



Characterization of an IgM Binding Soluble Fc Receptor in Channel Catfish, *Ictalurus punctatus*

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Abstract

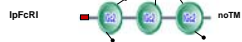
Recently, a channel catfish Fc receptor homolog, IpFcRI, was identified. The IpFcRI cDNA contained sequences for a leader, and three Ig-like domains, but did not contain sequences for either a transmembrane (TM) or a cytoplasmic tail (CYT). Genomic DNA analyses confirmed its lack of both transmembrane and cytoplasmic regions and mining of the rainbow trout and pufferfish genomic databases revealed the presence of similar FcR-like sequences. Since it was found that recombinant IpFcR proteins, made in a prokaryotic system, could bind to both affinity-purified catfish IgM and reduced IgM heavy chains immobilized onto nitrocellulose membrane, IpFcR is considered to be a homofide tetosol FcR. Thus, IpFcRI most likely represents a high affinity soluble FcR for IgM. Notably, the use of recombinant proteins containing various constant region domains of the catfish IgM chain (C_μ2, C_μ3, C_μ4, C_μ 2-3 and C_μ3-4) demonstrated that recombinant IpFcRI bound to the C_μ3 and C_μ4 domains. Currently, work is underway to map the binding site residues within both catfish IpFcRI and IgM.

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Are there Fc receptors in catfish?

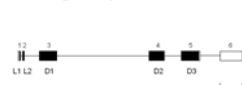
Protocol:

- FcR sequences CD16, CD32, and CD64 were used as queries to search the catfish EST databases.
- A single EST (BE469704) from a catfish pronephros (head kidney) cDNA library was identified.
- RACE protocols were used to obtain a full-length sequence now termed IpFcRI.

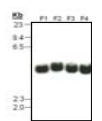


Nucleotide and predicted amino acid sequence of IpFcRI. IpFcRI consists of 1409 nucleotides encoding 311 amino acids. The predicted polypeptide is composed of a signal peptide, three Ig-like domains followed by a region of low compositional complexity. Five potential N-glycosylation sites (—●) are present. The mature protein has a predicted weight of 32.77 kDa. Notably, IpFcRI does not encode a TM segment or CYT region and there are no predicted GPI-anchorage sites. Catfish database searches show that all IpFcRI cDNAs identified lack TM and/or CYT.

IpFcRI gene structure



Southern blot



The IpFcRI gene spans 3944 bp and consists of 6 exons. Exons are numbered and labeled with the regions they encode: L1 and L2 for split leader and D1, D2 and D3 for Ig-outbound catfish (F1-F4) was digested with like domains 1, 2 and 3, respectively. White boxes represent 5' and 3' untranslated regions.

Southern blot analysis shows that IpFcRI is a single copy gene. Genomic DNA from three outbred catfish (F1-F4) was digested with EcoRI, electrophoresed on a 1% agarose gel, transferred to Hybond filter and hybridized at high stringency with an IpFcRI D3 probe.

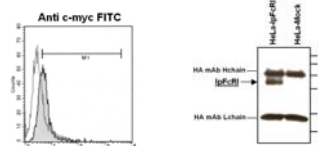
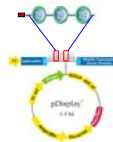


Database searches and genome mining identified IpFcRI-related sequences in pufferfish (*Fugu rubripes*) and Tetraodon nigroviridis) and rainbow trout (*Oncorhynchus mykiss*). These related sequences were 21-33% identical to IpFcRI; the highest amino acid identities/similarities were present in the D1 and D2 regions. Both the trout and *F. rubripes* FcRI-like sequences appeared to encode full-length proteins that lacked TM and CYT regions. The *T. nigroviridis* FcRI (CA397406) was truncated within D3. The trout FcRI sequence was generated by aligning the following ESTs: CA359052, CA350202, CA354438, CA356126, CA354773, CA344819, CA345217, CA347628, CA359710, CA366579, CA371062, CA372019, CA372368, CA374824, CA381028, CA381525, CA388014, CA366714, CA375361, CA381310, BX082867, BX082868, BX075495, BX075496, CR374266. The *F. rubripes* FcRI was found within Scaffold_610.12.

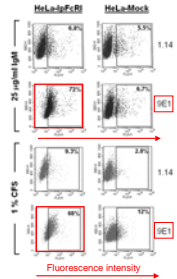
Does IpFcRI bind to Immunoglobulin?

Protocol:

- IpFcRI recombinant (r) protein was generated using an eukaryotic expression system.
- The three IpFcRI Ig domains were cloned into the pDisplay vector that introduces a N-terminal Ig K-chain leader and C-terminal phage-derived growth factor receptor (PDGFR) TM segment to target the protein to the cell surface.
- pDisplay r proteins contained hemagglutinin A (HA) and myc epitopes for detection by Ab.
- The IpFcRI-PDGFR construct was transiently expressed in HeLa cells and after 48 hrs, surface expression and IgM binding was detected by immunoprecipitation and flow cytometry.



Transfected HeLa cells express IpFcRI on surface. Cell surface expression by HeLa-IpFcRI was confirmed by flow cytometry using anti-c-myc FITC mAb and by immunoprecipitation using anti-HA mAb followed by Western blotting with anti-HA.

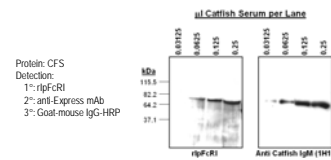
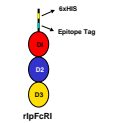


Cell surface IpFcRI binds catfish IgM. HeLa cells were incubated with media supplemented with 25 μg/ml catfish IgM or 1% catfish serum (CFS) for 20 hrs. Surface bound catfish IgM was detected by staining with anti-catfish IgM mAb (9E11) and an anti-mouse Ig-biotin conjugated secondary Ab followed by streptavidin-PE. Anti-treat IgM mAb 1.14 was used as a negative control. Percent gated cells are indicated. X axis represents Fluorescence intensity and Y axis represents side scatter.

rIpFcR binds to denatured and reduced catfish IgM

Protocol:

- An IpFcRI recombinant (r) protein was made in *E. coli* using the pET100/ D-TOPO vector expression system that introduces N-terminal 6xHis and Express epitope tags onto the protein.
- IpFcRI containing the three FcR Ig domains and was ~34.6 kDa, including the ~4.1 kDa Epitope tag.
- rIpFcRI binding to IgM was assessed by Western blot analyses.

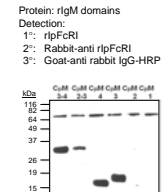
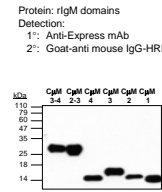


rIpFcR binds Ig μ chain. Various amounts of catfish serum (CFS) were electrophoresed in SDS-PAGE under reducing conditions, transferred to Hybond-ECL membranes and analyzed by Western blot using rIpFcR as the primary "binding protein" or anti-catfish IgM mAb 1H12. Blots were then developed using either the anti-Express mAb and goat anti-mouse IgG-HRP or goat anti-mouse IgG-HRP, respectively.

What portion of catfish IgM does IpFcRI bind to?

Protocol:

- Catfish IgM recombinant (r) proteins were made in *E. coli* using the pET100/ D-TOPO vector expression system that introduces N-terminal 6xHis and Express epitope tags onto the protein.
- Catfish rIgM proteins were produced either as individual domains, C_μ1, C_μ2, C_μ3, C_μ4 or in combinations as C_μ2-3 and C_μ3-4.
- rIpFcRI was used to generate polyclonal rabbit antisera.
- rIpFcRI binding to different regions of IgM was assessed by Western blots.



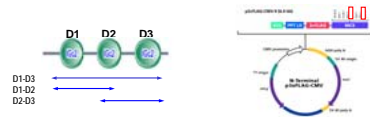
rIgM heavy chain domains C_μ2-3, C_μ3-4, C_μ1, C_μ2, C_μ3 and C_μ4 proteins can be detected in Western blots. The rIgM proteins were electrophoresed in SDS-PAGE, transferred to Hybond-ECL membranes and analyzed by using rIpFcR as the primary "binding protein". The binding rIpFcR was then detected using the polyclonal rabbit anti-IpFcRI and visualized Goat-anti rabbit IgG-HRP.

IpFcRI likely binds a combination of domains C_μ3 and C_μ4. IgM proteins were electrophoresed in SDS-PAGE, transferred to Hybond-ECL membranes and analyzed by using rIpFcR as the primary "binding protein". The binding rIpFcR was then detected using the polyclonal rabbit anti-IpFcRI and visualized Goat-anti rabbit IgG-HRP.

Which domains of IpFcRI bind Immunoglobulin?

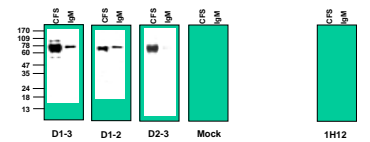
Protocol:

- IpFcRI recombinant (r) proteins were made using the p3xFLAG-CMV vector that introduces a FLAG tag onto the protein. This vector is suitable for transfections in mammalian cells.
- Catfish rIpFcR domain proteins were produced in combinations as D1-3, D1-2, and D2-3.
- rIpFcRI constructs were transiently expressed in Human Embryonic Kidney 293T cells.
- Binding of the different rIpFcR proteins to IgM was assessed by Western blots.



Protein: CFS or catfish IgM
Detection:
1^o: rIpFcRI
2^o: Anti-3x FLAG mAb
3^o: Goat-mouse IgG -HRP

Protein: CFS or catfish IgM
Detection:
1^o: 1H12
2^o: Goat-mouse IgG -HRP



IpFcRI D2 appears to be involved in IgM binding. Catfish serum (CFS) and affinity purified IgM were electrophoresed in SDS-PAGE under reducing condition, transferred to Hybond-ECL membranes and analyzed by Western blotting using the different rIpFcR proteins as a primary "binding protein" or anti-catfish IgM mAb 1H12. Blots were then visualized using either the anti-3x FLAG mAb and goat anti-mouse IgG-HRP or goat anti-mouse IgG-HRP, respectively.

Conclusions

- IpFcRI is a soluble Fc receptor consisting of three Ig-like domains.
- Unlike mammalian soluble FcRs, IpFcRI is not produced by alternative splicing or enzymatic cleavage from the cell surface.
- Soluble IpFcRI-like sequences are also found in rainbow trout and pufferfish.
- IpFcRI binds both native and denatured catfish IgM.
- Functional mapping shows IpFcRI binds to C_μ3 and C_μ4 domains.
- IpFcRI represents the first non-mammalian FcR identified.
- Future:
 - Finish functional mapping studies
 - Determine *in vivo* functions of IpFcRI
 - Identify sequence of surface bound FcRs present in catfish.