Localization of the Binding Site for the Monocyte Immunoglobulin (Ig) A-Fc Receptor (CD89) to the Domain Boundary Between Cα2 and Cα3 in Human IgA1

By Leonidas Carayannopoulos,* J. Mark Hexham,‡ and J. Donald Capra§

*From the *Program in Molecular Biophysics and Medical Scientist Training Program, the ‡Department of Microbiology, and the §Departments of Microbiology and Internal Medicine, The Molecular Immunology Center, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9140*

Summary

Immunoglobulin (Ig) A serves as the first line of humoral defense at all mucosal surfaces and is present in large quantities in blood. In playing its role in humoral immunity, IgA interacts with a variety of effector molecules present both in serum and on the surfaces of immune and inflammatory cells. To study these interactions, we previously established expression of human IgA1 in insect cells using recombinant baculoviruses and showed that the expressed antibody is a structurally and functionally intact polypeptide useful for examining the molecular properties of IgA. Indeed, since the Cα2 N-linked glycosylation site lies near the Fab-distal pole of Cα2, the inability of a mutant IgA1 lacking Cα2 N-glycosylation to bind its cognate receptor suggested that the monocyte Fcα receptor (mFcαR) recognizes IgA at a hinge-distal site encompassing the boundary between the Cα2 and Cα3 domains. In this report, we utilize both domain-swapped IgA/IgG and point-mutated IgA chimeras to verify the above hypothesis. Using an antigen-specific rosetting assay and a mFcαR-expressing cell line, we show that: (*a*) Cα2 and Cα3 together are necessary and sufficient for binding; (*b*) neither the IgA hinge nor the tailpiece is necessary for binding; (*c*) mutations away from the interdomain boundary do not affect binding; and (*d*) mutations located near the three-dimensional boundary between Cα2 and Cα3 completely disrupt binding. Taken together, these results localize the mFcαR recognition site on IgA to the boundary region between the second and third constant domains — a site analogous to that recognized by *Staphylococcus aureus* protein A on IgG. The use of this hinge-distal site is, to date, unique among Fc receptors of the Ig superfamily.

IgA is the dominant Ig at mucosal surfaces and in exocrine secretions; thus, in intact mammals, this molecule serves as the first line of immunologic defense against pathogenic invasion. Moreover, its high concentration in serum (1) and its ability to recruit neutrophils (2), eosinophils (3), and macrophages (4) suggest a role in immune responses against pathogens that have already penetrated mucosal barriers. In fulfilling its functions, IgA participates in a variety of intermolecular associations including those with the intracellular folding/assembly apparatus, the C3Bb convertase of complement (5), the poly-Ig receptor (pIgR) (6), and the surface receptors of cells that respond to IgA-coated antigens. Each of these interactions depends on the ability of the relevant effector molecules to recognize specific and as yet undefined surfaces on the IgA constant region (Cα). To fully understand and to potentially beneficially modulate immunity mediated by IgA, the specific features of these interactions should be investigated; in particular, the interaction between IgA and CD89 is of interest. CD89 is the monocyte/granulocyte Fcα receptor (mFcαR)[1] that serves to trigger inflammatory cell responses against IgA-coated antigens (2, 4). It is a single-pass transmembrane protein possessing two Ig-type extracellular domains. The molecule shows similarity to the high-affinity FcεR and the three FcγR (CD 64, 32, and 16) but is more distantly related to these receptors than they are to one another (7).

Previously (8), recombinant baculoviruses were shown to be useful for the expression of antigen-specific, human IgA1 whose in vitro functional and immunologic properties were largely identical to those of the natural protein. Using that system, it was also shown that mutant hapten-specific IgA lacking Cα2 N-linked glycosylation fails to mediate interaction between hapten-coated targets and cells expressing the monocytic/granulocytic FcαR CD89. However,

[1] *Abbreviations used in this paper:* ars, p-azophenylarsonate; mFcαR, monocyte/granulocyte Fcα receptor; Vars, V region of hybridoma 93G7.

wild-type IgA expressed in insect cells can mediate that interaction even though its N-linked sugars differ in composition from those found in natural human IgA. It was postulated that the sugar per se does not participate in the interaction between Cα and its cognate receptor (since to a first approximation, sugar composition appeared irrelevant). Rather, it was hypothesized that the mere presence of Cα2 glycosylation serves to maintain a functional binding site through conformational effects. Based on the location of the sugar distal to the classical intradomain disulfide bond in Cα2, it was further hypothesized that the site on Cα recognized by mFcαR lay at the intradomain boundary between Cα2 and Cα3 (8), and not at proximal Cα2 abutting the hinge as would be expected given the paradigm established in the Cγ and Cε systems (9–12, and reviewed in 13).

To test the above prediction, various domain-swapped and point-mutated IgA1 molecules with hapten specificity were expressed in insect cells and assayed for their ability to mediate interaction between hapten-coated targets and mFcαR-expressing cells. The results of these assays affirm the hypothesis.

Materials and Methods

Synthesis of Coding Regions. DNA fragments corresponding to the heavy and light chain V regions of the hybridoma 93G7 (Vars) were amplified using PCR from the previously described plasmids pHγ1-360E and pHκ-360E (14), respectively; these V regions encode *p*-azophenylarsonate (ars) specificity. The oligonucleotide primers for these amplifications included a 5′ NcoI site at the initiation codon and a 12-nucleotide antisense overlap with Cα at the 3′ end. The coding regions of human Cα1, Cγ1, and Cκ were obtained from human peripheral blood leukocyte RNA by reverse transcription–PCR as previously described (15). In this case, the primers included a 12-nucleotide sense overlap with the appropriate V region at the 5′ end and an XbaI site at the 3′ end. The appropriate V and C regions were joined by PCR overlap extension (16) using a total overlap of 24 bp at the highest annealing temperature that still yielded a product. A similar approach was used to create domain-swapped mutants. For example, to create a sequence with the Vars and Cγ1 domains coupled to Fcα, the 5′ Vars primer was used along with a 3′ Cγ1 primer containing an overlap with Cα2 to amplify a 5′ fragment; this was joined to an analogously generated 3′ fragment (made using a 5′ Cα2 primer with a Cγ1 overlap and the standard Cα 3′ primer) using overlap extension. Point mutants were generated using overlap primers that incorporate the point mutation. Residue locations as described in the text are numbered according to Kabat et al. (17). All amplifications were performed using PFU polymerase (2.5U; Stratagene Inc., La Jolla, CA) in order to minimize PCR errors.

Construction of Baculovirus Transfer Vectors. The resultant inserts were digested, purified on low-melt agarose gels, and ligated into the unique NcoI and XbaI sites in pH-360EX using standard techniques (18). All constructs were sequenced using T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) or Taq polymerase (Fisher Biosciences, Pittsburgh, PA) using the manufacturer's protocols.

Production of Recombinant Viruses. Transfer vector DNA was isolated by alkaline lysis "miniprep" except that DNA was also subjected to sequential protease K digestion, phenol/chloroform extraction, and chloroform extraction before final precipitation, all as described (18). 4 μg of transfer plasmid was cotransfected with linear wild-type baculoviral DNA into *Spodoptera frugiperidum* cells using cationic liposomes (Invitrogen, San Diego, CA) as instructed by the manufacturer, except that 0.25 μg of linear DNA was used. Growth, plaque purification, and titration of viruses was standard (19).

Isolation of Recombinant Proteins. Cell supernatants were adjusted to 20 mM Tris, pH 7.5, 10 mM EGTA, 10 mM EDTA, and 1 mM PMSF. They were then centrifuged at 90,000 *g* for 40 min. Resultant supernatant was then adjusted to 40% saturation with ammonium sulfate and left standing for more than 1 h at 25°C; the mixture was centrifuged at 2,500 *g* for 40 min at 25°C. The pellets were resuspended in Milli-Q water and reprecipitated. The final pellet was resuspended into PBS, pH 7.4/0.02% Na-azide and dialyzed into same.

Protein Analysis. Supernatants were analyzed by SDS-PAGE according to Laemmli (20) with and without reduction. Immunoblotting was performed in 10% methanol/10 mM sodium cyclohexylamino-propanesulfonate (CAPS), pH 11, onto polyvinylidene difluoride membranes (0.2 μm, Millipore Corp., Bedford, MA). Membranes were blocked with 2% nonfat dry milk in TBST (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% bovine albumin, and 0.05% Tween 20. Proteins were detected using appropriate antisera conjugated to alkaline phosphatase followed by nitro-blue tetrazolium and bromochloroindole phosphate (NBT-BCIP; GIBCO-BRL, Gaithersburg, MD) according to supplied instructions.

ELISA-based Assays. IgA concentrations were measured using sandwich ELISA. 200 ng of monoclonal anti-IgA1 antibody (courtesy of Roy Jefferis, University of Birmingham, UK [21]) was coated onto wells of Maxisorp (Nunc, Roskilde, Denmark) 96-well plates that were then blocked using 200 ml 2% nonfat dry milk in TBST (25°C for >2 h). Recombinant wild-type ars-specific IgA1 or purified serum IgA1 standard was then diluted into 100 ml TBST, applied to the coated wells, and detected using goat anti–human IgA coupled to alkaline phosphatase (The Binding Site Ltd., San Diego, CA). Plates were developed using *p*-nitrophenyl phosphate (1 mg/ml in 50 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$). Recombinant wild-type IgA was quantitated by comparison of sample absorbances to the standard dilution curve. Ars-specific mutant antibody was detected, assayed for reactivity with mAbs, and quantitated by sandwich ELISA as above except that ars-derivatized BSA was used as the capture reagent and anti–human Cκ coupled to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was used as the detecting reagent. Mutants were standardized by comparison to the previously quantitated wild-type recombinant IgA1. Standardized mutant and wild-type IgA1 molecules were also compared with regard to reactivity with mAbs M4C11 (anti-Fab), M4D8 (anti-Fc), and 2D7 (anti-Fc requiring intact interchain association) (21) using the technique described above with ars-BSA as the capture reagent. All steps were followed by three sequential washes with 200 μl TBST. All coating steps were at room temperature for over 1 h.

FcαR-expressing Cells. The promyelocytic leukemia line HL-60 (22) was obtained from the American Type Culture Collection (Rockville, MD) and propagated in SSFL medium. SSFL is a variant of a serum-free medium described by Baker and Knoblock (23) and consists of IMDM (Sigma Chemical Co.) reconstituted as per manufacturer and supplemented with 30 mg/l iron-saturated human transferrin (Boehringer Mannheim, Indianapolis, IN), 5 mg/l porcine insulin, 50 mg/l low-endotoxin bovine al-

bumin, 20 μM ethanolamine, and 5 ml/l cholesterol-rich lipid suspension (all from Sigma Chemical Co.). Cells were induced to express mFcαR/CD89 by growth in SSFL with 500 nM calcitriol (courtesy of Milan Uskokovic and Hoffman-La Roche, Nutley, NJ) for 5 d as described (24). Induction of CD89 on HL-60 cells was checked by standard flow cytometry using the My43 (IgM) anti-CD89 mAb (4).

Rosetting. Sheep E (Colorado Serum Co., Denver, CO) were derivatized with ars exactly as described (25) after which they were washed five times in 100 vol of HBSS. The haptenated E (HE) were then coated with sensitizing antibody or random serum Ig (in HBSS) for 12 h at 4°C, followed by further washing. 100 μl of 1% antibody-coated HE (AHE) was then mixed with 2×10^5 calcitriol-induced HL-60 cells, also in 100 μl of HBSS, with or without inhibitors. The mixture was gently agitated and incubated on ice for 20 min; the cells were then pelleted at 130 *g* in a swinging bucket microfuge and left on ice for a further 2 h. Supernatant was removed and the pellet was resuspended in 300 ml HBSS containing 0.0025% acridine orange. The cells were then dribbled on a slide, covered with a coverslip, and counted under UV illumination and low visible light such that rosettes and HL-60 nuclear fluorescence were simultaneously visible. 200 lymphocytes were counted and percent rosetting was expressed as percent rosetted HL-60 cells/total HL-60 counted (mean of three experiments). Rosettes were defined as fluorescent cells attached to four or more AHE. The specificity of all results was verified by the ability of both My43 and excess nonspecific IgA1 to individually inhibit rosetting.

Results

Cα2 and Cα3 But Not the Hinge Are Necessary and Sufficient for Binding. In an effort to grossly localize the binding site for mFcαR on Cα, mutant antibodies were generated whose C regions were composed of domains of Cα and Cγ in all possible permutations (Table 1). With the exception of the NH_2-terminal boundary of Cα2, the NH_2- and COOH-terminal boundaries of domains were determined by the 5′ and 3′ limits, respectively, of the corresponding genomic exons. In the case of Cα2, two NH_2-terminal limits were utilized, one at the beginning of the IgA1 hinge (the exonic 5′ limit) and the other at the end of the IgA1 hinge (substituting instead the IgG1 hinge). This was done so that the role of the IgA hinge was explicitly tested. An IgA lacking the COOH-terminal "tail" was also produced. A model of IgA monomer structure has been proposed (26) that postulates a disulfide bond between the tail and a site in Cα2. Given such a model, it was important to determine the necessity of the tail for receptor binding; since in reaching the Cα2 cysteine, the tail would necessarily span the Cα2-Cα3 boundary. Correct construction of each mutant was verified by complete sequencing of the coding regions and by immunoblotting of chimeric molecules with appropriate anti-Cα and anti-Cγ antisera (8, and data not shown). Binding of mutants to FcαR was tested using a previously described rosetting assay (4, 8) in which ars-derivatized E coated with anti-ars mutants were examined for their ability to rosette around HL-60 cells induced to express mFcαR (24). In all cases, specificity of rosetting was verified by the ability of anti-mFcαR mAb (My43) or a vast excess of soluble IgA to competitively inhibit rosetting.

The stated hypothesis involves an FcαR binding site at the boundary of the second and third constant domains, including surface provided by both domains, analogous to the site on Cγ recognized by the B fragment of protein A (27, 28). This hypothesis predicted that, of the IgA/G mutants listed in Table 1, only those with both Fcα domains present could have bound cognate receptor and thus mediated specific rosetting. Furthermore, the removal of the hinge (though genetically part of Cα2) should not have affected rosetting. No prediction was made regarding the tail first, because of the uncertainty regarding the existence of the tail-Cα2 disulfide, and second, because of the inability to predict whether the tail would actually block the postulated binding site were that disulfide to exist. The results of rosetting experiments utilizing the domain-swapped mutants appear in the last column of Table 1. As predicted, only the VAAA and VGAA chimeras were able to mediate rosetting through mFcαR despite the latter molecule's lack of an IgA hinge; thus, both Cα2 and Cα3 are necessary and sufficient for binding to CD89 irrespective of the hinge region. Removal of the tail failed to interfere with rosetting as demonstrated by the ability of the VAAA t− antibody to mediate rosetting.

This interpretation of the data is predicated on the assumption that individual Cα domains in the chimeras fold appropriately and somewhat independently despite substitution of wild-type neighboring domains with analogues from Cγ1. It is believed, however, that this assumption is reli-

Table 1. *Domain Composition of Chimeric Constructs and Their Capacity to Mediate CD89-specific Rosetting*

Construct	C_H1	Hinge	C_H2	C_H3	Tail	Binding*‡
VAAA	α	α	α	α	+	++
VAAG	α	α	α	γ	n/a§	−−
VAGA	α	α	γ	α	+	−−
VAGG	α	γ	γ	γ	n/a	−−
VGAA	γ	γ	α	α	+	++
VGAG	γ	α	α	γ	n/a	−−
VGGA	γ	γ	γ	α	+	−−
VGGG	γ	γ	γ	γ	n/a	−−
VAAA t−	α	α	α	α	−−	++

Domain boundaries are those of the genomic exons except for construct VGAA which incorporates the hinge of IgG1 rather than the IgA1 hinge encoded by the C_H2 exon of IgA1.

*++ refers to rosetting of >25% of assayed HL-60 cells taken as the average of three experiments; −− refers to rosetting of <5% of assayed HL-60 cells taken as the average of three experiments. Root-mean-squared deviations of positive and negative results were no greater than 10 and 50% of the means, respectively.

‡The specificity of all results was verified by the ability of anti-CD89 mAb as well as vast excess of free, nonspecific IgA1 to block rosetting.

§Not applicable since IgG has no terminal tail.

able for the following reasons. First, the Igs are secreted with good yield as disulfide-linked C_H dimers (i.e., as Ig monomers) that are recognized in solution by appropriate polyclonal and monoclonal reagents (8, and data not shown). A priori, it is unlikely that grossly malfolded molecules would be secreted from the endoplasmic reticulum to form stable, nonaggregating solutions. Also, the presence of interchain cystines and correct surface epitopes argues for, at the least, correct orientation of buried and exposed surfaces that likely constrain attainable backbone conformations to those close to wild type. Second, the robustness of Ig domain folding is strongly supported by the literature which demonstrates largely intact structure and/or function of mutant Igs in the face of (*a*) domain chimerism (28–30), (*b*) neighboring domain removal (27), and (*c*) unnatural linkages between domains (31). Lastly, the above data demonstrate the ability of Fcα to, at the very least, maintain receptor-binding conformation in spite of sequence changes at the NH_2 and COOH termini (e.g., deletion of the tail, substitution of the hinge and/or Cα1). Nevertheless, the above does not exclude the possibility that the CD89 binding site only requires one of the Fc domains, with the other domain merely stabilizing overall conformation.

Mutation of Residues Predicted to Lie at the Cα2-Cα3 Boundary, But Not Elsewhere, Abrogate IgA-mediated Rosetting. To more finely probe the binding site, point mutants of IgA1 were generated and assayed. Understanding of the rationale behind these mutations is aided by referral to Fig. 1. Fig. 1 shows an alignment of the sequences of Cα2 and Cα3 versus Cγ2 and Cγ3, respectively; secondary and tertiary structural characteristics of the two Cγ domains are indicated by symbols as explained in the figure legend and are derived from the data presented by Deisenhofer et al. (27, 28). As can be seen, sequence identity between the two C_H2 domains (25%) is much less than that between the two C_H3 domains (40%); thus, prediction of Cα2 structure by analogy to Cγ2 is a risky endeavor. Fortunately however, much of the area of interest lies either in Cα3 or in an area of Cα2 near conserved landmarks (e.g., domain boundaries, classical cysteine, strand-breaking prolines) that allow orientation. Specifically, the classical Ig cysteine at position 274 in Cα2 must lie in an "upgoing" β-strand as it does in all Ig domains (except, of course, those lacking the cysteine). Thus, the previously described N271Q mutation, which abrogated receptor binding, removed a carbohydrate chain from the NH_2-terminal (distal) end of the ascending strand containing the classical cysteine. By analogy to IgG, it had been predicted that a long loop located at the three-dimensional interdomain boundary preceded that strand; lack of the carbohydrate had presumably prevented the loop from attaining its wild-type, receptor-binding conformation. To directly test the role of the loop in receptor recognition, L266 was mutated to arginine. This site was selected because it lies in a hydrophobic stretch of residues whose analogue in Cγ interacts with *Staphylococcus aureus* protein A (SpA) (28, and Fig. 1). Furthermore, I266 in IgG is fully solvent exposed, is completely buried by protein A, and does not participate in interactions with Cγ3. Mutation to arginine was chosen because such a change was extreme in terms of charge, hydrophobicity, and in particular,

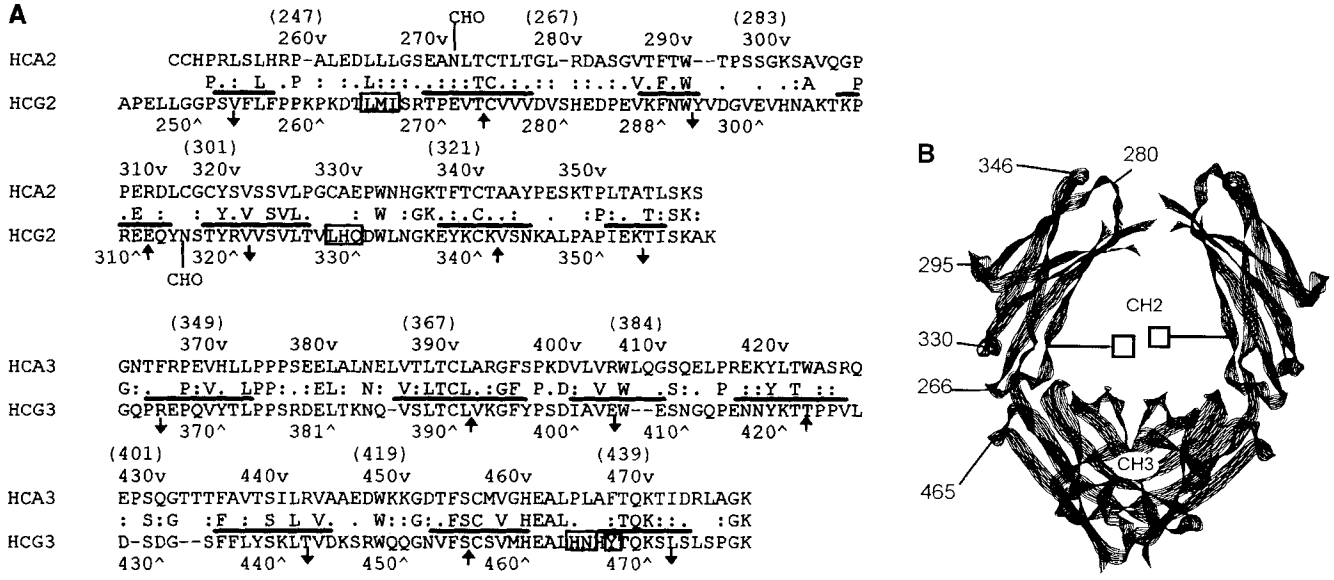

Figure 1. (*A*) Alignment of the Fc domains of human IgA1 and IgG1. Letters between the two sequences signify identities; (:) one-nucleotide differences in the respective codons; (- -) gaps. (*CHO*) N-linked carbohydrate positions; (*bars*) approximate β-strand locations in the IgG sequence; and (*boxes*) residues in Fcγ known to interact with the B fragment of protein A (26, 27). The directionality of the β-strands in three dimensions is indicated by arrows. (↑) Strands that ascend toward the hinge; (↓) strands that start more proximally and stretch away from the hinge. Primary numbering is according to Kabat et al. (17) with corresponding Eu residue numbering in parentheses. Alignments were performed using the DNASTAR™ software (DNASTAR, Madison, WI). (*B*) Ribbon drawing of the backbone of Fcγ based on the coordinates of Deisenhofer et al. (27). Positions of Fcα mutations described in the text are numbered and positioned by analogy to Fcγ. Predicted positions of the Cα2 N-linked sugar are marked by the boxes. Coordinates were downloaded from the Brookhaven database and rendered using HyperChem™ software (Hypercube Inc., Waterloo, ON, Canada).

Table 2. *IgA1 Point Mutants and Their Ability to Mediate Rosetting via the Macrophage/Monocyte Fcα Receptor*

Construct*	Predicted location by analogy to IgG‡	Binding§‖
N271Q (258)	Boundary of a distal loop and ascending strand of C_H2 domain	−−
L266R (253)	Solvent-exposed residue of loop influenced by 271 N-linked sugar	−−
L465R (434)	Proximal loop of C_H3 that contacts the L266 loop	−−
E330R (311)	Distal loop of C_H2; not part of the C_H2-C_H3 boundary	++ ++
R280H (267)	Proximal loop of C_H2 near the hinge	++
ESK346KLE (327)	Proximal loop of C_H2 near the hinge and distinct from that of R280	++
SSGKS295VEGHT (280)	Solvent-exposed distal loop of C_H2 away from domain boundary	++

*The letters preceding the numbers refer to wild-type residues; those following the numbers refer to the mutated residues; the numbers themselves refer to the position of the first mutated residue following the numbering scheme of Kabat et al. (17) with Eu numbering in parentheses. For example, ESK346KLE means that residues 346, 347, and 348 were mutated from ESK to KLE. Kabat numbering is used in the text.

‡Here, distal and proximal refer to the position of a loop in three-dimensional space relative to the V region.

§++ refers to rosetting of >25% of assayed HL60 cells taken as the average of three experiments; −− refers to rosetting of <5% of assayed HL60 cells taken as the average of three experiments. RMS deviations of positive and negative results were no greater than 10 and 50% of the means, respectively.

‖The specificity of all results was verified by the ability of anti-CD89 mAb as well as vast excess of free, nonspecific IgA1 to block rosetting.

size. Since the rosetting assay is insensitive to changes in affinity because of its multivalent nature (data not shown), a mutation had to be selected that would disrupt the binding surface sufficiently to cause the great drop in affinity necessary for detection by rosetting. As shown in Table 2, the L266R mutation abrogates binding as predicted. Since the domain-swapping experiments suggested a binding site composed of surfaces from both Cα2 and Cα3, an L465R mutant was also generated. This site, also by analogy to IgG, is predicted to lie in a Cα3 loop in contact with the Cα2 loop mutated above (see Fig. 1 *B*). Furthermore, N465 in IgG is solvent exposed, does not participate in interdomain contact, but does form part of the binding site for protein A (28). As shown in Table 2, the L465R mutation also abrogates binding to CD89. Mutations were also made at hinge-proximal locations; none of these influences binding as assessed by rosetting. The E330R mutation fails to interfere with rosetting despite its predicted location in a loop near that of L266 and despite the partial interaction of IgG Q330 with protein A.

In summary, mutations were made at positions predicted to lie in five loop regions of the Cα2 domain. Of these, only the mutation L266R, in the loop between the 4-1 and 4-2 strands (nomenclature of Edmundson et al. [32]), abrogates rosetting mediated by Fcα-FcαR interaction. Furthermore, a Cα3 mutation (L465R) in the loop predicted to contact the region of L266 also abrogates rosetting.

Discussion

Discussion of Results. The results presented above are as follows. (*a*) Cα2 and Cα3 are necessary and sufficient for binding of IgA to the mFcαR. Neither the Fab, the hinge, nor the tail appears to play a role in the interaction. (*b*) The N271Q mutation, which prevents glycosylation in Cα2, eliminates interaction between Fcα and CD89. (*c*) Of several mutations generated in the Cα2 interstrand loops, only a mutation (L266R) in the loop that bridges strands 4-1 and 4-2 abrogates mFcαR-mediated rosetting; by analogy to Cγ, this loop likely lies at the Cα2-Cα3 interface. (*d*) A mutation (L465R) in a proximal loop of Cα3 predicted to contact the region of L266 at the Cα2-Cα3 boundary also abrogates rosetting. Several caveats to these results come to mind; these are addressed as follows.

First, positional analogy to IgG may be flawed, resulting in uninterpretable placement of mutations; this is highly unlikely. The conserved features of the Ig domain and this motif's robustness to sequence alteration are amply described (33). Though there is no doubt that the details (e.g., the residues at which strands give way to loops, torsion angles of bonds, etc.) of IgA structure differ from those of IgG, the fundamental secondary structural assumptions employed in this study are likely valid, especially in C_H3 where sequence identity between IgA and IgG is high, and at locations in Cα2 close to well-conserved landmarks. For example, L266 appears before the first classical cysteine (which

must lie in upgoing strand 4-2); since L266 also follows a conserved proline (P259) that breaks the preceding and downgoing 4-1 strand, the position in question must lie in a loop between strands 4-1 and 4-2 at the lower end of the domain. The fact that L266 is also part of a well-conserved hydrophobic region in that loop (28) supports the argument. Similar logic can be applied to the other mutant sites described earlier. A second caveat can be raised stating that the above mutations do not represent precise changes in the binding site but rather gross distortions of domain conformation that necessarily abrogate receptor binding by virtue of their global nature. This also is improbable on a priori grounds alone since none of the mutations violate the β-sandwich core of the domains. The Cα2 domain would seem to be particularly difficult to deform since its backbone is constrained not only by the conserved cysteine, but also by an additional interdomain cystine (residue 251) and at least one "extra" intradomain disulfide bond. Two empirical arguments against this "global deformation" concern can also be made. First, the mutations that do not eliminate rosetting (especially the extensive SSGKS to VEGHT alteration at postion 295) serve as an internal control for the method since they fail to globally distort Fcα. Second, three anti-Cα mABs (M4C11, M4D8, and 2D7) (21) react similarly with wild-type, L266R, and L465R IgA1 molecules, indicating that structural distortion induced by these mutations fails to extend to the epitopes probed by these mAbs, and thus further arguing against global destruction of Cα conformation by these alterations.

Evolutionary Perspective. Taken in toto, therefore, the results presented here indicate that CD89 recognizes IgA at a site at the boundary between the two Fc domains. Such a site is analogous to that on IgG recognized by SpA (27), the neonatal rodent intestinal FcγR FcRn (34), and an as yet unidentified factor involved in IgG clearance (35). Except for the α3 domain of the MHC class I–like FcRn (36), neither of the above characterized molecules belongs to the Ig superfamily (IgSF), and none of the above is involved in antibody effector function. CD89 is the first IgSF FcR whose binding site lies at a hinge-distal position; FcγR I, II, and III (CD 64, 32, and 16, respectively), along with FcεRI, all recognize their cognate Fc at positions on the penultimate Fc domain just distal to and perhaps including the hinge (9–12, 30). This divergence in binding site is somewhat surprising on evolutionary grounds. Based on sequence analysis (7) and chromosomal location (37), CD89 is more distantly related to the other four IgSF FcR than they are to one another; nevertheless, they are related and likely evolved from a common ancestor. Simultaneously as these receptors diverged, they coevolved with their respective cognate Fc isotypic ligands, which similarly arose by duplication and adaptation from a common — likely four domain, μ-like (38 and references therein) — ancestral gene (39). If it is assumed that the primordial FcR recognized its respective primordial Fc at a site near the hinge, the CD89 protein must have undergone a quantum change in specificity from the primordial site to the present-day C_H2-C_H3 boundary site. Of course, the converse scenario where the primordial site was actually the C_H2-C_H3 boundary is also possible. Either way, an ancestral FcR probably had its Fc specificity suddenly translocated, but not eliminated, by reasonably small changes in sequence in the receptor itself and/or in the Fc region; however, further comparative/veterinary studies are necessary to shed light on this issue.

The teleologic consequences of this difference in site localization are perhaps more clear. The mucosal surfaces, especially those of the alimentary tract, are rich in nonspecific proteases (which are well known to cleave Igs at the hinge) as well as bacterial proteases whose only known substrate is human, gorilla, and chimpanzee IgA1 hinge (reviewed in 40–43). Heavy O-linked glycosylation of the IgA-extended hinge presumably serves as sufficient protection against attack by nonspecific proteases that evolve independently of IgA cleavage. In contrast to this relatively static relationship between IgA and host digestive enzymes, IgA of higher primates and bacterial IgA proteases are locked in an ongoing process of "evolutionary one-upmanship." The wide prevalence of IgA proteases in both gram-negative and -positive pathogenic bacteria, the lack of IgA protease in nonpathogenic strains, and the convergent functional evolution of unrelated IgA proteases, all demonstrate the adaptive benefit of IgA protease to bacteria that colonize hominoids. Retention (44) of the protease-resistant (45), hinge-deleted IgA2 isotype in hominoids whose IgA1 molecules are susceptible to the bacterial IgA proteases represents the host side of this coevolutionary process, the immediacy of which is further highlighted by the relatively recent appearance (based on isoallotypic studies of primate IgA [46]) of the A2m(2) allotype which resists cleavage by *Clostridium ramosum* IgA protease, the only IgA protease known to cleave IgA2m(1) (47). Accordingly, because of the selective pressure of pathogen proteases, IgA hinge variants prove advantageous to the host species. Given the apparent ability of hinge deletions to interfere with hinge-proximal FcR binding sites (9, 48), the location of the phagocyte FcαR binding site away from the hinge may thus allow the retention of resistant IgA hinge mutants that nevertheless preserve the ability to recruit effector cells.

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Address correspondence to Dr. J. Donald Capra, Department of Microbiology and Internal Medicine, The Molecular Immunology Center, University of Texas Southwestern Medical Center, Dallas, TX 75235-9140.

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