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B cell receptor accessory molecules in the channel catfish, *Ictalurus punctatus*

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Abstract

B cell receptor (BCR) accessory molecules CD79a and CD79b homologs were identified in the channel catfish, *Ictalurus punctatus*. Both are found as single copy genes that encode proteins containing a signal peptide, an extracellular Ig domain, a transmembrane region and a cytoplasmic tail containing an immunoreceptor tyrosine kinase activation motif (ITAM). IpCD79a and IpCD79b transcripts correlate well with IgM message expression. They are highly expressed in peripheral blood leukocytes (PBL) enriched in membrane (m) IgM⁺ cells and catfish clonal B cell lines, but not in catfish clonal T cells, indicating that IpCD79a and IpCD79b expression is B cell restricted. Studies using catfish clonal B cells (3B11) transfected with constructs encoding epitope-tagged IpCD79a and IpCD79b revealed that IpCD79a was expressed as a 45 kDa protein and IpCD79b was expressed as a 32 kDa protein. Furthermore, co-immunoprecipitations of epitope-tagged CD79 proteins demonstrate that these molecules are non-covalently associated with mIgM. These data correlate with some of the previous immunoprecipitation data demonstrating that catfish mIgM associates with proteins of 45 and 32 kDa.

Keywords

Channel catfish; B cell receptor; CD79a; CD79b; IgM; ITAM; recombinant proteins

1. Introduction

Most mammalian B cell receptor complexes are composed of membrane immunoglobulin (mIg), consisting of two Ig heavy (H) chains and two Ig light (L) chains, non-covalently associated with covalently associated Ig- α (CD79a)/Ig- β (CD79b) heterodimers ([1-9]; reviewed in [10-12]). The CD79 proteins are essential for mIg cell surface expression and B cell signal transduction. They are encoded by the B cell specific genes mb-1 [13,14] and B29 [15,16]. Both molecules consist of a single Ig-like domain, a transmembrane (TM) region and a cytoplasmic tail (CYT) containing an immune-receptor tyrosine-based activation motif (ITAM; [10]). ITAMs consist of the conserved sequence D/E-X₇-D/E-X₂-Y-X₂-L/I-X₇-Y-X₂-L/I and are also found in the CYTs of other TM signaling proteins (i.e. CD3, TCR ζ ,

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FcεRγ, DAP12; [17-23]. In B cells, the cross-linking of mIg results in the rapid phosphorylation of the ITAM tyrosine residues. In turn, this leads to the docking of the tyrosine kinase Syk and its activation. Syk then phosphorylates other targets, including the adaptor B-cell linker protein (BLNK), which forms a scaffold for other adaptor proteins and enzymes that initiate multiple B cell signaling cascades including the phosphatidylinositol-bisphosphate second messenger pathway leading to protein kinase C (PKC) activation and intracellular calcium flux (reviewed in [24-26]). If either one of the ITAM's tyrosines is mutated, that specific ITAM activity is lost [27,28].

While several studies have confirmed that the chicken BCR complex also contains a homolog to CD79b, which is structurally and functionally equivalent to the mammalian BCR complex [29-33], little is known about B cell signaling and B cell accessory molecules in ectothermic vertebrates. However it is well established that these animals produce antibodies and have mIg on the surface of their B cells, also data-mining has identified CD79a and CD79b sequences in the pufferfish, *Takifugu rubripes* and rainbow trout, *Oncorhynchus mykiss* [34]. Briefly, the existence of CD79 proteins in teleosts was inferred at a functional level in the catfish. For example, it was shown that peripheral blood leukocytes (PBL) exhibit rapid intracellular phosphorylation events, calcium flux and proliferation in response to anti-IgM stimulation [35]. This observation together with the short, three amino acid, CYT of catfish mIg [36] was consistent with the notion of catfish B cell accessory molecules. In addition, surface radioiodination of catfish B cells followed by lysis in the mild detergent octyl-β-glucoside and immunoprecipitation with antibodies to catfish Igμ chain revealed the presence of Ig associated molecules of 45, 32 and 25 kDa, which became tyrosine phosphorylated following stimulation by anti-Ig crosslinking [37]. Two-D gel analyses of these precipitates demonstrated the 32 kDa molecule to be either a homodimer or a heterodimer of similar sized molecules, while the 45 kDa molecule appeared covalently associated with an ~25 kDa molecule. Unfortunately attempts to isolate and obtain N-terminal sequences of these putative CD79 proteins were unsuccessful. More recently, at the molecular level, the pufferfish and trout CD79a and CD79b homologs were identified. They resembled both mammalian and chicken CD79a and CD79b molecules in sequence and genomic analyses showed conservation of synteny for the human and pufferfish molecules [34]. Here we report the identification of the catfish (Ip) CD79a and CD79b cDNA homologs, their expression in PBL and clonal leukocyte cell lines, correlate their protein expression patterns with the sizes of the previously reported immunoprecipitated IgM-associated molecules, and demonstrate their association with IgM using co-immunoprecipitation.

2. Materials and Methods

2.1 Animals and clonal cell lines

Channel catfish (1-2 kg) were obtained from a commercial source (ConAgra, Isola, MS) and maintained as previously described in individual tanks [38]. Catfish leukocyte cell lines were grown at 27°C in AL-3 medium consisting of equal parts AIM-V and L-15 (Invitrogen Life Technologies, Gaithersburg, MD) adjusted to catfish tonicity with 10% (v/v) deionized water and supplemented with 1 μg/ml NaHCO₃, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME, and 3% heat inactivated, pooled, normal catfish serum [39]. The 1G8 and 3B11 cell lines are cloned autonomous B cells generated from two different outbred catfish by mitogen stimulation [39,40]. 28S.3 is classified as a T cell line since it expresses TCRα and β message [41]. TS32.15 is a cloned nonautonomous antigen-dependent cytotoxic αβ T cell line, which requires weekly restimulation with irradiated catfish B cells for continuous proliferation [42, 43]. 42TA is a macrophage cell line [44]. Catfish PBL were isolated from heparinized blood by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical Corp. Westbury, NY) as described previously [45]. The 1G8 and 3B11 time course study was

performed by initiating cultures with approximately 2×10^5 /ml 1G8 and/or 3B11 B cells on day 0 and harvesting cells on days 1-8 for RNA.

2.2 Identification of IpCD79 homologs and sequence analyses

An IpCD79a 96 bp fragment was obtained by using short degenerate primers corresponding to a sequence in the pufferfish ITAM, ENIYQG, and a more 3' conserved sequence found in pufferfish, mouse and human CD79a cDNAs, YQDV. A full-length CD79a was then obtained using this sequence and 5' and 3'-RACE protocols and the complete IpCD79a was subsequently sequenced on both strands using universal forward and reverse primers and gene specific primers. IpCD79b was identified by searching the Catfish Gene Index in the TIGR database (now at <http://compbio.dfci.harvard.edu/tgi/>) using a rainbow trout CD79b cDNA sequence [34]. A single EST (accession number, CK421140) from a catfish spleen cDNA library was subsequently identified. Since the sequence was an unspliced transcript of the CD79b CYT and 3' UT region, 5'-RACE protocols were used to obtain the full-length sequence from pronephros (head kidney) mRNA. The full-length IpCD79b was then sequenced as described above.

Similarity searches were performed using BLAST analysis [46] against the National Center for Biotechnology Information (NCBI) non-redundant database. Immunoglobulin domains, TM segments, signal peptides and secondary structure were predicted using SOSUI (signal) Beta Version, SMART (<http://smart.embl-heidelberg.de/>) and CBS Prediction servers (<http://www.cbs.dtu.dk/services>). Nucleotide and amino acid sequences were analyzed using DNASTAR software (Madison, WI). Pairwise alignments were made using CLUSTALW [47] in the MegAlign module of DNASTAR and neighbor-joining trees with pairwise gap deletions were drawn using MEGA v3.1 [48]

2.3 Southern blotting

Genomic DNA was prepared using erythrocytes from outbred and homozygous gynogenetic [49] catfish as previously described [50]. Briefly, genomic DNA (10 μ g) was digested to completion with *Eco*R I, or *Pst* I restriction enzyme, separated on 1% agarose gels and transferred by capillary action onto Hybond-N+ membranes (GE Healthcare Bio-Sciences AB, Piscataway, NJ) using standard techniques. Hybridizations were performed in Rapid-hyb buffer (GE Healthcare Bio-Sciences AB) at 65°C according to manufacturer's instructions and membranes were washed at high stringency (65°C with 0.1X SSC, 0.1% SDS). The IpCD79a probe consisted of the full-length CD79a sequence and the IpCD79b probe encompassed the Ig domain plus the region directly 5' of the TM (see supplementary Table 1). Probes were amplified by PCR using IDPol DNA polymerase (ID Labs Biotechnology, London, Ontario) according to the manufacturer's protocol. Parameters were: 1 min 94°C, followed by 29 cycles of 94°C 30 sec, 61°C 30 sec, 72°C 1 min, then extension at 72°C for 5 min. The primers used are listed in supplementary Table 1; probes were random primed labeled with [³²P] dCTP by Megaprime labeling (GE Healthcare Bio-Sciences AB).

2.4 Magnetic cell sorting, RNA preparation and reverse transcription PCR (RT-PCR)

B cell enrichment from PBL was performed using magnetic activated cell sorting (MACs). Briefly, catfish PBL were isolated from heparinized blood as above and 2×10^8 PBL were incubated with 1ml of monoclonal antibody (mAb) anti-catfish IgM 9E1 [45] supernatant for 30 min at 4°C and washed with RPMI. Cells were then resuspended in 240 μ l of RPMI supplemented with 2 mM EDTA and 0.5% BSA. Sixty μ l of goat anti-mouse IgG microbeads (Miltenyi Biotec, Gladbach, Germany) was added and cells were incubated for 30 min at 4°C, washed with RPMI and separated into IgM (9E1) positive and IgM 9E1 negative fractions using MiniMACs separation columns (Miltenyi Biotec) according to the manufacturer's protocol. Fractions were washed with RPMI and RNA was prepared.

For RT-PCR, catfish PBL, sorted PBL and clonal cell line (3B11, 1G8, 28S.3, TS32.15, 42TA) RNA was prepared using RNA-Bee (Tel-test Inc, Friendswood, TX). Before being reverse transcribed RNA was treated with DNase I (Invitrogen Life Technologies) and 1 µg was subsequently converted into cDNA using an oligo-dT primer and 200 units of Superscript III RT (Invitrogen Life Technologies). Amplification was performed using catfish specific primers for CD79a, CD79b, the membrane forms of IgM and IgD, and elongation factor 1- α (EF1- α). Primer pairs are listed in supplementary Table 1. Typical parameters for PCR reactions were: 3 min 94°C, followed by 30 cycles of 94°C 30 sec, 61°C 30 sec, 72°C 1 min 30 sec, then a final extension at 72°C for 10 min. Annealing temperatures varied from 58°C to 61°C depending upon the specific primers used. Products were visualized following separation on 1.2% agarose gels.

2.5 Recombinant protein expression and Flow cytometry

IpCD79a, IpCD79b and catfish IgM H (μ) chain were individually cloned either into the pDisplay vector (Invitrogen Life Technologies), which introduces a hemagglutinin A (HA) epitope tag and/or the N-terminal p3XFLAG-CMV vector (Sigma), which introduces the FLAG epitope tag. Briefly, IpCD79a and IpCD79b fragments encoding the Ig domain through the CYT, including the stop codon, for each molecule were amplified from plasmids encoding the appropriate full-length cDNA. For catfish Ig μ chain, a fragment encoding the CH2, CH3, TM region and CYT including the stop codon was amplified from pronephros (head kidney) cDNA. Since C μ 1 contains the free cysteine, which forms the intrachain disulfide with IgL, it was decided not to include this domain in the Ig μ constructs to avoid any potential folding problems. Primers for cloning into pDisplay incorporated an *Xma* I site before the Ig domain and a *Pst* I site after the stop codon; primers for cloning into p3XFLAG incorporated an *Hind* III site before the Ig domain and a *Bam*H I site after the stop codon (see supplementary Table 1). All plasmids were purified using a Qiagen Endo-free Maxi kit (Qiagen Inc., Valencia CA) and the constructs were verified by PCR, restriction digestion and sequencing before transfection. All transfections were performed using a total of 6 µg of DNA. For example, for single transfections, 6 µg of one plasmid was used and for triple transfections, 2.0 µg of each of the three plasmids was used and the DNA was combined before the transfection. Routinely, transfections were performed using 5×10^6 3B11 B cells on day two (D-2; log phase) after passage by nucleofection using Kit-V and protocol T-20 of the Amaxa nucleoporator (Amaxa Biosystems, Gaithersburg, MD). Transfected 3B11 cells were then cultured for 36 h and aliquots of 5×10^5 cells were tested by fluorescence activated cell scanning (FACS; Becton Dickinson, San Jose, CA) for surface expression. Briefly, cells were washed and resuspended in 50 µl of ice cold RPMI-1640 medium (adjusted to catfish tonicity) containing 0.03% sodium azide and incubated with either 50 µl of anti-HA mAb (1:100), anti-FLAG M2 mAb (1:100), anti-catfish IgM 9E1 mAb or anti-trout IgM 1.14 mAb (isotype matched negative control; [51]) for 30 min on ice. After washing, the cells were incubated, 25 min on ice, with 50 µl goat anti-mouse Ig (H + L)-PE antibody diluted 1:200 (Southern Biotechnology, Birmingham, AL). Finally, the cells were washed, resuspended in 0.5 ml of RPMI and analyzed by FACS.

2.6 Western blotting and Co-immunoprecipitations

Transfected 3B11 B cells were harvested after 36 h, washed, and then lysed at 1×10^6 cells/10 µl lysis buffer (10mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40 with Complete Mini Protease Inhibitors (Roche Applied Science, 1 tablet per 10 ml lysis buffer)) for 1 h on ice. Nuclei and cell debris were then removed by centrifugation at $10,000 \times g$, 4°C for 15 min and lysate samples of 2.5×10^6 cells were analyzed under reducing or nonreducing conditions by 10% SDS-PAGE. Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) and incubated in Tris-buffered saline containing 5.0% BSA and 0.1% Tween 20 (TTBS-BSA) overnight at 4°C. The membranes were incubated with either anti-HA mAb (16B12; Covance, Princeton, NJ) diluted 1/7000 (v/v) followed by

an incubation with goat anti-mouse Ig (H+L)-HRP (Southern Biotechnology Associates) diluted 1/10000 (v/v) or anti-FLAG-HRP (M2: Sigma) diluted 1/2500 (v/v) for 1 h at room temperature. After washing 3 times with TTBS, immunoreactive bands were visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

Co-immunoprecipitations were performed 36 h post-transfection. Briefly, 3B11 B cells were transfected with plasmid constructs encoding HA-tagged catfish Igμ2-cyt, FLAG-tagged CD79a and HA-tagged CD79b and plated at 5×10^6 cells/well. After harvesting, 3×10^7 cells were washed $3 \times$ with ice cold phosphate buffered saline (PBS) adjusted to catfish tonicity and lysed in 1 ml lysis buffer (10mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% octyl-β-glucoside with Complete-Mini Protease Inhibitors (Roche Applied Science; 1 tablet per 10 ml lysis buffer) for 1 h at 4°C. The lysates were centrifuged at $10000 \times g$ for 15 min to remove the nuclear debris and the supernatant was precleared by rotating for 3 h at 4°C with Protein G Sepharose beads coated with anti-trout IgM 1.14 mAb (GE Healthcare Bio-Sciences AB). The preclearing beads were removed by centrifuging at $10000 \times g$ for 1 min and the supernatant was divided equally into three samples. One sample (330 μl) was incubated with 5 μg of anti-HA mAb (Covance) and the second was incubated with 5 μg of anti-FLAG mAb (Sigma). The third 330 μl sample was incubated with anti-trout IgM 1.14 as a negative control. After overnight incubation with rotation at 4°C, samples were incubated with 100 μl of Protein G Sepharose beads and mixed for 3 h at 4°C to select any immune complexes. The incubated beads were then washed and resuspended in SDS-PAGE sample buffer; the immunoselected proteins were separated by 10% SDS-PAGE under reducing and non-reducing conditions and transferred to nitrocellulose membranes (Amersham Biosciences). The blots were blocked by incubation in TTBS-BSA overnight at 4°C, incubated with either anti-HA-HRP (1:1000 v/v; GG8-1F3.3; Myltenyi Biotech) or anti-FLAG-HRP (1:2500 v/v; Sigma) and visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

3. Results

3.1 Catfish immunoglobulin accessory molecules

Both IpCD79a and IpCD79b encode for proteins containing a single Ig domain, a TM and a CYT with an ITAM. The full-length transcript of IpCD79a consists of 871 nucleotides with a 681 bp open reading frame encoding 227 amino acids. Four N-glycosylation sites are found and the un-glycosylated mature protein is predicted to have a molecular weight of ~23.6 kDa. Comparatively, the IpCD79b transcript consists of 911 nucleotides with a 645 bp open reading frame encoding 215 amino acids. A single N-glycosylation site, NQT, is encoded towards the 3'-end of the Ig domain and the un-glycosylated mature protein is predicted to be ~22.6 kDa (Fig. 1). The catfish CD79 sequences share several hallmark signatures that were previously noted for the pufferfish and rainbow trout CD79 sequences [34]. First, both encode a glycine bulge-like consensus motif (**W/Y/FGXGTXLXV**), a feature of J regions [52], near the 3' end of their Ig domains. The motif in IpCD79a, **FTPGTFLQV**, has a threonine in place of the first glycine, but otherwise matches the consensus; the IpCD79b motif is **WGRGTELQV**. Similar "J-like motifs" are found in the extracellular domains of novel immune type receptors (NITRs; [53-56]) and the *Xenopus* and chicken thymocyte receptor CTX/Cht1 [57,58] and authors have speculated that the presence of these "J-like motifs" reflects evolutionary relatedness. Second, similar to trout and pufferfish CD79 homologs, the IpCD79 sequences lack the cysteine residue found between the Ig domain and the TM that forms the CD79 heterodimer interchain disulfide bond in mammals (see Fig. 2; [59]). Third, similar to the spacing between the Y-X₂-L/I motifs in the pufferfish ITAMs, the spacing of the catfish Y-X₂-L/I motifs, D/E-X₈-D/E-X₂-Y-X₂-L/I-X₈-Y-X₂-L/I, is one amino acid longer than that of mammals, i.e. consists of eight amino acids instead of seven. In addition, both IpCD79a and trout CD79a contain the evolutionary conserved tyrosine residue 204 located toward at the carboxyl-terminal end of the CD79a CYT

domains. In mammals this tyrosine is phosphorylated upon antigen engagement and serves as a docking site for the adaptor BLNK [60-62].

As expected, phylogenetic analyses show that IpCD79a and IpCD79b cluster at high bootstrap values with their respective teleost counterparts (Fig. 3A). Among the three fish, CD79a amino acid sequence identities range from 41-44%, while the CD79b sequences exhibit slightly lower identity values with 35-43% (Fig. 3B). Comparably, mouse and human CD79a and CD79b molecules share ~69% amino acid identity, respectively. Most often the highest sequence identities are found in the TM and CYT regions, especially within the ITAM motifs (see Fig. 2).

3.2 Southern blot and message expression analyses

Southern blot analyses indicated that IpCD79a and IpCD79b are likely encoded by single copy genes since only a single hybridizing band was observed in restriction enzyme digests of genomic DNA from homozygous gynogenetic catfish with gene specific probes (Fig. 4). In contrast, when genomic DNA from outbred catfish was used, two or three hybridizing bands were observed with the IpCD79a probe and it is speculated that their presence may be due to allelic variations. For example, there is an *EcoRI* site with the Ig domain of the IpCD79a cDNA and there may be intronic *PstI* sites, which could account for the different bands in the *PstI* digest. Also, RT-PCR protocols using various primer pairs and mRNA from various fish and B cell lines have only yielded a single non-variant IpCD79a sequence.

At the message level, catfish mIgM, mIgD, IpCD79a and IpCD79b transcripts were readily detected by RT-PCR in catfish PBL and IgM⁺ B cells sorted from PBL of two different catfish. However, the IgM⁻ cells sorted from fish #103 had message for both mIgD and IpCD79a implying that IgM⁻/IgD⁺ B cells were present in PBL from this fish (Fig. 5). IpCD79a and IpCD79b message expression levels also appeared to differ in the catfish clonal B cell lines. Figure 6A shows the results from RT-PCR using RNA isolated from 1G8 and 3B11 B cells, 28S.3 and TS32.15 clonal T cells and 42TA macrophages. While IpCD79a message was detected in both 3B11 and 1G8, as well as in PBL, IpCD79b message was barely, if at all detectable. To further examine message levels for IpCD79b in clonal B cells, cells were harvested daily after a cell culture was initiated and RNA for RT-PCR was isolated. In both cell lines, message for mIgM and IpCD79a appear to be relatively constant (except for day 2 in 1G8 cells); detectable levels for both transcripts are apparent throughout the eight days of culture. In contrast, IpCD79b message levels are low immediately after the cells are passed and increase with time in culture up to day 8.

3.3 Association of IpCD79 molecules with catfish IgM on B cells

All protein expression studies were performed in catfish 3B11 B cells to ensure proper folding and glycosylation of the recombinant proteins and because they are more easily transfected as compared to primary lymphocytes. Also 3B11 B cells resemble catfish 1G8 B cells in that they express reduced levels of surface IgM as compared to freshly isolated B cells from PBL and have a lymphoblast morphology [39]. As expected, since the recombinant CD79 proteins introduced into 3B11 B cells by transfection included the tag sequences and have the opportunity to become glycosylated (just as endogenous 3B11 proteins), the overall size of the expressed proteins were larger than the predicted sizes of CD79a and CD79b based on their deduced amino acid sequences. Expression of HA-tagged CD79a and FLAG-tagged CD79b constructs produced bands at approximately 44 and 35 kDa, respectively, as assessed by Western blots of cell lysates (Fig. 7A). Neither of these recombinant proteins migrated as a dimer when electrophoresed under non-reducing conditions (data not shown). In comparison, catfish Igμ2-cyt, which contains 4 N-glycosylation sites also migrated slower in SDS-PAGE than expected from its predicted size of 32.5 kDa and appeared to run as a doublet (Fig. 7B).

In addition, under non-reducing conditions, I μ 2-cyt migrates as a dimer. Since C μ 2 and C μ 3 do not have extra cysteines that could form the intrachain disulfide bond [63,64], this indicates that the cysteine residue encoded at the very 5'-end of the TM exon is used to form the necessary disulfide bond.

In order to determine if catfish IgM can non-covalently associate with CD79a and CD79b, 3B11 B cells were co-transfected with I μ 2-cyt, CD79a and CD79b tagged constructs, lysed in 2% octyl- β -glucoside and immunoselected using anti-tag mAbs and Protein G Sepharose beads. Figure 8 shows the results from a representative experiment using 3B11 cells co-transfected with constructs encoding HA-tagged I μ 2-cyt, FLAG-tagged CD79a and HA-tagged CD79b. While untransfected 3B11 B cells express message for IgM, IpCD79a and IpCD79b (see Fig. 6), surface IgM levels as assessed by flow cytometry using anti-catfish IgM 9E1 mAb are low, i.e. only 16-35% of the cells are surface IgM positive. However, after transfection, 80% of the cells stain positive with anti-IgM mAb and it seems likely that this increase in staining can be attributed to the expression of the three introduced tagged proteins. For example, an increase in surface IgM can be due to expression of the endogenous IgM associating with the introduced tagged CD79 molecules or to expression of the introduced I μ 2-cyt associating with either the introduced tagged or endogenous CD79 proteins. Here it should be noted that anti-catfish IgM 9E1 mAb will react with both native IgM and tagged-I μ 2-cyt since previous analyses of 9E1 proteolytic fragments have shown that it reacts with either catfish C μ 2 or C μ 3 domains [65]. Also, the increase of surface IgM expression in the transfected cells is not likely due to the transfection protocol itself, since mock transfected cells exhibited no increase in surface IgM (data not shown). In comparison, only 16% of the cells stained positive with an anti-FLAG mAb, which detects tagged CD79a, while 25% of the cells stained positive when analyzed with anti-HA mAb, which detects both tagged I μ 2-cyt and tagged CD79b. This apparent low expression of tagged CD79a and tagged CD79b as compared to I μ may be a result of an antibody binding accessibility problem due to steric interference from the surface IgM covering a tag(s) or the tags themselves. This explanation also seems plausible since when co-transfection is performed using only FLAG-tagged CD79a and HA-tagged CD79b, anti-IgM mAb staining increased to ~60% while anti-FLAG and anti-HA mAb staining were between 3-10% depending upon the experiment. Similar results were obtained with single transfections using plasmids encoding either CD79a or CD79b (data not shown). Yet, because a percentage of 3B11 B cells were shown to be positive for the tagged proteins, they provide a means to demonstrate if I μ and CD79 proteins associate. This association of tagged I μ 2-cyt and tagged CD79a and CD79b was investigated using a combination of immunoprecipitation with one anti-tag mAb combined with Western blots using the other anti-tag mAb. Immunoselection of co-transfected 3B11 B cells with anti-FLAG mAb, yielded proteins corresponding to the HA-tagged I μ 2-cyt and HA-tagged CD79b mAb, as well as FLAG-tagged CD79a as visualized by Western blotting using anti-HA and anti-FLAG, respectively (Fig. 8B). Comparatively, cells immunoselected with anti-HA mAb and probed with anti-HA in Western blots yielded the same HA-tagged proteins, the 44-45 kDa I μ 2-cyt doublet and 35 kDa CD79b, but no FLAG-tagged CD79a. This failure to co-immunoprecipitate any tagged CD79a was consistent in several experiments and may in part be due to endogenous CD79a being more abundantly expressed in 3B11 cells as compared to CD79b allowing it to compete with the tagged CD79a (see Fig 6). Another explanation may be that the association of CD79a and CD79b is not very strong since sequencing shows they lack the required cysteines for interchain bonding. Also, it could simply be that anti-FLAG mAb is more efficient in immunoprecipitation than anti-HA mAb, at least in this system. Even so, these results demonstrate that I μ associates with both CD79a and CD79b in catfish B cells.

4. Discussion

In summary, sequence, RT-PCR and Southern blot analyses show that the catfish CD79a and CD79b molecules are quite similar to their mammalian counterparts and resemble the CD79 homologs described at the gene level in pufferfish and rainbow trout [34]. At the message level, both IpCD79a and IpCD79b are expressed by cells found in IgM⁺ cell fractions and in clonal B cells, but not in clonal T cells or macrophages. In this context it is also interesting that IpCD79a message (see Fig. 5) is found in IgM⁻/IgD⁺ B cells. Recently, we have been able to detect both IgM⁺/IgD⁺ and IgM⁻/IgD⁺ lymphocyte cell populations in PBL from some catfish by flow cytometry using an anti-Igδ monoclonal antibody. Moreover, the sizes of the IgM⁺/IgD⁺ and IgM⁻/IgD⁺ populations have been shown to vary from fish to fish (Edholm and Wilson unpublished). Whether or not these IgM⁻/IgD⁺ cells use IgCD79a, or IpCD79b as their accessory molecules, or if they use other as yet unidentified accessory molecules remains to be determined. Similarly, IpCD79b message in catfish clonal B cells, like that in B cells isolated from PBL, appeared to be expressed at a lower level when compared to IpCD79a message. As shown in Fig. 6, this differential expression of IpCD79b message during the culture of clonal B cell lines might suggest that CD79b is influenced by the cell cycle; i.e. quickly dividing cells express low levels of message and slowly dividing cells express higher levels. However, due to the lack of appropriate antibody reagents it cannot be determined if the protein levels for IpCD79a and IpCD79b in the clonal B cell lines change with time in culture. This lack of anti-CD79 antibodies also hindered us from obtaining sufficient amounts of associated proteins for amino acid sequencing that would link the catfish CD79 sequences with protein studies. For this reason, it was decided to use a transfection protocol with IpCD79 epitope-tagged constructs to determine if these encoded proteins associate with mIgM on the catfish B cell surface. Previously, using ¹²⁵I surface-labeled catfish PBL, it was found that under non-reducing conditions, mIgM is associated with two distinct sets of molecules which are ~64 and ~70 kDa in size [37]. When the 64 and 70 kDa bands were excised from the gel, reduced and separated by SDS-PAGE, the 64 kDa molecule was shown to consist of two 32 kDa proteins, which formed a dimer, and the 70 kDa molecule was a heterodimer formed by a 45 kDa protein and a 25 kDa protein. Here our transfection studies allow us to link the IpCD79a and IpCD79b cDNAs with some of this previous data. The Western blots with anti-tag mAbs indicate that IpCD79a encodes a protein of approximately 44-45 kDa and IpCD79b encodes a protein of 35 kDa, sizes that match two of the protein monomers shown to associate with catfish mIgM. However, our data cannot explain the 70 kDa and 64 kDa heterodimers observed under nonreducing conditions. Notably, sequencing shows that catfish CD79a and CD79b cDNAs, as well as trout and pufferfish CD79 molecules, lack the conserved cysteines found between the Ig domain and TM that form the interchain disulfide bond in mammals. In addition, even though there are “other” cysteines residues found in fish CD79 molecules, some of which are conserved, their location, either in the TM, CYT or adjacent to the first cysteine that forms the intrachain disulfide bond of the Ig domain makes it unlikely that they are involved in interchain disulfide bonding. Recently, transfection studies in *Drosophila* S2 cells and murine J558L B cells confirmed the cysteine residues adjacent to the TM in both CD79a and CD79b as being responsible for forming the interchain disulfide bond between mammalian CD79a and CD79b [66]. Interestingly, immunoprecipitation studies using mutant constructs also demonstrated that CD79 molecules could still associate with one another even in the absence of a covalent bond, i.e. the interchain disulfide bond between CD79a and CD79b is not necessary for assembly and transport of the mIgM-BCR complex. While expression of the mutant mIgM-BCR complex was consistently 40% lower than the wildtype complex in transfected B cells, such findings combined with the data presented here suggest that teleost B cell accessory molecules function by non-covalently forming heterodimers or homodimers. Is it always one IgM monomer noncovalently associated with one CD79a/CD79b heterodimer as in mammals, or can it be different? Also, it is intriguing to speculate that the catfish CD79 molecules could each form a heterodimer with another yet unidentified accessory molecule. However, at the

present, even though the 3B11 B cell immunoselection experiments demonstrate that the tagged CD79 proteins associated with tagged catfish Ig μ , future studies are needed to determine the stoichiometry of the catfish IgM-BCR complex and whether CD79 molecules can or cannot be covalently linked with other molecules. Additionally, without catfish anti-CD79 antibodies, the previously observed covalently linked proteins associated with mIgM [37] cannot be definitely identified. Nevertheless, the findings presented here indicate that fish B cells use signal transduction elements similar to those of mammalian B cells and represent an important step in understanding B cell signaling in ectothermic vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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	< SP	>	
IpCD79a:	ATGGAGGCAAGATTTGTGTTCTGTTTTGTTTCTTGGCAGCTGCTGCCAATGCTGCCACTTCTCCCAAAATACAACGT M E A R F V F L F C F L A A A A N A A T S P K I Q L	78	
	< Ig domain		
IpCD79a:	GGATTTGACGTTCCCGAGCTTACGTCTCGCCATCTCGCAGACAGCAAGTATGAGTTGCTGTTATACTGGCATTGTGCGT G F D V P S L R L A I S Q T A S M S C C Y T G I V R	156	
IpCD79a:	GTGCCAAGTCAATGGATTGGGCTTGTATGAATTCAGGAACACCACTAAACGTACTTCTATCAATGAGACAAATGCA V P S Q W I R L V M N S R N T T K R T S I N E T N A	234	
IpCD79a:	CGAGTGAACTTGACCAAGGCGAGAGAAACAGCAGCACTGTGAAATACTGATACTAAAGACATTGTGCTGAATGAC R V K L D Q G Q R N K Q H C E I L I L K D I V L N D	312	
	>		
IpCD79a:	ACAGGCTGTATCAATGCGTTCTAACAGAATCAAAACAACCGGTCTTTACACCTGGCACTTTTACAAGTATACGTG T G L Y Q C V L T E S N N T V E T P G T F L Q V Y V	390	
	< TM		
IpCD79a:	CCCGTACACATGATCCTGAACATCAATGAGAGGAGTAAGAACAGCATCATCACTGCTGAAGGTGTTCTGCTGCTGCTG P V H M I L N I N E R S K N S I I T A E G V L L L L	468	
	>< CYT		
IpCD79a:	CTGTGCTGATTCTCGCACCATGCTCATCTGCAAGTCAAAGGGATTAAATAACATAGAGAAAAGAAATGGGCAGAGAG L V L I P G T M L I C K S K G L N N I E K R M G R E	546	
IpCD79a:	GAAGAAAATATTTATGAGGGTCTAAATCTGGATGACTGCAATTCAACATATCATCAAATTCAGCGCTCTCTCGTCCAA E E N I Y E G L N L D D C N S T Y H Q I Q R S L V Q	624	
	>		
IpCD79a:	GGACCGTACCAAGATGTGATCAACAATGACGAAGATGACATTGAGCTGGAGAAGCCCTGAATAGGTGGTGTGAGGAG G P Y Q D V I N N D E D D I Q L E K P *	702	
IpCD79a:	TCAGGACCAATCCAATACAAATCATAGTTTCGCGCTGTTGCTATGTTTGCTTTTCTGTTGTTGGTATAAATTAGGGG	780	
IpCD79a:	TGTAACCGAATATGAATTGCTTATTGAGATTGAACAGATAATGCATCCTAAATGAATACAGATACAAAAAAGAAAAA	858	
IpCD79a:	AAAAAAAAAAAAA	871	
	<SP	>	
IpCD79b:	ATGAACATACATTTTTTAGGTTGTTTTCTACTGTCGCTGGTAAACCTCTCAGTCACGGCTGATTTTCAAGTTCAGCAG M N Y I F L G C F L L S L V N L S V T A D F Q V Q Q	78	
	< Ig domain		
IpCD79b:	AATCCCAGGTTTTACGGTGTAAACGTGGGAAAATTGGTGGGATTACATGTTCTGCTTCTGATCTCACCCCTTGGTGAG N P R F Y G V N V G K L V G F T C S A S D L T L G E	156	
IpCD79b:	GCAAAGTGACATGGTACAAGATTGATAAATATGACGAGGAGAAAGTGTCCAATGGTCAAATGCATACAGTCAAAAAT A K V T W Y K I D K Y D E E K V S N G Q M H T V K N	234	
IpCD79b:	GGCAGATCAGTGCAGCGTTCAGGTAACATGAAGGGTCACTTAACCATTAAGAATGTAGAGGTCCAAGACAGCGGCATT G R S V Q R S G N M K G H L T I K N V E V Q D S G I	312	
	>		
IpCD79b:	TACTACTGCAAGATGAACAGACTTGGGGACGTGGAACAGAGCTGCAAGTATTCAGACTGAGCAAAACAAAAGTGGTT Y Y C K M N Q T W G R G T E L Q V F R L S K P K V V	390	
	<TM		
IpCD79b:	GAAGGGAGGAACAACATGAAAGACATGATCATTTTATTCAGGGTTTTCTCCTTATGTTGTGTGAATCATTCCTTTG E G R N N M K D M I I L F Q G F L L M L C V I I P L	468	
	><CYT		
IpCD79b:	GTCTGGTATTACAGACTGGAGCAAAAAGAGGAGCCGTATATGAAGAGCCTGAGCGTGATCATACATATGAGGGTTTG V W Y Y R L E Q K E E A V Y E E P E R D H T Y E G I	546	
IpCD79b:	AGATCGAGCACTGTGGGACCTCTATGAAGACCTAACTGCATTTGCTCAAGAGCCAGACAGACGAGCCTGGGAA E I E H C G D L Y E D I T A F A Q E P D T D A A W E	624	
	>		
IpCD79b:	GTCGAGTCTCCAGAACAGAGTGATAAAGTCATGATGAGAATACATATCATGTAGTAGCCATCAGAATTACATGAT V E S P E Q E *	702	
IpCD79b:	TGTCTGGAGAAAGTTTTATTGTGGATTGAAAAATTAGGACTACATCACACATTACATATACAATATATAAAAAAATT	780	
IpCD79b:	AAGCAGAGTACACAAGGTATTTTATTTTATTTACATCATGTACCATGCTGAATTTTCTACATCCACTCTATATAA	858	
IpCD79b:	TATAATATGTAATATATAATATACAATATAACAAGCCTTGCACTTAAAAAAA	911	

Fig. 1.

Catfish IpCD79a and IpCD79b. Nucleotide and predicted amino acid sequences of IpCD79a and IpCD79b. The predicted signal peptide (SP), Ig domains, TM and CYT are labeled above the sequence. Potential N-linked glycosylation sites are underlined and the conserved charged/polar amino acid residue in the TM is in bold. The ITAM motifs are gray shaded and in bold, the J-like sequences are double-underlined and the stop (TGA) codons are marked with (*). In IpCD79a, Tyr 204 is also double-underlined. Nucleotide and amino acid numbers are at left. GenBank accession numbers are IpCD79a (**EF173897**) and IpCD79b (**EF173898**).

	< SP and Extracellular Region	▼	
IpCD79a:	-----MEARF--VPLFCFLAAANAATSPKIQLGFDVPSLRALASQTASMSCCY-TGIVRVPSQWIRLVMSNRNTTKRTSI		73
TrCD79a:	VVVAQDEVT.EA.K.Y..VRLHGV.EVE...-TSAEPLTAT.V.-----G.RGNKP		52
OmCD79a:	GDLSRIQVT.EP.R..Q.VQLSH..SLR...SVTGA.DTT.ATSPHVMVKILRGVDW		58
MmCD79a:	MPGGLEALR.LP----.LL..SY.CLGPGCQALRVBGGP...TVNLGEE.RLT.EN-N.-RNPNT.WF-----SLQSN.		69
HsCD79a1:	MPGGPGVLQALPATI..LFL.S.VYLGPGCQALMMHKVPA..MVSLGED.HFQ.PH-NSSNNANVT.W.-----VLHGNY		74
HsCD79a2:	MPGGPGVLQ.LPATI..LFL.S.VYLGPGCQALMMHKVPA..MVSLGED.HFQ.PH-NSSNNANVT.W.-----VLHGNY		74
	↓		
IpCD79a:	NETNARVKLDQGQRNKQHCBIILKDIVLNDTGLVQCVLTES--NNTVFTPGTFLQVY--VPVHMILNINERSKNSIITABGV		152
TrCD79a:	LNPSDH.TKGSKNMGTTK.SM.V..SVE.S.A....L.NN.--TEQLSY..Y.H..SKS.I.KM.T.S..T..N.L...I		133
OmCD79a:	RDNRTV.DGGNLTAAGVM.HT...REVR.....F.NH.ALRPS.Y.H...H.--M.IVK..D.S.ST...L...M		139
MmCD79a:	TWPFVPLGPG..TTGQ-----FFPEVNK.HR...W.QVI.N--ILKRSK..Y.R.RN-EVPRPF.DMG.GT..R.....I		144
HsCD79a1:	TWPFVPLGPGEDPNTG-----IQNVNKS.HG.I.V.RVQ.G-NESYQQSC..Y.R.RQ-P.PRPF.DMG.GT..R.....I		150
HsCD79a2:	TWPFVPLGPGEDPN-----EP.PRPF.DMG.GT..R.....I		112
	>> CYT	↑	>
IpCD79a:	LLLLLVLPGLTCLICKSKGLNNIEKR-MGR--EENIYEGLNDDCNSTYHQIQRLVQSPYQDVINNEDD-IQLEKP		227
TrCD79a:	...F..L.A.TILF.N.QVEL..KKVT--S...Q....E.CPK.D..E..HDH.....C.ASDEEFPH...		210
OmCD79a:	..MVA..LH..ML..T.K..QLK.KRVKE--.....EE.C...D.....		195
MmCD79a:	I..FCAVV...L.LPRKRMQ.EKFGVD.PDDY.D..L.....M.ED.S.G.QG-T...G.LHIG.-A....		220
HsCD79a1:	I..FCAVV...L.LPRKRMQ.EKGLDAGDEY.D..L.....M.ED.S.G.QG-T...GSLNIG.-V....		226
HsCD79a2:	I..FCAVV...L.LPRKRMQ.EKGLDAGDEY.D..L.....M.ED.S.G.QG-T...GSLNIG.-V....		188
	<SP and Extracellular Region	▼	
IpCD79b:	MNYIFLGC-----FLLSLVNLSVT-----ADFQVQNNRPFYGVNVUGKLVGFTCSASDLTLGEAKVTWYKIDKY		63
TrCD79b:	SSGAL.IR.C...I..GTRHQ.RIH..V.-----SGL.Q..RLN..		41
OmCD79b:	LN SPL..T.K.....KTNRT.AIF.VV..RN-OP.T.E...ASE.		45
GgCD79b:	.GDFC---RRLWVLQVN.WLMA.AAGGIPTDGNSTSNRTGSECVGMW.H..YLAACKNTS.H.I.YT.Q---PH.-MQ....		74
MmCD79b:	.ATLV.SSMPCHMLLF-.LLF.GEPVEAMTSSDLPLNFQSGPCS.IW.H...AAKRRSSM.K.H.YTNH---SGAL..FR---		77
HsCD79b:	.ARLA.SPVPSHMMVA.-.LL..AEPVPAARSEDYRNPKGS.CSRIW.S...IARKR.PT.KMH.YMNS---ASGN.S.LW----		78
	↓		
IpCD79b:	DEEKVNSGQMHTVKNGRSVQSRSGNM--KGHLTIKNVEVDSGIYCKM-----NQ-TWGRGTQLQVFLSKPKVVEGRNNMKDMI		140
TrCD79b:	N.DR-----NKADEVKAGG.ISVPSPTQ.F.SALR.E.K.V.F..V-----D...P.....SGNRMKACQY.T...GL		114
OmCD79b:	NTR-----KKIVGD.VTV.QESMLQNASIELRH..TE.T.F.F.LI-----NI..P.....L.P.NSENADR.SKL..AL		119
GgCD79b:	--ALNGKEF.VLDQSSDRFSIN.TNDRI SF..SRISYE.N...V.DSKNLTEEKRPNSC...IR.MSR.NIQIQN..TL..T.		158
MmCD79b:	--KRGSQ.PQELVSEEGRIVQQTQNGSVYT...Q.IQYE.N...F..QKCDSANHNVDSC...L.LGF.TLDQLKR..TL..G.		160
HsCD79b:	--KQEMDENPQQL.LEKGRMEESQNESLAT...QGIRFE.N...F.QQKCNNTSE-VYQ.C...R.MGF.TLAQLRQ..TL..G.		161
	TM	↓	>
IpCD79b:	ILFQGLMLMLCVIIPLVWYRLQ--KEEA VYEEPERDHTYEGLEIEHC-GDLYEDLTAFAPQEPD TDAWEV-ESPEQE		215
TrCD79b:	.M..AL..AV.IAALML---KHKL--NXD8LF..T..I...A..T.D.G...E.SVYTR-.EAB.PWK-----		181
OmCD79b:	.FL.AL..T.FALA..L---HQSLLK.ED.I...DH..I...DH..I...ISVY..-GPAB.AAA---WK..		191
GgCD79b:	.II.TI..VIPISV.MLLF-----LE.GDRK-.R..E.....V--QIAT..I.P.R---MKAK.T.G.H.GE.		226
MmCD79b:	.LI.TL.IILFV.V.IFLL-----LD.DDGK-AGM.E.....N.D--QTAT...IVTLR---TGEVK.S.G.H.G..		228
HsCD79b:	.MI.TL.IILFI.V.IFLL-----LD.DDGK-AGM.E.....D.D--QTAT...IVTLR---TGEVK.S.G.H.G..		229

Fig. 2.

CD79 amino acid comparisons. Amino acid alignment of IpCD79a with fish, mouse and human CD79a sequences and IpCD79b with fish, chicken, mouse and human CD79b sequences. The predicted extracellular, TM and CYT boundaries are labeled, conserved cysteine residues that form the intrachain disulfide bond are marked with (▼) and the cysteines that form the interchain disulfide bond between CD79a and CD79b in mammals are marked (↑). Other conserved cysteine residues in the fish CD79 Ig domains and TMs are marked with (↓). The ITAM motifs are gray shaded, tyrosine residue 204 in CD79a is marked by ‡, and (.) represent amino acid identities and (-) represent gaps in the alignment. The pufferfish (Tr) and rainbow trout (Om) CD79 sequences are from Guselnikov et al 2003 [34]. Abbreviations and GenBank accession numbers for the other CD79 sequences are: chicken (Gg CD79b, **AB062512**), mouse (Mm CD79a, **NP_031681**; CD79b, **CAJ18566**), human (Hs CD79a1, **NP-001774**; CD79a2, **NP-067612**; CD79b **P40259**). There is no available chicken CD79a sequence. Number of amino acids is listed at the right.

Fig. 3. Phylogenetic analyses of CD79 molecules. (A) Neighbor-Joining trees with pairwise gap deletions were drawn using MEGA3.1 [48] with 5000 bootstrap replications. (B) Comparison of CD79 percent amino acid identity values from pairwise alignments of full length sequences, Ig domains, TM and CYT regions. GenBank accession numbers and sequence references used are as in Fig. 2.

Fig. 4. Southern blot analysis of IpCD79a and IpCD79b. Genomic DNA from two gynogenetic (labeled G) and three out bred catfish was hybridized with either an IpCD79a or IpCD79b specific probe. Ten µg of genomic DNA was digested to completion with either *EcoR* I or *Pst* I. Kb Size markers are at left.

Fig. 5.

RT-PCR analysis of IpCD79 message expression in catfish PBL. Total RNA was obtained from catfish PBL and B cell enriched (IgM⁺) and depleted (IgM⁻) fractions from the same fish. RT-PCR was performed using primers specific for catfish membrane (m) IgM, mIgD, IpCD79a, IpCD79b and EF1α (see supplementary Table 1). Base pair markers are at left.

Fig. 6.

RT-PCR analyses of IpCD79 message in catfish clonal cell lines. **(A)** Total RNA was obtained from catfish PBL and log phase 3B11 and 1G8 B cells, 28S.3 and 32.15 T cells and 42TA macrophages. RT-PCR was performed using the same primers as described for fig. 4. **(B)** Time course of IpCD79 message expression in catfish 1G8 and 3B11 B cells. Days after culture initiation are labeled numerically. Base pair markers are at left.

Fig. 7.

Predicted sizes of catfish recombinant CD79a, CD79b and Igμ2-cyt using Western blots. Catfish recombinant proteins were expressed in 3B11 B cells and cell lysates (2.5×10^6 cells/lane) were analyzed by SDS-PAGE at 36 hrs post transfection. Proteins were transferred to nitrocellulose membranes and visualized using an anti-HA or anti-FLAG mAbs. **(A)** Recombinant HA-tagged CD79A and FLAG-tagged CD79B were expressed in 3B11 cells and cell lysates were electrophoresed under reducing conditions. **(B)** Recombinant HA-tagged Igμ2-cyt (containing Igμ domains 2-3, TM and CYT) was expressed in 3B11 cells and cell lysates electrophoresed using non-reducing and reducing conditions. Under non-reducing conditions, Igμ2-cyt dimers can be observed. In each panel, lysates from untransfected cells are used as controls. Arrows mark the identified proteins and molecular weight size markers are at left.

Fig. 8.

Recombinant catfish HA-tagged Ig μ 2-cyt, FLAG-tagged CD79a and HA-tagged CD79b are expressed on the surface of 3B11 B cells. (A) Schematic showing the expressed tagged proteins on the surface of 3B11 B cells (B) Surface expression of IgM, recombinant Ig μ 2-cyt, CD79a and CD79b molecules were analyzed by flow cytometry using anti-IgM (9E1), anti-FLAG, and anti-HA mAbs, followed by PE-conjugated goat anti-mouse secondary antibodies. 3B11 B cells transfected with all three constructs (shaded histograms) showed increased anti-IgM staining as compared to untransfected B cells (open histograms). Percentage of stained transfected cells are indicated on the individual histograms. (C) The transfected cells shown in A were lysed in 2% octyl- β -glucoside and the resulting lysates were immunoselected using anti-trout IgM 1.14 (negative control), anti-HA, or anti-FLAG mAbs coated Protein G Sepharose beads. The immunoselected proteins were separated by 10% SDS-PAGE under reducing conditions and visualized by Western blot using anti-HA-conjugated to horse radish peroxidase (HRP) and (D) anti-FLAG-HRP mAbs. Arrows mark identified proteins, IP indicates mAbs used for immunoselection and WB indicates mAb used in Western blotting.

The anti-HA-HRP mAb was reactive with the IgH chain of the immunoselecting mAb and is indicated by (*). Molecular weight size markers are at left.