Expression Cloning of a Human Fc Receptor for IgA
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Summary
IgA, the predominant isotype in secretions, mediates the neutralization and removal of environmental antigens from mucosal sites. Although cell surface receptors for the Fc region of IgA (FccαR) have been implicated in a variety of immune effector mechanisms, the molecular features of FccαR remain only marginally characterized. In this report, we describe the isolation of a clone from a myeloid cell line cDNA library that directs the expression of a cell surface molecule with IgA binding specificity. The cDNA encodes a peptide of Mr 30,000 including a putative transmembrane region with features atypical of conventional membrane-anchored proteins. Databank searches indicate that the human myeloid cell FccαR sequence is unique, is a member of the immunoglobulin gene superfamily, and is related to Fc receptors for IgG (FcγRI, II, and III) and IgE (FcεRI).

Materials and Methods

Cells.

The monocyte-like cell line U937 (15) was maintained in continuous culture in RPMI 1640 supplemented with 10% FCS. Differentiation was induced by culturing the cells overnight in the presence of 5 ng/ml of PMA (Sigma Chemical Co., St. Louis, MO). Monocytes and polymorphonuclear leukocytes were purified from heparinized peripheral blood by centrifugation through isoplymph (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY), followed by centrifugal elutriation, using a centrifuge (6 M/E; Beckman Instruments, Inc., Fullerton, CA) equipped with a JE-5.0 rotor. The resulting fractions were analyzed by Wright-Giemsa staining of cytocentrifuge preparations.

Human T lymphocytes were purified from tonsils by E-rosetting with 2-aminoethylisothio-uronium bromide (AET)-treated SRBC, followed by Ficoll-Hypaque density gradient centrifugation (16). Tonsillar B lymphocytes were isolated from the leukocyte layer resulting from AET-SRBC and Ficoll separation, with further depletion of non-B cells achieved by treatment with antibody and complement using Lympho-Kwik B (One Lambda, Los Angeles, CA). The purified preparations of T and B cells were maintained in a medium consisting of RPMI 1640/10% FCS (HyClone Laboratories, Logan, UT), sodium pyruvate (1 mM), nonessential amino acids (100 mM), penicillin/streptomycin (100 U/ml per 100 μg/ml), t-glutamine (2 mM), 2-ME (50 mM), and fungizone (1:2,000; Gibco Laboratories, Grand Island, NY). B cells or T cells were cultured either alone or, as indicated, in the presence of Con A (20 μg/ml; Sigma Chemical Co.), PWM (40 μg/ml; Sigma Chemical Co.), or PMA (5 ng/ml) plus ionomycin (500 ng/ml; Calbiochem-Behring Corp., San Diego, CA). After a 16-h incubation, cells were harvested for RNA preparation as described below.

cDNA Expression and Immunoselection.

The cDNA was synthesized from polyadenylated RNA extracted from 12-b PMA-stimulated U937 cells according to standard protocols (17). The cDNA was transferred to a standard vector, and the recombinant plasmids were transfected into programmed myeloid cell lines. The immortalized cell line, U937, was used for expression cloning. As described below, cDNA was synthesized and used to isolate a clone from the human myeloid cell cDNA library.
ligated into pDC303, a modified version of the pDC302 mammalian expression plasmid (18), using nonself-complementary linkers containing BglII restriction sites.

The cDNA library representing 600,000 independent clones was amplified by growing bacteria in liquid cultures in the presence of chloramphenicol overnight, then plating at a density of 5,000 colonies per plate. Colonies were scraped from plates and pooled, and plasmid DNA was recovered and purified by alkaline lysis and CsCl gradient centrifugation (19). Plasmid DNA from the cDNA library was used to transfect a subconfluent layer of monkey COS-7 cells using DEAE dextran followed by chloroquine treatment, as described (20). FcRα/β cells were selected by incubation in the My43 anti-FcRα mAb (14), followed by panning on anti-IgM-coated plates according to established procedures (21). Episomal DNA was prepared from the panned cells, amplified, and reintroduced into COS cells. After three such rounds of transfection (two DEAE dextran rounds and one protoplast fusion round) and immunoselection, 96 individual plasmid-transformed bacterial colonies were grown, collected into 20 pools, and pooled plasmids were extracted by alkaline lysis (19) and transfected into COS cells. Positive pools were selected by flow cytometric analysis of anti-FcRα/β (My43) binding to transfected cells. Individual clones containing FcRα/β cDNA were isolated from positive pools. One of these, pHuFccRα/β, was selected for characterization.

Sequence Analysis. DNA sequencing was performed on both strands as previously described (22). For alignment analysis, residues constituting the two extracellular Ig domains were aligned with other FcR Ig domains using computerized models (NBRF program ALIGN[23]) and by eye. The MD data matrix was used with a bias of +6, and a gap penalty of 6 was used. Scores are the SD from the indicated paired alignment sequences vs. the average scores from 100 randomized alignments of the same pair of sequences.

Immunoglobulins. The My43 anti-FcRα/β murine hybridoma cell line (IgM, λ) was maintained in Dulbecco's medium containing 10% FCS. Supernatants were collected at twice-weekly intervals, sterile filtered, and stored at 4°C. Human IgA1 and IgG1 paraproteins were purified as previously described (14). F(ab')2 anti-ox erythrocytes (OE) was prepared from rabbit anti-ox IgG (Cooper-Biomedical, Inc., Malvern, PA) by pepsin cleavage and passage through protein A-Sepharose CL-6B.

Flow Cytometry. For anti-FcR binding experiments, cells were incubated with hybridoma culture supernatants containing either My43 mAb or a nonselective isotype control mAb. After 30 min at 4°C, cells were washed and incubated with fluoresceinated second antibody (affinity-purified goat anti-murine IgM, μ chain specific [F(ab')2]) for 30 min at 4°C, then washed. For IgA binding experiments, cells were stained with 12.5 μg/ml FITC-conjugated human myeloma IgA1 or IgG1 (30 min at 4°C). All antibody dilutions and washes were performed in PBS/0.1% BSA/0.05% sodium azide. Cells were analyzed on a FACSscan flow cytometer using a logarithmic fluorescence intensity scale.

Rosette Assay. Conjugates of IgA or IgG linked to anti-OE were made by using the bifunctional reagent SPD (Pharmacia Fine Chemicals, Uppsala, Sweden) (24). IgA or IgG and F(ab')2 anti-OE (at 1–3 mg/ml) were treated separately with an eightfold molar excess of SPD for 2 h at 48°C. SPD-treated anti-OE F(ab')2 was dialyzed in PBS, pH 7.2, and SPD-treated IgA and IgG were dialyzed in 0.1 M acetate, 0.1 M NaCl, pH 4.5, treated with 0.02 M dithiothreitol (30 min), and passed through a G-25 Sephadex column (Pharmacia Fine Chemicals), equilibrated in 0.1 M phosphate, 0.1 M NaCl, pH 7.5. Equimolar amounts of the anti-OE F(ab')2 and IgA or IgG were then mixed and incubated at 18°C for 4 h, after which crosslinking was terminated with 2 mM iodoacetamide. Preparations contained <15% noncrosslinked Ig.

Packed OE (10 μl) were mixed for 16 h at 10°C with 25 μl of the F(ab')2 conjugates at concentrations previously determined to give maximal rosette formation. Equal volumes of cells (transfected COS-7 or U937 cells) at 2 x 10^6/ml and a 1% suspension of heteroantibody-coated OE were centrifuged together and incubated on ice for 90 min. In some cases, cells were preincubated on ice with human IgA, IgG, My43, or control IgM mAb before the addition of OE. After gentle resuspension, the percentage of rosette-forming cells, defined as cells binding greater than four OE, was determined by counting a minimum of 200 cells in duplicate samples using light microscopy.

Hybridization Techniques. Total RNA was isolated from cells as described (25), then subjected to oligo(dt)-cellulose chromatography to purify polyadenylated RNA (19). The mRNA was subjected to electrophoresis in a formaldehyde/12% agarose gel, blotted by capillary flow onto a Nytran filter (Schleicher & Schuell, Inc., Keene, NH) (26), and hybridized for 16 h at 63°C in Starks hybridization solution (50% formamide, 2x Denhardt's solution, 5x SSC, 0.1% SDS, 50 mM KH2PO4, 20 mM EDTA, 0.05% N-lauroylsarcosine, 150 μg sheared, denatured salmon sperm DNA per ml) containing 32P-labeled antisense riboprobe transcribed from the 5' portion (~430 bases) of pHuFccRα/β. Washes were carried out in 2x SSC, 0.1% SDS at 63°C. Dried filters were exposed to Kodak X-Omat AR film for 2 d and developed.

Human placental genomic DNA was isolated as described (27) and digested to completion with the indicated restriction endonucleases. 8 μg of digested DNA was subjected to electrophoresis on a 0.7% agarose gel, transferred to a Nytran filter, and hybridized for 16 h at 42°C in Starks solution containing a 32P-labeled 700-bp fragment encoding the 5' untranslated and extracellular coding region of FcRα/β. Hybridized blots were washed in 0.2x SSC, 0.1% SDS at 65°C, dried, and autoradiographed.

Results and Discussion

The My43 mouse mAb binds selectively to a cell surface receptor for IgA on human myeloid cells. We used this mAb as the basis for immunoselection of an FcRα/β cDNA clone, using a modification of the technique previously described by Seed and Aruffo (21). The U937 cell line represented a convenient source of mRNA for cDNA library construction, since FcRα/β expression as measured by My43 binding is inducible by PMA treatment. We found that maximal FcRα/β cell surface expression occurred after 24 h in the presence of PMA, suggesting that maximal mRNA levels are achieved at an earlier time point. Thus, polyadenylated RNA was extracted from U937 cells treated for 12 h with PMA, and a cDNA library was prepared in the pDC303 mammalian expression plasmid.

Purified plasmid DNA from the U937 cDNA library was transfected into COS-7 cells, FcRα/β-expressing cells were isolated by panning with the My43 mAb, and plasmid DNA was extracted from the immunoselected COS-7 cells. After additional rounds of transfection and immunoselection, an individual FcRα/β cDNA clone was selected by flow cytometric analysis of transfected COS cells using the My43 mAb. As shown in Fig. 1 A, My43 bound to a subpopulation of COS cells transfected with pHuFccRα/β, but failed to react with cells transfected with an insertless p125mAb. Besides expressing a cell surface epitope recognized by
My43, these cells were also capable of binding IgA. Thus, FcεR-transfected COS cells directly bound FITC-conjugated human IgA (Fig. 1 C), but not human IgG-FITC (Fig. 1 D). In contrast, control COS cells displayed only background binding of either Ig isotype. The receptor exhibited the expected binding specificity, since IgA-FITC binding was inhibited by unconjugated human IgA and by My43, but not by human IgG or an irrelevant murine IgM mAb (not shown).

FcεR+ COS cells were treated for 30 min at 4°C with hybridoma supernatants or purified Ig, or with medium alone. The cells were then examined for ability to form rosettes with OE coated with human myeloma IgA, as described in Materials and Methods. Results show mean and SD of duplicates.

FcεR expression was also measured by the ability of transfected cells to form rosettes with IgA-coated OE (Fig. 2). COS cells transfected with pHuFcεR formed distinct rosettes when incubated in the presence of human IgA-coated OE (A-D) or human IgG-OE (E), and microscopically analyzed for rosette formation. In some cases, transfected cells were incubated with "blocking" antibodies before addition of OE: (B) My43; (C) HuIgA; (D) HuIgG. In F, COS cells transfected with control plasmid were incubated with IgA-OE.

| Table 1. Inhibition of FcεR+ COS Cell IgA Rosette Formation |
|-----------------------------------------------|-----------------|-------------------|
| Antibody treatment | Concentration | % Rosette-forming cells |
|-----------------------------------------------|-----------------|-------------------|
| Medium | - | 10.6 (0.9) |
| My43 hybridoma supernatant | Undiluted | 1.5 (1) |
| 1:3 | 0.75 (0.75) |
| 1:9 | 8.3 (0.25) |
| 1:27 | 11.5 (0.15) |
| Control hybridoma supernatant | Undiluted | 11.4 (1) |
| Human IgA | 2 mg/ml | 0 |
| 666 μg/ml | 0.9 |
| 222 μg/ml | 6.2 (0.3) |
| 74 μg/ml | 9.3 (1) |
| Human IgG | 2 mg/ml | 9.7 (0.05) |
Figure 3. Human FceR cDNA. (A) Restriction map and schematic representation of FceR. Restrictionsites are indicated for the enzymes Bg III (B), Cl I (C), Hind III (H), and Pst I (P). The boxed coding region depicts the predicted domain structure, including signal sequence (stippled), extracellular (crosshatched), transmembrane (black), and intracellular (white) domains. (B) Human FceR nucleotide and predicted amino acid sequences. Nucleotides are numbered beginning at 5' terminus, and amino acids are numbered beginning with the predicted NH2 terminus (Gln 22) of mature FceR, which is marked with an arrowhead. Cysteine residues are boxed and potential N-linked glycosylation sites are marked with an asterisk. These sequence data are available from EMBL/GenBank/DDBJ under accession number X54150.

OE (Fig. 2 A), whereas control COS cells did not (Fig. 2 F). This activity was completely inhibited by the My43 mAb (Fig. 2 B) and human IgA (Fig. 2 C), but not by human IgG (Fig. 2 D). In a separate experiment, the inhibitory effects of My43 and human IgA were found to be dose dependent (Table 1). As a further indication of target specificity, the FceR-transfected COS cells failed to form rosettes with human IgG-coated OE (Fig. 2 E and Table 1). Thus, the recombinant receptor is capable of initiating the first step in IgA-mediated immune effector functions, i.e., specific binding of IgA-coated targets.

The structural features of the 1.6-kb cDNA insert in pHuFceR were determined by restriction endonuclease mapping (Fig. 3 A) and nucleotide sequence analysis (Fig. 3 B). The pHuFceR cDNA is composed of a 39-bp 5' untranslated region, a 861-bp open reading frame, and a 711-bp 3' untranslated region terminating in a poly-A tract. The 3' region also includes an Alu-sequence (1020-1296) (reference
28) and AT-rich, but lacks a polyadenylation signal. The open reading frame encodes a protein of 287 amino acids, the first 21 amino acids of which exhibit characteristics of a hydrophobic signal sequence (29). Thus, the predicted mature receptor sequence would begin at Gln22, resulting in a peptide of predicted Mi, \( \approx 29,900 \), a value that is consistent with the reported molecular mass (32–36 kD; reference 13) of deglycosylated U937 FcaR. The 206-amino-acid extracellular domain includes six potential sites for N-linked glycosylation, a feature that along with O-glycosylation could account for the significantly higher molecular mass, \( \approx 60 \) kD, observed for native FcaR.

When the amino acid sequence for the extracellular region was compared with existing protein sequence databases using the FASTA algorithm (30), homology was found with the other IgFcRs sequenced to date. Further analysis of the FcR sequences using the NBRF program ALIGN (23) revealed statistically significant alignments between the FcIIR and other members of this receptor family. Most notably, the FcIIR appeared, as do the other FcRs, to contain several conserved residues that are the hallmarks of Ig-like sequences (31) in two contiguous domains. These residues, including the two cysteines in each domain that form the disulfide bond that holds together the Ig fold (31), are indicated in an align-

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Table 2. Alignment Scores and Percent Sequence Identity for the Ig Fc Receptor Family

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|---------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Sequence | FcIIR | FcIIR | FcIIR | FcIIR | FcIIR |
|----------|-------|-------|-------|-------|-------|
| FcIIR    | EC1   | EC2   | EC1   | EC2   | EC1   | EC2   | EC1   | EC2   |
| EC1      | 7.0   | 3.4   | 4.1   | 4.5   | 5.5   | 4.6   | 3.6   | 2.4   | 3.1   |
| EC2      | 23.2  | –     | 4.8   | 5.2   | 5.1   | 4.2   | 4.0   | 3.5   | 3.6   | 2.6   |
| FcIIR    | 22.4  | 24.7  | –     | 4.8   | 15.9  | 5.2   | 14.6  | 4.6   | 15.3  | 3.2   |
| EC1      | 22.8  | 21.7  | 25.9  | –     | 4.9   | 4.2   | 5.3   | 3.1   | 5.3   | 4.6   |
| EC2      | 24.1  | 25.3  | 49.4  | 26.5  | –     | 6.0   | 17.2  | 5.6   | 13.0  | 3.4   |
| FcIIR    | 21.0  | 28.2  | 28.2  | 18.8  | 24.1  | –     | 4.7   | 17.1  | 3.9   | 13.7  |
| EC1      | 28.2  | 21.2  | 49.4  | 28.2  | 55.4  | 21.2  | –     | 4.5   | 12.3  | 3.1   |
| EC2      | 18.2  | 20.5  | 27.1  | 18.2  | 25.3  | 45.9  | 22.4  | –     | 4.3   | 13.1  |
| FcIIR    | 17.6  | 24.7  | 38.8  | 27.1  | 43.4  | 23.5  | 43.5  | 25.9  | –     | 3.3   |
| EC1      | 19.6  | 25.0  | 22.4  | 20.7  | 24.1  | 36.6  | 25.9  | 42.0  | 28.2  | –     |

Scores are the SD for the indicated pair of aligned sequences (top) vs. the average scores from 100 randomized alignments of the same pair of sequences (bottom).

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Figure 4. Alignment of FccR Ig domains. Bold residues indicate conserved residues typical of Ig family members; boxed residues indicate FccR domain residues common with five other FcR domains in this alignment; numbers indicate location of residues in the FccR sequence. EC1 and EC2 denote first and second extracellular domains of the indicated FcR protein.
ment of the FcR sequences in Fig. 4. Given the conservation of Ig domain structure, it is likely that Cys 28 is disulfide bonded to Cys 79 in domain 1, and Cys 125 and Cys 172 are paired in domain 2 (shown as boxed residues in Fig. 4). The resulting ALIGN scores from the alignment are shown in Table 2. It is clear from these alignments that although the FcαR is homologous to the other known FcRs and is more closely related to those proteins than to several other members of the Ig superfamily, it is somewhat more distantly related to other FcRs than are these receptors to each other. Thus, it is likely that the FcαR diverged from a common ancestor very early in the development of the Ig FcR gene family. The existence of a distinct family of FcRs is supported by previous work demonstrating that the genes for FcγRI, FcγRII, FcγRIII, and the α chain of FcεRI map to the long arm of chromosome 1 (32, 33). We are currently investigating the possibility that the FcαR, which shares structural and functional qualities with these other FcRs, maps to the same chromosomal region.

Hydropathicity analysis indicated that the extracellular region of the FcαR is followed by a 19-residue stretch of hydrophobic amino acids (Leu 207 to Val 225) corresponding to a potential transmembrane region. The intracytoplasmic region would thus be composed of 41 amino acids. Two features of the putative transmembrane region are atypical of "protein anchored" transmembrane proteins, including the presence of a charged residue (Arg 230) within the hydrophobic transmembrane domain and the lack of a cluster of basic residues immediately following the hydrophobic stretch (34). A number of proteins containing a charged residue in the transmembrane region have been reported to attach to the membrane either through a glycosyl-phosphatidylinositol (GPI) linkage (e.g., the Qa-2 MHC antigen; reference 35) or by association with another membrane-bound protein (e.g., the TCR, which requires association with the CD3 complex for cell surface expression; reference 36). With regard to Fc receptors, the α chain of the high affinity IgE receptor (FcεRI) contains an Asp residue in the transmem-
brane region and is attached by the latter mechanism, i.e., through interaction with the γ chain (37). The two genes for human FcγRIII also include an Asp residue in the middle of the membrane-spanning region (38). The FcγRIII isoform expressed by PMNs is GPI linked (38, 39), while the isoform expressed by NK cells and macrophages appears to require an additional peptide for cell surface expression, a function that can be provided by the γ chain of the FcεRI (40, 41). The critical difference between these two isoforms appears to reside in the presence or absence of a Ser 203 residue in the extracellular region, which signals GPI attachment (40-42). Although there is a Ser residue in an analogous site of the FcαR (Ser 197), it is unlikely that FcαR is GPI linked, since Monteiro et al. (13) have shown that FcαR on human monocytes, granulocytes, and U937 cells are resistant to cleavage with GPI-specific phospholipase C (PLC). We have made similar observations with these cell populations, and also found that the level of expression of FcαR on transfected COS cells was unaffected by PLC treatment. The presence of an associated protein cannot be ruled out, although results with transfected COS cells would require such a protein to be present in both myeloid cells and COS-7 cells. Clearly, additional studies are required to determine the nature of FcαR attachment and the possible relevance of the different forms of membrane attachment of Fc receptor function.

Using an RNA probe representing the 5' untranslated region and coding sequence for a portion of the extracellular region, we analyzed expression of FcεRI mRNA (Fig. 5). Two major hybridizing transcripts of ∼2.8 and ∼2.6 kb were observed in polyadenylated RNA preparations from U937 cells, peripheral blood monocytes, and PMN. PMA-stimulated U937 cells expressed significantly higher FcεRI message levels in comparison with unstimulated U937 cells, which is consistent with the finding that U937 cell surface FcεRI protein expression is PMA inducible (13). Subpopulations of human B and T lymphocytes have been reported to express cell surface FcεRI (43–45). Except for nonspecific binding to ribosomal RNA, the FcαR probe failed to hybridize with polyadenylated RNA from either untreated or mitogen-stimulated tonsillar B or T cells (Fig. 5, lanes 5–9), even after extended exposure of blots. This finding would suggest either that the receptor on these cells is structurally distinct or that the conditions used failed to induce receptor expression.

Human genomic DNA was digested with five different restriction endonucleases and subjected to Southern blot analysis, using as probe a 32P-labeled cDNA fragment representing the 5' untranslated and extracellular regions of the FcαR cDNA (Fig. 6). Single or double hybridizing bands were observed for each restriction digest, suggesting that the human myeloid FcαR gene exists as a single copy.

In conclusion, we have isolated a cDNA clone encoding a cell surface protein that displays IgA binding properties and that is structurally similar to FcRs for other isotypes. The availability of this cDNA will facilitate investigations into such issues as genomic organization, regulation of gene expression, and structure/function relationships. Furthermore, a plasmid that directs the synthesis of recombinant soluble FcαR was recently constructed (Maliszewski, C., unpublished results). Clearly, such a reagent will be valuable in dissecting the role of FcαR in IgA-mediated effector functions and in immunoregulation of IgA synthesis.

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