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Research Article

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Novel Fc γ Receptor I Family Gene Products in Human Mononuclear Cells

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Abstract

Unlike the human Fc γ RII and Fc γ RIII families, which exhibit considerable diversity at both the nucleic acid and protein levels, the human Fc γ RI family has only a single recognized product expressed as a 70-kD cell surface receptor with high affinity for monomeric IgG (hFc γ RIa1). Using both polymerase chain reaction-based amplification and Northern hybridization, we document multiple interferon- γ -inducible hFc γ RI RNA transcripts in human mononuclear cells and neutrophils. The sequences of two of these Fc γ RI related transcripts indicate that they are alternatively spliced products of a second Fc γ RI family gene, termed Fc γ RIB. The cDNA derived from the larger of these transcripts, termed hFc γ RIB1, encodes a surface molecule that is not recognized by Fc γ RI specific monoclonal antibodies when transfected into COS-7 cells. Unlike the interferon- γ -inducible hFc γ RIA gene product, hFc γ RIB1 does not bind monomeric IgG with high affinity. However, both hFc γ RIa1 and hFc γ RIB1 do bind aggregated human IgG. Previously unrecognized diversity within the hFc γ RI family includes an interferon- γ -inducible, putative low affinity Fc γ receptor that may play an important role in the mechanism by which Fc γ receptors participate in the humoral immune response. (*J. Clin. Invest.* 1992. 90:2102-2109.) Key words: immunoglobulin • interferon- γ • mRNA / cDNA • high affinity

Introduction

Among the three families of human Fc γ receptors (hFc γ Rs),¹ the hFc γ RI family is the most distinct (1-8). Although hFc γ RI appears to have diverged from a primordial Fc γ R gene early in evolution before the gene duplication and recombination leading to the hFc γ RII and hFc γ RIII families (8), Fc γ RI lacks the recognized structural diversity of these two families. The hFc γ RII family consists of at least three distinct genes, two

of which have multiple splice isoforms (6). The hFc γ RIII family has two distinct genes encoding highly homologous proteins which demonstrate cell type-specific expression and different membrane anchors (7). In contrast, hFc γ RI has only one recognized family member (9-11).

While the hFc γ RII and hFc γ RIII families encode receptors with low affinity for IgG (1-8), the hFc γ RI family contains the only Fc γ R with high affinity for monomeric IgG (9-11). This increased affinity presumably results from the presence of a third extracellular immunoglobulin-like domain (EC3) not present in other Fc γ Rs (12). Similarly, the cytoplasmic domain of hFc γ RI shows limited homology with other Fc γ Rs and appears to have a distinctive capacity to interact intracellularly with actin binding protein (13). Finally, the expression of hFc γ RI is regulated by IFN- γ (14-16) through a cis acting IFN- γ response region within the promoter (17). Each of these elements suggests an important role for the hFc γ RI family in the humoral immune system.

Preliminary evidence has suggested the possibility that our current understanding of the hFc γ RI family may be incomplete, and that one or two additional hFc γ RI genes may exist (hFc γ RIB, hFc γ RIC) (18, 19). Three highly homologous cDNA clones, each encoding 70-kD surface glycoproteins with three extracellular immunoglobulin-like domains and with high affinity for monomeric IgG have also been described (20, 21). However, there has been no evidence that any Fc γ RI gene product, other than the full-length transcript predicted by the hFc γ RIA gene, encodes a functional IgG receptor on the surface of cells.

We report the existence of three distinct transcripts from the hFc γ RI family in human monocytes. One transcript is a full-length product of the hFc γ RIA gene, encoding a surface molecule with high affinity for monomeric IgG and recognized by mAbs specific for the 70-kD Fc γ RI. Two other transcripts encode a number of nonconservative amino acid substitutions, matching preliminary reports of the hFc γ RIB genomic sequence (19). One of these hFc γ RIB gene transcripts lacks the entire exon encoding the third extracellular immunoglobulin-like domain (EC3), while the other transcript lacks sequences encoding both EC1 and EC3. When transfected into COS-7 cells, the longer of these two putative hFc γ RIB gene transcripts does not bind monomeric IgG, but does bind aggregated human IgG. This lower affinity Fc γ R, encoded by the IFN- γ inducible hFc γ RIB gene and sensitive to multivalent immunologic stimuli such as immune complexes, may play a significant role in triggering leukocytes and modulating immune effector functions.

Methods

Cell isolation and culture. Briefly, 15 ml of heparinized fresh human whole blood was diluted with 15 ml of HBSS (Gibco Laboratories,

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1. Abbreviations used in this paper: EA, erythrocytes coated with rabbit IgG; E_{BA}, streptavidin-coated erythrocytes; E-hIgG, erythrocytes coupled to human IgG; hFc γ R, human Fc γ receptor; hIFN- γ , human interferon- γ ; PCR, polymerase chain reaction; RT, reverse transcriptase.

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Grand Island, NY) and separated by density gradient centrifugation in sterile Ficoll-Hypaque (22). Mixed mononuclear cells were washed three times, resuspended in RPMI 1640 containing 20% heat inactivated autologous serum, plated on 100-mm plastic dishes (Becton Dickinson, Morristown, NJ) and incubated at 37°C for 6 h. Nonadherent cells were removed by gentle washing. Those cells receiving recombinant hIFN- γ (Genzyme Corp., Cambridge, MA) were treated with 200 U/ml for 6 h before harvest (16).

The human monocytoid cell line U937 (ATCC CRL 1593) was cultured in RPMI 1640 supplemented with IgG depleted 10% FCS (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 25 mM Hepes, and penicillin-streptomycin in a humidified atmosphere containing 5% CO₂. COS-7 cells (ATCC CRL 1651) were cultured under the same conditions in DMEM with the same supplements.

RNA extraction and polymerase chain reaction. 5×10^7 purified mononuclear cells or U937 cells were lysed with 4 M guanidinium thiocyanate (Boehringer Mannheim Biochemicals, Indianapolis, IN) buffer containing 1% β -mercaptoethanol and RNA was isolated by ultracentrifugation at 35,000 rpm for 18 h over a 5.7 M cesium chloride gradient (23). Total cellular RNA was then recovered by ethanol precipitation.

For cDNA synthesis, 10 μ g RNA was incubated with 50 pM an "antisense" oligonucleotide primer complementary to the 3' noncoding region of p135 (GAA GTC AGT TCA TTT ATT TGC) (20), 100 U MuMLV reverse transcriptase (RT) (U.S. Biochemical Corp., Cleveland, OH) and 200 μ M each dNTP for 60 min.

The polymerase chain reaction (PCR) was performed by adding to cDNA 50 pM "sense" primer from the 5' noncoding region of p135 (GAC AGA TTT CAC TGC TCC CAC CAG), 2 U *Taq* polymerase, and increasing reaction volume to 100 μ l with PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) and 1.5 mM MgCl₂ (24). In a thermal cycler, the mix then underwent 30 cycles of denaturation at 94°C for 1 min annealing at 50°C for 1 min and extension at 72°C for 3 min. No visible product was seen with ethidium bromide staining of products at the end of this first series of PCR amplifications.

To increase amplification, a nested PCR approach was used (Fig. 1 A). A second set of sense (AGA CAA CAT GTG GTT CTT GA) and antisense (CT TTA AGA GTT ACA TAC CAT) primers ~ 30 bp within the initial set of primers were synthesized. The product of the first 30 cycles of PCR was diluted 1:50 in 1 \times PCR buffer, plus 200 μ M free nucleotides, 50 pmol of each of the inner primers, 1.5 mM MgCl₂, and 2 U *Taq* polymerase. Thermal cycling was then performed for 20 cycles under similar conditions, except that annealing was performed at 54°C to increase specificity.

For direct DNA sequencing of PCR products, several other primers were selected to amplify smaller cDNA fragments. These primers were homologous to each of the three extracellular domains of p135 (EC1 sense: ACA GCC ACT CAG ACC TCG AC, EC1 antisense: TGT GGA TTT CCA GCT GTA TG, EC2 sense: GTA CAA TGT GCT TTA CTA TCG, EC2 antisense: GAG GTT AGA ATT CCA GTG GA, EC3 sense: GCT CCA GTG CTG AAT GCA TC, EC3 antisense: ACT CAG GGC TGC GCT TAA GG). When used in combination, these primers generated overlapping 300–700 bp cDNA products.

Cloning and sequencing. PCR-amplified products from freshly isolated human mononuclear cells were separated by electrophoresis through 2% low melting point agarose, and each band was excised and purified using a silica matrix based GeneClean kit (BIO 101, Inc., La Jolla, CA). Each PCR product was phosphorylated with T4 polynucleotide kinase (U.S. Biochemical Corp.) and ATP. Vector pRc/CMV (Invitrogen, San Diego, CA) was linearized, converted to blunt ends with T4 polymerase, dephosphorylated with calf intestinal phosphatase, and ligated to PCR products using T4 DNA ligase (25). Ligated vectors were used to transform competent *Escherichia coli*.

For sequencing, cloned PCR products were denatured in 0.2 M NaOH for 30 min, annealed to 0.5 pmol of primer, and then DNA synthesis with dideoxynucleotides, ³⁵S-dATP and recombinant DNA polymerase (Sequenase kit; U.S. Biochemical Corp.), followed by polyacrylamide gel electrophoresis (26). Direct sequencing of the smaller

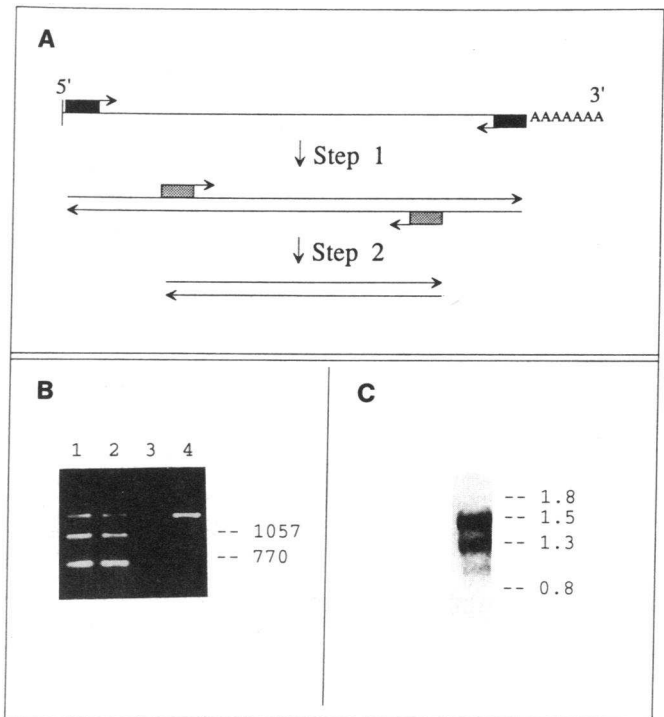


Figure 1. Identification of hFc γ RI transcripts of three distinct sizes. (A) PCR amplification strategy, schematic diagram. cDNA was synthesized using reverse transcriptase, followed by 30 cycles of PCR using human Fc γ RI specific primers (shown as dark rectangles). In step 2, a second set of hFc γ RI specific primers 30 bp inside of the initial primers (shaded rectangles) was used in a "nested" supplemental 20 cycles of PCR. (B) PCR-amplified cDNA, separated by DNA gel electrophoresis. Amplification of hFc γ RI cDNA clone p135 (lane 4) (20, 21) resulted in a single 1,200-bp product, as predicted. Amplification of cDNA from human mononuclear cells (lane 1) or U937 cells (lane 2) led to products of 1,200, 1,000, and 750 bp. Without the addition of mRNA (lane 3), no product was observed. DNA size standards, in basepairs, are shown to the right. (C) Northern hybridization of IFN- γ -stimulated U937 RNA with a hFc γ RI specific probe also showed three distinct transcripts. Strong bands were identified at 1,500 and 1,250 bp, and a weaker band was seen at 1,000 bp. RNA size standards in kilobase are shown to the right.

PCR amplified products was performed using the same technique after purification over Sepharose CL-4B columns (Boehringer Mannheim Biochemicals).

Northern hybridization. 15 μ g of RNA was separated on 1.4% agarose containing 2.2 M formaldehyde by electrophoresis at 120 V for 3 h, and blotted onto a 0.45- μ m nylon membrane (Schleicher & Schuell, Inc., Keene, NH). After ultraviolet crosslinking, the nylon was prehybridized in 50% formamide containing 100 μ g/ml denatured salmon sperm DNA and yeast tRNA at 62°C for 18 h.

A [³²P]UTP (NEN, Wilmington, DE) labeled RNA probe was synthesized using SP6 RNA polymerase (Promega, Madison, WI) (27) from the pRc/CMV construct containing the smallest Fc γ RI (750 bp) PCR product. 10⁶ cpm/ml of probe was added to the prehybridization solution, and hybridization was performed for 24 h at 62°C. Hybridized blots were washed at 60°C.

Transfection of COS-7 cells. 10 μ g of DNA was precipitated onto a 40% confluent monolayer of COS-7 cells using calcium phosphate (28). After 4 h, the cells were glycerol shocked, and exposed to 100 μ M chloroquine for 3 h. After 48 h of culture, the cells were harvested by vigorous aspiration.

Flow cytometry. 2×10^5 cells in 0.05 ml were incubated 30 min at room temperature with first antibody (saturating doses of mouse mono-

A

1	ATGTGGTTCTTGACAACCTCGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATC ATGTGGTTCTTGACAACCTCGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATC ATGTGGTTCTTGACAACCTCGCTCCTTTGGG-----	hF γ RIa1 hF γ RIb1 hF γ RIb2
73	<u>ACTTTGCAGCCTCCATGGGTCAGCGTGTTC</u> AAGAGGAAACCGTAACCTTGCAC'TGTGAGGTGCTCCATCTG ACTTTGCAGCCTCCATGGGTCAGCGTGTTC <u>AAGAGGAAACCGTAACCTTGCAC'TGTGAGGTGCTCCATCTG</u> -----	hF γ RIa1 hF γ RIb1 hF γ RIb2
145	CCTGGGAGCAGCTCTACACAGTGGTTTCTCAATGGCACAGCCACTCAGACCTCGACCCCCAGCTACAGAAT CCTGGGAGCAGCTCCACACAGTGGTTTCTCAATGGCACAGCCACTCAGACCTCGACCCCCAGCTACAGAAT -----	hF γ RIa1 hF γ RIb1 hF γ RIb2
216	CACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCA CACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCA -----	hF γ RIa1 hF γ RIb1 hF γ RIb2
289	TACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCA <u>CGGAAGGAGA</u> ACCT TACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCA <u>CGGAAGGAGA</u> ACCT -----GCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCA <u>CGGAAGGAGA</u> ACCT	hF γ RIa1 hF γ RIb1 hF γ RIb2
360	CTGGCCTTGAGGTGTCATGCGTGGAAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGC CTGGCCTTGAGGTGTCATGCGTGGAAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGC CTGGCCTTGAGGTGTCATGCGTGGAAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGC	hF γ RIa1 hF γ RIb1 hF γ RIb2
431	CTTTAAGTT <u>TTT</u> CCACTGGAATTCTAACCTC <u>ACC</u> ATTCTGAAAACCAACATAAGTCACAATGGCACCTACC CTTTAAGTT <u>TTT</u> CCACTGGAATTCTAACCTC <u>ACC</u> ATTCTGAAAACCAACATAAGTCACAATGGCACCTACC CTTTAAGTT <u>TTT</u> CCACTGGAATTCTAACCTC <u>ACC</u> ATTCTGAAAACCAACATAAGTCACAATGGCACCTACC	hF γ RIa1 hF γ RIb1 hF γ RIb2
502	ATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCT <u>T---GTC</u> ACTGTGAAAGAGCTATTT ATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATC <u>ACAATAC</u> ACTGTGAAA----- ATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATC <u>ACAATAC</u> ACTGTGAAA-----	hF γ RIa1 hF γ RIb1 hF γ RIb2
570	CCAGCTCCAGTGCTGAATGCATCTGTGACATCCCCACTCCTGGAGGGGAATCTGGTCACCCTGAGCTGTGA ----- -----	hF γ RIa1 hF γ RIb1 hF γ RIb2
641	AACAAAAGTTGCTCTTGCAGAGGCCTGGTTTGCAGCTTACTTCTCCTTCTACATGGGCAGCAAGACCCCTGC ----- -----	hF γ RIa1 hF γ RIb1 hF γ RIb2
712	GAGGCAGGAACACATCCTCTGAATACCAAATACTAACTGCTAGAAGAGAAGACTCTGGGTTATACTGGTGC ----- -----	hF γ RIa1 hF γ RIb1 hF γ RIb2
783	GAGGCTGCCACAGAGGATGGAAATGTCTTAAGCGCAGCCCTGAGTTGGAGCTTCAAGTGCTTGGCCTCC -----GGCCTCC -----GGCCTCC	hF γ RIa1 hF γ RIb1 hF γ RIb2
853	AGTTACCAACTCCTGTCTGGTTTCATGTCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACAC AGTTACCAACTCCTGTCTGGTTTCATGTCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACAC AGTTACCAACTCCTGTCTGGTTTCATGTCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACAC	hF γ RIa1 hF γ RIb1 hF γ RIb2
923	TGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGAAAGAAAAAGTGG <u>GA</u> ATTTAGAAATCTCTTTGGAT TGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGAAAGAAAAAGTGG <u>GA</u> ATTTAGAAATCTCTTTGGAT TGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGAAAGAAAAAGTGG <u>GA</u> ATTTAGAAATCTCTTTGGAT	hF γ RIa1 hF γ RIb1 hF γ RIb2
993	TCTGGTTCATGAGAAGAAGGTAAT <u>TT</u> CCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTC TCTGGTTCATGAGAAGAAGGTAAT <u>TT</u> CCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTC TCTGGTTCATGAGAAGAAGGTAAT <u>TT</u> CCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTC	hF γ RIa1 hF γ RIb1 hF γ RIb2
1063	AGGAACAAAAAGAAGAACAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAG AGGAACAAAAAGAAGAACAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAG AGGAACAAAAAGAAGAACAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAG	hF γ RIa1 hF γ RIb1 hF γ RIb2

Figure 2. Sequences of three hF γ RI transcripts. (A) Aligned nucleotide sequences of the three PCR-amplified hF γ RI cDNAs (hF γ RIa1, hF γ RIb1, and hF γ RIb2). Nucleotide differences between hF γ RIa1 and p135 are overlined. Nucleotide differences between hF γ RIa1 and hF γ RIb1,2 (*shadowed*). Nucleotide differences between hF γ RIb1 and hF γ RIb2, (*underlined*). (B) Predicted amino acid sequences of the three PCR amplified hF γ RI cDNAs. Amino acid substitutions between hF γ RIa1 and p135, (*overlined*). Amino acid differences between hF γ RIa1 and hF γ RIb1,2, (*shadowed*). Amino acid differences between hF γ RIb1 and hF γ RIb2, (*underlined*). GenBank accession numbers L03418, L03419, and L03420.

B

hFcγRIa1	- MWFLT ¹ TLLLWVPVDGQVD ² TTKAVITL ³ QPPWVS ⁴ VFQEETVTLHCEVLH ⁵ HLPG -50
hFcγRIb1	- MWFLT ¹ TLLLWVPVDGQVD ² TTKAVITL ³ QPPWVS ⁴ VFQEETVTLHCEVLH ⁵ HLPG -50
hFcγRIb2	- MWFLT ¹ TLLLW----- -10
hFcγRIa1	- SSSTQWFLNGTATQTSTPSYRITSASV ¹ NDSGEYRCQRGLSGRSDPIQLEI -100
hFcγRIb1	- SSSTQWFLNGTATQTSTPSYRITSASV ¹ NDSGEYRCQRGLSGRSDPIQLEI -100
hFcγRIb2	- ----- -10
hFcγRIa1	- HRGWLLLQVSSRVF ¹ TEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWN -150
hFcγRIb1	- HRGWLLLQVSSRVF ¹ TEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWN -150
hFcγRIb2	- --GWLLLQVSSRVF ¹ TEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWN -58
hFcγRIa1	- SNL ¹ TILKTNISHNGTYHCSGMGKHRYTSAGIS-VTVKELFPAPVLNASVT -199
hFcγRIb1	- SNL ¹ TILKTNISHNGTYHCSGMGKHRYTSAGISQYTVK----- -187
hFcγRIb2	- SNL ¹ A ² ILKTNISHNGTYHCSGMGKHRYTSAGISQYTVK----- -95
hFcγRIa1	- SPLLEGNLVTLSCETKLLLQRPGLQLYFSFYMGSKTLRGRNTSSEYQILT -249
hFcγRIb1	- ----- -187
hFcγRIb2	- ----- -95
hFcγRIa1	- ARREDSGLYWCEAATEDGNVLKRSPELELQVLG ¹ LQLPVWFHVL ² FYLAV -299
hFcγRIb1	- -----GLQLP ¹ TPVWFHVL ² FYLAV -205
hFcγRIb2	- -----GLQLP ¹ TPVWFHVL ² FYLAV -113
hFcγRIa1	- GIMFLVNTVLWVTIRKELKRKKK ¹ WDL ² EISLDSGHEKKVISSLQEDRHLEE -349
hFcγRIb1	- GIMFLVNTVLWVTIRKELKRKKK ¹ WDL ² EISLDSGHEKKVISSLQEDRHLEE -255
hFcγRIb2	- GIMFLVNTVLWVTIRKELKRKKK ¹ WDL ² EISLDSGHEKKVISSLQEDRHLEE -163
hFcγRIa1	- ELKCQE ¹ QKEEQ ² LQEGVHRKEPQ ³ GAT -374
hFcγRIb1	- ELKCQE ¹ QKEEQ ² LQEGVHRKEPQ ³ GAT -280
hFcγRIb2	- ELKCQE ¹ QKEEQ ² LQEGVHRKEPQ ³ GAT -188

Figure 2 (Continued)

clonal, or dilutions of aggregated human IgG prepared by heat aggregating human Cohn fraction II (Miles Labs, Kanakee, IL) at 63°C for 20 min) in PBS and 0.1% BSA and then washed twice. Cells were subsequently incubated with fluorochrome conjugated F(ab)₂ second antibody (goat anti-mouse or goat anti-human) (Tago, Burlingame, CA) at 4°C for 30 min and washed twice again before flow cytometry with a dual laser Cytofluorograf IIS with a 2151 computer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Preparation of IgG-opsonized erythrocytes. Erythrocytes were coupled to human IgG (E-hIgG) by a biotin-avidin technique, as described previously (29). 5 × 10⁸ erythrocytes were incubated with sulfo-*N*-hydroxysuccinimide-biotin (Pierce Chemical Co., Rockford, IL) (500 μg/ml) for 20 min at 4°C, followed by three washes. Cells at 10⁹/ml were mixed with an equal volume of streptavidin (250 μg/ml) for 30 min at 4°C. The streptavidin coated cells (E_{BA}) were then washed and resuspended at 10⁹ E/ml for immediate use.

Human IgG was biotinylated with *N*-hydroxysuccinimide-long chain-biotin (0.01 mg biotin/mg protein) for 60 min at room temperature. To bind the biotinylated hIgG to the E_{BA}, E_{BA} (12.5 μl at 10⁹/ml) were combined with biotinylated IgG (20 μg) for 30 min at 4°C with mixing. After three washes, the human IgG-coated E_{BA} (E-hIgG) were resuspended in 125 μl (10⁸ E/ml) and used immediately.

Alternatively, antibody-coated erythrocytes (EA) were prepared by incubating bovine E with a subagglutinating titer of rabbit IgG anti-bovine E antibody (Cappel Laboratories, Cochranville, PA) for 1 h at 37°C. The cells were washed and resuspended at 10⁸ cells/ml in RPMI and 20% FCS.

Rosette formation with IgG-opsonized erythrocytes. COS-7 cells were resuspended in RPMI and 20% heat-inactivated IgG free FCS at 5

× 10⁶ cells/ml, as described previously (30). Cells (100 μl) were combined with EA or E-hIgG (250 μl), centrifuged at 150 *g* for 3 min, incubated at 37°C for 1 h, and then gently resuspended. Adherence of EA or E-hIgG to COS-7 cells was assessed by light microscopy.

Results

Amplification of FcγRI transcripts. Using oligonucleotide primers complementary to a known hFcγRI cDNA (20), RT-PCR amplification of RNA extracted from U937 cells and from resting human mononuclear cells of two healthy donors produced little or no product after 30 cycles of amplification. This result probably reflects low hFcγRI mRNA levels in resting cells. To increase sensitivity, a nested PCR strategy was used (Fig. 1 A). This technique, with both U937 cell RNA and human mononuclear cell RNA, consistently resulted in three distinct amplified DNA products (Fig. 1 B). The longest of these three products, 1,200 bp, was identical in size to the single product resulting from PCR amplification of p135 cDNA. The two smaller amplified DNA products were ~ 1,000 and 750 bp in length.

Northern hybridization. To confirm the existence of these smaller hFcγRI related transcripts, nonamplified RNA from U937 cells was analyzed on Northern blots. To enhance the ability to detect low levels of message, a ³²P-labeled antisense RNA probe with high avidity for mRNA was used instead of a cDNA probe. IFN-γ-treated U937 cells showed transcripts of

1,500 and 1,250 bp, with a weaker third transcript at 1,000 bp (Fig. 1 C). These messages most likely correspond to the 1,200-, 1,000-, and 750-bp PCR amplified cDNAs, respectively.

Characterization of PCR amplified transcripts. To characterize each amplified cDNA, each product was cloned into vector pRc/CMV and sequenced by the dideoxy technique. The coding cDNA and predicted protein sequences of these three products are shown in Fig. 2.

The 1,200-bp PCR amplified cDNA is similar but not identical to previously described hFc γ RI gene transcripts described by Allen and Seed (20). It differs at nucleotide positions 73 and 1,024 from the p135 cDNA clone (20), resulting in nonconservative amino acid substitutions at positions 25 and 338. However, the coding sequence exactly matches the nucleotide and predicted protein sequences described for the Fc γ RIA gene by van de Winkel (18). Direct sequencing of all 1,200-bp PCR products from two healthy individuals showed both individuals to be homozygous for the same hFc γ RIA sequence.

The 1,000- and 750-bp cDNA sequences both lack the exon encoding EC3, and the 750-bp cDNA additionally lacks the exon encoding EC1. Both of these transcripts also have a series of seven consecutive divergent nucleotides (nucleotide positions 544–550), which result in an inserted amino acid and a nonconservative amino acid substitution near the end of the exon encoding EC2 (amino acid positions 183–184, Fc γ RIA = Val, Fc γ RIB = Gln, Tyr). These amino acid substitutions agree with a preliminary, partial sequence of a second hFc γ RIB gene reported recently (19), and therefore both the 1,000- and 750-bp cDNAs probably represent transcription products of a hFc γ RIB gene. These putative hFc γ RIB gene products both differ from the A gene at nucleotide positions 346 and 981 and at predicted amino acid (hFc γ RIa1) positions 115 (Fc γ RIA = Thr, Fc γ RIB = Met) and 324 (Fc γ RIA = Asp, Fc γ RIB = Asn). In addition to the presence or absence of the exon encoding EC1, which is presumably caused by alternative splicing, the two hFc γ RIB gene product sequences also differ from each other at nucleotides 440 (silent) and 462, the latter predicting an amino acid substitution at position 154 in the larger gene product.

Using primers complementary to both hFc γ RIA and hFc γ RIB, direct sequencing of 1,200-bp PCR amplified cDNA from mononuclear cells of two individuals showed no evidence of a B gene transcription product that would include the exon encoding EC3 and lead to a full-length B gene product with three extracellular immunoglobulin-like domains.

Expression of Cloned Fc γ RI Transcripts. COS-7 cells transiently transfected with pRc/CMV vector constructs containing hFc γ RIA cDNA express a surface protein recognized by Fc γ RI-specific mAbs (10.1, 22.2, 32.2, 44, 62, and 197) as measured by flow cytometry (Fig. 3 A). These cells bind both monomeric and heat-aggregated human and murine IgG (Fig. 3 C), and form rosettes with E-hIgG (Fig. 4 A). Therefore, the transfected full-length hFc γ RIA cDNA encodes a surface molecule with binding properties and epitopes matching those of the high affinity IgG receptor.

COS-7 cells transfected with plasmid containing the larger hFc γ RIB cDNA have very different properties. These cells are not recognized by any of the above Fc γ RI-specific mAbs (Fig. 3 B), nor by several mAbs specific for Fc γ RII (IV.3, 41H16) and Fc γ RIII (3G8). These cells have no appreciable binding of monomeric IgG, as measured by flow cytometry.

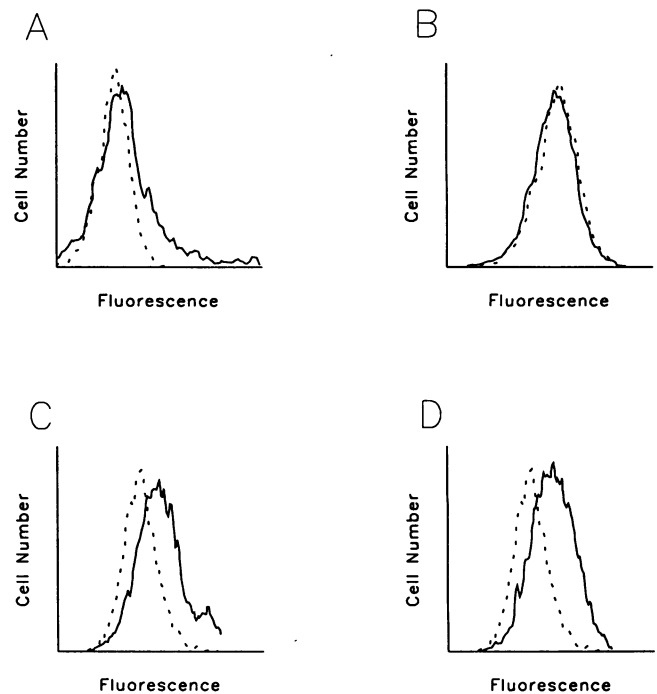


Figure 3. Binding properties of COS-7 cells transfected with hFc γ RI isoforms. (A–D) Indirect immunofluorescence of transfected cells, as measured by flow cytometry. Cells transfected with plasmid alone are shown as a dashed line. Fluorescence intensity is plotted on a logarithmic scale. (A, C) Cells transfected with plasmid containing Fc γ RIa1 cDNA are shown as a solid line. (B, D) Cells transfected with plasmid containing Fc γ RIB1 cDNA are shown as a solid line. (A, B) Staining with mAb 32.2. Results with mAbs 10.1, 22.2, 32.2, and 197 were similar. Cells transfected with hFc γ RIa1 are recognized by Fc γ RI specific monoclonal antibodies (A), but cells receiving hFc γ RIB1 cDNA were not recognized (B). (C, D) Staining with heat-aggregated human IgG. Cells transfected with either hFc γ RIa1 (C) or hFc γ RIB1 (D) bind heat-aggregated human IgG.

However, expression of the larger hFc γ RIB cDNA on COS-7 cells did result in binding of multimeric IgG. hFc γ RIB-transfected cells bind E-hIgG and reproducibly form rosettes, although with lower efficiency compared with cells transfected with Fc γ RIA (Fig. 4 B). Results with EA, using rabbit IgG, were similar. Furthermore, cells expressing the larger hFc γ RIB are able to bind heat-aggregated human IgG, as measured by flow cytometry (Fig. 3 D). Interestingly, no measurable binding of murine heat-aggregated murine IgG could be detected, suggesting that binding may show species specificity.

Regulation of message levels by IFN- γ . Since the Fc γ RIA gene mRNA level and protein product can be induced by IFN- γ , we sought evidence that transcripts for the two alternative splice products of the putative Fc γ RIB are similarly regulated by performing Northern hybridizations using a 32 P-labeled RNA probe. Unstimulated human mononuclear cells and U937 cells show low levels of at least two transcripts, one of 1,500 and one of 1,250 bp (Fig. 5). The 1,250-bp product is less prominent in human mononuclear cells, while unstimulated human neutrophils have no detectable Fc γ RI related transcripts. Treatment of all cell types with recombinant human IFN- γ markedly increases levels of Fc γ RI transcripts. In both human mononuclear cells and U937 cells, an increase in the level of both 1,500- and 1,250-bp transcripts was seen, al-

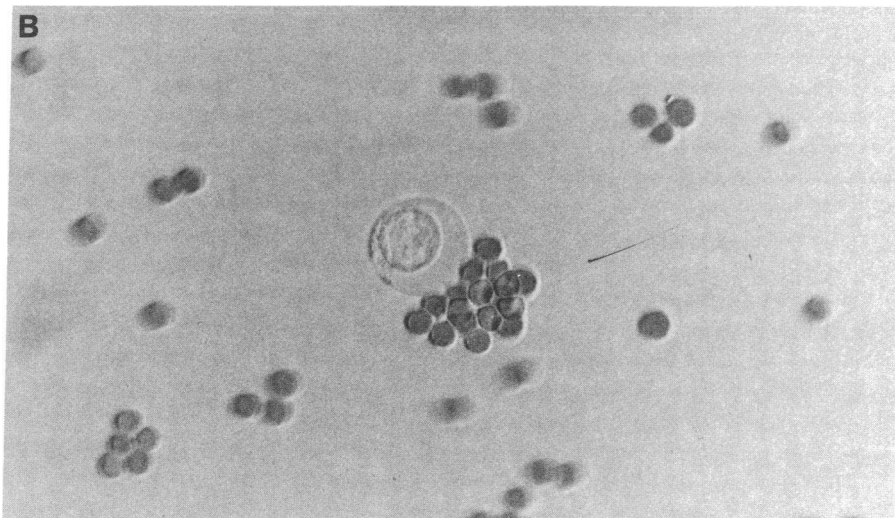
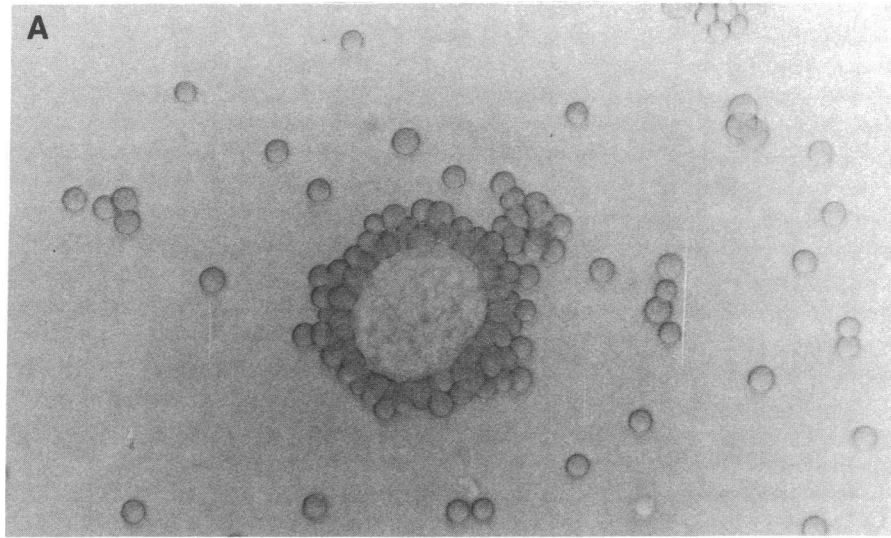


Figure 4. Rosetting patterns of transfected COS-7 cells. Erythrocytes are coated with human IgG via a biotin-avidin linkage. (A) COS-7 cells transfected with hFc γ RIa1. Rosetting of IgG coated RBCs is observed. (B) COS-7 cells transfected with hFc γ RIb1. A less vigorous rosetting pattern can be observed with transfection of the hFc γ RIb1 cDNA.

though the smaller band was less enriched by IFN- γ stimulation in mononuclear cells. In the case of U937 cells, a third 1,000-bp transcript could also be clearly identified. For IFN- γ treated neutrophils, enrichment of all three transcription products was even more pronounced.

Discussion

The role of receptors from the Fc γ RI family in modulating the immune response has been enigmatic, in part because structural diversity seen in other Fc γ R families (Fc γ RII and Fc γ RIII) has not been previously identified in the human Fc γ RI family. The ability of IFN- γ to regulate quantitative expression of the high affinity Fc γ RI implies an important role in immune regulation. However, some investigators have argued that the constitutive univalent occupation of the Fc γ RI ligand binding site precludes its participation in the handling of many immune stimuli, including immune complexes. Much of the debate about the role of the Fc γ RI family, however, is obviated by this and other (19) demonstrations of multiple transcripts in the hFc γ RI family, and by our recognition of both high and low affinity Fc γ RI cell surface receptors.

Both PCR amplification and Northern hybridizations of nonamplified cellular RNA from human mononuclear cells, the human monocyte-like cell line U937, and human neutrophils have provided evidence for at least three distinct hFc γ RI-related transcripts. Direct sequencing of the largest PCR product has demonstrated it to be identical to the full-length sequence of the Fc γ RIA gene described by van de Winkel (18). This product differs from the p135 cDNA at nucleotide positions 73 and 1,024, resulting in nonconservative amino acid substitutions at positions 25 and 338 (20). These differences in the p135 sequence may represent allelic polymorphisms or amplification artifact. It is interesting to speculate whether the single amino acid difference in EC1 of p135 might explain the lower than expected affinity for monomeric IgG of the p135 product expressed in COS cells (21).

The intermediate-sized PCR product corresponds to the 1,250-bp transcript on our Northern hybridizations of human mononuclear cells, U937 cells, and IFN- γ stimulated neutrophils, and perhaps corresponds to the second transcript band in other Northern hybridizations with hFc γ RI (p135) probes (21). This product shows several features that distinguish it from the Fc γ RIA gene product, and which indicate that it corresponds

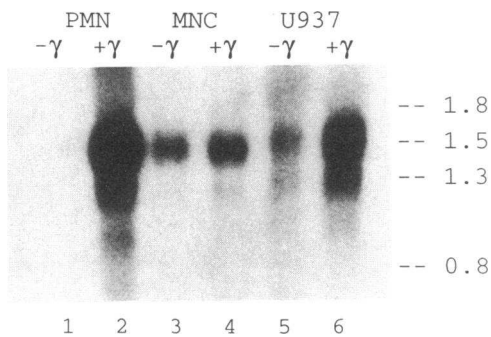


Figure 5. Northern hybridization using a hFc γ RI specific probe under high stringency. (Lanes 1, 2) Neutrophil RNA. (Lanes 3, 4) Mixed mononuclear cell RNA. (Lanes 5, 6) U937 cell RNA. (Lanes 2, 4, 6) IFN- γ (200 U/ml) added for 6 h before RNA extraction. Molecular weight standards are shown on the right. Neutrophils cultured without IFN- γ showed no Fc γ RI related transcripts, but IFN- γ inducible bands can be seen at 1,500, 1,250, and 1,000 bp. Cultured mononuclear cells show a 1,500-bp band, and a weakly IFN- γ inducible 1,000-bp band. The unstimulated monocytoid cell line U937 demonstrates 1,500 and 1,250 bp transcripts, with enhancement of 1,500, 1,250, and 1,000 bp transcripts after culture with IFN- γ .

to a product of the Fc γ RIB gene (18, 19). Two nonconservative amino acid substitutions found in this product at the end of the exon encoding EC2 are characteristic of the hFc γ RIB gene. Interestingly, this transcript, named hFc γ RIB1, lacks the entire exon encoding EC3. Since this exon in the hFc γ RIB gene contains a stop codon (19), the absence of this exon at the message levels allows the translation of a mature protein with both a transmembrane and a cytoplasmic domain. This protein can be expressed in COS-7 cells. Much like the murine Fc γ RI chimera, which contained EC1 and EC2 but not EC3 and had the properties of a low affinity receptor (12), hFc γ RIB1 does not bind monomeric IgG but does have the capacity to bind human IgG aggregates.

The transcript on Northern hybridization corresponding to the smallest PCR product was not easily visualized in resting mononuclear and U937 cells, but like the two larger products it was IFN- γ inducible, especially in human neutrophils. Sequence analysis showed that this product has nucleotide substitutions matching the Fc γ RIB gene (18, 19), but unlike the Fc γ RIB1 product, this transcript (Fc γ RIB2) lacks both EC1 and EC3. Clones of hFc γ RIB2 also have two nucleotide (and one amino acid) substitutions relative to hFc γ RIB1 clones. While both of these transcripts probably arise through alternative splicing of hFc γ RIB mRNA, we cannot exclude the possibility that the "hFc γ RIB2" transcript results from a distinct ("hFc γ RIC") gene. Alternatively, the two nucleotide substitutions may represent allelic polymorphisms, or perhaps cloning artifact. The properties of the expressed hFc γ RIB2 protein are currently unexplored.

COS-7 cells transfected with hFc γ RIB1 cDNA confirmed that the encoded protein has binding properties analogous to other low affinity receptors for human IgG. hFc γ RIB1-transfected COS-7 cells formed rosettes with antibody-coated erythrocytes, although more weakly than cells expressing hFc γ RIa1. hFc γ RIB1-transfected COS-7 cells were unable to bind monomeric IgG, but were able to bind heat-aggregated human IgG. Thus, hFc γ RIB1 has the capacity to recognize immune com-

plexes. The hFc γ RI family is therefore unique in having both high and low affinity members.

The identification of hFc γ RIB1 and hFc γ RIB2 demonstrates that the hFc γ RI family has significant structural diversity, both at the genomic and mRNA levels. The Fc γ RII and Fc γ RIII families also demonstrate extensive structural diversity. Results from Northern hybridization confirm that each of these hFc γ RI transcripts is IFN- γ inducible. Given the distinct cytoplasmic domain of the Fc γ RI family members, one can anticipate unique roles for hFc γ RIB1, hFc γ RIB2, and probably other unrecognized members of the hFc γ RI family in the humoral immune response.

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