Alternative splicing of the human IgA Fc receptor CD89 in neutrophils and eosinophils

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Receptors for the Fc portion of IgA (FcαR) trigger important immunological elimination processes against IgA-coated targets. Investigation of human FcαR (CD89) transcripts in neutrophils, eosinophils and a monocyte-like cell line, THP-1, with the use of reverse transcriptase PCR, Northern blotting and RNase protection analysis, has provided evidence in these cell types for at least two distinct transcripts generated by alternative splicing. The cDNAs derived from the two major transcripts of both neutrophils and eosinophils have been cloned and sequenced. For both cell types, the larger clone represents the previously described full-length receptor, whereas the second, shorter, splice variant lacks the entire second, membrane-proximal, Ig-like domain. Stable CHO-K1 transfectants have been obtained for both full-length and truncated variant neutrophil receptors. Whereas the full-length receptor is recognized by a panel of five anti-FcαR monoclonal antibodies (mAbs), the shorter variant is bound weakly by only two of the antibodies, suggesting that the epitopes recognized by the majority of the mAbs lie at least in part in the second Ig-like domain of FcαR. Both full-length and splice variant forms of the receptor bind secretory IgA, but the weak binding to serum IgA seen with the full-length receptor is not evident with the shorter variant. Alternative splicing might therefore serve as a means of diversifying FcαR structure and function.

INTRODUCTION

Human Fcα receptors (FcαR) are present on a number of cell types, including neutrophils, monocytes, macrophages and eosinophils. The interaction of FcαR with aggregated IgA, such as IgA coated on the surface of invading micro-organisms, mediates several important immunological defence processes, including phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and stimulation of the release of superoxide and inflammatory mediators [1,2]. FcαR on neutrophils and monocytes has been characterized as a glycoprotein of 50–70 kDa, showing considerable size diversity on different cell types [3–5].

A myeloid FcαR (CD89) cDNA clone has been isolated and sequenced [6], which encodes a membrane-spanning 30 kDa peptide with six potential sites for N-linked glycosylation. It has two extracellular Ig-like domains and displays homology with the ligand-binding chains of IgG Fc receptors (FcγR) and the high-affinity receptor for IgE (FcϵRI). The FcαR on eosinophils seems to represent a glycoform of this receptor [7]. Northern blot analysis, with an FcαR probe representing the 5′ untranslated region and coding sequence for part of the extracellular region, has demonstrated two major transcripts of approx. 2.8 and 2.6 kb in polyadenylated RNA from monocytes and neutrophils, but no transcripts were evident in tonsillar B or T lymphocytes. No further analysis of the transcripts was reported. Southern blot analysis has suggested that the human myeloid FcαR gene exists as a single copy [6,8]. Recently the myeloid FcαR gene has been isolated and shown to consist of five exons spanning approx. 12 kb [8]. The first two exons, S1 and S2, encode the leader sequence, the third (EC1) and fourth (EC2) exons each encode a homologous Ig-like domain, and the final exon (TM/C) encodes a short extracellular region, a transmembrane segment and a short cytoplasmic tail.

It has been established that for the human IgG Fc receptors, FcγRI and FcγRII, alternative splicing results in the expression of several closely related but functionally distinct receptor variants, which in some cases lack whole Ig-like extracellular domains [9,10]. A preliminary report has been made of alternative splicing of FcαR resulting in deletion of the 36 nt second leader sequence exon, but the function of this splice form is unknown [8]. In the present study, we sought to determine whether, as for FcγR, alternative splicing might serve as a means of diversifying FcαR structure and function through deletion of entire domains. We investigated human FcαR expression in neutrophils, eosinophils and a monocytic-like cell line THP-1. Using Northern blot and RNAse protection analysis, we found evidence for the existence of at least two distinct transcripts generated by alternative splicing, the products of which have been assessed for functional differences.

EXPERIMENTAL

Materials

RAMOS cells derived from a human Burkitt's lymphoma, obtained from the European Collection of Cell Cultures (ECACC no. 85030802), and control oligonucleotides for prohibitin, were provided by Dr. P. Coates (University of Dundee, Dundee, Scotland). Cell culture components were purchased from Life Technologies (Paisley, Scotland). Oligonucleotides as follows were synthesized by the Department of Biochemistry Oligonucleotide Laboratory (University of Dundee): 5′-GCGCGCG-

Abbreviations used: FcαR, receptor for the Fc region of IgA; FcϵRI, high-affinity receptor for IgE; FcγR, receptor for the Fc region of IgG; FITC, fluorescein isothiocyanate; HBSS, Hanks balanced salt solution; mAbs, monoclonal antibodies; RT, reverse transcriptase.

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C**GAATTC**GCCGCCACCATGGACCCCAAACAGACC-3′ (primer RP1) incorporating an underlined portion complementary to nt 40–57, a Kosak sequence, and an *Eco*RI site in bold; 5′-CCGGCCGCGCG**CTCGAG**CTATTACTTGCAGACACTT-GGTGT-3′ (primer RP2) incorporating an underlined portion complementary to nt 883–903 and a *Xho*I site in bold; and 5′-GACCACATGGACGCA-3′ (primer RP3) complementary to nt 301–315. Nucleotides are numbered in accordance with the FcαR cDNA previously described [6]. The plasmid pHuIgAR containing the original FcαR cDNA clone [6] was a gift from Dr. C. Maliszewski (Immunex Corporation, Seattle, WA, U.S.A.).

Antibodies

Pooled human IgG was prepared from serum by standard methods by using ammonium sulphate precipitation and ion-exchange and gel-filtration chromatography [11]. Human serum IgA1 was similarly purified from normal serum by using modifications of these techniques, followed by affinity chromatography on Jacalin-Sepharose (Vector Laboratories, Burlingame, CA, U.S.A.) [12]. Dimeric secretory IgA was a gift from Dr. M. Glennie (Tenovus Research Laboratory, Southampton, Hants., U.K.). Monoclonal antibodies (mAbs) 3G8, A77, My43 and three other anti-CD89 antibodies (A3, A62 and A59) were gifts from Professor J. Unkeless (Mount Sinai School of Medicine, New York, NY, U.S.A.), Dr. J. G. J. van de Winkel (Utrecht University, Utrecht, The Netherlands), Dr. L. Shen (Dartmouth Medical School, Lebanon, NH, U.S.A.) and Professor M. D. Cooper (University of Alabama at Birmingham, AL, U.S.A.) respectively.

Cell isolation

Neutrophils were isolated from heparinized blood taken from healthy volunteers by sedimentation of erythrocytes in 6 % (w/v) dextran T70 (Pharmacia Biotech, St. Albans, Herts., U.K.) at 37 °C for 30 min, followed by leucocyte separation on a discontinuous density gradient of Lymphoprep ($\rho = 1.077$ g/cm^3; Nycomed, Birmingham) over Ficoll-Hypaque ($\rho = 1.119$ g/cm^3), centrifuged at 700 ***g*** for 20 min at room temperature. Eosinophils were isolated by dextran sedimentation as above, followed by discontinuous Percoll (Pharmacia) gradient sedimentation [13] and negative indirect selection to remove contaminating neutrophils. Briefly, 1 vol. of anti-FcγRIII mAb 3G8 supernatant was mixed with 19 vol. of magnetic polymer beads precoated with sheep anti-mouse IgG (Dynal, New Ferry, Wirral, U.K.) and incubated overnight at 4 °C on a rotary mixer. The beads were then washed three times in Hanks balanced salt solution (HBSS) containing 0.1 % gelatin and retrieved magnetically. Eosinophil-enriched cell fractions were washed twice in HBSS and re-suspended in HBSS/0.1 % gelatin to which 3G8-coated beads were added (at a bead-to-neutrophil ratio of 1 : 1), and the mixture was incubated for 30 min at 4 °C on a rotary mixer. Beads with attached neutrophils were removed by multiple rounds of magnetic extraction until the eosinophils were 98–99 % pure as judged by Eosin/thiazine (Diff-Quik; Baxter Diagnostics, Dudingen, Switzerland) staining. Lymphocytes from buffy coat preparations were depleted of monocytes by adherence to plastic Petri dishes at 37 °C for 1 h [14].

Cell culture

The promyelomonocytic cell line THP-1 (European Collection of Cell Cultures ECACC no. 88081201) was maintained in RPMI-1640 supplemented with 2 mM glutamine, 20 μM 2-mercaptoethanol, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10 % (v/v) fetal calf serum at 37 °C in a humidified 5 % CO_2/air incubator. CHO-K1 cells were maintained as described previously [15].

RNA extraction, reverse transcriptase PCR (RT–PCR), cloning and sequencing

Total RNA was extracted from 5×10^7 neutrophils, THP-1 cells, RAMOS cells or untransfected CHO-K1 cells, or from 10^6 eosinophils, in a guanidium thiocyanate/phenol/chloroform single-step extraction with an RNA Isolation Kit (Stratagene, Cambridge, Cambs., U.K.). Before use, the integrity of the RNA was checked by electrophoresis on Mops/formaldehyde/1.4 % (w/v) agarose gels [16]. cDNA was synthesized with either 2 μg (for eosinophils) or 10 μg (for other cells) of total RNA, 300 ng of oligo(dT) primer, 4 mM dNTPs and 20 units of Moloney murine leukaemia virus reverse transcriptase (1 unit catalyses the incorporation of 1 nmol of dNTP into product in 10 min) with a Stratascript RT–PCR Kit (Stratagene). PCR reactions were performed by mixing 10 μl of cDNA, 10 μl of 10 × *Taq* DNA polymerase buffer (Promega, Southampton, Hants., U.K.), 1 μl of 100 mM dNTPs, 10 pmoles of each primer, 5 units of *Taq* DNA polymerase (Promega) (1 unit catalyses the incorporation of 10 nmol of dNTP into product in 30 min) and sterile water to 100 μl. Samples were denatured for 5 min at 94 °C before 30–35 cycles of amplification in a thermal cycler with a step programme of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. Individual bands generated by PCR with primers RP1 and RP2 and neutrophil cDNA were purified from Tris/acetate/EDTA gels by using the Geneclean II extraction kit (Anachem, Luton, Beds., U.K.) and subcloned into the unique *Eco*RI and *Xho*I sites in pcDNA3 (Invitrogen, Leek, The Netherlands) to produce plasmids pRP7, encoding the full-length receptor (FcαRIa1), and pRP8, encoding a splice variant (FcαRIa2). Amplified bands similarly generated from eosinophil cDNA were also subcloned in this way. Sequencing of the double-stranded vector templates was performed by the dideoxy chain-termination method [17] using SP6 and T7 oligonucleotides (Promega), complementary to sites flanking the multiple cloning site, and an internal sense primer, RP3.

Northern blot analysis and RNase protection assay

Total RNA (10 μg) was subjected to electrophoresis on 1.4 % (w/v) agarose gels and transferred to nitrocellulose membranes (Hybond-C; Amersham, Bucks., U.K.) by capillary action overnight in 20 × SSPE (3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7). The filters were baked at 80 °C for 2 h and hybridized by standard methods [16] to a 900 bp single-stranded anti-sense full-length FcαR riboprobe [specific radioactivity $(1–3) \times 10^8$ c.p.m./μg] generated by a Riboprobe System (Promega) by using SP6 RNA polymerase and [α-^{32}P]CTP (approx. 3000 Ci/mmol; Amersham, Little Chalfont, Bucks., U.K.) with *Eco*RI-linearized pRP7 as template. RNase protection assays were performed with an RNase Protection Assay System (Promega). Briefly, full-length FcαR probe (5×10^5 c.p.m.) was incubated with 10 μg of total RNA in 30 μl of hybridization buffer [40 mM Pipes, pH 6.4, 0.2 M sodium acetate, 1 mM EDTA, 80 % (v/v) formamide] for 16 h at 50 °C. Unhybridized RNA was digested by dilution of the reaction mix in 270 μl of 10 mM Tris/HCl (pH 7.6)/5 mM EDTA/200 mM sodium acetate followed by treatment with 10 units of RNase One® (1 unit is the amount of enzyme required to completely degrade RNA at the rate of 100 ng per s) at 25 °C for 1 h. The sizes of protected

fragments were determined on a denaturing polyacrylamide gel [6 % (w/v) acrylamide/7 M urea] alongside riboprobe markers of 2566, 900, 720, 600, 432 and 373 bp, each generated by appropriate restriction-enzyme digestion of pRP7 and pRP8 templates before synthesis with the Riboprobe system. Gels were dried and exposed to X-ray film with intensifying screens at −70 °C.

CHO-K1 transfection

Transfections were carried out by calcium phosphate precipitation as described previously [15], and positive transfectants were selected in medium supplemented with 250 μg/ml Geneticin (Life Technologies). Resistant colonies were picked and expanded in culture.

Immunofluorescence microscopy

Positive CHO-K1 transfectants were screened by immunofluorescence microscopy. Cells were seeded into chamber slides (Nunc; Life Technologies) and grown until nearly confluent. Slides were air-dried for 10 min, fixed in ice-cold methanol/acetone (50:50, v/v) for 20 min, then washed three times in PBS for 10 min. Non-specific binding was blocked by incubating slides in PBS containing 5 % (v/v) goat serum (Scottish Antibody Production Unit, Carluke, Scotland, U.K.) in a humid chamber for 30 min. The slides were drained and mAbs (A77, A3, A59 or A62) added at a 1:100 dilution in PBS/5 % goat serum and incubated for 1 h. Slides were washed three times in PBS as above before the application of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG γ chain (Sigma, Poole, Dorset, U.K.) diluted 1:30 in PBS/5 % goat serum. Slides were incubated for 1 h in the dark and washed as before; they were then mounted with anti-fade fluorescence mounting medium (Dako, High Wycombe, Bucks., U.K.). Slides were viewed on the same day with an Olympus BH-2 fluorescence microscope. Clones showing strongest mAb reactivity were expanded in culture and further analysed by flow cytometry.

Flow cytometry

Cells were washed and incubated in ice-cold PBS containing 1 mM EDTA, 0.2 % BSA and 5 % (v/v) goat serum (blocking buffer) for 30 min on ice. After washing in PBS containing 1 mM EDTA and 0.2 % BSA and centrifugation at 400 ***g*** and at 4 °C for 5 min, cells were resuspended at 5×10^6/ml in one of the following in blocking buffer: mAb My43 supernatant diluted 1:20, mAb A77 supernatant diluted 1:20, 1 mg/ml serum IgA, 1 mg/ml IgG or 0.32 mg/ml dimeric secretory IgA. After incubation for 2 h on ice, cells were washed as above and resuspended in a 1:100 dilution in blocking buffer of an appropriate FITC-conjugated antibody [goat anti-mouse IgM (Sigma) for My43; goat anti-mouse IgG (Sigma) for A77, A59, A3 and A62; goat F(ab′)2 anti-human IgA α-chain for IgA (Bradsure Biologicals, Loughborough, Leics., U.K.); and sheep anti-human IgG (The Binding Site, Birmingham, U.K.) for IgG]. After 1 h on ice, the cells were washed as above and resuspended in 2 % (w/v) paraformaldehyde solution in PBS before analysis on a FACScan flow cytometer.

RESULTS

Amplification of FcαR transcripts

With oligonucleotide primers complementary to known human FcαR cDNA [6], RT–PCR amplification of RNA extracted from both human neutrophils and eosinophils consistently resulted in two prominent amplified DNA products of approx. 870 and 580 bp (Figure 1a, lane 6, and Figure 1b, lanes 1–4). A number of further minor bands were also amplified from neutrophil RNA. Similar amplification of RNA from the human monocyte-like cell line THP-1 also generated two products of the same sizes, but in this case the larger band was by far the more prominent (Figure 1a, lane 7). The larger of these two major products was identical in size with the single product resulting from PCR amplification of the full-length FcαR cDNA in pHuIgAR (Figure 1b, lane 6). No products were amplified from RNA extracted from a human Burkitt's lymphoma cell line RAMOS, untransfected CHO-K1 cells, or human lymphocytes (Figure 1a, lanes 8–10), nor as a negative control, in the absence of any cDNA (Figure 1a, lane 1). A positive control amplification with primers specific for mRNA encoding the ubiquitously expressed protein, prohibitin [18], served to demonstrate that the neutrophil cDNA preparation was a suitable template for PCR (Figure 1a, lane 3).

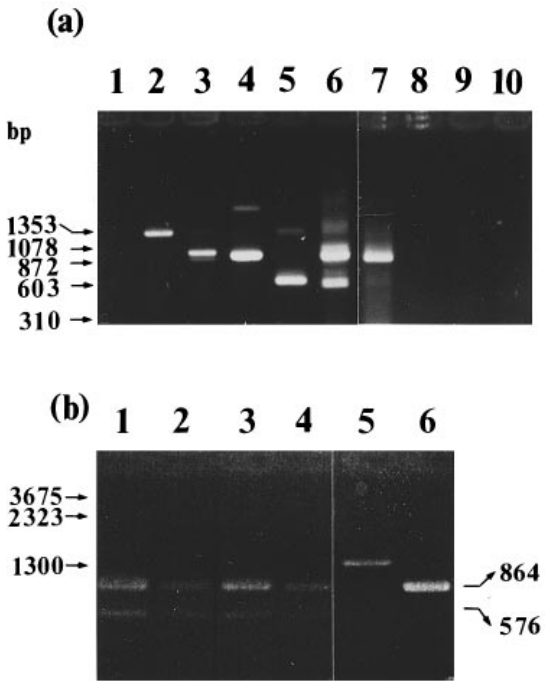

Figure 1 RT–PCR analysis of neutrophil, eosinophil and THP-1 mRNA

Agarose gel electrophoresis of RT–PCR products. (**a**) Lanes 1 and 4–10, with primers RP1 and RP2 and the following templates: lane 1, no cDNA (negative control); lane 4, plasmid pRP7 (giving 864 bp band); lane 5, pRP8 (giving 576 bp band); lane 6, neutrophil cDNA; lane 7, THP-1 cDNA; lane 8, RAMOS cDNA; lane 9, CHO-KI cDNA; lane 10, lymphocyte cDNA. Lane 2, electrophoresis with kit control primers and kit cDNA (positive control giving a 1.3 kb band). Lane 3, with prohibitin-specific primers and neutrophil cDNA (positive control giving a band of 976 bp). (**b**) With primers RP1 and RP2 and the following templates: lane 1, neutrophil cDNA (10 μl); lane 2, neutrophil cDNA (5 μl); lane 3, eosinophil cDNA (10 μl); lane 4, eosinophil cDNA (5 μl); lane 6, pHuIgAR (positive control of 864 bp). Lane 5, With kit control primers and kit cDNA (positive control of 1.3 kb). The positions of molecular-mass markers are indicated in bp at the left, and in (**b**) the sizes of the amplification products in bp are indicated at the right.

Northern hybridization and RNAse protection

To confirm the existence of the smaller FcαR-related transcript, non-amplified RNA from neutrophils was analysed on Northern blots, with a full-length ^{32}P-labelled anti-sense riboprobe. Neutrophils showed two major transcripts (labelled I and II in

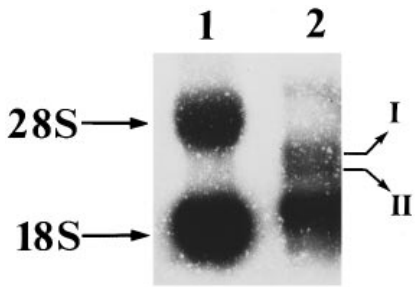

Figure 2 Northern blot analysis

Autoradiogram showing the hybridization of a full-length FcαR riboprobe with a blot of 10 μg of total RNA from untransfected CHO-K1 cells (lane 1) and neutrophils (lane 2). The two neutrophil transcripts are labelled I and II.

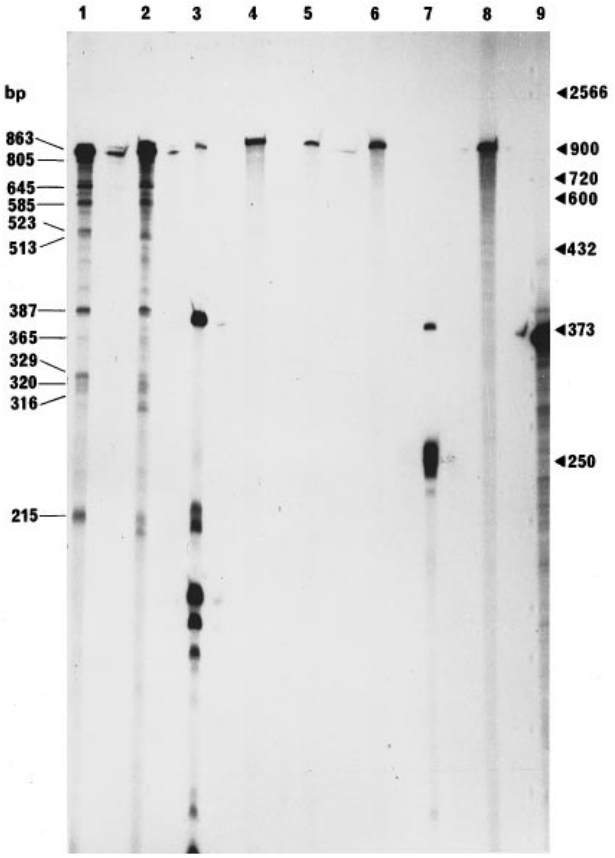

Figure 3 RNase protection analysis of total RNA

Autoradiogram showing RNA fragments protected from RNase digestion by hybridization to single-stranded anti-sense riboprobes. Lanes 1 and 2, neutrophil RNA from two normal donors probed with the full-length FcαRI probe; lane 3, neutrophil RNA probed with a β-actin probe; lanes 4, 5 and 6, RAMOS RNA, untransfected CHO-K1 RNA and yeast tRNA respectively, probed with the FcαRI full-length probe; lane 7, kit control RNA (mouse liver) probed with a 373 bp β-actin probe (kit positive control) to give a protected fragment of 250 bp; lane 8, undigested FcαRI probe (900 bp); lane 9, undigested β-actin kit control probe (373 bp). Markers at the right are in bp.

Figure 2) running between the 28 S and 18 S ribosomal RNA species, which are absent from the untransfected CHO-K1 controls (Figure 2) and from RAMOS cells (results not shown).

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                                        FcαRIa1      39 A TGGACCCCAA ACAGACCACC
                                        FcαRIa2      39 A TGGACCCCAA ACAGACCACC

  61 CTCCTGTGTC TTGTGCTCTG TCTGGGCCAG AGGATTCAGG CACAGGAAGG GGACTTTCCC
  61 CTCCTGTGTC TTGTGCTCTG TCTGGGCCAG AGGATTCAGG CACAGGAAGG GGACTTTCCC

 121 ATGCCTTTCA TATCTGCCAA ATCGAGTCCT GTGATTCCCT TGGATGGATC TGTGAAAATC
 121 ATGCCTTTCA TATCTGCCAA ATCGAGTCCT GTGATTCCCT TGGATGGATC TGTGAAAATC

 181 CAGTGCCAGG CCATTCGTGA AGCTTACCTG ACCCAGCTGA TGATCATAAA AAACTCCACG
 181 CAGTGCCAGG CCATTCGTGA AGCTTACCTG ACCCAGCTGA TGATCATAAA AAACTCCACG

 241 TACCGAGAGA TAGGCAGAAG ACTGAAGTTT TGGAATGAGA CTGATCCTGA GTTCGTCATT
 241 TACCGAGAGA TAGGCAGAAG ACTGAAGTTT TGGAATGAGA CTGATCCTGA GTTCGTCATT

 301 GACCACATGG ACGCAAACAA GGCAGGGCGC TATCAGTGCC AATATAGGAT AGGGCACTAC
 301 GACCACATGG ACGCAAACAA GGCAGGGCGC TATCAGTGCC AATATAGGAT AGGGCACTAC

 361 AGGTTCCGGT ACAGTGACAC CCTGGAGCTG GTAGTGACAG GCTTGTATGG CAAACCCTTC
 361 AGGTTCCGGT ACAGTGACAC CCTGGAGCTG GTAGTGACAG .......... ..........

 421 CTCTCTGCAG ATCGGGGTCT GGTGTTGATG CCAGGAGAGA ATATTTCCCT CACGTGCAGC
 421 .......... .......... .......... .......... .......... ..........

 481 TCAGCACACA TCCCATTTGA TAGATTTTCA CTGGCCAAGG AGGGAGAACT TTCTCTGCCA
 481 .......... .......... .......... .......... .......... ..........

 541 CAGCACCAAA GTGGGGAACA CCCGGCCAAC TTCTCTTTGG GTCCTGTGGA CCTCAATGTC
 541 .......... .......... .......... .......... .......... ..........

 601 TCAGGGATCT ACAGGTGCTA CGGTTGGTAC AACAGGAGCC CCTACCTGTG GTCCTTCCCC
 601 .......... .......... .......... .......... .......... ..........

 661 AGTAATGCCT TGGAGCTTGT GGTCACAGAC TCCATCCACC AAGATTACAC GACGCAGAAC
 661 .......... .......... ........AC TCCATCCACC AAGATTACAC GACGCAGAAC

 721 TTGATCCGCA TGGCCGTGGC AGGACTGGTC CTCGTGGCTC TCTTGGCCAT ACTGGTTGAA
 721 TTGATCCGCA TGGCCGTGGC AGGACTGGTC CTCGTGGCTC TCTTGGCCAT ACTGGTTGAA

 781 AATTGGCACA GCCATACGGC ACTGAACAAG GAAGCCTCGG CAGATGTGGC TGAACCGAGC
 781 AATTGGCACA GCCATACGGC ACTGAACAAG GAAGCCTCGG CAGATGTGGC TGAACCGAGC

 841 TGGAGCCAAC AGATGTGTCA GCCAGGATTG ACCTTTGCAC GAACACCAAG TGTCTGCAAG
 841 TGGAGCCAAC AGATGTGTCA GCCAGGATTG ACCTTTGCAC GAACACCAAG TGTCTGCAAG

 901 TAA
 901 TAA
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Figure 4 Nucleotide sequence comparison of two PCR-amplified FcαRI cDNA species

Aligned nucleotide sequences of the full-length receptor (FcαRIa1) and the splice variant (FcαRIa2). The start of the coding region of exon S1, the end of exon TM/C, and the four boundaries between exons S1, S2, EC1, EC2 and TM/C are indicated by open triangles. Nucleotides absent from FcαRIa2 are marked by dots. The nucleotides are numbered in accordance with the previously described FcαR cDNA [6].

The more prominent ribosomal RNA in CHO-K1 cells presumably results from the high level of protein synthesis necessary to maintain active division in culture in comparison with the neutrophils, which are not undergoing cell division.

RNase protection analysis with a radiolabelled anti-sense RNA probe of the full-length FcαR was used to establish the presence of spliced transcripts in neutrophils. A control probe for β-actin was also used. Probes were hybridized with total RNA, treated with RNAse One® to digest single-stranded RNA, then subjected to high-resolution denaturing gel electrophoresis and autoradiography. The undigested control β-actin probe gave a band of 373 bp (Figure 3, lane 9), and digestion after hybridization with control mouse liver RNA decreased the size of most of the probe to 250 bp, as expected (Figure 3, lane 7), indicating that the conditions used gave almost complete digestion. After hybridization to total neutrophil RNA the β-actin probe was digested, as expected, to a number of smaller fragments (Figure 3, lane 3), indicating that the neutrophil RNA preparation served as a good substrate for the assay. The untreated FcαR probe (Figure 3, lane 8), which contained insert and 36 bp of flanking plasmid sequence, had the expected size of 900 bp. After RNase digestion of the FcαR probe hybridized with neutrophil RNA from two separate donors, a number of smaller fragments, as well as full-length protected probe (864 bp), were

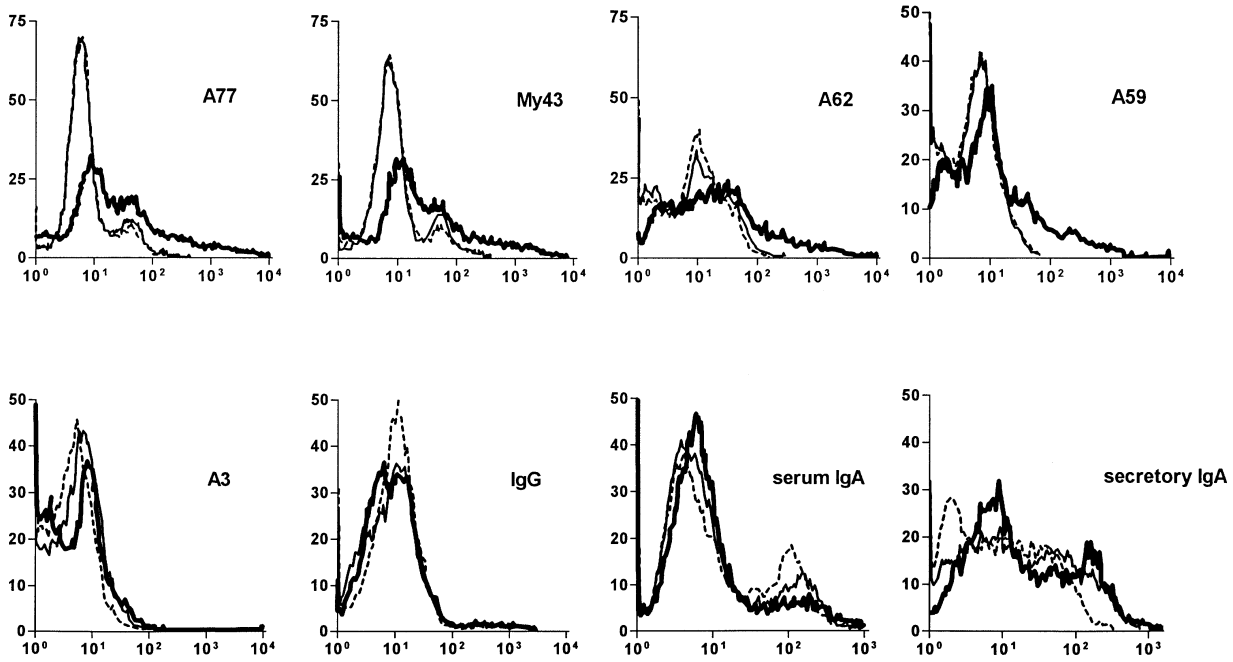

Figure 5 Analysis, by flow cytometry, of the reactivity of transfected CHO-K1 cells with CD89 mAbs and human IgA

The cell number is shown on the abscissa; the relative fluorescence intensity is shown on the ordinate. Cells were incubated with the indicated antibody and subsequently with the appropriate FITC-conjugated second antibody. Each panel includes profiles for CHO-K1 cells transfected with the full-length FcαRIa1 (thick unbroken line), CHO-K1 cells transfected with the splice variant FcαRIa2 (thin unbroken line) and untransfected CHO-K1 cells (broken line).

evident (Figure 3, lanes 1 and 2), verifying that alternative splicing had occurred. Neutrophil RNA from both donors showed the same major protected bands, although some degree of polymorphism was suggested by the slight size variations seen between some of the smaller bands. In contrast, hybridization of the FcαR probe to total RNA from RAMOS or untransfected CHO-K1 cells, or to yeast tRNA, did not offer protection, with most of the probe being digested: thus the intensity of the band at 900 bp was greatly reduced (Figure 3, lanes 4, 5 and 6) compared with undigested probe (Figure 3, lane 8). This lack of protection suggests that these cell types do not express this FcαR and is consistent with the non-amplification with RT–PCR seen earlier.

Characterization of PCR-amplified transcripts

To characterize the amplification products, the major neutrophil and eosinophil products were individually excised from agarose gels and subcloned into expression vectors. PCR amplification from the two neutrophil-derived vectors confirmed that the inserts of approx. 870 and 580 bp respectively were amplified by FcαR-specific primers (Figure 1a). For both neutrophil- and eosinophil-derived cDNAs, DNA sequencing revealed that the longer insert (864 bp) was identical with the previously cloned full-length FcαR cDNA [6], with the exception of one base difference at position 363, where a G replaced the A originally reported (Figure 4). This change does not, however, result in any amino acid change in the protein product. For both cell types, the shorter insert (576 bp) was found to lack the entire exon encoding the second (membrane-proximal) extracellular domain (EC2). Each clone again had G at position 363 (Figure 4).

Expression of cloned FcαR transcripts

CHO-K1 cells stably transfected with a vector containing the FcαRIa1 cDNA (pRP7) express a surface protein recognized by FcαR-specific mAbs (My43, A77, A62, A59 and A3) as assessed by flow cytometry (Figure 5). Reactivity with mAb A77 was also observed by immunofluorescence microscopy (results not shown). These cells seem to bind weakly to serum IgA at 1 mg/ml (Figure 5). A more marked reactivity with secretory IgA at 0.32 mg/ml is seen (mean fluorescence intensity of 58.4 compared with 27.6 for untransfected cells in a typical experiment), but serum IgG does not bind, as expected.

CHO-K1 cells transfected with plasmid containing the splice variant FcαRIa2 were selected on the basis of their reactivity with mAbs A3 and A59 by immunofluorescence microscopy. These cells show weak positivity with mAb A3, as assessed by flow cytometry, but binding to the other anti-FcαR mAbs is undetectable by analysis by flow cytometry (Figure 5). These transfectants do not show appreciable binding to serum IgA at 1 mg/ml but do bind secretory IgA at 0.32 mg/ml (mean fluorescence intensity of 69.2 compared with 27.6 for untransfected cells in a typical experiment). No reactivity with serum IgG at 1 mg/ml was evident.

DISCUSSION

A cDNA clone for the human myeloid FcαR has been described earlier [6] that encodes a transmembrane glycoprotein with two extracellular Ig-like domains, displaying homology with members of the FcγR family and with FcεRI. Here we demonstrate that in addition to expression of this full-length receptor, which we term FcαRIa1, neutrophils and eosinophils also express a trunc-

ated version, termed FcαR1a2, that lacks the entire second (membrane-proximal) extracellular domain. RT–PCR with FcαR-specific primers suggests that neutrophils might in fact express a number of other minor FcαR-related transcripts. One of these might represent the alternative transcript, noted in preliminary observations by others, which is reported to lack the second leader exon [8]. RT–PCR suggests that the THP-1 cell line also expresses both full-length receptor and the FcαRIa2 variant.

FcαRIa1 and a2 were cloned from neutrophils and were found in both cases to have sequences identical with those of the equivalent species cloned from eosinophils. For the full-length receptor we noted a silent nucleotide sequence difference from the clone originally documented [6] in that nucleotide 363 was G rather than A. An identical change was also seen in the truncated variant. It is possible that this difference has arisen as an artifact of amplification, but the fact that it was found in all four independently isolated clones suggests that this is not so. An alternative explanation is that this point difference represents an allelic polymorphism.

Northern blot analysis confirmed the existence of two major transcripts in neutrophils, a finding consistent with the earlier description of 2.8 and 2.6 kb FcαR-related mRNA species in these cells [6]. The two bands (labelled I and II in Figure 2) run between the 28 S and 18 S ribosomal RNA species, indicating sizes consistent with the earlier report. An estimated difference of approx. 200 bp between the two major transcripts is broadly in keeping with the size difference of approx. 280 bp noted with RT–PCR to assess FcαR transcripts.

Sequence analysis revealed that FcαRIa2 lacked the second extracellular domain and suggested that it was generated by splicing out the entire EC2 exon, maintaining exon–intron boundaries conforming to the GT–AG rule. RNase protection analysis of neutrophil total RNA was used to provide evidence for the existence of transcripts in which this entire domain was lacking. A radiolabelled full-length FcαR anti-sense probe was protected from digestion by an RNase specific for single-stranded RNA by annealing to complementary stretches of RNA. Using neutrophil RNA, the probe was evidently protected by a number of differently sized transcripts, resulting in several radiolabelled bands when run on a polyacrylamide gel. This was obviously FcαR-specific protection, because the vast majority of probe was digested in the absence of complementary transcripts as seen with RAMOS and untransfected CHO-K1 cells. With neutrophils a major protected band of 864 bp was seen, indicative of probe protection by mRNA encoding the full-length receptor. Attempts to explain the other band sizes seen demand knowledge of the relative sizes of the FcαR exons. These have been defined as follows: coding sequence of S1, 34 bp; S2, 36 bp; EC1, 291 bp; EC2, 288 bp; TM/C, 215 bp [8]. Deletion of the exon encoding the second receptor domain would be expected to result in looping out of the portion of the probe complementary to EC2, rendering this region susceptible to cleavage by RNase. Two protected bands of 361 bp (from protection afforded by contiguous S1, S2 and EC1 message) and 215 bp (from protection by TM/C message) would therefore result. RNase protection analysis of neutrophil RNA did indeed yield fragments of these sizes, although the band at approx. 361 bp is fainter than the 215 bp band. This form of analysis therefore supports our proposal that the FcαRIa2 variant is generated by alternative splicing. The other protected bands seen in the neutrophil lanes might indicate a number of other splice variants perhaps lacking S2, EC1 or TM/C, or combinations of exon deletions.

Deletions of entire Ig-like domains from other Fc receptors have been documented. Full-length human FcγRI possesses three Ig-like extracellular domains, but splice variants lacking either the third or both the first and third domains are expressed in mononuclear cells [10]. The latter variant serves as an example of an Fc receptor isoform with a single Ig-like domain, as proposed here for FcαRIa2.

The panel of five anti-FcαR mAbs all recognized FcαRIa1 expressed on CHO-K1 transfectants. With FcαRIa2 transfectants, although the immunofluorescence microscopy seemed more sensitive at detecting reactivity with the mAbs than flow cytometry, these assays indicated that only A3 and to a smaller extent A59 were able to recognize the shorter receptor variant. Thus mAbs My43, A77 and A62 presumably recognize epitopes lying wholly or in part in the second extracellular domain, whereas A3 and A59 might bind to epitopes located at least in part in the first domain.

In terms of function, we found that CHO-K1 transfectants stably transfected with either FcαRIa1 or FcαRIa2 bound secretory IgA well, suggesting that the interaction site for secretory IgA might lie in the first Ig-like domain of the receptor. Binding to serum IgA was much weaker for FcαRIa1, possibly owing to an avidity effect, and undetectable for FcαRIa2, whereas neither receptor bound human IgG. To mimic the neutrophil surface, it might be of interest to co-express both receptor variants in CHO-K1 and assess whether affinity for serum IgA is enhanced. It is possible that the receptors work in concert, particularly because synergism has already been noted between FcαR and FcγR [19]. FcαR on neutrophils and the monocyte-like cell line U937 have recently been shown to associate with FcR γ chain, originally described as a component of FcεRI, and also known to associate with the ligand-binding chains of FcγRI and FcγRIIIa [20,21]. Therefore it might also be illuminating to co-express γ chain with both FcαRIa1 and FcαRIa2, and re-assess binding and signalling characteristics.

In conclusion, we describe a truncated variant form of human FcαR, present on neutrophils and eosinophils, generated as an alternative splice product of the FcαRI gene. This shortened form of the receptor is still capable of binding secretory IgA. The precise roles in immunity of this and the full-length receptor remain to be elucidated. The existence of receptor splice variants might hint at a family of related FcαR molecules, each with subtle variations in structure and function, mirroring the diversity of the FcγR family.

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