequences.

Because the extra piece (137 residues) of the loop present in mammalian ECL2 does not seem to play a role in C3a binding, it has been proposed that it might bind to additional ligands and/or it might associate with surrounding cell surface proteins (53). Combined with our results, these findings suggest that the ancestral molecular architecture of C3aR did not include this extra piece of sequence, which was probably acquired later in evolution, after the appearance of amphibians, but before the emergence of mammals.

Northern blot analysis showed that PBLs were the most plentiful source of TC3aR mRNA. In mammals, C3aR is expressed in a wide variety of organs, although tissue distribution varies considerably between species. In guinea pigs, C3aR has been found to be expressed primarily in macrophages and spleen, with residual expression in liver, brain, and lung (36). However, in mice, C3aR is expressed mainly in heart and lung tissue, with no significant expression in spleen (22). This contrasts with human C3aR, which is found to be primarily expressed in placental, heart, and lung tissues, with no appreciable levels found in brain (17). The high expression of C3aR in PBLs was also confirmed at the protein level, using Abs against TC3aR. Significantly, flow cytometric analysis showed a high degree of TC3aR expression in trout B cells, which suggests an important role for this receptor in fish immunity. The presence of C3aR in mammalian B cells is inconclusive at this time. Although two studies using anti-human C3aR Abs showed no C3aR staining in circulating B cells (35, 61), another study demonstrated the presence of C3aR at the cDNA and protein levels in human activated-tonsil derived B cells (9). A similar situation was shown with regard to C3aR expression in human T cells. Although unchallenged circulating T cells were shown to be devoid of C3aR (35, 55), activated human T cells were demonstrated to express a functional C3aR (64). Thus, it is possible that the expression of C3aR in mammals depends on the activation state of lymphocytes. Our data, however, seem to suggest that all circulating lymphocytes in trout express TC3aR. The staining results obtained for TC3aR are very similar to those previously reported for TC5aR (42). Like the anti-TC3aR, anti-TC5aR Abs were shown to stain all B cells as well as the granulocyte population of PBLs. In addition, anti-TC5aR, similar to anti-TC3aR, did not stain the thrombocyte population, a finding in agreement with the lack of staining of thrombocytes in humans when using anti-human C3aR (55). It is worth noting that although several studies have convincingly demonstrated that human platelets do not express C3aR (65, 66), C3a has been reported to activate guinea pig platelets (67), implying the presence of such receptors in the platelets of these animals. It is therefore possible that in mammals, the expression of C3aR in platelets is species specific.

In conclusion, our findings represent the first structural and functional characterization of a C3aR in a nonmammalian species. The data presented in this study support the hypothesis that if C3aR and C5aR diverged from a common ancestor, then this event occurred before the emergence of teleost fish. Given the new array of roles recently demonstrated for C3a and C3aR in mammals (13–16), one anticipates that the study of these molecules in fish may identify unexpected functions of these molecules in higher vertebrates.

**Acknowledgments**

We thank Xinyu Zhao (Biomedical Imaging Core, School of Medicine, University of Pennsylvania, Philadelphia, PA) for her excellent technical assistance with the IF analysis for Fig. 9.

**Disclosures**

The authors have no financial conflict of interest.
References


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