PERGAMON

Molecular Immunology 35 (1998) 943–954

Molecular Immunology

Molecular characterization of six variant Fcγ receptor class I (CD64) transcripts[1]

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Received 10 April 1998; accepted 10 August 1998

Abstract

In humans, three distinct but closely related classes of receptors that bind the Fc portion of IgG (FcγRI, II, and III) have been identified. FcγRI can bind monomeric IgG with high affinity and has a unique third extracellular domain (EC3). Three very similar genes have been characterized for FcγRI (A, B, C). Although the sequences are remarkably similar, a number of coding-region differences discriminate between the genes and amongst their transcripts. Six distinct FcγRI transcripts were analysed. Three transcripts, one from each gene, contain all six exons. Only the gene A transcript appears to encode a bona fide high affinity receptor, a three Ig-domain membrane spanning receptor that can bind monomeric IgG. Stop codons in the EC3 domains of the gene B and gene C isoforms would be predicted to generate secreted receptors. Three transcripts are alternatively spliced isoforms, one from gene A and two from gene B. One gene B transcript encodes a two Ig-domain transmembrane receptor which has structural characteristics of a low affinity FcγR. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: IgG; mRNA; Macrophage

Introduction

Antibodies are bifunctional molecules comprised of highly specific antigen binding portions (Fab) linked to effector domains (Fc) that elicit host defense mechanisms. Immunoglobulin G (IgG) initiates several distinct functions by first binding and then clustering Fc receptors (FcγR) on cells of the immune system. These immune responses, such as endocytosis, phagocytosis, cytolysis, and inflammatory mediator release, function to clear antigen from the host.

The human FcγR consists of three distinct but closely related structural classes (Hulett and Hogarth, 1994; van de Winkel and Anderson, 1991). All receptors are members of the Ig superfamily of genes, and their extracellular (EC) regions consist of two (FcγRII and III) or three (FcγRI) domains of the C2 set of Ig domain structural types (Powell et al., 1996). These receptors are integral membrane glycoproteins that consist of a leader peptide, 2–3 EC domains, a hydrophobic transmembrane (TM) domain, and a cytoplasmic (C) region of variable length (Hulett and Hogarth, 1994). FcγRI is unique in that it can bind monomeric IgG with relatively high affinity (Ka $10^{8–9}$ M^{-1}) (Anderson, 1982; Canfield and Morrison, 1991; Miller et al., 1996), contains a third EC domain (Allen and Seed, 1989), and is expressed only on monocytes and macrophages while its expression is inducible by IFNγ (Perussia et al., 1983) and G-CSF (Repp et al., 1991) on polymorphonuclear leukocytes (PMN). On monocytes and monocytic cell lines, expression of FcγRI can be increased 20-fold by IFNγ (Perussia et al., 1983; Pan et al., 1990).

There are five genes for the low affinity FcγR (Qui et al., 1990); they all localize to chromosome 1, bands q23–24 (Ravetch, 1994; Hulett and Hogarth, 1994). Three human FcγRII genes (A, B, C) have been described that encode multiple transcripts expressed on a variety of cells (Hulett and Hogarth, 1994; Brooks et al., 1991; Metes et al., 1998). Two human FcγRIII genes, A and B, each encodes a single transcript that is expressed in a cell-specific manner (Ravetch and Perussia, 1989). We have

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Abbreviations: C, cytoplasmic region exon; CYT, cytoplasmic tail; cRNA, complementary RNA; EA, antibody-coated erythrocytes; EC, extracellular Ig-like domain/exon; GSO, gene-specific oligonucleotide; IFNγ, interferon-γ; IVT, in vitro translation; mAb, monoclonal antibody; nt, nucleotide; PBMC, peripheral blood monocytes; PMN, polymorphonuclear leukocyte; RT, reverse transcription; S, signal; YMF, yeast mating factor.

[1] The gene sequences modified in this article are contained in the GeneBank/EMBL Data Bank with accession numbers M91645, M91646, and M91647.

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PII: S0161–5890(98)00079–0

identified three genes for the human high affinity receptor for IgG (FcγRIA, B, and C) (Ernst et al., 1992). All three genes are located on chromosome 1; genes A and C localized to 1q21 while gene B is located at 1p12 (Maresco et al., 1995; Ravetch, 1994). They are remarkably similar in gene structure with each consisting of six exons that span 9.4 kb with similar intron distances and a few distinguishing restriction enzyme sites. Although they are very similar, genes B and C are distinctly different from gene A. The most important differences occur in the third Ig-domain (EC3); gene B has a single nucleotide change and gene C has a single nucleotide deletion that both result in stop codons.

In this study, we continue to analyse the products from these three genes and describe a total of six transcripts. Three of these products, one from each gene, are full-length transcripts consisting of all six exons; however, two transcripts contain stop codons in the EC3 domain. The other three transcripts are alternatively spliced products from genes A and B. One of the gene B transcripts encodes a receptor that is structurally similar to the low affinity FcγR.

2. Materials and methods

2.1. Cells and cell culture

U937, a monocyte line; KG1, a myeloblast line; and K562, an erythroblast line were all maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. COS-7 (COS) cells were grown in Dulbecco's Modified Eagle Medium with the supplements listed above. U937 cells cultured with IFNγ used 100 U/ml human rIFNγ (Genentech, San Francisco, CA, U.S.A.) for the times indicated in each experiment; for each 24 h time period, fresh IFNγ was added. PMN and peripheral blood monocytes (PBMC) were isolated from whole blood samples by Mono-Poly Resolving Medium (ICN, Irvine, CA, U.S.A.) as previously described (Anderson et al., 1990).

2.2. Antibodies, cell labeling, immunoadsorption, and indirect immunofluorescence

Purified IgG of anti-FcγRI mAbs 32.2, 22, 197, anti-FcγRII mAb IV3, and anti-FcγRIII 3G8 were obtained from Medarex (West Lebanon, NH, U.S.A.). The anti-FcγRI mAbs 44.1 and 62.2 were the generous gift of Dr Paul Guyre and the anti-FcγRI mAb 10.1 was the kind gift of Dr Nancy Hogg. The goat anti-FcγRI serum has been described previously (Anderson et al., 1984). The rabbit anti-FcγRI cytoplasmic tail (CYT) peptide antiserum was prepared in collaboration with Dr Pravin Kaumaya (Ohio State University). The 18-residue peptide (H_3N^+-VTI RLE LKR KKK WNL EIS-COO-, Fig. 2, residues 312–329) was linked by a four-residue spacer to an 18-residue measles virus promiscuous epitope (Kaumaya et al., 1992). Two rabbits were injected with the peptide in Freund's complete (first immunization) and incomplete adjuvant four times at 4- to 6-week intervals. Sera from the rabbits were titered against a dilution series of peptide in an enzyme-linked immunosorbent assay. After three injections, serum samples from both rabbits bound picogram quantities of peptide at a dilution of 1 : 3200. Cells were radioiodinated by the chloroglycouril (CGU) method (Fraker and Speck, 1978) by incubating 10^7 cells in 0.7 ml PBS with 50 μl borate buffered saline, and 1 mCi Na ^{125}I (500 mCi/ml) (Amersham, Arlington Heights, IL, U.S.A.) in a vial coated with 50 μg of CGU for 30 min at 4°C. Cells were washed with 5 mM KI in PBS and then lysed in TritonX-100 buffer (1% TritonX-100, 10 mM EDTA, 2 mM PMSF in PBS). For immunoadsorbant preparation (Ernst et al., 1993), mouse mAbs (100 μl, 10 μg/ml) were coupled to 25 μl of Sepharose-goat *F*(ab′)2-anti-mouse beads; the goat (200 μg IgG) and rabbit (100 μl, 1 : 200 dilution) antibodies were coupled to 25 μl of Sepharose-Protein G beads (Pharmacia LKB, Piscataway, NJ, U.S.A.). For preclearing experiments, the cell lysate was adsorbed for 3 h at 4°C on the first set of antibody-coated beads. The supernatant was transferred to the second set of antibody-coated beads and incubated overnight at 4°C. Adsorbed material was eluted from the beads by boiling in an equal volume of Laemmli sample buffer containing SDS with 2-mercaptoethanol as a reducing agent. Indirect immunofluorescence was performed as described (Ernst et al., 1993). The flow cytometry analysis set the gates to include cells that were brighter than 97% of COS cells stained with the secondary FITC-antibody only. The results are expressed as the percentage of cells brighter than the negative controls.

2.3. Reverse transcription (RT)/PCR amplification, cloning, and analysis by GSO probe hybridization

RNA was isolated using the RNazol B reagent and method (Tel-Test, Friendswood, TX, U.S.A.). Oligonucleotides for sequencing and PCR were synthesized by Oligos Etc., Wilsonville, OR, U.S.A. RT and PCR amplification conditions have been previously described (Ernst et al., 1992). FcγRI-specific primers were 1A (5′-ATG TGG TTC TTG ACA-3′) corresponding to S1 nucleotides (nt) 1–15 (Fig. 2) and 2A (5′-ACT GAG CCG CTG CTA CGT-3′) corresponding to the reverse complement of TM/C nt 1120–1137 (Fig. 2, a1). FcγRI-specific primers 9 and 10 correspond to EC1 nt 53–70 (5′-ACA CCA CAA AGG CAG TGA-3′, Fig. 2) and to the reverse complement of TM/C nt 919–936 (5′-CAC CCA GAG AAC AGT GTT-3′, Fig. 2). Primer pair 1A and 2A were annealed at 52°C. GSO probes 37A (5′-GAA TAT CTG TCA CTG TGA-3′) and 39B/C (5′-GAA TAT

CAC AAT ACA CTG-3′) corresponding to EC2 nt 539-556, a1 and b1, respectively, in Fig. 2 were 5′-end phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase. These end-labeled GSO probes were used in Southern blots and slot blots to differentially hybridize to gene A or gene B/C sequences. Hybridization conditions have been described (Ernst et al., 1992). The RT/PCR products were purified and concentrated by using an Elutip-D column (Schleicher and Schuell, Keene, NH, U.S.A.); they were cloned into pBluescript KS that was digested with EcoRV and incubated with dTTP and DNA Taq polymerase (Marchuk et al., 1991) to generate a *t*-tailed vector. The nucleotide sequence of cloned RT/PCR fragments was determined by the chain termination method (Sanger et al., 1977) using a modified T7 DNA polymerase (Sequenase; United States Biochemicals, Cleveland, OH, U.S.A.). The FcγRI cDNA, p135, was provided by Dr B. Seed (Allen and Seed, 1989).

2.4. RNase protection analysis

A 855 bp pIb2 cDNA (Fig. 2, b2) was used to synthesize a riboprobe for RNase protection analysis of RNA from K562, U937, and COS pIb2 transfectants. The pIb2 cDNA was inserted into pBluescript for synthesis of an antisense RNA probe using T3 polymerase and [γ-^{32}P]UTP in an RNA transcription reaction (van de Winkel et al., 1991) (Stratagene, La Jolla, CA, U.S.A.). The riboprobe (5×10^5 cpm in 30 μl of 80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4), and 1 mM EDTA) was hybridized with 30 μg of total cellular RNA at 45°C overnight. The samples were digested as described (van de Winkel et al., 1991), size separated on a 6% polyacrylamide, 7 M urea denaturing gel, and analysed by autoradiography.

2.5. In vitro transcription/translation

Plasmids containing a1, a2, b2, and b3 cDNAs in pBluescript were linearized downstream of the 3′-end of the cDNA by restriction enzyme digest of a unique vector site (either X*ho*I or X*ba*I depending on the orientation of the cDNA in the vector). Capped complementary RNA (cRNA) transcripts were then generated from this linearized template using T7 or T3 (depending on the insert orientation) polymerase promoter-based in vitro transcription (Stratagene). cRNA transcripts produced in vitro were used to program a rabbit reticulocyte lysate in vitro translation system (Promega, Madison, WI, U.S.A.), in the presence of ^{35}S-methionine (Amersham), according to the manufacturer's directions. Reactions were performed in the absence or presence of canine pancreatic microsomal membranes (Promega) for 1 h at 30°C. Samples to be analysed by immunoprecipitation were lysed in TritonX-100 lysis buffer prior to adsorption onto antibody-coated beads. The various translated products were placed in Laemmli sample buffer containing SDS, boiled for 3 min, and fractionated by SDS–PAGE. ^{35}S signals were enhanced by fluorography (Entensify; DuPont NEN, Boston, MA, U.S.A.) and analysed by autoradiography.

2.6. COS transfection and rosette assays

For transfection, FcγRI cDNAs were transferred from pBluescript to the expression vector pCDM or pcDNA1 (Invitrogen, San Diego, CA, U.S.A.). The FcγRIa1 cDNA (pIa1) with sequence identical to the exon sequence of a gene A genomic clone (Ernst et al., 1992) and FcγRIb2 cDNA (pIb2) were used in transfection studies. COS cells were transiently transfected with plasmid DNA (2 μg/ml) by the diethylaminoethyl-dextran method (Seed and Aruffo, 1987) and harvested 48 h later for analysis. Antibody-coated erythrocytes (EA) were prepared by washing 100 μl of packed sheep or human E with PBS and incubating with sub-agglutinating concentrations of rabbit IgG or IgM (anti-sheep E), human IgG (anti-Rh, RhoGam), or mouse IgG switch variant mAb (mIgG1, 2a, or 2b) anti-human glycophorin A ((Boot et al., 1989), kindly provided by Dr Jan van de Winkel) at 37°C for 45 min. EA were washed three times in PBS. For rosette assays in situ, opsonized EA (0.05% hematocrit) were added to 24-well plates containing transfected COS cells. Plates were centrifuged at 400 ***g*** for 3 min and incubated at room temperature for 1 h. Unattached EA were removed by washing with PBS three times; COS cells were fixed with 1% paraformaldehyde and examined under an inverted microscope. The percentage of FcγR-expressing cells represents the number of cells binding EA vs total number of cells counted. Alternatively, for rosetting in suspension, trypsinized COS cells were pelleted with EA (as above) and incubated for 1 h at room temperature. The cells were centrifuged at 400 ***g*** for 3 min in an excess of PBS, gently resuspended, and counted. Typically, percentage EA binding values were greater when performed in suspension ($\sim$20–30%) than when performed in situ in 24-well plates ($\sim$5%).

3. Results

3.1. Description of six FcγRI transcripts

We previously identified four transcripts that are the gene products of three FcγRI genes (A, B, C) (Ernst et al., 1992). One of these transcripts (a) encodes three EC Ig-domains, two (b1 and c) contain a stop codon in the EC3 domain, while the fourth transcript, the product of gene B (b2), is the result of alternative splicing. In the b2 transcript the domain unique to the FcγRI class, EC3, has been spliced out leaving only EC1, EC2 and TM/CT domains. These four transcripts had been amplified by

RT/PCR using primers from the start of the EC1 domain (primer 9) to the middle of the TM/C (primer 10) (Materials and methods). In our new study, we wanted to determine if all FcγRI transcripts had been identified; therefore, new primers were used that would amplify nearly full-length transcripts. The new primer pair (1A/2A) was designed to amplify from the start to stop codons (Materials and methods); only the 5′- and 3′-untranslated regions are not amplified.

RNA from a variety of FcγRI-expressing cells cultured without or with IFNγ was used in an RT/PCR assay with primers 1A/2A. From all cell types, three bands of 1.1, 0.87 and 0.6 kb in size were visible in ethidium bromide stained agarose gels; with the primers 9/10 only two sized (0.88 and 0.6 kb) bands had been amplified. No products were amplified from K562, an FcγRI-negative cell line. When the PCR products were hybridized with the FcγRI cDNA p135, the first-cloned FcγRI cDNA (Allen and Seed, 1989), all three bands hybridized this probe.

To distinguish which gene products were represented in the three bands, we end-labeled two gene-specific oligonucleotides (GSO) for hybridization to the PCR amplified products. The GSO sequences were derived from sequence differences in the EC2 domain. GSO 37A, which consists of gene A sequence, hybridized to the 1.1 and 0.87 kb bands (Fig. 1A) with the predominant product being the 1.1 kb full-length size. The FcγRI cDNA p135 provides a full-length (a1 isoform) amplification control and serves as a GSO probe hybridization standard. The second GSO probe, 39B/C which corresponds to both gene B and C sequences, hybridized to all three band sizes (Fig. 1B/C) with the predominant product being the 0.87 kb sized band. It did not hybridize to the p135 amplification product. Culture of cells with IFNγ apparently increased the expression of all transcripts recognized by both GSO probes.

Five distinct types of transcripts were detected by the GSO hybridization, two products from gene A and three products from genes B/C. From our previous RT/PCR studies using primers 9/10, we had discovered that the full-length B/C hybridizing band consisted of a mixture of b1 and c transcripts (Ernst et al., 1992). We would expect that the corresponding 1.1 kb full-length hybridizing band from primers 1A/2A would also be a mixed population. Also, any transcripts that splice out the EC2 domain would not be detected by the GSO probes. We therefore analysed individual PCR products to determine the transcript diversity.

RT/PCR products from U937 cells cultured in IFNγ for 6 h exhibited the three sized banding pattern (Fig. 1, U937 + IFN); cDNAs from this sample were cloned into the vector pBluescript. Individual FcγRI clones were first identified by hybridization to the p135 cDNA probe and then categorized according to hybridization with the 37A and 39B/C GSO probes. All clones tested hybridized to one of these two probes; therefore, we did not identify transcripts that spliced out the EC2 domain. Nucleotide sequence analysis of representative clones identified six distinct transcripts (Fig. 2). For each of the six FcγRI isoforms, a minimum of two clones were sequenced. In addition to the nucleotide changes attributable to gene-specific differences, several other random nucleotide changes were found in the cDNAs. Each noted difference was not represented in any other clone; these unique changes can represent either allelic polymorphisms or a PCR amplification artifact.

Figure 3 illustrates the structural diversity represented by these six FcγRI gene products. Two transcripts derive from gene A (a1 and a2), three from gene B (b1, b2, b3), and one from gene C (c). The 1.1 kb a1 transcript contains all six exons and defines a membrane-spanning receptor with three Ig-like EC domains; the 0.87 kb a2 transcript

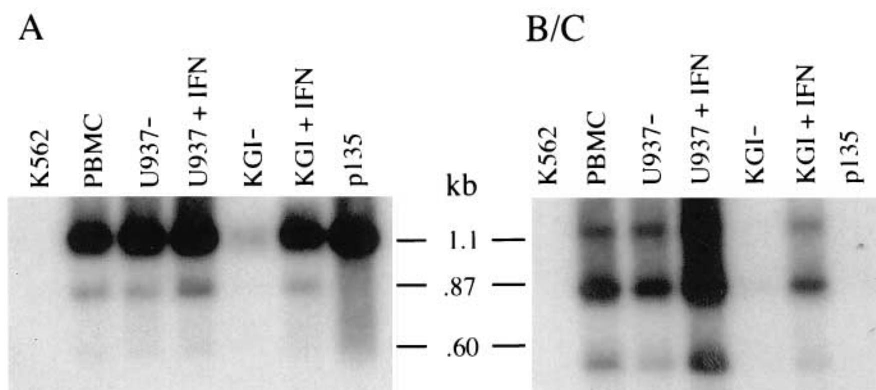


Fig. 1. Southern blot of FcγRI RT/PCR amplification products using gene-specific oligonucleotide (GSO) probes. RNA was made from FcγRI expressing cells (PBMC, U937, KG1) cultured without (−) or with IFNγ (+IFN) for 6 h and from the non-expressing control cell K562. RT/PCR was performed using primer pair 1A/2A to generate cDNAs containing full-length coding sequence. The cDNA p135 (a1 isoform) was used as a PCR and GSO hybridization control. The samples were electrophoresed in an agarose gel, transferred to Hybond-N, and hybridized with end-labeled GSO 37A (panel A) or GSO 39B/C (panel B/C). The four oligonucleotides used in this experiment are defined in Fig. 2. Hybridizing fragments are shown with sizes (kb).

```
    S1       .         .         . ■S2     .     +1  . ■EC1   .         .         .         .         .         .         .
    MetTrpPheLeuThrThrLeuLeuLeuTrpValProValAspGlyGlnValAspThrThrLysAlaValIleThrLeuGlnProProTrpValSerValPheGlnGluGluThrValThr
a1  ATGTGGTTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATCACTTTGCAGCCTCCATGGGTCAGCGTGTTCCAAGAGGAAACCGTAACC 120
a2  -------------------------------
b1  ------------------------------------------------------------------------------------------------------------------------
b2  ------------------------------------------------------------------------------------------------------------------------
b3  -------------------------------
c   ------------------------------------------------------------------------------------------------------------------------

             .         .         .         .         .         .         .         .         .         .         .         .
    LeuHisCysGluValLeuHisLeuProGlySerSerSerThrGlnTrpPheLeuAsnGlyThrAlaThrGlnThrSerThrProSerTyrArgIleThrSerAlaSerValAsnAspSer
a1  TTGCACTGTGAGGTGCTCCATCTGCCTGGGAGCAGCTCTACACAGTGGTTTCTCAATGGCACAGCCACTCAGACCTCGACCCCCAGCTACAGAATCACCTCTGCCAGTGTCAATGACAGT 240
a2                                        c
b1  --------------------------------------C---------------------------------------------------------------------------------
b2  --------------------------------------C---------------------------------------------------------------------------------
b3
c   ------------------------------------------------------------------------------------------------------------------------

             .         .         .         .         .         .       ■EC2        .         .         .  Met    .         .
    GlyGluTyrArgCysGlnArgGlyLeuSerGlyArgSerAspProIleGlnLeuGluIleHisArgGlyTrpLeuLeuLeuGlnValSerSerArgValPheThrGluGlyGluProLeu
a1  GGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCATACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCACGGAAGGAGAACCTCTG 360
a2                                                                     -----------------------------------------------------
b1  -------------------------------------------------------------------------------------------------------T----------------
b2  -------------------------------------------------------------------------------------------------------T----------------
b3                                                                     ------------------------------------T----------------
c   -------------------------------------------------------------------------------------------------------t----------------

             .         .         .         .         .         .         .         .         .         .         .         .
    AlaLeuArgCysHisAlaTrpLysAspLysLeuValTyrAsnValLeuTyrTyrArgAsnGlyLysAlaPheLysPhePheHisTrpAsnSerAsnLeuThrIleLeuLysThrAsnIle
a1  GCCTTGAGGTGTCATGCGTGGAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGCCTTTAAGTTTTTCCACTGGAATTCTAACCTCACCATTCTGAAAACCAACATA 480
a2  ------------------------------------------------------------------------------------------------------------------------
b1  ------g-----------------------------------------------------------------------------------------------------------------
b2  ------------------------------------------------------------------------------------------------------------------------
b3  ------------------------------------------------------------------------------------------------------------------------
c   ------------------------------------------------------------------------------------------------------------------------

                       .         .Lys      .  His    .         .      GlnTyr       .  ■EC3   .         .                   .
    SerHisAsnGlyThrTyrHisCysSerGlyMetGlyLysHisArgTyrThrSerAlaGlyIleSer***ValThrValLysGluLeuPheProAlaProValLeuAsnAlaSerValThr
a1  AGTCACAATGGCACCTACCATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCT***GTCACTGTGAAAGAGCTATTTCCAGCTCCAGTGCTGAATGCATCTGTGACA 600
a2  ------------------------------------------------------------------------------------------------------------------------
b1  -----------------------------------------------------------------ACAATA-------------------------------------------------
b2  -----------------------------------------------------------------ACAATA-----------
b3  -----------------------------------------------------------------ACAATA-----------
c   -------------------------------A-----------A/g-------------------ACAATA-------------------------------------------------

             .  G*lyGlyIleTrpSerProTer     .         .         .         .  Ter    .         .         .         .         .
    SerProLeuLeuGluGlyAsnLeuValThrLeuSerCysGluThrLysLeuLeuLeuGlnArgProGlyLeuGlnLeuTyrPheSerPheTyrMetGlySerLysThrLeuArgGlyArg
a1  TCCCCACTCCTGGAGGGGAATCTGGTCACCCTGAGCTGTGAAACAAAGTTGCTCTTGCAGAGGCCTGGTTTGCAGCTTTACTTCTCCTTCTACATGGGCAGCAAGACCCTGCGAGGCAGG 720
a2  ------------------------------------------------------------------------------------------------------------------------
b1  ----------------------------------------------------c-------------------T-----------------------------------------------
b2
b3
c   -------------*----------------------------------------------------------------------------------------------------------

             .         .         .         .         .         .         .         .         .         .         .         .
    AsnThrSerSerGluTyrGlnIleLeuThrAlaArgArgGluAspSerGlyLeuTyrTrpCysGluAlaAlaThrGluAspGlyAsnValLeuLysArgSerProGluLeuGluLeuGln
a1  AACACATCCTCTGAATACCAAATACTAACTGCTAGAAGAGAAGACTCTGGGTTATACTGGTGCGAGGCTGCCACAGAGGATGGAAATGTCCTTAAGCGCAGCCCTGAGTTGGAGCTTCAA 840
a2  ------------------------------------------------------------------------------------------------------------------------
b1  -------------------------------------------------------------------------------------------------A----------------------
b2
b3
c   ------------------T-----------------------------------------------------------------------------------------------------

           ■TM/C       .         .         .         .         .         .         .         .         .         .         .
    ValLeuGlyLeuGlnLeuProThrProValTrpPheHisValLeuPheTyrLeuAlaValGlyIleMetPheLeuValAsnThrValLeuTrpValThrIleArgLysGluLeuLysArg
a1  GTGCTTGGCCTCCAGTTACCAACTCCTGTCTGGTTTCATGTCCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACACTGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGA 960
a2  ------------------------------------------------------------------------------------------------------------------------
b1  ------------------------------------------------------------------------------------------------------------------------
b2         -----------------------------------------------------------------------------------------------------------------
b3         -----------------------------------------------------------------------------------------------------------------
c   ------------------------------------------------------------------------------------------------------------------------

             .  Asn    .         .         .         .         .         .         .         .         .         .         .
    LysLysLysTrpAspLeuGluIleSerLeuAspSerGlyHisGluLysLysValIleSerSerLeuGlnGluAspArgHisLeuGluGluGluLeuLysCysGlnGluGlnLysGluGlu
a1  AAGAAAAAGTGGGATTTAGAAATCTCTTTGGATTCTGGTCATGAGAAGAAGGTAATTTCCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTCAGGAACAAAAAGAAGAA1080
a2  ------------------------------------------------------------------------------------------------------------------------
b1  ------------A-----------------------------------------------------------------------------------------------------------
b2  ------------A-----------------------------------------------------------------------------------------------------------
b3  ------------A-----------------------------------------------------------------------------------------------------------
c   ------------A-----------------------------------------------------------------------------------------------------------

             .         .         .         .         .         .
    GlnLeuGlnGluGlyValHisArgLysGluProGlnGlyAlaThrTer
a1  CAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAGCGGCTCAGT                                  cDNA sizes:   a1 1137
a2  ------------------------------------------------------------                                                a2  861
b1  ------------------------------------------------------------                                                b1 1140
b2  ------------------------------------------------------------                                                b2  855
b3  ------------------------------------------------------------                                                b3  579
c   ------------------------------------------------------------                                                 c 1139
```

Fig. 2. Nucleotide sequence comparison of six transcripts derived from three human FcγRI genes. RT/PCR products resulting from amplification with FcγRI-specific oligonucleotide primers 1A (*S*1, underlined 1–15) and 2A (*TM*/*C*, reverse complement of underlined nt 1120–1137, a1) were analysed. The cloned inserts were ~1100, 870, 600 bp in size and were screened for differential hybridization to end-labeled GSO 37A (EC2, underlined nt 539–556, a1) and GSO 39B/C (EC2, underlined nt, 539–556, b1). Representative clones were sequenced and found to consist of six types of transcripts originating from the three genes; the six transcript names are listed in the left margin (lower case letters are used in accordance with accepted nomenclature rules). Dashed lines indicate nucleotide identity with the FcγRIa1 isoform sequence. Base pair substitutions are shown by the nucleotide change; nucleotide changes corresponding to changes found in the gene B and gene C sequences (Ernst et al., 1992) are shown in upper case letters. Those nucleotides that are unique to a single cDNA clone are shown in lower case letters. Nucleotide deletions are indicated by asterisks. The amino acid sequence is indicated by three-letter code; gene-specific nucleotide changes resulting in amino acid differences are indicated by listing the new code above the code for the FcγRIa1 transcript. Potential *N*-linked glycosylation sites (NXS/T) are overlined. Exon-intron boundaries are indicated by a black box above the first nucleotide of the exon along with the exon name. The three nucleotides and amino acids that differ among the FcγRI isoforms and the cDNAs p135 and p90 (Allen and Seed, 1989) are double underlined.

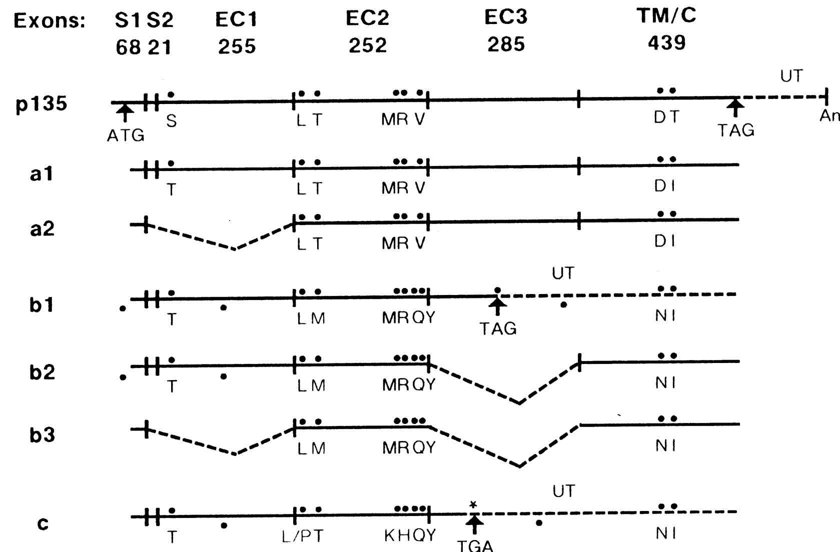


Fig. 3. Diagram of six FcγRI transcripts. The signal sequences (S), extracellular domains (EC), and the transmembrane (TM)/cytoplasmic (C) exons are shown. The six exon lengths were determined by the sequence of FcγRI genes A, B, and C (Ernst et al., 1992); the map is drawn to scale based on a full-length cDNA p135 (1321 bp). Exon-intron boundaries are indicated by vertical lines. Lower case letters in the left column refer to transcript names. Dots above the line maps indicate nucleotide changes that result in amino acid changes; those below the line are silent mutations. Start (ATG) and stop (TAG, TGA) codons are indicated with arrows. Dotted v-shaped lines indicate alternative splicing of the exon. Dotted straight lines indicate 3′-untranslated (UT) regions.

results from alternative splicing that removes the S2 and EC1 exons. Two full-length 1.1 kb transcripts, b1 and c, consist of all six exons; however, both have gene-specific nucleotide changes that result in premature stop codons in EC3. The 0.87 kb b2 transcript is an alternatively spliced product of gene B that is missing EC3 and encodes a transmembrane receptor with two EC domains. The 0.6 kb b3 product is also an alternatively spliced product that is missing S2, EC1, and EC3.

3.2. *Analysis of FcγRIb2 transcript expression*

The b2 transcript predicts a two EC domain transmembrane receptor. This is an interesting transcript since it is structurally similar to the two EC domain low affinity receptors, FcγRII and FcγRIII. The 0.87 kb b2 product appears to be the most abundant B/C gene product detected by hybridization analysis with the GSO 39B/C probe (Fig. 1B/C). We wanted to confirm that the b2 transcript is expressed in RNA from FcγRI-expressing cells and determine relative abundance to the a1 transcript. Therefore, we used a riboprobe synthesized from the b2 cDNA in an RNase protection assay to assess the presence of this transcript in RNA from the human monocytic cell line U937. It would be expected that b2 transcripts would fully protect the b2-riboprobe (855 bp). In contrast, a1 transcripts would allow digestion at the position of the 3-base to 6-base substitution near the C terminus of EC2 (nt 545, Fig. 2), and the lengthy EC3 domain of a1 would leave unprotected the EC–TM juncture of the b2-riboprobe. Therefore, it would be predicted that a1 would allow the b2 riboprobe to be digested into two major protected bands of 545 bp (S1 to near the C terminus of EC2, nt 1–545, Fig. 2) and 290 bp (all of TM/C, nt 848–1137).

The RNase protection assay was performed by hybridizing the b2-riboprobe to RNA from FcγRI negative (K562) and positive (U937) cell lines (Fig. 4). Three protected bands of predicted size (~290, 550, and 860 bp) were observed in U937 cell RNA. The intensity of all three bands was increased by culturing U937 cells with IFNγ for 6 h prior to RNA isolation (Fig. 4). Additional bands seen in this sample may be the result of digestion due to the differences of the b2 riboprobe and the other FcγRI isoforms (single nucleotide and alternate exon differences). It is apparent that b2 transcripts are expressed in U937 RNA and are up-regulated by culture of cells with IFNγ although they are less abundant than a1 transcripts.

3.3. *Protein products of the FcγRI transcripts*

Having described six transcripts for the three genes, we next wanted to characterize their translation products. We focused our attention on the four transcripts that contain a TM region (a1, a2, b2, b3) and would therefore be expected to be cell surface expressed. Beginning with in vitro translation using a rabbit reticulocyte lysate system with dog pancreas microsomes, we found the glycosylated a1 protein to be ~65 kDa as judged by sizing on SDS–PAGE gels. The other three translated proteins ranged in size from ~35 (b3) to 45 (a2, b2) kDa (Fig. 5A).

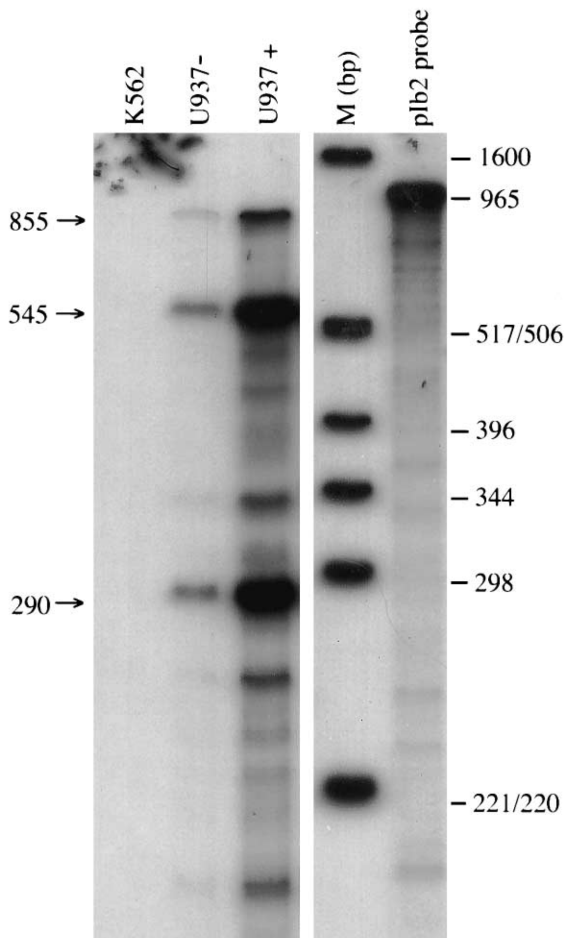


Fig. 4. Ribonuclease protection assay of RNA from FcγRI-expressing U937 cells. 30 μg of total RNA from FcγRI-negative (K562) or from FcγRI-positive U937 cells cultured without (−) or with (+) IFNγ were hybridized to a riboprobe synthesized from an FcγRIb2 (pIb2) (Fig. 2, Materials and methods). Arrows indicate major protected bands. Sizes (bp) of protected fragments were determined by comparison with band sizes of pBR322 digested with H*inf I*(*m*).

The a1 protein increased in molecular mass when translated in the presence of microsomes (65 kDa, Fig. 5A, a1+) compared to translation performed without microsomes (42 kDa, Fig. 5A, a1−). These sizes are comparable to the FcγRI sizes before (67 kDa) and after (50 kDa) *N*-glycanase treatment of U937 cells described by Peltz et al. (1988). There are different numbers of potential asparagine-linked glycosylation sites for the FcγRI isoforms that can contribute to the apparent size differences of these proteins (Fig. 2).

We next wanted to test the ability of anti-FcγRI monoclonal antibodies (mAbs) to bind to these proteins. If we could first establish the pattern of reactivity of these several mAbs with the in vitro expressed FcγRI isoforms, we could assess the potential of these mAbs to recognize the isoforms on FcγRI-expressing cells. Unfortunately, the three mAbs tested (22, 32.2, 197) and FcγRI ligand (HOPC1, mIgG2a) failed to immunoadsorb the translated products, even the a1 product, probably due to a lack of perfect folding fidelity of the in vitro translation system. However, an anti-FcγRI polyclonal antiserum (raised in goats against ligand purified FcγRI (Anderson et al., 1984)) was capable of immunoadsorbing all four translation products tested (Fig. 5B). This reagent was able to bind both the unglycosylated and glycosylated a1 product (Fig. 5B, lanes a1− and a1+). The goat antibody shows FcγR specificity since the control yeast mating factor (YMF) protein was not adsorbed by this antiserum (Fig. 5B, YMF). The in vitro translation (IVT) products did not bind to pre-immune goat serum in control adsorptions (not shown).

We have also prepared a rabbit anti-FcγRI cytoplasmic tail (CYT) peptide antiserum that may prove useful in analysing the cellular expression patterns of the FcγRI isoforms. Preliminary results with this reagent showed that it was capable of specifically immunoadsorbing the a1 translation product. However, only a small fraction of the a1 product was immunoadsorbed ($R >$ CYT vs IVT, Fig. 5C); therefore, the antibody affinity or titer may be low. The antibody does appear to exhibit specificity since the control yeast mating factor (YMF) protein was not adsorbed by this antiserum (Fig. 5C). The a1 IVT product does not bind to pre-immune normal rabbit serum (NRS, Fig. 5C).

We have assessed whether additional proteins other than the classical 70 kDa FcγRI can be detected on U937 cells using anti-FcγRI mAbs; these proteins would be candidate translation products of the FcγRI isoforms. We showed that preclearing a radioiodinated U937 (IFNγ-cultured) detergent lysate with immobilized anti-FcγRII mAb (IV3) does not remove all 40 kDa material. A second-step adsorption of the precleared lysate with anti-FcγRI mAb 32 shows both a 70 and a 40 kDa protein (Fig. 6, lane 1). Thus, a molecule of the mass predicted for the a2 and b2 translation products is specifically recognized by the anti-FcγRI mAb in lysates of IFNγ-cultured cells. In parallel conditions, preclearing the same cell lysate with the IV3 mAb almost completely removes any 40 kDa protein recognizable by the anti-FcγRII mAb in the second adsorption step (Fig. 6, lane 3). The IgG2b preclearing conditions (Fig. 6, lanes 2 and 4) provide an isotype control for the IV3 mAb. In lane 2, both the 70 and 40 kDa band intensities are reduced as compared to lane 1 so it appears that recovery of material is reduced, but the 40 kDa protein persists.

3.4. *Transfection analysis of FcγRI isoforms*

Initially, our attention has focused on comparing the characteristics of the receptors encoded by the two most prominent transcripts detected in our RNase protection assay, namely a1 and b2. These two are particularly interesting since a1 encodes the three Ig-domain, high affinity, TM receptor and b2 encodes the two Ig-domain TM

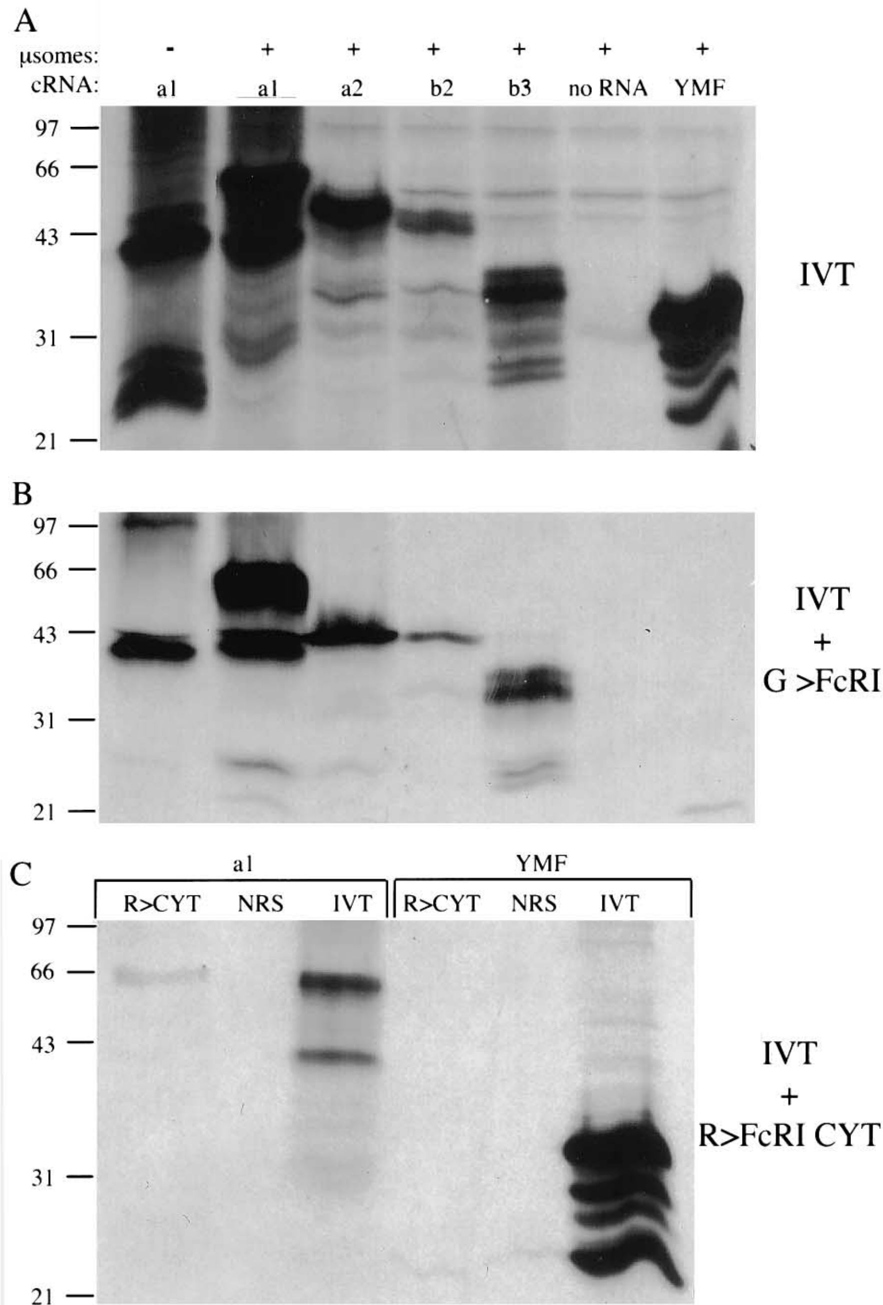


Fig. 5. In vitro translation of FcγRI variant transcripts. (A) Autoradiograph of ^{35}S-methionine (Met)-labeled in vitro translation (IVT) products fractionated by SDS–PAGE. In vitro-generated RNA transcripts complementary to the four FcγRI cDNA inserts (a1, a2, b2, b3) in pBluescript were used to program a rabbit reticulocyte in vitro translation cocktail containing ^{35}S-Met. Translations were done in the presence (+) or absence (−) of canine pancreas microsomes. Products generated in the absence of added FcγRI cRNA (no RNA) or with yeast mating factor RNA (YMF) are shown. (B) Immunoadsorption of in vitro translated products using goat anti-FcγRI antiserum. The in vitro translation products (shown in panel A) were lysed in TritonX-100 buffer and then adsorbed onto goat anti-FcγRI antibody-coated beads (IVT+G > FcRI). Adsorbed material was eluted under reducing conditions and then analysed by SDS–PAGE and autoradiography. (C) Immunoadsorption of in vitro translated products using rabbit anti-FcγRI cytoplasmic tail (CYT) antiserum (R > FcRI CYT). The in vitro translation (IVT) products from FcγRIa1 (a1) and yeast mating factor (YMF), a control protein, were lysed in TritonX-100 buffer. The products were adsorbed onto either rabbit anti-FcγRI CYT (R > CYT) antibody or normal rabbit serum (NRS) coated beads. Adsorbed material was eluted under reducing conditions and analysed by SDS–PAGE and autoradiography along with the original IVT product (IVT). For all panels, molecular mass markers are indicated in kDa.

form predicted to be a low affinity receptor. An FcγRIa1 cDNA (pIa1) that is identical to the exon sequence of gene A (differing by two amino acids from the cDNA p135; see Fig. 2 and Materials and methods) has been used to study the expression of FcγRI in COS cells. Previously (Ernst et al., 1993), we showed that cells trans-

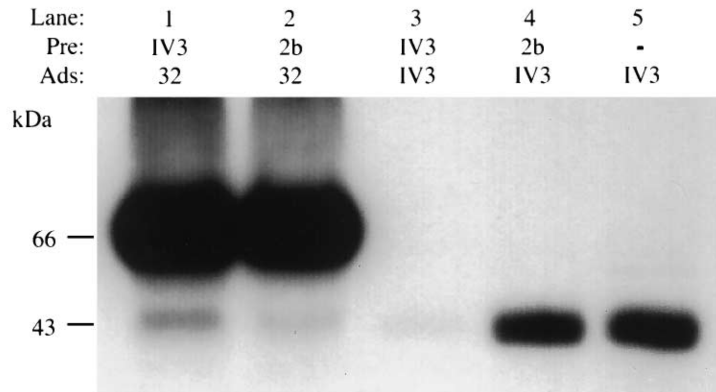


Fig. 6. Presence of a 40 kDa protein in anti-FcγRI adsorbed U937 lysates after preclearing of FcγRII. U937 cells cultured with IFNγ for 48 h were radioiodinated and lysed in TritonX-100 buffer. Lysates were adsorbed onto antibody coated-Sepharose beads in a two step process. The first preclearing (Pre) adsorption was with Sepharose beads coated with anti-FcγRII mAb (IV3) (lanes 1 and 3) to remove FcγRII from the lysate, with MOPC141 (mIgG2b (2b), lanes 2 and 4), an isotype control for IV3, or with no (−) antibody (lane 5). The material that did not bind to the first adsorbant was removed and placed on a second set of antibody coated beads, either anti-FcγRI mAb 32 (lanes 1 and 2), or anti-FcγRII mAb IV3 (lanes 3, 4, and 5). Adsorbed material on the second (Ads) set of beads was eluted under reducing conditions and was analysed by SDS–PAGE.

fected with pIa1 were able to bind anti-FcγRI mAbs 32.2 and 197, but not anti-FcγRII mAb IV3.

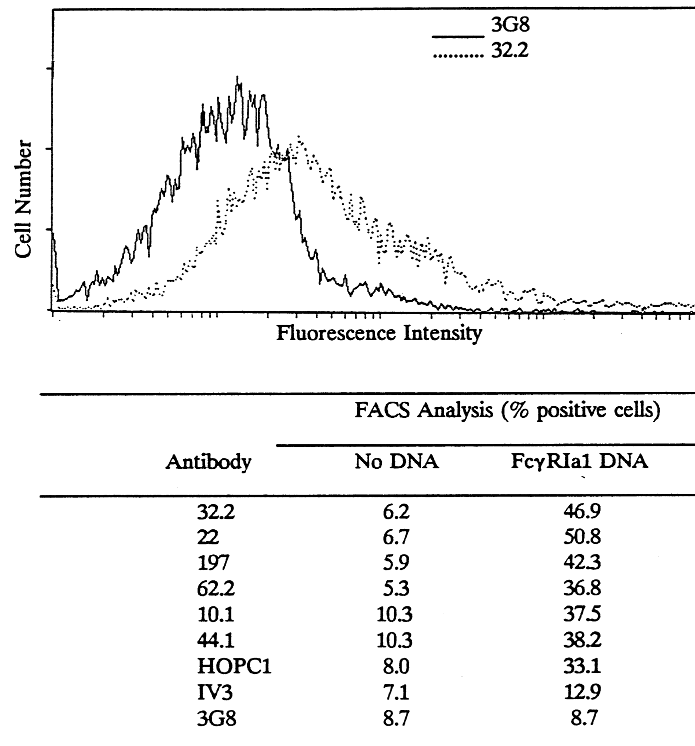
In these studies, we analysed the ability of COS cells transfected with either pIa1 or pIb2 cDNAs to bind a panel of six anti-FcγRI mAbs (32.2, 22, 197, 62.2, 10.1, 44.1) and IgG ligand (HOPC1, mIgG2a). The pIa1 transfectants were able to bind all six anti-FcγRI mAbs and the FcγRI ligand (Fig. 7). In contrast, the pIb2 transfectants were unable to bind either the FcγRI mAbs or ligand; they also did not bind anti-FcγRII mAb (IV3) or anti-FcγRIII mAb (3G8) (not shown). In addition, we saw no enhancement of mAb or ligand binding with co-transfection of COS with pIa1 and pIb2 from cells transfected with pIa1 alone. Cells co-transfected with pIb2 and FcR γ-chain, a subunit found to associate with the 70 kDa FcγRIa1 (Ernst et al., 1993), were also negative for anti-FcγRI mAb or ligand binding.

Both the pIa1 and the pIb2 COS transfectants were also tested for their ability to bind an immune complex by using antibody-coated erythrocytes (EA) in a rosetting assay. Several ligands were tested. Rabbit IgG, human IgG, and mouse IgG2a EA formed rosettes on pIa1 COS transfectants (5–30%); mIgG1, mIgG2b, and rabbit IgM EA were not bound by the pIa1 transfectants. In contrast, the pIb2 transfectants were unable to form rosettes consistently with any of the EA tested. However, an occasional cell (0.05–0.1%) could be found that vigorously bound EA (rabbit IgG). Co-transfection of COS with pIb2 and FcR γ-chain did not improve the efficiency of EA binding. Analysis of RNA made from the pIb2-COS transfectants in an RNase protection assay, using b2 as a riboprobe, determined that the cDNA was efficiently being transcribed. Although we have tried several different approaches to show ligand interaction with this receptor, the functional capabilities of the two Ig-domain FcγRIb2 isoform remain to be determined.

4. Discussion

With our initial characterization of the three FcγRI genes (A, B, and C), we had defined four distinct transcripts (Ernst et al., 1992). In this later study, we now identify a total of six transcripts: two from gene A (a1, a2), three from gene B (b1, b2, b3), and one from gene C (c). These isoforms result from alternative splicing (a2, b2, b3) and from gene-specific nucleotide differences that change amino acids and a reading frame. Figure 3 shows the structural diversity represented by these six FcγRI gene products. An interesting feature of the FcγR family gene structure is the cassette-like nature of their exons. All intron-exon junctions occur between the first and second nucleotide of a codon triplet (Qiu et al., 1990; Ernst et al., 1992); therefore, whenever an exon is alternatively spliced to a non-sequential exon, the protein reading frame is maintained in the utilized exon. These six transcripts fall into four major categories discussed below.

First, FcγRIa1 encodes a membrane spanning receptor that contains three EC domains. This is the only full-length gene product that has an intact EC3 domain, the exon unique to FcγRI. In vitro translation of the cDNA pIa1 identifies the protein product size as 65 kDa with a core protein of 42 kDa. In addition, anti-FcγRI mAb 197 immunoadsorbs a 65 kDa protein from radioiodinated pIa1-COS transfectant cell lysates (not shown). All six anti-FcγRI mAbs and several FcγRI IgG ligands bind to pIa1-COS transfectants. Therefore, this cDNA for the



Antibody	FACS Analysis (% positive cells)	
	No DNA	FcγRIa1 DNA
32.2	6.2	46.9
22	6.7	50.8
197	5.9	42.3
62.2	5.3	36.8
10.1	10.3	37.5
44.1	10.3	38.2
HOPC1	8.0	33.1
IV3	7.1	12.9
3G8	8.7	8.7

Fig. 7. Flow cytometry analysis of FcγRI a1- and b2-transfected COS cells. COS cells were transfected and indirect immunofluorescence staining of the cells was performed in preparation for flow cytometric analysis. No DNA, FcγRIa1, or FcγRIb2 cDNA transfected cells were incubated with anti-FcγRI mAbs (32.2, 22, 197, 62.2, 10.1, 44.1), FcγRI ligand (mIgG2a, HOPC1), anti-FcγRII mAb (IV3), or anti-FcγRIII mAb (3G8) followed by staining with FITC-*F*(ab′)2 goat anti-mouse IgG. An example of a fluorescence histogram of FcγRIa1 transfected cells is shown (32.2 compared to 3G8 staining). FcγRIb2 transfectants resulted in values no greater than the secondary (FITC)-antibody alone with all the listed antibodies and are not shown. The results are expressed as the percentage of cells brighter than the negative controls within each transfection condition.

FcγRIa1 isoform appears to encode a bona fide high affinity FcγR. It is noteworthy that FcγRIa1 differs from the archetype cDNA (p135) by 2 amino acids, at amino acid 10 in EC1 (Thr vs Ser) and at amino acid 324 in the CT domain (Ile vs Thr). To our knowledge no differences in function have been ascribed to these alterations. We would presume they are due to allotypic differences.

A second isoform, FcγRIb2, is an alternatively spliced product of gene B that encodes a transmembrane receptor with two EC domains. Although it has the structural hallmarks of a low affinity FcγR, cells transfected with a cDNA for this receptor in our hands have yet to bind ligand in a convincing manner, yielding only a rare rosette (0.05–0.1% of targets). Experiments (Hulett et al., 1991) using equivalent chimeras of murine FcR origin have shown that after removing the EC3 of murine FcγRI, the remaining EC1 and EC2 domains retained the capacity to bind immune complexes (EA) although the ability to bind monomeric IgG was reduced. We had expected that the b2 transcript would behave in a similar manner, but it did not. Porges (Porges et al., 1992) also identified an FcγRIb2 transcript by RT/PCR and reported that the b2-COS transfectants can form rosettes with human IgG EA, although more weakly than cells expressing FcγRIa1. We can only surmise that their transfection method and their FcR binding assay may have been more efficient than ours.

We searched for other molecules that might associate with the b2 isoform to form a more efficient ligand binding complex, considering among others the FcR γ-subunit that is shared among several FcR (Ernst et al., 1993; van Vugt et al., 1996; Hulett and Hogarth, 1994). We co-transfected COS cells with the pIb2 cDNA and the γ-chain cDNA to determine if the γ-subunit would aid the expression and function of FcγRIb2. No detectable expression was rescued by this co-transfection strategy. However, the low number (0.05–0.1%) of pIb2 COS transfectants that form rosettes is reminiscent of the studies with FcεRI and FcγRIIIA multiunit receptors. Experiments with these receptors showed the need to co-transfect all subunits of a receptor complex to get cell surface expression and function (Kuster et al., 1990; Hibbs et al., 1989). Therefore, it is possible that an additional subunit(s), not yet identified, is required to facilitate surface expression of FcγRIb2. Likewise, co-transfection with pIa1 did not enhance ligand binding

A third category of FcγRI isoform, represented by b1 and c, has stop codons in the EC3 domain. The b1 transcript has a C to T transition (Fig. 2, nt 673) that results in a TAG termination codon. The c transcript has

a single nucleotide deletion (Fig. 2, nt 614) that results in a frameshift and a stop codon six residues later. The predicted result of these changes would be the generation of secreted receptors containing the first two EC domains. Soluble FcR have indeed been reported by a number of investigators and are postulated to have immunoregulatory properties (Fridman and Sautes, 1990).

The fourth category of FcγRI transcript is represented by a2 and b3 which splice out the sequence encoded by the mini exon S2 (21 bp) in addition to one or two EC domains (EC1 for a2; both EC1 and EC3 for b3). The missing S2 sequence encodes the peptidase cleavage site (predicted between glycine-15 and glutamine-16, Fig. 2). No other potential cleavage site can be readily identified downstream (Gunnar von Heijne, personal communication). Two other examples of transcripts lacking this S2 exon are found in related FcR families (Brooks et al., 1989; Tepler et al., 1989). The protein product of the FcγRIa2 and b3 receptors would be expected to be integrated into the membrane at both the leader and the TM hydrophobic regions. Thus, an extracellular loop would form unless an alternate cleavage site is utilized. The fate of this type of receptor is unknown although an FcγRIIb3 transcript (Brooks et al., 1989), lacking the S2 exon, was transfected and found able to bind EA with equivalent density to transfected full-length FcγRIIb1. These forms thus appear to be potentially functional.

With the identification of these six FcγRI transcript variants, it is provocative to question whether these isoforms are expressed in a wider range of cells than originally defined for FcγRI. It is possible that the current anti-FcγRI mAbs only detect epitopes on the 70 kDa glycoprotein that is the product of the FcγRIa1 isoform. By using RT/PCR coupled with GSO probe hybridization, a survey of different immune system cells (i.e. T and B cells, NK cells, platelets) may reveal the presence of FcγRI isoforms in cells previously categorized as FcγRI-negative. Additionally, cytokines known to affect FcγRI expression (i.e. IFNγ, G-CSF, IL-4, IL-10) may also be important to the regulation of these isoforms. Furthermore, the location of the FcγRIB gene on the p arm of chromosome 1 (1p12), removed from the FcγRIA and C genes (1q21) (Maresco et al., 1995), may result in differential regulation of this gene from the others. We suggest that a systematic analysis of the FcγRI transcripts in a variety of cells and tissues and the effect of cytokines on their expression may give insights into the function of these newly identified isoforms.

Acknowledgments

This work was supported in part by an award from the United States Public Health Service (RO1-CA44983). The authors would like to thank Dr Fredika Robertson for flow cytometric analysis and Ms Jeanne Osborne and Ms Stacey Garber for expert assistance.

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