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The Binding Affinity of Human IgG for its High Affinity Fc Receptor Is Determined by Multiple Amino Acids in the C2 Domain and Is Modulated by the Hinge Region

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Summary

A family of chimeric immunoglobulins (Igs) bearing the murine variable region directed against the hapten dansyl linked to human IgG1, -2, -3, and -4 has been characterized with respect to binding to the human high affinity Fc γ receptor, FcγR1. Chimeric IgG1 and -3 have the highest affinity association (K_a = 10^9 M^-1), IgG4 is 10-fold reduced from this level, and IgG2 displays no detectable binding. A series of genetic manipulations was undertaken in which domains from the strongly binding subclass IgG3 were exchanged with domains from the nonbinding subclass IgG2. The subclass of the C2 domain was found to be critical for determining IgG receptor affinity. In addition, the hinge region was found to modulate the affinity of the IgG for FcγR1, possibly by determining accessibility of FcγR1 to the binding site on Fc. A series of amino acid substitutions were engineered into the C2 domain of IgG3 and IgG4 at sites considered potentially important to Fc receptor binding based on homology comparisons of binding and nonbinding IgG subclasses. Characterization of these mutants has revealed the importance for FcγR1 association of two regions of the genetic C2 domain separated in primary structure by nearly 100 residues. The first of these is the hinge-link or lower hinge region, in which two residues, Leu(234) and Leu(235) in IgG1 and -3, are critical to high affinity binding. Substitution at either of these sites reduces the IgG association constant by 10-100-fold. The second region that appears to contribute to receptor binding is in a hinge-proximal bend between two β strands within the C2 domain, specifically, Pro(331) in IgG1 and -3. As a result of β sheet formation within this domain, this residue lies within 11 Å of the hinge-link region. Substitution at this site reduces the Fc receptor association constant by 10-fold.

The antibody molecule serves as an immunologic bridge, combining in one polypeptide sites responsible for recognition of foreign pathogens and others responsible for triggering of host response systems. IgG, the predominant form of serum antibody, interacts through its Fc region with a variety of host effector molecules, among which are a group of surface receptors found on cells predominantly of hematopoietic origin. This Fc receptor family can be divided based on IgG subclass specificity and mAb recognition into three groups: FcγR types I, II, and III. FcγR1 is distinctive in being the only receptor capable of binding monomeric IgG with high affinity (1).

The human FcγR1 receptor binds ligand with the subclass preference: IgG1, -3 > IgG4 >> IgG2 (2). It binds murine IgG2a, but not muIgG1 or muIgG2b, with affinity equivalent to that of human IgG (3). It has a valence for IgG of one (4), and association of IgG with huFcγR1 appears to be mediated by a single heavy chain (5), although there are two binding sites on each IgG molecule. FcγR1 functions demonstrated in vitro include phagocytosis (6), and antibody-dependent cellular cytotoxicity (ADCC) (7–11). Evidence supports a unique role for FcγR1 early in the immune response before antigen-specific IgG reaches high levels.

Early attempts to localize the site on IgG responsible for associating with huFcγR1 using proteolytic fragments of Ig were inconclusive (12, 13). Analysis of mutant Igs was more informative and suggested that at least a number of the residues required for association are located in the C2 domain, potentially in the NH2-terminal end of the domain (3).

Examination of a number of IgG subclasses for amino acids
conserved among strong (Kₐ > 10⁸ M⁻¹), but not among weak binders (Kₐ < 10⁸ M⁻¹) revealed two homology regions potentially involved in FcγR₁ binding. The first is a hexapeptide sequence in the hinge-link region immediately before the β strand formation of the C₂₂ domain. This peptide segment, conserved among huIgG1 and -3, muIgG₂a, and rabbit IgG, consists of the sequence Leu - Leu - Gly - Gly - Pro - Ser (residues 234-239, EU numbering [14]). In huIgG₄, an intermediate affinity IgG, the sequence contains a single amino acid substitution: Phe for Leu at position 234. Murine IgG₂b, a weakly binding IgG, likewise contains a single residue substitution: Glu for Leu at position 235. In human IgG₂, which shows no binding activity, there are two substitutions and a deletion leading to the sequence Val(234) - - - - Ala(236) - Gly - Pro - Ser (Fig. 1). The significance of this region in mediating binding to the high affinity Fc receptor was recently demonstrated by Duncan et al. (15), who reported conferring IgG₂a-like binding properties on an IgG₂b transfectoma protein by using oligonucleotide-directed mutagenesis to change Glu(235) to Leu.

A second region of the C₂₂ domain identified by homology as a potential contributor to FcγR₁ binding is located near the COOH-terminal end of the domain's primary structure. This broad stretch of residues extends over two of the three β strands in the Y-face of the domain, including a hinge-proximal bend between the strands, and contains 16 residues perfectly conserved among strongly binding IgGs. Of particular interest is the fact that among the four human IgG subclasses, there is only one substituted site within these conserved residues: a pair of serine residues in IgG₄ replaces Ala(330) and Pro(331) (Fig. 1) (present in the other three subclasses); this difference is located in the bend between the two β strands and is predicted to fold into close proximity to the hinge-like region, although the latter is not strictly resolved in the crystallographic Fc model due to a high degree of disorder in this region of the crystal (16).

We have taken two approaches to defining the structural features of IgG that influence, either directly or via conformational effects, the molecule's association with the human FcγR₁. To examine the contributions made by individual domains and by the hinge, gene manipulation has been used to exchange exons between the four cloned human IgG constant regions. Expression of these genes in a chimeric antidansyl system in association with the human C₅ light chain allowed the examination of intact human IgG "variant" proteins with a series of domain interchanges that do not suffer from the drawbacks of the proteolytic cleavage studies or the uncertainties of heterologous IgG receptor systems. Studies of natural variant proteins to date had indicated a role for C₂₂ in the binding to FcγR₁, but have not ruled out a contribution from the C₃ domain, nor had the role of the hinge been addressed, beyond establishing that its presence is required for full strength binding.

On a sub-domain level, oligonucleotide-directed site-specific mutagenesis has been used within the C₂₂ domain to better define the amino acid residues that account for the FcγR₁ affinity differences between IgG subclasses. By this approach, we have examined residues at the NH₂- and COOH-terminal ends of the domain sequence indicated by homology patterns to be potentially important for receptor binding. These mutant IgG genes, like those of the domain-altered proteins, have been expressed in the context of the chimeric antidansyl system in order that homologous (human) receptor IgG interactions may be investigated. These studies have indicated the importance of residues at both ends of the C₂₂ domain linear sequence in either directly contacting the receptor, or maintaining the functional conformation of the binding site (or both), and have shown that the hinge modulates the affinity of IgG for FcγR₁.

Materials and Methods

Vectors. The SalI-BamHI cassette containing the constant region gene was subcloned into either pBR322 for exon exchange (EE), or M13mp19 for site-directed mutagenesis (SDM). EE was performed using appropriate intronic restriction sites (e.g., PvuII), and was confirmed by restriction digest analysis and double-stranded DNA sequence analysis. SDM was performed using the double-primer method (17) using single-mismatch oligonucleotides, and was confirmed by sequence analysis. Mutated constant region genes were cloned as SalI-BamHI fragments into the mammalian expression vector pSV2ΔHneo (18) with the expressed V₅ gene from a mouse anti-DNS hybridoma (Fig. 2).

Transfectoma Production. The heavy chain was transfected into the nonproducing myeloma cell line P3X63Ag8.653 along with the chimeric anti-DNS κ light chain gene carried in the compatible expression vector pSV184ΔHneo by protoplast fusion, as previously described (18). Transfectants were selected with G418 at 1.0 mg/ml, and surviving clones screened for antibody (Ab) production by ELISA using DNS/BSA-coated plates. The amount of bound chimeric Ab was determined using alkaline phosphatase-conjugated polyclonal goat Ab (Sigma Chemical Co., St. Louis, MO) against human IgG constant regions. Clones producing large quantities of anti-DNS Ab were expanded and maintained in IMDM containing 5% calf serum.

Characterization of Chimeric Ab. To characterize the assembly, secretion, and molecular weight of IgG, cells were labeled with 3⁵S-methionine (3⁵S-met). Ab molecules in the supernatants were immunoprecipitated with polyclonal rabbit Ab against human Fc and Staphylococcus aureus protein A (IgG sorb; The Enzyme Center, Malden, MA) and analyzed by SDS-PAGE. Antibodies were purified from culture supernatants as previously described (19).

FcR Binding. An enzymatic assay was developed to quantitate the binding affinities of chimeric Ab for FcγR₁ (20). Human monocye-like U937 cells (2) were incubated with chimeric IgG molecules at various concentrations. After a 2-h incubation, β-galactosidase-conjugated DNS was added and incubated for another 2 h. The cells were then washed by centrifugation through a sucrose pad and β-galactosidase bound to IgG quantitated using the substrate o-nitrophenyl galactoside. Scatchard analysis (21) was used to determine the association constant of IgG to the receptor and the number of receptors per cell.

Results

Antibody Characterization. The size of the H and L chains produced by the transfectants and their assembly into H₅L₂ tetramers was assessed by metabolic protein labeling, followed
of particular interest with respect to assembly were the two IgG molecules deleted for Cα1, since they might be expected to be severely compromised conformationally. As is evident under reducing conditions (Fig. 3 B), both IgG1ΔCα1 and IgG3ΔCα1 make H chains with the expected decrease in size from their wild-type counterparts. From Fig. 3 A, it appears that IgG1ΔCα1 is secreted primarily in the form of H2 and L, whereas IgG3ΔCα1 appears to assemble normally, secreting predominantly H2L2 tetramers. All transfecants not represented in Fig. 3 make H and L chains of the anticipated size and secrete only full H2L2 covalent tetramers.

Binding of Wild-type IgG Subclasses to FcγR1. In initial experiments, the equilibrium binding constants of the four wild-type chimeric IgG subclasses to FcγR1 on the human monocytic cell line U937 were determined (Fig. 4 and Table 1). Fig. 4 shows representative Scatchard plots for IgG1, -3, and -4 (each curve represents a single assay). In Fig. 4 A, the binding of IgG3 to U937 cells pretreated with human IFN-γ (100 U/ml, 18 h), which increases the expression of the FcγR1, is compared with the binding of the same protein to untreated cells. Treatment with IFN-γ causes a shift in the Scatchard curve toward the right, increasing the x-intercept of the plot, in this case from 3,400 to 19,000 (representing an increase in receptor number per cell); the slope of the Scatchard plot, on the other hand, remains unchanged by treatment with IFN-γ, indicating that there is no effect on the affinity of the IgG receptor interaction. In Fig. 4 B, the Scatchard plots of IgG1 and IgG3 assayed at different times on IFN-γ-treated cells are compared in order to demonstrate the equivalent binding affinities of the two proteins. The apparent number of receptors per cell was seen to vary between assays even when the same protein preparation was assayed repeatedly, presumably as a consequence of uncontrolled variation in cell culture conditions. In Fig. 4 C, the binding of IgG3 and IgG4 to untreated U937 cells is compared in order to demonstrate the reduced affinity of IgG4 for FcγR1 (represented by the slope of the Scatchard curve). It is worth noting that although the plot for IgG4 appears nearly flat, this is a function of the dimensions of the y-axis (chosen in order that both curves could be represented in one plot); in fact, the slope is only 10-fold reduced.

Figure 2. Limited restriction map of heavy chain construct used for expression of antitrypsin chimeric antibodies. IgG2 constant region, hatched; IgG3 constant region, dark stippled, except the first, unique hinge exon, distinguished by light stipple; L, exon for hydrophobic signal peptide; V-DNS, exon for variable region domain; Cα1-3, exons for heavy chain constant region domains; H, hinge exon; PDNS, endogenous murine Ig promoter; IgEnh, murine Ig heavy chain enhancer, 234, 331, sites of oligonucleotide-directed mutagenesis. Restriction sites: R1, EcoRI; S, SalI, P, PvuI; B, BamHI. The vector pSV2AHgpt is represented by a thickened line. The drawing is not to scale.
Figure 3. SDS-PAGE analysis of 35S methionine-labeled chimeric IgGs. Secreted Ig proteins were metabolically labeled with 35S-Methionine (15 μCi/ml) as described, then immunoprecipitated using rabbit anti-human IgG Fc and S. aureus Protein A. (A) SDS-PAGE analysis of nonreduced IgGs on a 5% acrylamide gel. Each lane is labeled above, and the migration positions of whole IgG (H2L2) and half-molecules (HL) are indicated to the side of the gel. (B) SDS-PAGE analysis of reduced IgGs on a 12.5% acrylamide gel. Lanes are labeled as in A, and the migration positions of heavy (H) and light (L) chains are indicated to the side of the gel.

Table 1. Equilibrium Binding Constants of Domain-exchanged Chimeric IgGs

| Immunoglobulin | Structure | Binding Constant $K_b$ (M⁻¹) |
|---------------|-----------|-----------------------------|
| IgG1          | -         | 1.2 (±0.3) x 10⁹             |
| IgG2          | -         | <1 x 10⁶                     |
| IgG3          | -         | 1.2 (±0.5) x 10⁹             |
| IgG4          | -         | 1.4 (±0.2) x 10⁹             |
| 2-2-2-3       | -         | <1 x 10⁶                     |
| 3-3-3-2       | -         | 1.8 (±0.2) x 10⁹             |
| 2-3-3-2       | -         | 2.8 (±1.1) x 10⁹             |
| 3-3-3-2       | -         | <1 x 10⁶                     |
| 3-3-2-3       | -         | <1 x 10⁶                     |
| IgG3 ΔH      | -         | <1 x 10⁶                     |
| IgG1 ΔCH1     | -         | 3.0 (±1.0) x 10⁷             |
| IgG3 ΔCH1     | -         | 2.0 (±1.2) x 10⁹             |
| IgG3h1        | -         | 2.2 (±1.3) x 10⁸             |
| IgG3h1(2,3,4)  | -         | 1.8 (±0.8) x 10⁸             |

IgG1–4, wild-type antidansyl chimeric antibodies. Mutants with exon exchanges are given a four-digit name: the first digit refers to the subclass of the C1 domain, and the second, third, and fourth digits indicate the subclasses of the hinge, Cα2, and Cα3 domains, respectively. IgG3 ΔH, hinge-deleted IgG3; IgG3h1, IgG3 bearing a hinge encoded by the first (unique) γ3 hinge exon; IgG3h1(2,3,4), IgG3 with extended hinge (containing a duplication of the three reiterated γ3 hinge exons). The structure of each recombinant antibody is shown diagrammatically. Each $K_b$ value represents the mean of at least three independent trials (± SD). $V_w$, exon for the heavy chain variable domain directed against the hapten dansyl.

closely with previous reports in which the binding constants were determined using 125I-IgG (23, 24).

Binding of Exon Exchange Mutant to FcγR1. The role of the constant region domains in FcγR1 binding was evaluated by exchanging these regions between IgG2 and IgG3 (which display opposite extremes of binding activity). Although the presence of a Cα3 domain is required for Fc receptor binding, as indicated by the fact that a myeloma protein lacking this domain shows no affinity for FcγR1 (25), this domain does not appear critical in determining the isotype-specific binding pattern (Table 1). The proteins denoted 2223 and 3332 represent the products of Cα3 exon exchange between IgG2 and IgG3 subclasses. IgG2 bearing the Cα3 domain of IgG3 (2223) showed no detectable binding to U937 cells ($K_b < 10^8$ M⁻¹). The reciprocal exchange, IgG3 bearing the Cα3 domain of IgG2 (3332), bound to U937 cells with a $K_b$ equivalent to that of IgG3 (1.8 × 10⁹ M⁻¹), indicating that the Cα3 domain is not responsible for the subclass-specific affinity difference between nonbinding IgG2 and the strongly binding IgG3. Although the presence of a Cα3 domain is required for maximal binding FcγR1 (as discussed earlier), the subclass of this domain does not determine the
isotype-specific binding phenotype, suggesting that either this domain does not directly interact with the Fc receptor or that the interacting residues are conserved between these subclasses.

By contrast, the subclass of the C\(_{2}\) domain does appear to determine, by a first approximation, the binding phenotype of the molecule. Hence, IgG3 with an Fc region almost completely from IgG2 (3322), and IgG3 bearing only the C\(_{2}\) domain of IgG2 (3323), show no detectable binding to U937 cells. Moreover, IgG2 with a y3 C\(_{2}\) domain (2232) binds FcγR\(_{j}\) with an affinity nearly that of IgG3 (\(K_a = 2.8 \times 10^8\) M\(^{-1}\)). Interestingly, the \(K_a\) value for the protein 2232 was slightly but reproducibly lower than that of 3332 (three-fold decreased) suggesting that the hinge and/or C\(_{1}\) domain may contribute to overall IgG affinity, possibly through conformational effects on the C\(_{2}\) domain. Deletion of C\(_{1}\) altogether from IgG1 results in a decrease in association constant of ~30-fold (see Table 2), whereas the equivalent deletion from IgG3 results in only a 10-fold reduction in \(K_a\).

The hinge region plays a role both as a spacer region between the Fab and Fc regions and in imparting the molecule with segmental flexibility. The presence of the hinge region is necessary for the interaction of IgG FcγR\(_{j}\), and hinge-deleted human IgG1 myeloma proteins Dob and Lec display little binding to the mouse analogue of this receptor (26). IgG3 contains an extended hinge of 62 amino acids, encoded genetically by four exons (one unique, followed by three reiterated exons). As the subclass with the greatest degree of segmental motion in the Fab region, IgG3 may require the extended hinge in order to prevent steric hindrance to close approach of effector molecules. As shown in Table 1, deletion of the entire hinge (IgG3\(_{AH}\)) results in complete loss of binding to the human FcγR\(_{j}\), in agreement with the studies of Dob and Lec. Partial replacement of the hinge with a 17-residue segment encoded by the first (unique) IgG3 hinge exon results in restoration of receptor binding, however, this protein (IgG3\(_{h1}\)) displays a \(K_a\) value 10-fold reduced from wild-type (\(K_a = 2.2 \times 10^8\) M\(^{-1}\)) (Table 1). Therefore, IgG3\(_{h1}\), which exhibits segmental flexibility equivalent to wild-type IgG3, is reduced in ability to fix complement and bind FcγR\(_{j}\) supporting a role for the extended hinge in preventing steric hindrance. Somewhat surprisingly, IgG3 bearing a hinge extended by duplication of its reiterated exons (IgG3\(_{h1(2,3,4)^3}\)) also exhibits ~10-fold reduced affinity for FcγR\(_{j}\) relative to IgG3; therefore, there is an optimal spacing between Fab and Fc.

**Amino Acid Substitutions.** Having established the importance of the C\(_{2}\) domain of IgG in the interaction with FcγR\(_{j}\) on U937, we next investigated the contributions to

| Table 2. Equilibrium Binding Constants of Amino Acid-substituted Chimeric IgGs |
|-----------------------------|-----------------------------|
| IgG                        | \(K_a\) (M\(^{-1}\)) |
| IgG3(Glu[235])              | <1 \times 10^7           |
| IgG3(Phe[234])              | 1.9 (± 0.8) \times 10^8   |
| IgG4(Leu[234])              | 3.8 (± 1.4) \times 10^8   |
| IgG3(Ser[331])              | 3.1 (± 2.6) \times 10^8   |
| IgG4(Leu[234], Pro[331])   | 1.3 (± 0.4) \times 10^8   |
| IgG4(Leu[234])Hyγ3          | 1.0 (± 0.2) \times 10^8   |

The name of each mutant indicates its subclass, the position of the amino acid substitution (EU number system), and the amino acid created at that site. IgG4 (Leu[234]) Hyγ3 represents IgG4 substituted at position 234 (Leu for Phe) and bearing the hinge of IgG3. Each \(K_a\) value, represents the mean of at least three independent trials (± SD).
binding made by selected residues within the domain, using oligonucleotide-directed mutagenesis to produce amino acid substitutions. Table 2 lists a series of site-specific mutant IgG proteins and the associated $K_v$ values for binding U937 cells.

The first substitutions introduced were designed to examine the role of the homology region (identified by Woof et al. [25]) encoded at the 5' end of the C2 exon, and falling in the hinge-link region of the molecule (Fig. 1). As discussed earlier, this conserved hexapeptide consisting of Leu(234) - Leu - Gly - Pro - Ser(239) is present in human IgG1 and IgG3, is substituted once at residue 234 by Phe in IgG4, and once at residues 235 by Glu in muIgG2b. A single amino acid substitution in the hinge-link region of mouse IgG2b, Glu(235) to Leu, increased the binding of this Ig $>$100-fold, to a level equivalent to that of human IgG1 (15). We have found that the reciprocal substitution of Glu for Leu(235) in IgG3 leads to a significant decrease in $F_c$ receptor affinity (Table 2). However, our studies make it clear that in human IgG more than one residue determines $F_c$ receptor binding affinity, and that the critical residues are not all contiguous in the primary sequence.

Human IgG4 differs in the hinge-link region from the high affinity subclasses (IgG1 and -3) at only one position: Phe replaces Leu(234). To evaluate the role played by this amino acid in determining the relatively low binding affinity of IgG4 for the receptor, the reciprocal substitution of Leu and Phe was performed between IgG3 and IgG4 at this position. IgG3 (Phe234) is reduced in association constant ($K_v = 1.9 \times 10^8$ M$^{-1}$), indicating that residue 234, like residue 235, is important in receptor binding. The reciprocally substituted protein, IgG4 (Leu[234]) exhibits increased affinity for $F_c\gamma R_1$, however, the binding affinity does not reach the level of IgG3 (Table 2). Apparently, other aspects of the IgG4 molecule, potentially masked by the negative contribution of Phe(234), affect antibody receptor interactions. Sequence comparison reveals that among the C2 residues conserved in strongly binding IgG subclasses (human IgG1, -3, mouse IgG2a, IgG4 also differs at residues 330 and 331. These residues are within a hinge-proximal bend between two $\beta$ strands of the C2 domain predicted to fold in close proximity to the hinge-link region (Fig. 5). In IgG3, residues 330 and 331 are Ala and Pro, respectively; in IgG4, these are replaced by Ser-Ser, and in mouse IgG2b, by Ser-Pro. In as much as the affinity of IgG2b can be increased to the level of IgG3 by a single amino acid substitution in the hinge-link region, the presence of serine at position 330 does not appear detrimental to receptor interaction. To evaluate the role of Ser(331) in the binding affinity for $F_c\gamma R_1$, a second substitution was made in the mutant IgG4(Leu[234]) generating IgG4(Leu[234], Pro[331]); a reciprocal mutation was generated in IgG3 yielding IgG3(Ser[331]).

IgG3(Ser[331]) is reduced in affinity for $F_c\gamma R_1$ by a factor of 10, indicating that Pro(331), like Leu(234) and Leu(235), is important for high affinity receptor association (Table 2). Curiously, the double-mutant IgG4(Leu, Pro) shows no improvement in $K_v$ relative to IgG4(Leu[234]), and in fact appears reduced in affinity, indicating that the appropriate combination of factors for high affinity binding still has not been achieved.

Another feature of the IgG4 molecule that might contribute to its reduced affinity relative to IgG3 is its hinge, which is relatively rigid. To evaluate the possibility that the mutant IgG4(Leu[234]) is reduced in $K_v$ relative to IgG3 due to the stiff nature of its hinge region, the hinge of IgG4(Leu[234]) was replaced with a $\gamma_3$ hinge, yielding the antibody IgG4(Leu[234])Hy3. As indicated in Table 2, however, the

Figure 5. Graphic representation of the Fc region of IgG. C2 is shown in yellow, C3 and the C2-linked carbohydrate are in white. Pro 238 is red, Pro 331 is magenta, with the rest of the loop from 328 to 333 shown in blue. Ala 327 is light blue-green. The coordinates are from Diesenhofe (16); the graphic was made using the Macimad computer graphics package. The two Fc regions face in opposite directions, therefore, the residues visible on the right domain are invisible on the other side of the left domain.
hinge substitution does not improve binding affinity. Like the double-mutant IgG4(Leu, Pro), IgG4(Leu[234]), Hγ3 exhibits a Kd value reduced 10-fold from the level of IgG3.

There are several conclusions that can be drawn from these results. The association of IgG with FcγRI is directly influenced by multiple amino acid residues in the C2 domain. These residues are not limited to the peptide segment defined by Duncan et al. (15), but are also found in a hinge-influenced by multiple amino acid residues in the C3 domain. The association of IgG with FcγRI, is directly determined by the double-mutant IgG4(Leu, Pro), IgG4(Leu[234]), Hy3 hinge substitution does not improve binding affinity. Like the Fab and Fc regions, determines the accessibility of the main. These residues are not limited to the peptide segment allowing physical isolation (e.g., by fragmentation) allows for mutagenesis to isolate regions functionally without requiring physical isolation (e.g., by fragmentation) allows for the correlation of structure with function without artifacts introduced by loss of native conformation. Chimeric Igs have normal Ig function and, indeed, the values we obtain for the association constants of IgG1, -3, and -4 binding to the monocline receptor on U937 cells are in agreement with previous published reports (23).

The relative FcγRI affinities of the mutant antibody proteins examined here offer a number of insights into the role of IgG structure in effector function. The association constants of IgG2 and IgG3 are unaltered by exchange of the Cα3 domain between the subclasses, indicating that the structural basis for the nonbinding phenotype of IgG2 does not reside in the COOH-terminal domain. Likewise, the features of the IgG3 molecule that confer on it high affinity for FcγRI are not contained within the Cα3 domain. Admittedly, this analysis is limited to localizing the structural differences between proteins responsible for variations in functional efficiency. To the extent that the Cα3 domains of IgG2 and IgG3 contribute equally to receptor binding, the degree to which they contribute may not be determined. Comparison of the amino acid sequences of the Cα3 domains in IgG1, -2, -3, and -4 (14) reveals 11 positions at which the four subclasses are not identical. However, only one site of sequence difference between subclasses, located three residues from the COOH terminus of the Cα3 domain, is likely to be solvent accessible. As this residue is shared by IgG1, -2, and -3 (Pro) but differs in IgG4 (Leu), it could conceivably contribute to the reduced affinity of IgG4. Though it is impossible to rule out this contribution without specifically substituting this residue, its position relative to Cα2 residues, shown by us and others (15) to be critical to FcγRI binding, makes it an unlikely candidate for a role in receptor binding. We therefore conclude that the Cα3 domain of IgG does not contribute to the FcγRI affinity differences displayed by the subclasses.

The hinge region imparts segmental flexibility to IgG. Among the human IgG subclasses, IgG3 stands out as possessing the most radically different hinge structure and displays the greatest segmental flexibility (27); the exceptional flexibility of IgG3 is mediated by that region encoded by the first (unique) hinge exon (22). The role of a hinge per se for full effector function activity has been demonstrated for IgG1 using the hinge-deleted myeloma variants Dob and Lec; in these proteins, the close contact of Cα and Cα2 domains emphasizes the role of the hinge as a spacer between Fab and Fc. Likewise, in IgG3, deletion of the hinge region altogether abrogates FcγRI binding (as well as complement fixation [22]). Unlike IgG1, however, IgG3 appears to require a long spacer for full effector function. Hence, IgG3 bearing a hinge of 17 amino acids encoded by hinge exon 1, but lacking the rigid spacer encoded by the repetitive exons 2, 3, and 4, is reduced in affinity for FcγRI. We propose that the added Fab flexibility mediated by the IgG3 upper hinge would permit close approximation of the Fab arms and the FcγRI contact site were it not for the added distance provided by the extended hinge; thus, high Fab flexibility in IgG3h1 may sterically compromise Fc receptor access to its binding site. It should be noted that the amino acid composition of hinge segment 1 is different from that of the reiterated segments normally abutting the hinge-like region, however, five amino acids most proximal to the hinge-link are identical in all four hinge segments, and in fact, represent the conformationally constrained spacer unit (Cys - Pro - Arg - Pro). Interestingly, when the hinge reaches a certain length, added spacing appears detrimental to effector function, perhaps because of flexion in the polyproline helix. Hence, IgG3 with a hinge of 117 amino acids is compromised in its affinity for FcγRI, as well as in its ability to consume complement (22).

The role of the Cα1 domain in the association of IgG with human high affinity receptor appears to be limited to maintenance of overall quaternary structure since the Fc region alone shows full binding capacity. The requirement for this domain for full strength binding to FcγRI may represent the effects of steric hindrance on antibody receptor interaction by the Cx domain, without an analogous Cα domain with which to pair in these mutants. Both IgGΔCα1 and IgG3ΔCα1 showed reduced affinity for FcγRI with IgG1ΔCα1, showing the most profound reduction. The pattern of H-L disulfide bridging differs between IgG1 and IgG3:
the light chain in IgG1 bonds to the NH₂-proximal cysteine residue in the hinge region, whereas in IgG3 this bond is to a cysteine residue in the C₄₁ domain. Based on these patterns, it seemed likely that the assembly of IgG3 would be more disturbed by the C₄₁ deletion than that of IgG1. In fact, the contrary was observed. Apparently, in IgG3ΔC₄₁, the L chain is able to use one of the 11 cysteine residues in the extended hinge to form the H-L bridge. Electromicroscopic studies (V. Schumaker, unpublished results) have shown that the extended hinge of IgG3 is about the same size as a domain, and apparently the hinge can assume some of the roles played by C₄₁ in the C₄₁-deleted molecule. In IgG1ΔC₄₁, on the other hand, the hinge is much shorter, and apparently Cₓ cannot assume a conformation suitable for disulfide bonding with this hinge. Hence, IgG3ΔC₄₁ is secreted predominantly as covalent H₂L₂ tetramers, whereas IgG1ΔC₄₁ is secreted primarily as covalently linked H chain dimers.

The IgG region most clearly implicated in direct binding of the human FcγR₁ is the C₂₂ domain. Binding phenotype is transferred with this domain in exchanges between binding and nonbinding subclasses. Hence, IgG2 bearing the C₂₂ domain of IgG3 is improved at least 300-fold in receptor affinity. Though the mutant 2232 did not display IgG3-level FcγR₁ affinity, this is not entirely surprising considering the conformational contributions to Fc function made by the hinge region.

Intradomain substitutions within C₂₂ itself identify two regions of the primary structure as contributing to the differential subclass affinities for FcγR₁. The pair of leucine residues in the hinge-link region (positions 234 and 235, EU numbering system) shared among all high affinity IgGs examined are critical to Fc receptor association, since substitution of either of these residues with the amino acid present in subclasses of reduced affinity results in reduction of Kᵣ. Leu (bearing a branched hydrocarbon side chain) is replaced by a Glu residue (bearing a negatively charged side chain) at position 235 in muiIgG2b. The introduction of a charged residue at this site may interrupt hydrophobic interactions critical to receptor association, resulting in the severely compromised binding affinity of this Ig (>100-fold reduced relative to the strongly binding subclasses, huIgG1, -3, muiIgG2a). In human IgG4, on the other hand, Leu is replaced by Phe (bearing an aromatic side chain) at position 234. The introduction of a bulky aromatic group in this position may cause steric hindrance of the IgG-FcγR₁ interaction, however, the nonpolar environment of the region is maintained. This disturbance appears less severe than that caused by the Glu for Leu substitution, since IgG4 is reduced in binding affinity only 10-fold relative to the strongly binding subclasses.

Residue 331 (Pro in IgG1 and -3), near the COOH-terminal end of the domain sequence, also appears directly involved with receptor interaction, since FcγR₁ association is highly sensitive to substitution at this site. Though separated by 96 residues in linear sequence, these two sites are in fact quite close in space due to the tertiary structure of the C₂₂ domain (Fig. 5). Examination of the crystallographic structure of FcγR₁ by computer graphics using the atomic coordinates of Deisenhofer (16) reveals that residue 331 lies in a peptide loop between two β strands, at the hinge-proximal end of the domain. Though the hinge-link region is not resolved due to a high degree of disorder at this site in the crystal structure, Pro(331) folds into close proximity with Pro(238) (the first resolvable residue in the Fc) of the hexapeptide L - L - G - G - P - S discussed earlier (<11 Å between α-carbon atoms) (see Fig. 5). Our findings represent the first experimental evidence that the hinge-link region and hinge-proximal bend may function together in binding the human high affinity Fc receptor. These two regions may serve as contact points for receptor association, or alternatively, one region may be critical for stabilizing peptide conformation in the other region, allowing FcγR₁ proper access. A puzzling issue is why the Phe(234) and Pro(331) substitutions do not restore maximal binding to IgG₄. IgG₂ and IgG₄ differ from IgG₁ and IgG₃ at amino acid 327 (light blue-green in Fig. 5); this residue is Gly in IgG₂ and -4, and Ala in IgG₁ and -3. Residue 327 is not completely conserved for it is Asp in murine IgG₂b, and that substitution does not appear to compromise FcγR₁ binding. The Ala, Gly substitution would be highly conservative, however, the Gly substitution may impart additional mobility to the 328–333 bend and this mobility may impair binding.

Our results do not delimit the extent of FcγR₁-interacting residues. Indeed, Woof et al. (25) have pointed out that the COOH-terminal 20–30 residues of C₂₂ show numerous sites of identity between IgGs with high affinity for FcγR₁. Most of these sites extend along two of the three β strands in the Y-face of the domain, with Pro 331 falling at the bend between the strands. Thus, with regard to proximity of these residues to the hinge-link region, residue 331 and those neighboring it (Fig. 5) appear the most likely candidates for receptor interaction. Clearly, more site-specific substitution will be required to further delimit the residues critical to high affinity binding.

Clearly, multiple features of the IgG molecule influence its interaction with FcγR₁. In the divergent evolution of the four human IgG subclasses, factors making negative and positive contributions to binding have been sorted to yield the present binding phenotypes. Reduction of affinity may follow a single alteration in a structural feature required for receptor association, as demonstrated by the Glu(235) or Ser(331) substitutions of IgG3. On the other hand, increasing binding affinity may require an appropriate combination of features (e.g., the high segmental flexibility of IgG3 matched with the long hinge of this molecule) before an observable improvement is achieved. This may explain the seemingly paradoxical results obtained with the IgG₄ substitutions mutants. Engineering antibodies with desired receptors binding properties may require a combination of the proper structural alterations.
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