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*J Immunol* 2006; 177:2505-2517; doi: 10.4049/jimmunol.177.4.2505

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Identification and Characterization of a FcR Homolog in an Ectothermic Vertebrate, the Channel Catfish (Ictalurus punctatus)1,2

James L. Stafford,* Melanie Wilson,* Deepak Nayak,* Sylvie M. Quiniou,† L. W. Clem,* Norman W. Miller,* and Eva Bengtén3,*

An FcR homolog (IpFcRI), representing the first such receptor from an ectothermic vertebrate, has been identified in the channel catfish (Ictalurus punctatus). Mining of the catfish expressed sequence tag databases using mammalian FcR sequences for CD16, CD32, and CD64 resulted in the identification of a teleost Ig-binding receptor. IpFcRI is encoded by a single-copy gene containing three Ig C2-like domains, but lacking a transmembrane segment and cytoplasmic tail. The encoded Ig domains of IpFcRI are phylogenetically and structurally related to mammalian FcR and the presence of a putative Fc-binding region appears to be conserved. IpFcR-related genomic sequences are also present in both pufferfish and rainbow trout, indicating the likely presence of a soluble FcR in other fish species. Northern blot and qualitative PCR analyses demonstrated that IpFcRI is primarily expressed in IgM-negative leukocytes derived from the lymphoid kidney tissues and PBL. Significantly lower levels of IpFcRI expression were detected in catfish clonal leukocyte cell lines. Using the native leader, IpFcRI was secreted when transfected into insect cells and importantly the native IpFcRI glycoprotein was detected in catfish plasma using a polyclonal Ab. Recombinant IpFcRI binds catfish IgM as assessed by both coimmunoprecipitation and cell transfection studies and it is presumed that it functions as a secreted FcR akin to the soluble FcR found in mammals. The identification of an FcR homolog in an ectothermic vertebrate is an important first step toward understanding the evolutionary history and functional importance of vertebrate Ig-binding receptors. The Journal of Immunology, 2006, 177: 2505–2517.

R eceptors specific for the Fc portion of Ig (i.e., FcR) are expressed by a wide variety of mammalian cells of hematopoietic origin. In general, FcR participate in activation or inhibition of immune responses following the recognition of monomeric Ig or Ig in the form of immune complexes. Since early reports describing the presence of FcR on macrophages (1) and lymphocytes (2–4), more recent studies have expanded the understanding of the genomics and functional significance of these important innate immune receptors.

In humans, eight genes found on chromosome 1q21-23 encode for the IgG FcR family (FcγRI) (5–7). These include the high-affinity FcγRI (RIIA, RIB, and RIC) as well as the low-affinity FcγRII (RIIB, RIB, and RIIIC) and FcγRIII (RIIA and RIIB). Comparatively, only three genes, split between two different chromosomes, encode for the mouse FcγR (5, 8). Recently, a murine IgG FcR (FcγRIV) with preferential specificity for IgG2a and IgG2b has been described, and it appears to be conserved in all mammalian species (9). Receptors for IgE (FcεR) are present in both humans and mice (10, 11), and a novel FcR that binds to both IgM and IgA isotypes (Fcα/μR) has also been reported in mammals (12, 13).

In most instances, the classical mammalian FcR genes encode for immune receptors consisting of extracellular C2 Ig domains (D), a transmembrane (TM) segment, and a cytoplasmic tail (CYT) that may contain signaling motifs. The membrane distal D1 and D2 Ig domains of FcγRI are structurally related to the two Ig domains of the low-affinity receptors, albeit the acquisition of a third membrane proximal domain (D3) is necessary for the high-affinity Ig binding exhibited by FcγRI (14–17). Depending on the type of FcR engaged, cellular activation or inhibition occurs through association with adaptor molecules or signaling motifs present within the CYT. In addition to the positive and negative regulation of cellular responses, FcR also participate in the uptake and clearing of immune complexes as well as the transport of Ig (18).

Alternative splicing and proteolytic cleavage of membrane-associated FcR have been shown to generate soluble forms of these receptors (sFcR) (19). For example, alternative splicing results in the generation of a TM-deleted FcγRIIb2 mRNA (termed FcγRIIib3), providing a third isoform encoded by the single murine FcγRII gene (8, 20). This FcγRIIib3 is a secreted protein and has been identified in culture supernatants of macrophage cell lines

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1 Department of Microbiology, University of Mississippi Medical Center, Jackson, MS 39216; and U. S. Department of Agriculture/Agricultural Research Service, Catfish Genetics Research Unit, Stoneville, MS 38701
2 Received for publication November 17, 2005. Accepted for publication June 6, 2006.
3 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked
4 Abbreviations used in this paper: D, domain; TM, transmembrane; CYT, cytoplasmic tail; FCRL, FcR-related protein; XFL, Xenopus leukocyte FcR-like protein; Ip, Ictalurus punctatus; LITR, leukocyte immune-type receptor; sFcR, soluble FcR; EST, expressed sequence tag; LRC, leukocyte receptor complex; CFS, catfish serum; UT, untranslated; qPCR, quantitative PCR; PDGFR, platelet-derived growth factor receptor; BLAST, basic local alignment search tool; BAC, bacterial artificial chromosome.

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expressing FcyRIIb2 (20). A similar mechanism also has been identified in humans for the FcyRIla gene, which is secreted by Langerhans cells and K562 and U937 cell lines (21). In activated neutrophils and macrophages, proteolytic cleavage of the membrane bound FcyRIII releases sFcyRIII, which represents the major form of sFcy found in human plasma (22, 23). Although the biological significances of sFcy are not entirely understood, mammalian sFcy can inhibit the binding of immune complexes to FcyR-positive cells, down-regulate B cell proliferation and Ab production, and trigger cellular activation by binding to complement receptors (19, 21, 24–26).

To date, Ig-binding FcR homologs have not been identified in any ectothermic vertebrate, although it is well established that these animals produce Abs (reviewed in Ref. 27). Several studies suggest that immune cells in amphibians, fish, and sharks bind to Ig, presumably through Fc-like receptors (28–37), and it is speculated that the transfer of Abs from mother to eggs in teleosts and elasmobranches is also a FcR-mediated process (38, 39). However, despite this functional evidence, an Ig-binding FcR homolog in these animals has not been identified, which has limited the understanding of not only the evolutionary history of Ig-binding receptors but also their functional significance in ectotherms. In this study, we report the initial identification and preliminary functional characterization of a novel Ig-binding soluble FcR homolog from the channel catfish.

Materials and Methods

Experimental animals, cell lines, and mAbs

Catfish (1–2 kg) were maintained in individual tanks as described previously (40). The 1G and 3B11 lines are cloned autonomous B cells generated from two different out-bred catfish by mitogen stimulation (41, 42). The 42TA is a macrophage cell line (42), and TS32.17 is a cloned non-autonomous Ag-dependent cytotoxic T cell line, which requires weekly stimulation with irradiated allogeneic cells for continuous proliferation (43). An MLP was developed by in vitro stimulation of naive catfish PBL with irradiated 3B11 cells and maintained by weekly stimulation (44). Catfish cell lines were grown at 27°C in a modified mixture of AIM-V and L-15 (Invitrogen Life Technologies) supplemented with 3% normal catfish serum (CFS) as described previously (45). The HeLa cell line was obtained from American Type Culture Collection and grown in DMEM (Sigma-Aldrich) with 10% FBS.

Mouse mAbs 9E1 (IgG1, κ) and H12 (IgG1, κ) react with catfish IgM H chain (45); mAb 9E1 was used for flow cytometry and H12 for Western blot analyses. mAb 1.14 is an isotype control that reacts with rainbow trout (Oncorhynchus mykiss) IgM H chain, but not with catfish IgM (46). Hybridoma cell lines were grown at 37°C in DMEM supplemented with 10% FBS. Hybridoma culture supernatants were used directly as the mAb source and contained equivalent IgG concentrations as determined by ELISA and Western blot analyses.

Fractionation of catfish leukocytes

Leukocyte isolation and fractionation were performed using a combination of Percoll gradients (Sigma-Aldrich) and MACS. Briefly, catfish leukocytes were isolated from heparinized blood and homogenized tissues (splen, pronephros, and mesonephros) by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep; Accurate Chemical) (42). Leukocytes were washed twice and resuspended in PBS containing 0.1% Glutamax (Invitrogen) with 10% FBS. 0.2 ml of each sample were electrophoresed on 1.5% formaldehyde-agarose gels and transferred onto Hybond-N+ (Amersham Biosciences). Hybridization and washing procedures were performed using Rapid-Hyb buffer (Amersham Biosciences) at 65°C and membranes were exposed at high sensitivity (65°C with 0.1X SSC, 0.1% SDS). The 5′ IpFcRI probe consisted of the first two Ig domains of IpFcRI, and was PCR amplified using primers I317F and I318R (Table I) and IPFcRI probe (primer pairs I265F and I271R; Table I) with IDPol DNA polymerase (ID Labs Biotechnology) and random primed with (A)^12-labeled dCTP by Megaprime labeling (Amersham Biosciences). The 3′ IpFcRI probe (primer pairs I265F and I271R; Table I) contained part of the third Ig domain plus the untranslated (UT) region. For Northern blots, total RNA from catfish PBL, cell lines, and various tissues was prepared using RNA-Bee (Tel-Test). 10 μg of each sample were electrophoresed in 1.5% formaldehyde-agarose gels and transferred onto Hybond-N+ (Amersham Biosciences), hybridized, and washed as above.

For RT-PCR, freshly isolated RNA was treated with DNase I (Invitrogen Life Technologies), and 1 μg was converted into cDNA using an oligo-T primer and 200 U Superscript III RT (Invitrogen Life Technologies). Oligonucleotide primers were designed using catfish-specific primers listed in Table I. Typical parameters were as follows: 3 min 94°C, followed by 30 cycles of 94°C 30 s, 58°C 30 s, and 72°C 1 min 30 s, with a final 10 min extension at 72°C. Products were visualized on 1.2% Agarose gels. Reactions contained 12.5 μL of 1:100 diluted cDNA and 0.2 units of Bio-Rad Taq DNA polymerase (Bio-Rad). 1 μL of each reaction was electrophoresed on a 1.5% agarose gel in TAE buffer. Products were visualized by ethidium bromide staining.

Sequence analyses

Nucleotide and amino acid sequences were analyzed using DNAStar software and aligned using CLUSTAL W (49). Neighbor-joining trees with pairwise gap deletions were drawn using MEGA v3.0 (50). Similarity searches were performed using BLAST (47) against the NCBI nonredundant protein database and the Protein Data Bank (PDB). Ig domains, TM segments, and secondary structure were predicted using Simple Modular Architecture Research Tool (51), Pfam databases (52), and the 3D-PSSM server (53). Signal peptide and predicted signal peptide cleavage site was performed using the SignalP 3.0 server (54). Secondary structure was performed using GeneDoc (http://pesc.edu/biomed/genedoc). Protein modeling was performed using SWISS-Model (http://swissmodel.expasy.org/SWISS-MODEL.html). The GPI-anchorage prediction program, bigPPI Predictor, was used to locate potential GPI-anchorage sites (http://mendel.imp.univie.ac.at/gpi/gpi.server.html).

Southern blot and expression analyses

Genomic DNA was prepared using erythrocytes from outbred catfish (54), which was digested with PstI or EcoR I, separated on 1% agarose gels, and transferred to Hybond-N+ membranes (Amersham Biosciences). Hybridizations were performed in Rapid-Hyb buffer (Amersham Biosciences) at 65°C and membranes were exposed at high sensitivity (65°C with 0.1X SSC, 0.1% SDS). The 5′ IpFcRI probe consisted of the first two Ig domains of IpFcRI, and was PCR amplified using primers I317F and I318R (Table I) with IDPol DNA polymerase (ID Labs Biotechnology) and random primed with (A)^12-labeled dCTP by Megaprime labeling (Amersham Biosciences). The 3′ IpFcRI probe (primer pairs I265F and I271R; Table I) contained part of the third Ig domain plus the untranslated (UT) region. For Northern blots, total RNA from catfish PBL, cell lines, and various tissues was prepared using RNA-Beet Test), 10 μg of each sample were electrophoresed in 1.5% formaldehyde-agarose gels and transferred onto Hybond-N+ (Amersham Biosciences), hybridized, and washed as above.
Recombinant protein expression and binding of IgM to IpFcRI

To assess IgM binding capabilities of IpFcRI, recombinant proteins were generated using both prokaryotic and eukaryotic expression systems and tested in Ig-binding assays. rIpFcRI was produced in Escherichia coli by cloning the three IpFcRI Ig domains into the pET 100 Directional TOPO expression vector (Invitrogen Life Technologies). The rIpFcRI, containing an N-terminal 6xHis epitope tag, was purified using MagNeHis Ni-Particles (Promega) according to the manufacturer's protocol, and refolding was performed according to the protocol given in the protein reflowing kit (Novagen). The rIpFcRI was ~34.6 kDa, including an ~4.1-kDa epitope tag, and was detected by the anti-Xpress mAb specific for the N-terminal Xpress epitope.

For the common precipitation of catfish IgM with rIpFcRI (or rIpFcRI with catfish IgM), 2.5 μl of CFS was diluted in 500 μl of immunoprecipitation (IP) buffer (50 mM Tris-HCL, 300 mM NaCl, 0.1% SDS pH 7.5) and precleared with 20 μl of Sepharose G beads (Amersham Pharmacia Biotech) for 30 min at 4°C. Two μg of rIpFcRI, 25 μl of Sepharose G beads, and 1 μg of anti-Xpress mAb or 1 μg of anti-V5 mAb (Invitrogen Life Technologies) were added to the precleared CFS and incubated for 14 h at 4°C on a rotary shaker. Immunoprecipitations were also performed as described above but with 50 μl of anti-catfish IgM mAb 9E1 or 50 μl of the isotype control mAb (1:14). After three washes, the Sepharose G beads were re-suspended in 75 μl of IP buffer and 25 μl of 4X SDS-PAGE reducing buffer and boiled for 5 min. Twenty μl samples were electrophoresed on 10% SDS-PAGE gels, transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences) and incubated in Tris-buffered saline supplemented with 1.0% BSA and 0.1% Tween 20 (TTBS-BSA) for 30 min at room temperature. Duplicate membranes were incubated in Tris-buffered saline containing 1.0% BSA and 0.1% Tween 20 (TTBS-BSA) for 30 min at 4°C. Two μg of rIpFcRI, 25 μl of Sepharose G beads, and 1 μg of anti-Xpress mAb or 1 μg of anti-V5 mAb (Invitrogen Life Technologies) were added to the precleared CFS and incubated for 14 h at 4°C on a rotary shaker. Immunoprecipitations were also performed as described above but with 50 μl of anti-catfish IgM mAb 9E1 or 50 μl of the isotype control mAb (1:14). After three washes, the Sepharose G beads were re-suspended in 75 μl of IP buffer and 25 μl of 4X SDS-PAGE reducing buffer and boiled for 5 min. Twenty μl samples were electrophoresed on 10% SDS-PAGE gels, transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences) and incubated in Tris-buffered saline supplemented with 1.0% BSA and 0.1% Tween 20 (TTBS-BSA) for 30 min at room temperature. Duplicate membranes were incubated overnight with either anti-catfish IgM mAb 1H12 (1/2 v/v) or anti-Xpress mAb (1/1000 –1/10000 (v/v)) were screened by Western blots against the protein according to the protocols of Cocalico Biologicals. Rabbit sera (1/1000–1/10000 (v/v)) were screened by Western blots against the protein to the cell surface. The IpFcRI-PDGFR construct was then transiently expressed in HeLa cells using GeneJuice Transfection Reagent (Novagen) according to the manufacturer's protocol. After 48 h, surface expression was detected by flow cytometry using anti-c-myc-FITC conjugated mAb (2 μg/ml; Sigma-Aldrich) and by immunoprecipitation using the anti-HA mAb (HA.11 ascites fluid; Covance) as described previously (55). To determine whether surface expressed IpFcRI binds catfish Ig, IpFcRI-transfected HeLa cells were incubated with either 1% CFS or 25 μg/ml purified catfish IgM (56) for 20 h at 37°C. Cells (1 × 10⁶/ml) were harvested and washed with ice-cold RPMI 1640 medium containing 0.3% sodium azide, and 100 μl of the cell suspension was incubated with 100 μl of a 1/2 dilution of mAb 9E1 (for surface IgM detection) or mAb 1.14 (negative control) for 30 min. After washing, cells were incubated with 15 μg/ml goat anti-mouse Ig (H+L)-biotin (Southern Biotechnology Associates) and 10 μg of streptavidin-PE (BD Biosciences) for 30 min each. Detection of IgM (9E1) reactive HeLa cells was determined by analyzing samples on a BD Biosciences FACScan.

Recombinant protein expression and binding of IgM to IpFcRI

To test whether the native leader of IpFcRI could generate a secreted protein, IpFcRI was amplified using primers incorporating a SacI site before the native leader and an EcoRV site after the third Ig domain (Table I). The product was cloned into the pDisplay vector (Invitrogen Life Technologies) using Cellfectin reagent (Invitrogen Life Technologies) and transfected into Sf9 cells in the presence of Cellfectin reagent (Invitrogen Life Technologies) and transfected into Sf9 cells (Invitrogen Life Technologies) using Cellfectin reagent (Invitrogen Life Technologies). After 72 h, supernatants (30 μl) and cell lysates were examined for rIpFcRI by Western blot using the anti-V5 mAb.

A rabbit IpFcRI Ab was produced using the E. coli-generated rIpFcRI protein according to the protocols of Cocalico Biologicals. Rabbit serum (1/1000–1/10000 (v/v)) was screened by Western blots against the protein at 100 ng per lane using goat anti-rabbit IgG-HRP (1/5000 (v/v); Southern Biotechnology Associates) as the secondary Ab. The prebleed

Table I. IpFcRI Primers

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<th>Primer</th>
<th>Sequence 5' to 3'</th>
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<td>TTAGTACCTCATACCCATC N/A</td>
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a Refers to nucleotide number in GenBank DQ286289.
b s, Sequencing; h, hybridization; c, protein expression; p, RT-PCR; n, nested PCR; i, insect expression; q, qPCR.

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FIGURE 1. Comparison of catfish IpFcRI and other teleost FcR-like sequences. A, Nucleotide and predicted amino acid sequence of IpFcRI. Predicted signal peptide and Ig domains are labeled, the signal peptide cleavage site between residues 19 (M) and 20 (D) is underlined, potential N-linked glycosylation sites are gray shaded, and the stop (TAG) codon is underlined and marked (>). Polyadenylation sites are underlined and nucleotide numbers are at left.

B, Schematic IpFcRI gene representation. Exons are numbered and labeled with the regions that they encode: L1 and L2, leader sequence; D1–D3, domains 1–3; 5′ and 3′ untranslated regions. The GenBank accession numbers for IpFcRI cDNA and the IpFcRI gene are DQ286290 and DQ286289, respectively.

C, Amino acid alignment of IpFcRI with related fish FcR-like sequences. The predicted signal peptide and domains are labeled. Residues similar/identical with IpFcRI are gray shaded; conserved cysteines are boxed, and gaps (−) are indicated. Amino acid numbers are at right.
sera demonstrated no immunoreactivity at all dilutions tested, whereas immune sera readily detected the recombinant protein (data not shown). The IpFcRI pAb (1/3000 (v/v)) was then used to detect IpFcRI in catfish plasma samples (5 μl per lane) by Western blot analyses. For some experiments, the plasma proteins were deglycosylated using PNGaseF (0.01 U; QA-Bio, CA) for 3 h at 37°C according to the manufacturer’s instructions.

**Results**

**IpFcRI sequence analyses**

By searching the catfish EST databases using the mammalian FcR sequences CD16, CD32, and CD64 as queries, a catfish FcR homolog (IpFcRI) was identified. IpFcRI cDNA consists of 1409 nucleotides with a 933-bp open reading frame encoding 311 aa (Fig. 1A). The polypeptide is composed of a signal peptide sequence containing a signal peptide cleavage site between residues 19 and 20 (predicted using SignalP 3.0 software) and a 292-aa region containing three Ig-like domains (D1–D3). The mature protein has a predicted weight of 32.77 kDa. Five potential N-glycosylation sites were found, two within the first Ig domain, one in the second Ig domain, and two in the third. The IpFcRI transcript does not encode a TM segment or CTY region. The third Ig domain ends at leucine 290, and the remaining 19 C-terminal amino acids encode a region of low compositional complexity with no identifiable motifs or domains. Notably, no GPI-anchorage prediction sites are predicted within the sequence, suggesting that IpFcRI is secreted. Five additional IpFcRI cDNAs also were identified from the known catfish databases in an attempt to find an IpFcRI-like transcript containing a TM and/or CTY segments. A total of 44,037 ESTs were screened, and all 5 IpFcRI-like sequences were identical with the original IpFcRI and lacked a TM and CTY. Furthermore, cDNA library screens, nested PCR, and 3’RACE protocols yielded only IpFcRI transcripts that lacked TM segments and CTY regions (data not shown).

The IpFcRI gene spans 3944 bp and consists of 6 exons (Fig. 1B). The first 33 nucleotides of exon 1 are UT, and the remaining 25 nucleotides plus the first 36 nucleotides of exon 2 encode the predicted leader sequence. Exons 3–5 contain 300 bp, 277 bp, and 328 (305 coding) bp, respectively, each encoding a single Ig domain. The last 60 nucleotides of exon 5 encode the low-complexity region and part of the 3’ UT region. All of the IpFcRI exon/intron boundaries follow a phase 1 splicing pattern. Exon 6 encodes the remainder of the 3’ UT. The longest intron, 1596 bp, is between exons 3 and 4.

Database searches and genome mining identified IpFcRI-related sequences in pufferfish and rainbow trout (Fig. 1C). These related sequences were 21–33% identical with IpFcRI and the highest amino acid identities/similarities were present in the D1 and D2 regions. The IpFcRI predicted signal peptide was not similar with those of the other fish species and the Tetraodon nigroviridis sequence was truncated within D3. Both the trout and Fugu rubripes IpFcR-like sequences appeared to encode full-length proteins, and like catfish, they lacked TM and CTY regions, suggesting that FcR homologs lacking TM and CTY are not unique to catfish.

**Phylogenetic analysis and similarity searches**

Database searches using the IpFcRI predicted amino acid sequence identified various mammalian FcR and FCRLs as potential relatives (E values ranging from 4e-19 to 1e-10; ~25–35% amino acid identity; Table II) a relationship reinforced by phylogenetic analyses. As shown in Fig. 2A, the IpFcRI D1 and D2 Ig-domains (encoded by exons 3 and 4) cluster with high bootstrap values with their mammalian FcR Ig-domain counterparts. IpFcRI D2 also appears to be related to the D2 of a new FcR-related molecule (FcRX) that is also known as the FcR homolog expressed in B cells (FREB; 57, 58). However, mammalian FcRX/FREB does not contain a D1 similar to those of the classical FcR or IpFcRI, a notion further reinforced by phylogenetic analysis (Fig. 2A). Neither IpFcRI D1 nor D2 clustered with FcRI D3 domains or with the two Ig domains of FcR (CD89), a receptor encoded within the LRC on chromosome 19q13.4. The IpFcRI D3 Ig-domain also was not phylogenetically related to any of the mammalian FcR Ig domains (data not shown). When the IpFcRI D1/D2 sequence was compared with the D1/D2 aa sequences of representative mammalian FcR, FCRL, and with receptors found within the LRC, it was clear that the putative IpFcRI Ig-binding unit was significantly related to the mammalian FcR and FCRL families (bootstrap value = 94; Fig. 2B). Furthermore, IpFcRI D1/D2 is a closer relative to mammalian FcR than are the newly described IpLITRs from catfish, which also encode FcR-related D1 and D2 Ig domains (59). Schematic comparisons of IpFcRI Ig domains with representative mammalian FcR and FCRL (60) as well as IpLITRs are summarized in Fig. 2C.

**IpFcRI structural analysis**

The predicted β-strand in IpFcRI are present in similar positions as the β-strand found in the mammalian FcR family members with the exceptions of an additional β-strand predicted within the D1 between the F and G strands as well as a missing C’ β-strand within the D2 (Fig. 3A). In some instances, the predicted IpFcRI β-strand were present in regions of high amino acid identity/similarity, and five of the FcR residues known to contact the Fc portion of mammalian Ig were either conserved or similar in the catfish sequence (Fig. 3A; note that human FcεR was the FcR used for comparison of contact residues). Similarity searches against NCBI’s PDB also revealed that IpFcRI D1 and D2 are structurally related to the corresponding Ig domains of several mammalian FcR. Human FcεRIα (1889; score 63, E value 8e-11) was identified as the closest structural relative to IpFcRI; Fig. 3B depicts a tube diagram comparing IpFcRI and FcεRIα. A protein model of IpFcRI generated using the SWISS-Model program is also shown (Fig. 3B). Similar to FcR, IpFcRI D1/D2 folded into two Ig-like domains consisting of eight β-strand that were arranged perpendicular to each other. Such a
structure is reminiscent of the tertiary protein structure of mammalian FcR determined by crystallization studies (61–63).

Southern blot and expression analyses

Southern blot analysis indicated that the IpFcRI is likely a single copy gene (Fig. 4A). Only one hybridizing band of ~4kb was observed in EcoRI digests with both an IpFcRI D1D2 and a 3′-end specific probe. Comparatively, three bands are observed in PstI digests, which reflect the presence of intronic PstI sites (data not shown). At the message level, IpFcRI transcripts were readily detected by Northern blot (Fig. 4B) in spleen, hematopoietic kidney tissues, and PBL. The lower levels of IpFcRI

**FIGURE 2.** Phylogenetic analyses reveal IpFcRI contains Ig domains related to mammalian FcRs. A, Comparisons of IpFcRI individual D1 and D2 sequences (gray, underlined) with representative mammalian FcR and FcRX/FREB D1 and D2 sequences. B, Comparisons of IpFcRI D1D2 (gray, underlined) with representative D1D2 of FcR, FCRL, IpLITRs (gray) and genes encoded by the mammalian and avian LRC. Accession numbers for the Ig domain sequences are: human (hu)FcγRI (CAI12557), huFcγRII (CAA35642), huFcγRIII (CAA36870), huFcλRI (AAH05912), huFCRL3 (AAH28933), huFCRL4 (AAK93970), huFCRL5 (NP.112571), huFcrR (AAH27953), huIL1R1 (AAH15731), huKIR3DL1 (AAC83928), huKIR3DS1 (AAV32446), human NKp46 (AJ001383), mouse (mo)FcγRI (AAD34931), moFcγRII (AA37608), moFcγRIII (NP.034318), moFcεRI (NP.034314), moFCRL3 (AA91578), moNKp46 (AJ23765), rat (ra)PIR1 (XP.341773), CHIR1 (CAG33731), catfish (Ip)LITR1 (AAW82352), IpLITR2 (AAW82353), and IpLITR3 (AAW82354). C, Schematic representation of IpFcRI compared with representative catfish IpLITR, human FcR and selected FCRL. The individual Ig domains for each receptor are shaded according to their relatedness with huFcγRI. Domains colored black have no phylogenetic relationship with huFcγRI.
message expressed in heart, gill, and liver may be due in part to residual PBL in these tissues. Because sFcRs in mammals are released from activated neutrophils, catfish neutrophils were enriched from freshly isolated PBL by depleting IgM-positive cells using MACS and fractionation on a discontinuous 34 and 51% Percoll gradient (Fig. 4). FACs analysis demonstrated an enrichment of the granular cell subpopulation from \( \frac{65}{100} \) in total leukocytes to \( \frac{88}{100} \) following enrichment (Fig. 4). The cells present in the lymphocyte gate (based on forward and side scatter analyses) were reduced from 30 to 5% (Fig. 4). Staining of the neutrophil enriched population with Sudan Black B and enumeration confirmed that these cells were \( \frac{90}{100} \) neutrophils. Importantly, analysis by RT-PCR demonstrated that although levels of TCR and IgM message were greatly reduced in the neutrophil-enriched fraction, equivalent levels of IpFcRI message were found when compared with the unfractionated PBL. qPCR was performed to assess the expression of IpFcRI in fractionated PBL as well as in various tissue subpopulations. Overall, kidney leukocytes expressed the highest levels of IpFcRI message when compared with PBL, spleen, gill, and liver (Fig. 5A). Because this expression was only partially attributed to the IgM-positive subpopulation, the IgM-negative leukocytes were subsequently examined for IpFcRI expression following isopycnic separation on Percoll gradients (Fig. 5B). These experiments demonstrated that IgM-negative lymphocytes (p34 layer) derived from the kidney tissues expressed the majority of IpFcRI with lower levels of expression in the granulocytes (p51 layer). Conversely, within PBL, the granulocytes present at the p51 layer predominantly expressed IpFcRI, whereas IgM-positive cells and the IgM-negative lymphocytes from the p34 layer expressed lower levels of message. Therefore, depending on the tissues examined, the various leukocyte subpopulations differentially expressed IpFcRI.

IpFcRI message expression also was determined in the catfish clonal cell lines, including those representing B cells (3B11 and 1G8), macrophage (42TA), T cells (TS32.17), and a polyclonal MLC (SV12). As opposed to freshly isolated PBL, all cell lines, except for the cytotoxic T cell line TS32.17, did not express detectable message for IpFcRI, nor did a polyclonal MLC (Fig. 5C). However, reamplification from the diluted PCR templates

FIGURE 3. Sequence and structural conservation between IpFcRI and mammalian FcRs. A. Amino acid alignment of IpFcRI D1 and D2 with representative murine and human FcR D1 and D2 Ig domains. Residues similar/identical with IpFcRI are gray shaded. Residues known to make contact with Ig for the huFcRo (62) are boxed and (*) marks residues in IpFcRI that are similar/identical with those contacts. Black and gray arrows represent the predicted \( \beta \)-strand for IpFcRI and huFcRo, respectively. Hatched boxes indicate conserved cysteines and dashes (--) represent gaps. B. Structural comparison of IpFcRI D1D2 with huFcRI (PDB: 1RPQ, left panel). Colors indicate positions where IpFcRI is identical (red), nonidentical (blue), and the location of conserved or similar amino acids involved in contacting the Fc portion of Ig between IpFcRI huFcRo (yellow). Predicted tertiary structure model of IpFcRI was generated using SwissModel with mouse FcεRI (SwissProt Accession No. P20489) as template (right panel). Accession numbers for the FcRs used in amino acid alignment are as in Fig. 2.
revealed that low levels of IpFcRI message could be amplified from 42TA, 3B11, and 1G8 cells, as well as the polyclonal MLC, indicating that in vitro IpFcRI expression is significantly down-regulated, compared with in vivo expression levels (described above).

In vitro secretion of IpFcRI and detection of the native protein

To address the possibility that IpFcRI is a secreted protein it was cloned into an insect expression vector with its native leader peptide sequence and then transiently transfected into an insect cell line (Sf9). As shown in Fig. 6, rIpFcRI (52 kDa) was detected in the supernatant of IpFcRI-transfected cells but not in the control, indicating that the native leader can indeed be used to generate a soluble IpFcRI.

Two immunoreactive bands were readily detected in catfish plasma using a rabbit polyclonal anti-IpFcRI with approximate weights of 64 and 90 kDa (Fig. 6B, left panel). Both proteins are larger than the predicted weight of the polypeptide (33 kDa); however, 5 N-linked glycosylation sites identified within the IpFcRI may account for this discrepancy. Following PNGase F treatment, the 64-kDa band was reduced to ~40, 50, and 55 kDa, which is remarkably similar to the pattern observed following deglycosylation of mammalian soluble FcR (21). The ~90-kDa protein was reduced to ~70 kDa following treatment with PNGase F. Whether or not this is a highly glycosylated form of IpFcRI, a related protein, or a product of nonspecific reactivity to the pAb is presently unknown. Similar-sized bands were detected in PBL lysates (data not shown).

Ig-binding assays

Normal CFS was incubated with E. coli generated rlpFcRI and the anti-Xpress mAb in the presence of Sepharose G beads. As demonstrated by Western blot analyses of the immuno-selected material using anti-catfish IgM, anti-Xpress mAb coimmunoprecipitated rIpFcRI with more catfish IgM than did the irrelevant anti-V5 mAb used as a control indicating that rlpFcRI associated with catfish IgM (Fig. 7A, left panel). The weak catfish IgM signal observed in the control sample may be due to nonspecific association of residual IpFcRI to the Sepharose G beads because low levels were detected in this sample when developed with the anti-Express mAb (Fig. 7A, right panel). Immunoprecipitations using 9E1 mAb not only selected catfish IgM (Fig. 7B, left panel), but

FIGURE 4. IpFcRI gene and expression analyses. A, Southern blot analysis using two different IpFcRI-specific probes is shown. Genomic DNA from four outbred catfish (F1–F4) were hybridized with either an IpFcRI D1D2 (probe 1) or the IpFcRI 3'-end (probe 2). B, Northern blot analyses of IpFcRI tissue expression. Total RNA from spleen, pronephros (HK), mesonephros (TK), heart (H), gill (G), liver (L), muscle (M) and PBL (P) were examined. RNA integrity and load levels were determined by using a catfish EF1α probe as a representative housekeeping gene. Kb markers are at the left margin. (*) marks nonstripped (residual) EF1-positive band. C, FACs analysis, Sudan Black B staining of catfish PBL and neutrophil-enriched fractions (40×) and IpFcRI message expression in catfish PBL and neutrophils. RNA was obtained from freshly isolated PBL and neutrophils that were enriched from the same PBL. RTPCR was performed using primers specific for IpFcRI (I317 F and I316 R), catfish TCRα, IgM H chain (membrane form, μM) and IpEF1α. The panels represent results from two different catfish. Reactions performed without the addition of template failed to amplify products for any of the primer pairs used (data not shown).
also coimmunoprecipitated rIpFcRI (Fig. 7B, right panel), again suggesting that rIpFcRI binds to catfish IgM. Neither catfish IgM nor rIpFcRI were detected when replicate experiments using 1.14 (isotype control) were performed (Fig. 7B).

To verify the binding results obtained with prokaryotic produced protein, IpFcRI also was cloned into the eukaryotic pDisplay expression vector. The IpFcRI-PDGFR construct, which contains myc and HA tags, was transiently expressed in HeLa cells, and the cells were then incubated with CFS or purified catfish IgM. Surface expression of IpFcRI was demonstrated by increased anti c-myc reactivity and an average of 40% of the cells exhibited increased staining (i.e., percent increase of FL1 signal) in cells incubated with CFS and IgM or 1% CFS resulted in increased and equivalent intensity of IgM on rIpFcRI and that other proteins found in CFS likely do not compete with the transfected cells by reacting with the surface expressed IpFcRI. Individual bars represent the mean plus SD calculated from three replicate experiments using cDNA from an individual fish. C, IpFcRI RT-PCR and nested PCR expression analyses in various catfish clonal cell lines, PBL, and MLC (SV12). IpFcRI specific primers I317 F and I316 R were initially used in PCR (top panel). The PCR were diluted 1/100 (v/v) and then re-amplified with the specific primers I317 F and I319 R (see Table I). PCR products were verified by sequencing and PCR performed without the addition of template failed to amplify products for any of the primer pairs used (data not shown).

Discussion
In the present study, a novel catfish FcR cDNA, termed IpFcRI, is characterized. It consists of three predicted C2 Ig-like domains with no identifiable TM/CYT segments or GPI-linkage motif(s), suggesting that IpFcRI is secreted and/or intracellularly expressed. The IpFcRI gene consists of six exons that encode a split leader peptide (exons 1 and 2), three Ig-like domains (exons 3–5), as well as a UT region (exon 6). The first five exons follow a phase 1 splicing pattern and in combination with the split leader peptide; the IpFcRI gene shares features characteristic of other FcR family members (5, 20). Although IpFcRI lacks TM/CYT, it may have significant functional roles in teleosts as a sFcR. Soluble FcR in mammals inhibit the binding of immune complexes to FcγR-positive cells, down-regulate B cell proliferation and Ab production, and trigger cellular activation by binding complement receptors (19, 21, 24–26). Both alternative splicing mechanisms and proteolytic cleavage of the membrane FcR have been shown to produce sFcR. At present, the functional significance of IpFcRI as a soluble protein is unknown; however, we have demonstrated that IpFcRI can be secreted in vitro using its own leader peptide, and the endogenous protein was detected in catfish serum/plasma with a pAb. This evidence reinforces that IpFcRI does indeed exist in vitro as a soluble protein. As in mammals, several N-linked glycosylation sites are present throughout the IpFcRI sequence. Phylogenetic analyses and similarity searches revealed that in addition to an unnamed pufferfish sequence (CAF97406), the majority of IpFcRI-related proteins were mammalian FcR and FCRL family members (e.g., mouse FcγRI, E value 4e-19; 26% amino acid identity) as well as several XFL and the three IpLITR-types. However, unlike the multigene and highly diversified XFL and IpLITR gene families (59, 64), only a single gene encodes IpFcRI.

IpFcRI is expressed primarily in catfish lymphoid tissues, with much lower levels of expression found in nonhemopoietic tissues such as heart, gill, liver, and muscle. Following separation into
IgM-positive and IgM-negative fractions, it was demonstrated that within PBL, neutrophils appeared to predominantly express IpFcRI. In addition, IgM negative lymphocytes from the anterior and posterior kidney expressed more IpFcRI message than lymphocytes from the peripheral blood. Therefore, the differential expression of IpFcRI in vivo may be in part due to the regionality of the leukocyte subpopulations and/or their activation state. In the absence of available catfish lymphocyte-specific mAbs other than anti-IgM, precise determination of the cells responsible for IpFcRI expression cannot be accurately determined. However, our expression analysis provides some insights into IpFcRI expression by leukocyte subpopulations in vivo. Several catfish clonal cell lines were also examined, and only the nonautonomous TS32.17 CTL cell line expressed IpFcRI. Following nested PCR, IpFcRI message was also detectable in 3B11 and 1G8 B cells, 42TA macrophages, and within PBL, neutrophils appeared to predominantly express IpFcRI. In addition, IgM-negative lymphocytes from the anterior and posterior kidney expressed more IpFcRI message than lymphocytes from the peripheral blood. Therefore, the differential expression of IpFcRI in vivo may be in part due to the regionality of the leukocyte subpopulations and/or their activation state. In the absence of available catfish lymphocyte-specific mAbs other than anti-IgM, precise determination of the cells responsible for IpFcRI expression cannot be accurately determined. However, our expression analysis provides some insights into IpFcRI expression by leukocyte subpopulations in vivo. Several catfish clonal cell lines were also examined, and only the nonautonomous TS32.17 CTL cell line expressed IpFcRI. Following nested PCR, IpFcRI message was also detectable in 3B11 and 1G8 B cells, 42TA macrophages, and a polyclonal MLC, suggesting that the expression levels in vitro are significantly lower than those observed in vivo. At present the factors responsible for regulating IpFcRI expression are unknown. However, increased expression in hemopoietic tissues, freshly isolated leukocyte subpopulations, and enriched PBL-derived neutrophils, suggest that this FcR homolog is widely expressed in vivo.

Coimmunoprecipitation experiments indicated that rIpFcRI produced in E. coli recognized native catfish IgM directly in the serum. It also appears that N-linked glycosylation of IpFcRI is not necessary for IgM binding although it is possible that glycosylation could influence affinity. In humans, prokaryotic derived soluble rFcRIIB was generated and refolded using a similar procedure described in this study (65). After refolding, the unglycosylated rFcRIIB bound to immobilized Ig as well as Ig in solution. Furthermore, mutant mammalian FcR devoid of N-linked glycosylation sites exhibited increased binding to monomeric IgG, compared with the wild-type glycosylated receptors (66). How N-linked glycosylation effects IpFcRI binding properties is presently unknown, but it appears not to be a requirement for the binding observed in this study. IgM binding by IpFcRI also was confirmed using a eukaryotic pDisplay system where transfected HeLa cells expressing surface targeted IpFcRI were shown to bind catfish IgM by FACS analysis. A similar experimental approach using the pDisplay vector has been used to determine whether selected mammalian FCRL (i.e., FREB/FcRX) bind Ig (58). Although FREB/FcRX is similar to IpFcRI in that it does not contain a TM and is related to FcR, it also is quite different. This member of the FCRL lacks any identifiable Fc fragment binding residues, as well as the corresponding D1 Ig-like domain present in the classical FcRs and IpFcRI. Because FREB/FcRX does not bind Ig, this further reinforces the notion of the evolutionary importance of the FcR D1D2 Ig domains required for Ab binding.

Amino acid alignments, PDB searches, and protein modeling demonstrated a high degree of structural conservation between IpFcRI and mammalian FcRs, in particular, the D1 and D2 domains. Eight of the predicted β-strand in the D1 of human FcR are present in similar positions in the corresponding regions of the
FIGURE 8. Cell surface IpFcRI binds catfish IgM. A. Surface expression and increased 9E1 staining of IpFcRI-pDisplay transfected HeLa cells. Mock cells were treated with all transfection reagents without plasmid DNA and cell surface expression by HeLa-IpFcRI was confirmed by flow cytometry using Anti c-myc FITC mAb (upper panel) and by immunoprecipitation using anti-HA mAb followed by Western blotting with anti-HA mAb (lower panel). B. Forty eight hours after transfections, both IpFcRI-transfected and mock-transfected HeLa cells were incubated with DMEM supplemented with either 25 µg/ml catfish IgM or 1% CFS for 20 h at 37°C. Surface bound catfish IgM was then detected by staining with the anti-catfish IgM mAb (9E1) and the secondary anti-mouse Ig-biotin conjugated followed by streptavidin-PE (right panel). Percentage of gated cells (FL2) stained with 9E1 and with 1.14 (isotype control) are indicated in the top right corner of the individual histograms.

catfish sequence and amino acid identity/similarity among the predicted β-strand was observed. An additional β-strand was predicted in the IpFcRI D1 sequence between the F-G loops of human FcR. Likewise, a similar degree of conservation of β-strand positions and amino acid identity/similarity was observed in the D2 with the exception that the C′ β-strand was not in the IpFcRI sequence. The contention that IpFcRI D1 and D2 were structurally conserved with the corresponding Ig domains of mammalian Ig-binding receptors was further supported by the PDB and SwissProt database searches. The most significant PDB matches were FcR with human FceRI being identified as the top scoring structural IpFcRI relative with an E value of 8e-11. Comparatively, the SwissProt database identified mouse and human FcγRI, FcγRII, FcγRIII, and FcεRI, with E values ranging from 4e-7 to 1e-19. Based on this information, an IpFcRI protein model was generated that revealed a folding pattern and overall tertiary structure similar to that of mammalian FcR (61–63). In mammalian FcR, the Ig binding residues are primarily located in the D2 loop regions (62, 63). Specifically, the contact residues are scattered throughout D2 in areas that consist of the B-C, C′-E, F-G loops, and the Cβ strand of the D2 domain. Five of the 15-aa residues known to make contact with the Fc portion of Ig in mammalian FcRs are either identical or conserved within IpFcRI D2. The conservation of all the Fc contact residues is unlikely due to the recognizable variability between mammalian and teleost Ig at both the amino acid and structural levels. However, the structural conservation of the D1D2 Ig-binding domains between mammalian FcRs and the novel IpFcRI does suggest an evolutionary importance of the D1D2 unit for Ig-binding among the vertebrates.

Located among the classical FcR on human chromosome 1q21-23 is a family of FcR relatives recently identified by using a consensus FcγR Fc-binding sequence motif to screen the databases (60). These receptors, known as the Fc-related proteins (FCRL), also have been identified in mice (chromosome 3; 67) and, although little is known about their functional roles and specific ligands, their identification has provided insights into the evolution of FcR-like receptors (68). FCRL genes appear to be members of a phylogenetically conserved immune receptor family. Evidence for this notion comes from the recent identification of novel IgSF receptors in ectothermic vertebrates. Of note is the discovery of the Xenopus leukocyte FcR-like proteins (XFL; Ref. 64) and the leukocyte immune-type receptors in channel catfish (IpLITR; Ref. 59). These amphibian and teleost molecules represent large and highly diversified families of putative immunoregulatory receptors that are composed of Ig domains related to those of the mammalian FCRL and FcR. Discovery of FCRL, XFL, and IpLITR in combination with detailed phylogenetic analysis of Ig domains encoded by receptors within the mammalian and avian LRC (69) represents the first molecular evidence that FcR-like receptors are present in amphians and fish. However, the chicken (Gallus gallus) is the only nonmammalian species where bona fide Ig-binding FcR homologs have been described (70).

In summary, IpFcRI appears to be a bona fide teleost FcR capable of binding Ig. It is structurally conserved with members of the classical FcRs, and maintains the D1D2 unit and Fc-binding sites required for Ab recognition. Moreover, preliminary functional analysis suggests that IpFcRI binds to catfish Ig as a soluble protein in the serum, or as a receptor when expressed on the cell surface. Although a membrane form of this receptor or other catfish putative Ig-binding molecules have yet to be identified, the discovery of IpFcRI is the first report of an FcR homolog in ectothermic vertebrates. Thus, it represents an important step toward understanding the evolutionary history and functional significance of Ig-binding proteins throughout vertebrates.

Acknowledgments
We thank Robin Chandler, Cecile Snell, and Aihua Tang for excellent technical assistance.

Disclosures
The authors have no financial conflict of interest.

References


