Molecular Basis for a Polymorphism of Human Fcγ Receptor II (CD32)

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Summary

The IgG Fc receptor II on human monocytes is polymorphic in its ability to bind mIgG1, and its isoelectric focusing pattern. To study the molecular basis of this polymorphism, a cDNA library from cell line K562, expressing two different allelic forms (high responder [HR] and low responder [LR]) of FcyRII, was used for cDNA cloning. We report the isolation and identification of different FcyRII cDNA clones, comprising the LR form of FcyRII, as was evident from studies using a new HR-specific anti-FcyRII mAb 41H16, and from rosetting experiments. Sequence analysis revealed that HR and LR forms differ by two amino acids, both located in the external domain. In the cloned LR form, a glutamine is substituted by a tryptophan residue at aa position 27, located in the first Ig-like domain, and an arginine residue by a histidine residue at aa position 131 in the second Ig-like domain. Furthermore, an FcyRII cDNA clone was isolated with a deletion of 123 bp, overlapping the predicted transmembrane segment. Data showing the presence of an alternatively spliced mRNA detected by using polymerase chain reaction (PCR) might suggest the existence of a soluble form of the human FcyRII, in addition to the membrane-bound forms.

Receptors for the Fc region of IgG, FcyR, mediate important regulatory and effector functions within the immune system. FcyR are heterogeneous and three classes have been described on human leukocytes, FcyRI (CD64), FcyRII (CD32), and FcyRIII (CD16). They can be distinguished on the basis of molecular weight, cellular distribution, affinity and specificity for IgG isotypes, and recognition by mAbs (1, 2). During the last few years knowledge about these receptors has increased rapidly, and cDNAs have been cloned for all types (3-8). FcyRII is a 40-kD glycoprotein with low affinity for human IgG (K, ~10-5-10-6 M-1), which also binds mouse IgG1 (mIgG1).1 Until now two allelic forms of the human FcyRII could be distinguished based on differences in interaction with mouse IgG1, as observed in studies on the induction of T cell proliferation, antibody-dependent cellular cytotoxicity, and in rosetting experiments. In these studies monocytes from different individuals interact either strongly or weakly with mouse IgG1 (high or low responder individuals; HR or LR) (9-11).

To study the molecular basis of the functional polymorphism of FcyRII, a library from cell line K562, which expresses two different allelic forms of FcyRII (apparent from isoelectric focusing studies) (12), was used for cDNA cloning. We obtained different cDNA clones comprising the LR form of FcyRII, and found that LR FcyRII differed by two amino acids (aa), located in the extracellular domain of the HR form. Furthermore, one cDNA clone was isolated with a 123 bp deletion, overlapping the entire transmembrane segment, which might encode a soluble form of FcyRII.

Materials and Methods

Cell Culture. K562, U937, and Jurkat cells were cultured in RPMI 1640 medium supplemented with 5% FCS. Mouse L cells and 3T6 were grown in DMEM/F10 (vol 1:1) containing 5% FCS.

Monoclonal Antibodies. Anti-FcyRII mAb used were 32.2 (culture supernatant) provided by Dr. C. Anderson (1), and 197.1 (purified Ig) from Medarex (Lebanon, NH) (13). Four mAb with reactivity to FcyRII were used, IV.3 (purified Ig), a gift from Dr. C. Anderson (1), KuFc79 (ascites) from Dr. T. Mohanakumar (14), C22M5 (purified Ig) from Dr. G. Pilkington (15), and 41H16 (purified Ig) from Dr. T. Zipf (16). We also tested anti-FcyRIII mAb 3G8 (purified Ig) obtained from Medarex (Lebanon, NH) (17).

cDNA Clone Isolation and Characterization. A human K562 cDNA, Agt10 library (a generous gift of Drs. Hoeijmakers and Van Duin, Erasmus University, Rotterdam) was plated on Esche-
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...method (21). After overnight exposition of the DNA, cells cotransfected with PSV3 gpt (2, ug) by using the CaP0c precipitate...mouse 3T6 cellswere seeded inculture flasks 1 day before DNA transfection in DMEM/F10 with 5% FCS. Cellswere harvested on day 4 and plates were washed twice, and cellswere allowed to grow for 48 h.

FCS supplemented with chloroquine (final concentration 50 ug/ml) were added per well. After 30 min at 37°C, 0.5 ml DMEM/F10/5% FCS and 2 ug plasmid DNA were added. The cellswere washed twice with TBS (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.25 mM MgCl₂, 0.6 mM Na₂HPO₄), and 2 ug plasmid DNA were added per well. After 30 min at 37°C, 0.5 ml DMEM/F10/5% FCS supplemented with chloroquine (final concentration 50 ug/ml) was added, and the mixture was incubated for 3 h at 37°C, the plates were washed twice, and cellswere allowed to grow for 48 h in DMEM/F10 with 5% FCS. Cellswere harvested on day 4 and used in immunofluorescence experiments.

Transfection of 3T6 Cells. To obtain stable transfectants, 10⁶ mouse 3T6 cellswere seeded inculture flasks 1 day before DNA transformation. Cellswere grown in F10/DMEM/5% FCS. The pcDX vector containing pPW3 or pCd-16.2 as an insert (10 ug) was cotransfected with pSV3gpt (2 ug) by using the CaPO₄ precipitation method (21). After overnight exposition of the DNA, cells were treated with DMSO (10%, 30 min) and grown for 24 h on nonselecting culture medium to allow expression of the transfected markers. Selection for the dominant marker was performed in modified MPA medium containing: F10/DMEM 1:1, 5% FCS, aminopterin (0.2 ug/ml), thymidine (5 ug/ml), xanthine (10 ug/ml), hypoxanthine (15 ug/ml), mycophenolic acid (MPA) (20 ug/ml), and deoxyctydine (2.3 ug/ml). Selection medium was replenished every 3-4 d. Cellswere isolated after ~2-3 wk and subcloned by limiting dilution. Expression of FcγRII was determined by staining with mAb IV3. Stable transfectants expressing FcγRII were maintained in MPA medium and used in rosetting assays.

Immunofluorescence. Expression of FcγR on cellswas assayed by indirect immunofluorescence, using different anti-FcγR mAb and FITC-labelled F(ab')₂ fraction of goat anti-mouse Ig antibody (Tago, Burlingame, CA). Fluorescence was measured with a FACStar flow cytometer (Becton & Dickinson).

EA-mIgG1 Rosetting Assay. EA rosette formation between stable transfectants expressing either pcD16.2, or pPW3, or K562 cellswere assayed by enzyme-linked immunosorbent assay to determine the expression of the receptor.

Results and Discussion

The external domain of human FcγRII is homologous with the mouse FcγR reactive with IgG2b and IgG1 (22). Oligonucleotides were constructed on the basis of the highest number of homologous sequences between mouse (22) and human (3-6) FcγRII with a maximum length of 30 nt. Several FcγRII cDNA clones were isolated from a K562 Agt10 library using these probes, plaque purified, and subcloned. K562 cDNA clones pPW3 and pPW6 were found to contain 1.4- and 1.0-kb inserts, respectively (Fig. 1). Their specificity was confirmed by sequence analysis.

Fig. 1 A shows a physical map of two cDNA clones and the location of the FcγRII coding region. An almost full-length clone (pPW3) is identical to FcγRII cDNA clone pcD-16.2 (3), except for 8 bp in the coding region and 5 bp in the 3' untranslated region, and clone pPW3 is 37 bp longer at the 5' end (Fig. 1 B). A nt substitution (A into T) and a deletion of 3 bp, resulting in the absence of an alanine (A) residue, are located in the signal sequence. The site is mismatched at aa position 27 in the extracellular domain and encodes an aa change of glutamine (Q) into tryptophan (W) provoked by the substitution of two nt, CA into TG (Fig. 1 B). In the second Ig-like domain of the receptor a point mutation (G into A) was found in clone pPW3, resulting in an aa change of arginine (R) into histidine (H) (position 131). Finally, in the coding region at position 179 a silent mutation has been found. Another isolated clone (pPW16; data not shown) is not full-length at the 5' end, but contains the same mutations at positions 27, 131, and 179 as clone pPW3. Furthermore, pPW16 includes a much longer 3' untranslated region showing an alternate poly(A) site selection, which is analogous to an FcγRII isolated from a pheochromocytoma library (4).

Sequence analysis revealed an interesting deletion of 123 bp in clone pPW6 (Fig. 1). Unfortunately, this clone is not full-length at the 5' end, but starts at aa 53. Therefore, only the second mutation site is known encoding an arginine res-
Figure 1. Physical map of K562 FcγRII cDNA clones showing their relation to the restriction map and coding sequence of human FcγRII (A), and part of the nucleotide sequence of clone pPW3 (B). (A) Arrows indicate polyadenylation signals (AATAAA), and open triangles designate deletions determined by sequence analysis. The open triangle marked in clone pPW6 represents a deletion of 123 bp compared with pPW3, and previously published human FcγRII cDNAs (3-6). The open triangle marked in clone pPW3 represents a deletion of three bp relative to other FcγRII cDNAs (3-6). Closed triangles in clone pPW3 point at mismatches compared to pcD-16.2, as detailed in (B). The upper row in B shows sequence, and deduced aa, of clone pPW3 compared with homologous regions in pcD-16.2 (lower row). The coding sequence of FcγRII is depicted as an open box (A), wherein S is signal peptide, E-C is extracellular domain, TM is transmembrane segment, and C is intracellular domain. The putative W-linked glycosylation sites are depicted with asterisks, and cysteine residues with a small c. Abbreviations used for the aa are: L, leucine; T, threonine; C, cysteine; Q, glutamine, G, glycine; A, alanine, P, proline; H, histidine; R, arginine; S, serine; M, methionine; and W, tryptophan.

To exclude a cloning artifact for clone pPW6, we evaluated the existence of corresponding mRNA in K562 cells. Two oligonucleotides were synthesized, one overlapping 10 nt at both sides of the deleted region, and a second one 221 bp upstream of this deletion. Plasmid DNAs containing clones pPW3 or pPW6 as inserts were used as controls. Agarose gel electrophoresis of the PCR-amplified reaction products shows a clear band of 231 bp using the plasmid-containing clone pPW6 (with deletion; Fig. 3, lane 2), and no specific band using pPW3 (Fig. 3, lane 3). Obviously, these primers can discriminate between the presence and absence of this deletion. Expression of mRNA transcripts encoding an FcγRII analogous to clone pPW6 in K562 cells is demonstrated by a specific band of 231 bp (Fig. 3, lane 4), which is absent in Jurkat cDNA (Fig. 3, lane 5). Because the deletion of 123 bp excludes a transmembrane coding region, we postulate that this mRNA encodes a soluble human FcγR type II, most likely created by alternative splicing. The possibility that alternative splicing might be involved is supported by recent studies showing an exon border located at position 212 (6).

Soluble FcγRII molecules have previously been shown to be present in normal murine serum (23). Furthermore so-called IgG-binding factors are reported, which are implicated to play an important role in isotypic regulation (24).
To analyze whether the isolated clones were encoding the HR or LR FcγRII allelic form, murine L cells were transiently transfected with cDNA clone pPW3, using the pcDX expression vector (19). We assessed the ability of these transfectants, as well as two human cell lines U937 and K562, to bind several anti-FcγR mAbs. One of the antibodies used was mAb 411416, which is an FcγRII-specific mAb (16) with a unique specificity, in that it recognizes an epitope expressed only on the HR allelic form of FcγRII. This has recently been demonstrated by one of us, using human cells from well-defined HR and LR individuals, typed by isoelectric focusing analysis. It was possible to discriminate between LR and both heterozygous and homozygous HR monocytes, platelets, as well as PMNs using this mAb (25). mAb IV.3 is also FcγRII specific, but interacts with monocytes from both HR and LR individuals with similar intensity (1).

Isoelectric focusing data (Fig. 4) show similar fluorescence intensity of U937 cells, expressing FcγRII in the homozygous HR form, after incubation with mAbs IV.3 or 41H16. K562 cells express FcγRII in a heterozygous manner, indicated by the fluorescence level of 41H16, which was ~60% of that obtained by using mAb IV.3. These findings are in accordance with earlier isoelectric focusing studies of FcγRII on these cells (12). L cells transiently transfected with K562 cDNA clone pPW3 show 2.1% positive transfectants, as determined by reactivity with mAb IV.3. These transfectants, however, were much less reactive with mAb 41H16 (Fig. 4 B), indicating that K562 cDNA clone pPW3 encodes the LR form of the polymorphic human FcγRII. Indirect isoelectric focusing showed, furthermore, that L cells transfected with pPW3 bound anti-FcγRII mAbs CIKM5, and KuFc79, but not anti-FcγRII mAbs 32.2 or 197.1, or anti-FcγRIII mAb 3G8. Transient transfection of the U937 cDNA clone pcD-16.2 revealed 2.8–2.9% positive transfectants as determined by reactivity with both IV.3 and 41H16 mAbs, which is consistent with the HR phenotype of this FcγRII (Fig. 4 B).

The functional activity of FcγRII was evaluated by rosetting experiments. Stable transfectants expressing equivalent amounts of FcγRII encoded by pPW3 or pcD-16.2, as determined by FACS analysis (data not shown), K562 cells, and mock-transfected cells were used in these studies. Rosetting with HRBC sensitized with graded amounts of mIgG1 anti-
glycophorin A mAb resulted in striking differences between the transfected HR (pcD-16.2) and LR (pPW3) receptors. K562 cells expressing both HR and LR FcγRII showed intermediate rosetting (Fig. 5). Using F(ab')2 fragments of the anti-glycophorin A mAb no rosetting was observed, nor with mock-transfected cells. To exclude that the observed differences are due to a dysfunction of expressed receptors, similar rosetting experiments were performed using mlG2a and mlG2b anti-glycophorin A switch variant mAb. Strong rosetting was observed without differences between either the FcγRII transfectants, or K562 cells (data not shown), indicating the functionality of the receptors. Moreover, FcγRII expressed by the three cell types were able to bind heat-aggregated human IgG, as evaluated using immunofluorescence analysis (data not shown). These results establish the functional polymorphism of FcγRII with respect to mlG1, and show that this polymorphism does not result in differential interaction with mlG2a or mlG2b antibodies.

From sequence analysis, immunofluorescence data obtained with mAb 41H16, and rosetting experiments, we conclude that the HR and LR forms of human FcγRII differ by three aa. Since data on the NH₂ terminus of the FcγRII glycoprotein are lacking at present, we do not know whether the missing alanine residue is located either at the end of the signal peptide, or at the beginning of the external domain. Several identical clones have been reported (3–6), each starting at another aa for the external domain, due to differences in computer analysis. Comparison of these clones with clone pPW3 shows that the NH₂-terminal part of FcγRII encoded by pPW3 is identical to FcγRII cDNA clone HFc3.0 (5).

The differences responsible for the LR allelic form of FcγRII, are most likely the conversions of a glutamine into a tryptophan at position 27, and an arginine into a histidine (position 131). These changes must constitute the basis for the observed polymorphic behavior in functional assays, isoelectric focusing, as well as for a conformational epitope change, leading to an altered reactivity with mAb 41H16. The theoretical change in isoelectric point by these aa differences of the unglycosylated protein is 0.16 (6.54 to 6.38). This difference between HR and LR forms correlates with the observed change in isoelectric focusing pattern (12).

Changing a strong into a weak basic aa (position 131) might be important for binding of mlG1. The extent to which each of these differences accounts for the functional or anti-

**Figure 4.** Expression of HR and LR forms of human FcγRII on K562, U937, murine L cells, and L cells transfected with clones pPW3 or pcD-16.2. Relative immunofluorescence (A) of K562, U937, and L cells after binding mAb IV.3 (crosshatched bars), mAb 41H16 (black bars), or second antibody only (control; open bars). L cells transiently expressing clones pPW3 or pcD-16.2 in the pcDX expression vector (B) incubated with mAb IV.3, mAb 41H16, or second antibody, indicated as above. Data are representative of five independent experiments.

**Figure 5.** Rosette formation by transfectants expressing pPW3 (□; LR), or pcD-16.2 (■; HR), or K562 cells (○) with HRBC sensitized with different amounts mlG1 anti-glycophorin A mAb.
genic differences is currently being investigated. Recently, the aa change at position 131 has been reported by others as the only basis for the polymorphism of FcyRII on human monocytes (26). Provided this indeed is the only change in the individuals studied by these authors, the functional polymorphism of FcyRII is composed of multiple allelic forms.

In conclusion, our study shows that the functional polymorphism of membrane-bound human FcyRII is based on at least two amino acid differences, a glutamine or tryptophan residue at aa position 27, and an arginine or histidine residue at position 131 in the extracellular domain.

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