

Domain One of the High Affinity IgE Receptor, FcεRI, Regulates Binding to IgE through Its Interface with Domain Two*

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The high affinity receptor for IgE, FcεRI, binds IgE through the second Ig-like domain of the α subunit. The role of the first Ig-like domain is not well understood, but it is required for optimal binding of IgE to FcεRI, either through a minor contact interaction or in a supporting structural capacity. The results reported here demonstrate that domain one of FcεRI plays a major structural role supporting the presentation of the ligand-binding site, by interactions generated within the interdomain interface. Analysis of a series of chimeric receptors and point mutants indicated that specific residues within the A' strand of domain one are crucial to the maintenance of the interdomain interface, and IgE binding. Mutation of the Arg¹⁵ and Phe¹⁷ residues caused loss in ligand binding, and utilizing a homology model of FcεRI-α based on the solved structure of FcγRIIa, it appears likely that this decrease is brought about by collapse of the interface and consequently the IgE-binding site. In addition discrepancies in results of previous studies using chimeric IgE receptors comprising FcεRIα with either FcγRIIa or FcγRIIIa can be explained by the presence or absence of Arg¹⁵ and its influence on the IgE-binding site. The data presented here suggest that the second domain of FcεRI-α is the only domain involved in direct contact with the IgE ligand and that domain one has a structural function of great importance in maintaining the integrity of the interdomain interface and, through it, the ligand-binding site.

The high affinity IgE receptor, FcεRI, is a tetrameric complex composed of an IgE-binding α subunit associated with a tetraspan β subunit and homodimeric γ subunits and is a key player in IgE dependent effector mechanisms. The α subunit, FcεRI-α, is the ligand-binding chain and is composed of two Ig-like domains. The role of the second domain has been clearly defined as containing the IgE-binding region. However, the role of

the first domain is not clear in FcεRI nor indeed in any Fc receptor. Analyses to date have variously indicated that domain one is necessary for optimal binding (1–3), that it has a possible role in direct interaction with IgE (4, 5), and that it provides a supportive role in maintaining receptor integrity (1, 2). The structural reasons for this are not apparent. FcεRI, however, is related to FcγRIIa, and the recent description of the three dimensional structure of FcγRIIa (6), FcγRIIb (7), and FcεRI-α (8) may provide a basis for the understanding of the roles of the individual domains in FcεRI and other Fc receptors.

In the crystal structure of FcγRIIa the extracellular domains are “bent” to form an acute angle (52°) between domains 1 and 2. In this orientation, the IgG-binding site of domain 2 points away from the cell in such a manner as to be accessible to ligand, and domain 1 is angled away from the binding site and down toward the cell membrane. The acute angle is dictated by interactions within the interdomain interface, and the structural studies indicate that domain 1 is likely to support domain 2 providing an architectural role in the positioning of the binding site. Because FcεRI and FcγRIIa show 40% amino acid identity and considerably higher amino acid homology, it is probable that FcεRI has a similar structure to that of FcγRIIa, confirmed by the recent publication of the solved FcεRI structure (8).

In the study described herein we have utilized a model of FcεRI-α (see Fig. 1) based on the solved crystal structure of FcγRIIa (6) and undertaken a mutagenesis study of domain 1 to define its role in the interaction with IgE. The solved x-ray structure of FcεRI-α (8) strongly resembles that of FcγRIIa and therefore that of the FcεRI-α homology model. Indeed, the FcεRI-α homology model and the x-ray structure of FcεRI-α, as described by Garman *et al.* (8), show compelling concurrence in comparisons of structure and molecular interactions. Here, data from the chimeric Fc receptors and alanine mutants have been used together with molecular modeling to propose a functional structure of FcεRI-α.

EXPERIMENTAL PROCEDURES

Production and Nomenclature of FcεRI-α Chimeric cDNA Receptor Constructs—Two previously produced chimeric cDNA receptor constructs (1) were used as templates in the construction of this series of FcRs. The amino acid sequences of the chimeras and chimera nomenclature are displayed in Table I. The first template was designated εεγ and comprised domain one (D1)¹ and domain two (D2) of FcεRI-α linked with the transmembrane region and cytoplasmic membrane anchor of

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¹ The abbreviations used are: D1, domain one; D2, domain 2; SOE-PCR, splice overlap extension-polymerase chain reaction; TNP, trinitrophenol; mIgE, mouse anti-TNP IgE; mIgG, mouse anti-TNP IgG; mAb, monoclonal antibodies; EA, erythrocyte-antibody.



FIG. 1. Homology model of the extracellular region of Fc ϵ RI- α , based on the solved crystal structure of Fc γ RIIa. The β strands of each domain are labeled, as are the carboxyl (c-) and amino (n-) termini. The IgE-binding region is indicated in pink, and the A' strand of domain one is indicated in green.

Fc γ RIIa. The second chimeric template was based on a simple domain exchange and comprised D1 of Fc γ RIIa and D2 of Fc ϵ RI- α , also with the transmembrane region and cytoplasmic sequence of Fc γ RIIa, and was designated $\gamma\epsilon\gamma$. Chimeric receptors were generated using the template receptor $\gamma\epsilon\gamma$ or $\epsilon\epsilon\gamma$. Specific loops, strands, or regions of the Fc γ RIIa D1 were replaced with the equivalent portion of Fc ϵ RI (or *vice versa*) to produce a series of chimeric receptors using splice overlap extension-polymerase chain reaction (SOE-PCR) using the method previously reported (9). A further template receptor was constructed with a glycosyl-phosphatidylinositol membrane anchor of Fc γ RIIIB replacing the Fc γ RIIa cytoplasmic tail of the $\gamma\epsilon\gamma$ construct. This chimera was designated $\gamma\epsilon$ RIII and was generated by SOE-PCR. Substitution into domain one of the $\gamma\epsilon$ RIII template receptor of the A strand of Fc ϵ RI D1 produced the γ (A ϵ)RIII chimera. The Fc ϵ RI D1 A' strand point mutants, R15A and F17A, were made using SOE-PCR and incorporated into $\epsilon\epsilon\gamma$. The Fc ϵ RI D1 A' strand point mutants N14A and R15L were constructed using the QuikChangeTM site-directed mutagenesis kit (Stratagene). cDNA was purified by centrifugation in a CsCl gradient (10), and the mutations were verified by nucleotide sequencing.

Production and Purification of IgE—Human IgE (hIgE) (ATCC clone TIB196) was affinity purified over an anti-human IgE affinity column (ATCC clone HB121 purified supernatant, coupled to cyanogen bromide-activated Sepharose beads (Amersham Pharmacia Biotech). Bound IgE was eluted in 1-ml fractions with 0.5 M sodium citrate/0.5 M NaCl (pH 2.7) into tubes containing 50 μ l 1 M Tris (pH 9.0), buffer exchanged to phosphate-buffered saline (7.6 mM Na₂HPO₄/3.25 mM NaH₂PO₄/145 mM NaCl, pH 7.4) on a Sephadex G-25 PD-10 column (Amersham Pharmacia Biotech) and concentrated in a centrifugal concentrator (Macrosep, Filtron, Life Technologies, Inc.).

Detection of IgE Activity by Enzyme-linked Immunosorbent Assay—High bind EIA/RIA plates (Costar 3690) were coated with 8 μ g/ml of anti-human IgE mAb HB121 in phosphate-buffered saline overnight. The plates were blocked prior to the addition of serially diluted monoclonal IgE in phosphate-buffered saline containing 1.5% bovine serum albumin (50 μ l/well, 60 min). rsFc ϵ RI² was then added (50 μ l/well, 60 min). Bound rsFc ϵ RI was detected using horseradish peroxidase-conjugated 3B4, a nonblocking anti-human Fc ϵ RI mAb (1 μ g/ml, 50 μ l/well, 60 min). The assay was carried out at 20 °C, and the plates were washed seven times with water between each incubation step. Color development was with *o*-phenylenediamine (Sigma) and stopped after 15 min with 25 μ l of 4 M sulfuric acid. The optical density was measured at 490 nm.

Transfection of Mammalian Cells with cDNA—COS-7 cells were maintained (1) in Dulbecco's modified Eagles medium (Life Technologies, Inc.). For transient transfection LipofectAMINE (Life Technologies, Inc.) reagent was used, with plasmid DNA of interest, according to the manufacturer's instructions.

Immune Complex Binding—The binding of IgE or IgG immune complexes to cells transfected with chimeric or mutant cDNA was determined by erythrocyte-antibody (EA) rosetting, which was assayed and scored according to the method previously reported (1). Briefly, mouse anti-TNP IgE or IgG (mIgE or mIgG) was incubated with TNP-coated sheep red blood cells to form complexed IgE or IgG. These antibody sensitized erythrocytes were mixed with transfected cells, and the binding of these complexes to cells was determined microscopically (1). The

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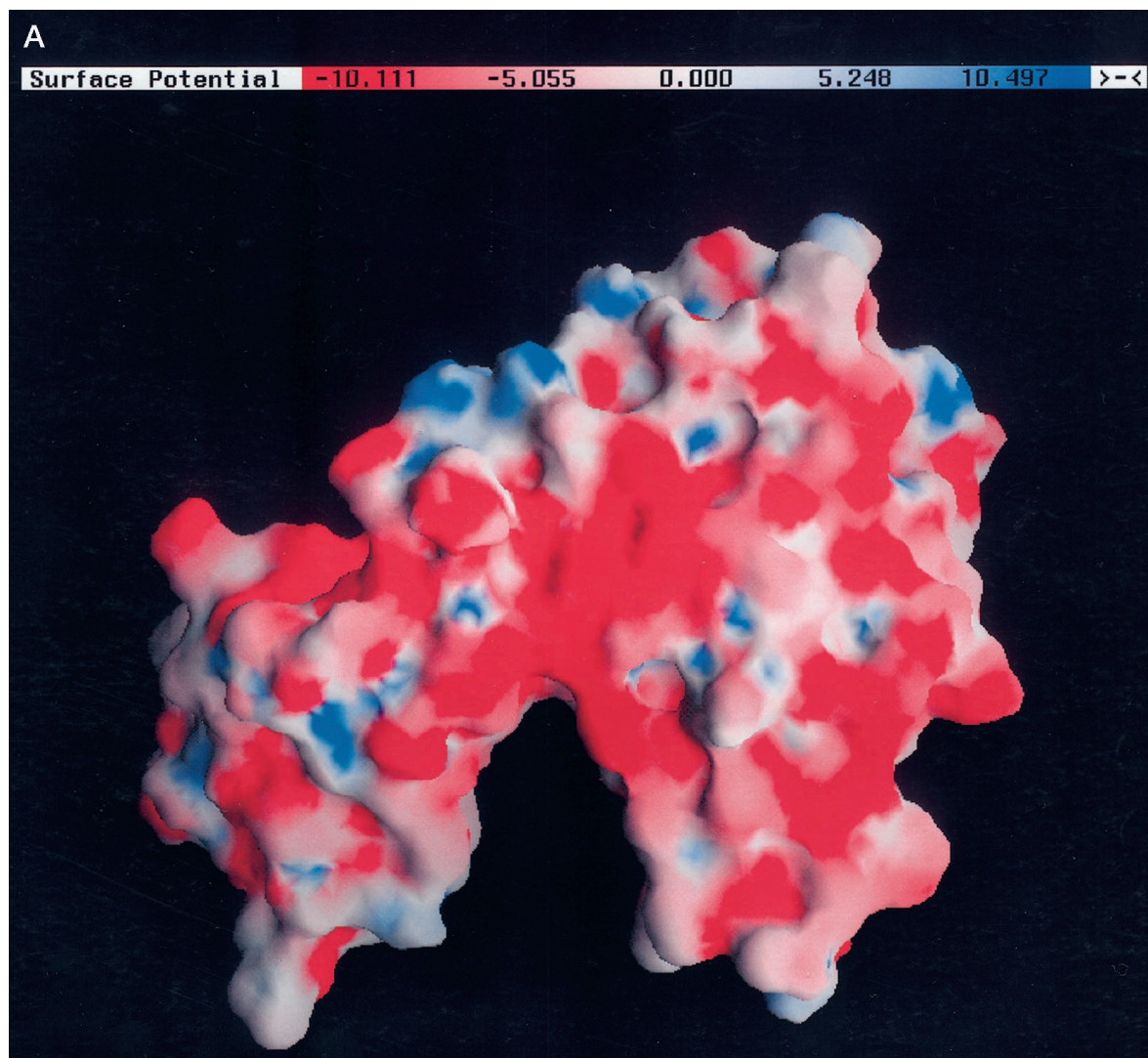


FIG. 2. **FcεRI-α homology model with electrostatic potential displayed.** The electrostatic potential of FcεRI-α model was calculated and mapped onto the molecular surface, with red indicating a negative, and blue indicating a positive electrostatic potential. It can be seen that the face of FcεRI-α comprising the C/F/G strands of domain 1 and domain 2 (A) has a considerably more negative electrostatic potential than the face comprising the A/B/E strands of domain 1 and domain 2 (B).

utilization of avidity in this way permits the determination of low affinity binding.

Measurement of IgE/FcεRI by Equilibrium Binding—Equilibrium binding was determined by the method previously reported (1). IgE was radioiodinated using IODO-GEN (Pierce) according to the manufacturer's instructions. The ^{125}I disintegrations/min were determined separately for the cell pellets (bound IgE) and the supernatant (free IgE) in a WALLAC 1470 WIZARDTM automatic γ counter. Nonlinear regression analysis was performed by plotting IgE free versus IgE bound in the program "Curve Expert" using the formula for single site binding, $y = (a * x)/(b + x)$; where $y = \text{IgE bound}$ and $x = \text{free IgE}$. The equilibrium binding dissociation constant (K_D) was obtained from three experiments with a correlation coefficient of >0.99 (see Table II). The maximum binding (B_{max}) of IgE was also determined and used to estimate receptor expression.

Detection of Membrane-bound FcεRI by Monoclonal Antibodies Using Flow Cytometry—COS-7 cells were transiently transfected with rFcεRI cDNA as above. Approximately 40 h post-transfection the COS-7 cells were incubated with saturating amounts of antibody, on ice, for 45 min; the cells were washed, resuspended in a 1:100 dilution of anti-mouse Ig F(ab')₂-fluorescein isothiocyanate (Silenus), and incubated for 30' on ice. The cells were washed and resuspended in phosphate-buffered saline containing 0.5% bovine serum albumin, 0.1% glucose, 3 $\mu\text{g}/\text{ml}$ propidium iodide and analyzed in a FACScalibur (Becton Dickinson). All washes and dilutions were in phosphate-buffered saline containing 0.5% bovine serum albumin, 0.1% glucose. Analysis was conducted on live (propidium iodide negative) cells.

Monoclonal Antibodies—Anti-human FcεRI-α monoclonal antibodies from hybridoma cell lines X52-47-5.4 (mAb47), X52-54.1 (mAb54), and 3B4 (mAb3B4), all mouse IgG1, were used to determine FcεR expression. These antibodies recognize separate epitopes: mAb 47, which recognizes an epitope in the G' strand D2; mAb54, an epitope in the BC loop of D1; and mAb3B4, an epitope in the C'EF region of D1. Anti-human FcεRI-α mouse monoclonal antibody 15-1 was generously supplied by J.-P. Kinet. Anti-mouse FcγRI (IgG1) monoclonal allo-antibody from the hybridoma cell line X54-5/7.1 was kindly provided by Peck Sze Tan for use as an isotype control antibody. The anti-TNP mouse monoclonal antibodies were mIgE anti-TNP (ATCC clone TIB142) and mIgG1 anti-TNP (A3), and the latter was the gift of Dr. A. Lopez.

Modeling of FcεRI, Chimeras, and Mutants—The extracellular regions of the α-chain of the human Fcε receptor type I (FcεRI-α) and the human Fc γ Receptor type II (FcγRIIa) show a sequence identity of about 40% for 172 residues (this consists of a sequence identity of about 45% for the first domain and about 36% for the second domain). FcγRIIa is the protein most homologous to FcεRI, for which the three-dimensional structure is known (6). With the significant sequence identity, even higher sequence similarity, and the conservation of several important amino acid residues between the two proteins (see the sequence alignment given in Table I), clearly FcγRIIa is the most appropriate three-dimensional structural template to use in modeling FcεRI, more suitable than the structures of CD2 or CD4, which have been used in the past to construct models of FcεRI (12, 13). The recently solved crystal structure of FcεRI (8) confirmed the similarity of the two structures, including the C2 sub type of the Ig-like domains and the acute

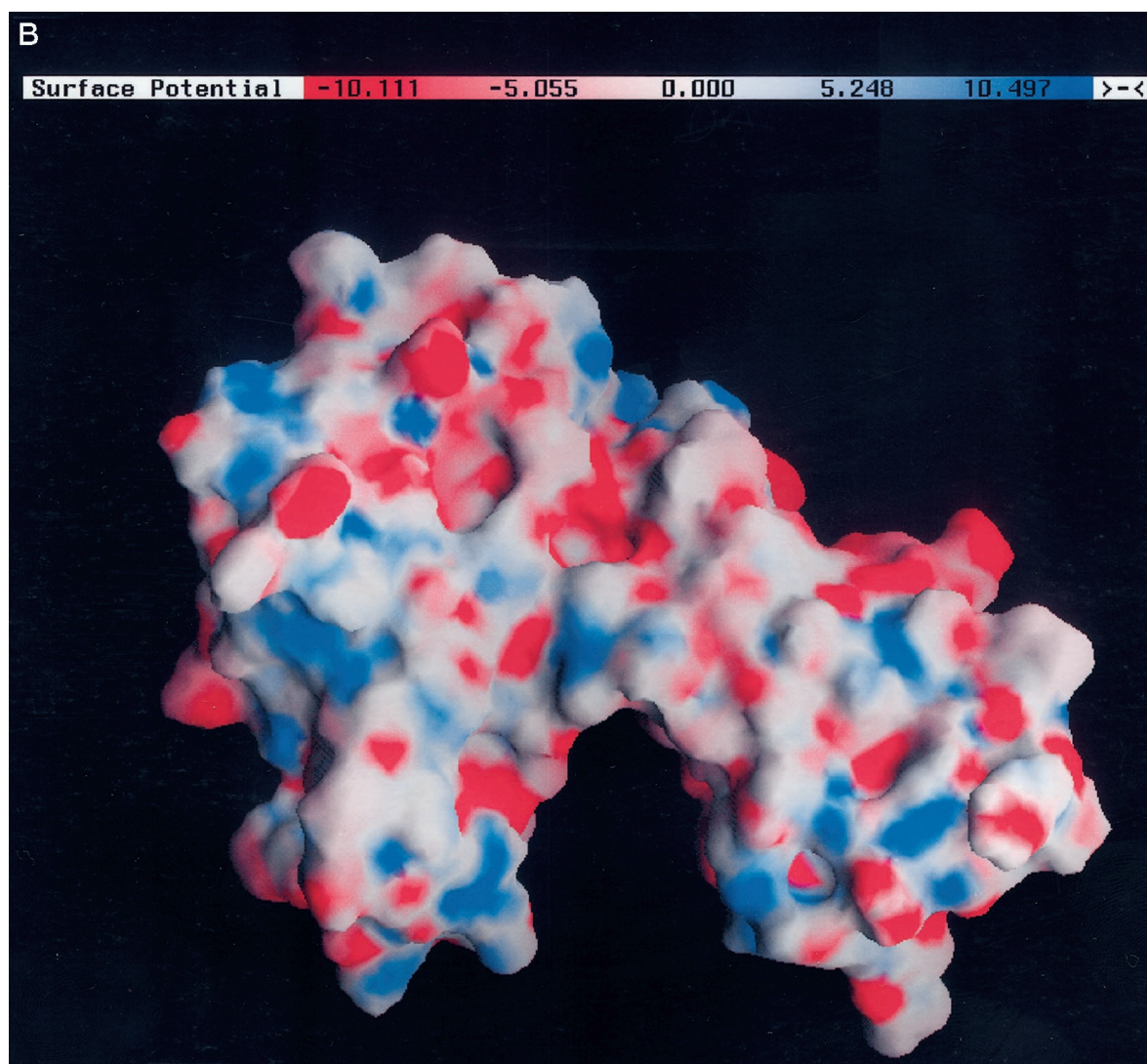


FIG. 2—continued

angle between the two domains. However, the cartesian coordinates of the crystal structure of FcεRI were not available, and therefore we made use of the homology model of FcεRI built in this work.

FcεRI-α Model—Modeler (14) as implemented in the InsightII_Homology software package (Insight II (97.0), Molecular Simulations Inc., San Diego, CA) was used to build three-dimensional models of FcεRI-α using a number of different initial sequence alignments and two structural templates of FcγRIIa. One of the structural templates was the three-dimensional coordinates of FcγRIIa where for the residues that had alternative side chain conformations (residue numbers 10, 21, 33, 57, 60, 61, 65, and 89) the conformations labeled A were selected, whereas in the other template the conformations labeled B were selected. In each Modeler run, five structural models of FcεRI-α were generated. The following parameter values or options were used: library_schedule of 1, max_var_iterations of 300, md_level of refine1', repeat_optimization of 3, and max_molpdf of 10^6 . The best model from these runs had the sequence alignment given in Table I and used the structural template of FcγRIIa where residues 10, 21, 33, 57, 60, 61, 65, and 89 had side chains in the A conformation. The criteria for judging the best model included the lowest value of the Modeler objective function (or $-1.0 \times \ln$ (molecular probability density function), well behaved ProsaII (15) residue energy plot for the model (for example, negative residue energy scores throughout the sequence), and well behaved Profiles-3D (16) local 3D-1D compatibility score plot (for example, positive plot scores throughout the sequence).

Next, Modeler was used to generate 20 different structural models of FcεRI-α using the sequence alignment and template selected above and using the parameter values and options listed above. From these, the model with the lowest $-\ln$ (molecular probability density function) value was then further improved (as measured by ProsaII, Profiles-3D,

and Procheck (17)) by being selected as the template to generate structural models of the FcεRI-α sequence in the next cycle of Modeler runs. At the end of four such cycles, the best three-dimensional model of the FcεRI-α structure (*i.e.* the model with the lowest value of the Modeler objective function) was selected as the final structural model of the FcεRI-α monomer. Secondary structure prediction performed on FcεRI-α sequence confirmed the validity of the alignment given in Table I and showed the pattern of β strands is the same in both FcεRI-α and FcγRIIa. The secondary structure prediction methods used were PHD (18) and PREDATOR (19). The model is displayed in Fig. 1.

Mutant and Chimeric Receptors—The R15A and the F17A point mutants of FcεRI-α were modeled from the above FcεRI-α model by mutating the R15 and F17 residues to alanines with InsightII_Homology module (MSI), adding hydrogens to the two models, and energy minimizing the structures, keeping all heavy atoms fixed except for the Ala¹⁵ and Ala¹⁷ residues, respectively. The program Discover v. 2.98 (MSI) was used for the energy minimization with the CFF91 force field and a distance-dependent dielectric constant of 1.0xr, and the minimization was done with the conjugate gradients method until the maximum energy gradient was less than 0.01 kcal/Å.

Three chimera structures of FcεRI that were experimentally constructed and the binding to IgE investigated were modeled based on the structural template of FcγRIIa. The sequences of these three chimera, labeled $\gamma\epsilon\gamma$, $\gamma(A'B\epsilon)\epsilon\gamma$, and $\epsilon(A'B'\gamma)\epsilon\gamma$, respectively, are shown in Table I. The same sequence alignment as shown in Table I and the same Modeler parameter values and options as were used to generate the model of FcεRI-α (as described earlier) were used to construct these chimera models. Again, out of 20 models generated for each chimera, the model with the lowest Modeler objective function was selected, and the model structure was validated with ProsaII, Profiles-3D, and Procheck.

TABLE I
Amino acid sequences of FcγRIIa, FcεRIα, hFcγRIIIa, and chimeric receptors

a) 'Wild-type' receptor sequences with identity indicated between hFcγRIIa and hFcεRI-α.									
	A	A'	B	C	C'	E	F	G	
DOMAIN 1									
hFcγRIIa	1	10	20	30	40	50	60	70	80
hFcεRI	1	10	20	30	40	50	60	70	80
DOMAIN 2									
hFcγRIIa	90	100	110	120	130	140	150	160	169
hFcεRI	90	100	110	120	130	140	150	160	169
b) Domain 1 amino acid sequences of chimeric clones									
CLONE	A	A'	B	C	C'	E	F	G	
hFcγRIIa	1	10	20	30	40	50	60	70	80
γγ	1	10	20	30	40	50	60	70	80
γ(Aε)εRIII ^a	1	10	20	30	40	50	60	70	80
γ(A'Be)εγ	1	10	20	30	40	50	60	70	80
γ(AA'Be)εγ	1	10	20	30	40	50	60	70	80
γ(ABCCε)εγ	1	10	20	30	40	50	60	70	80
γ(ABCCε)εγ	1	10	20	30	40	50	60	70	80
γ(Gε)εγ	1	10	20	30	40	50	60	70	80
ε(A'By)εγ	1	10	20	30	40	50	60	70	80
εγ	1	10	20	30	40	50	60	70	80
hFcεRI	1	10	20	30	40	50	60	70	80
c) Amino acid sequences of membrane proximal (MP), transmembrane (TM), and membrane anchor (MA) regions of chimeric constructs with FcγRII or FcγRIIIb.									
εγ	159	D2 ε	MP γ	TM γ	MA γ				
εγ	159	D2 ε	MP γ	TM γ	MA γ				
εεRIII	159	D2 ε	MP γ	TM γRIII	MA γRIII				

^a indicates mutant clone nomenclature, initial character (γ or ε) designates the receptor of D1 origin (FcεRI and FcγRIIa); the portion in parentheses indicates the region exchanged (e.g. A'B loop) and its receptor of origin. The possible sites for N-linked glycosylation have been underlined. 0 indicates position of intradomain disulphide bonds.

Finally the electrostatic potential was calculated and mapped onto the molecular surface (Fig. 2) of the constructed FcεRI-α model using the program GRASP (20). The PARSE3 (21) charge set was used in computing the electrostatic potential. It can be seen that one face of FcεRI-α has a considerably more negative electrostatic potential than the other face.

The co-ordinates of the FcεRI-α model are available on request.

RESULTS AND DISCUSSION

Analysis of Chimeric Receptors to Establish the Role of D1 FcεRI-α in IgE Binding—Examination of the FcεRI-α homology model (shown in Fig. 1 and fully described below) and the model of the γγ chimera indicates that the two major regions of D1 that impinge on the D1/D2 interface are, first, the A strand, A' strand, and A'B loop, and second, the G strand. Utilizing a chimeric FcR (γγ) comprising FcγRIIa D1 and FcεRI D2 (IgE-binding domain), those segments that form part of the D1/D2 interface of D1 FcγRIIa cDNA were replaced with the equivalent portion of FcεRI cDNA (Table I). The receptors were assayed to determine which segments conferred a gain of function. Four chimeric receptors were constructed that encompassed the A' strand A'B loop region, namely, γ(ABCCε)εγ, γ(A'Be)εγ, γ(AA'Be)εγ, and γ(A'Be)εγ. Analysis of the transfected receptors by EA rosetting (1) indicated, surprisingly, that none of these chimeric receptors bound complexed mIgE as IgE-coated erythrocytes; as expected, none bound mIgG.

To determine whether the receptors were expressed on the cell surface, the chimeras were tested by flow cytometry, using a panel of four anti-FcεRI-α mAbs that recognized separate nonoverlapping epitopes, two (mAb3B4 and mAb54) in D1 and two (mAb47 and mAb15-1) in D2.² Two of the four chimeras tested, γ(ABCCε)εγ and γ(ABCCε)εγ, were not detected on the cell surface and thus were assumed not to be expressed. However, the γ(AA'Be)εγ and γ(A'Be)εγ chimeras were detected on the cell surface by mAb and were then tested for their ability to bind monomeric hIgE in an equilibrium binding assay (Table II). Both chimeras failed to bind monomeric hIgE, which is

TABLE II
Binding and expression of chimeric and mutant receptors of the A strand A'B loop or G strand regions of D1

	mIgE EA rosetting (Avidity) ^a	Relative expression detected by		K_D^d	Relative affinity ^e
		IgE ^b	mAb ^c		
γγ	3+	1	1	2.1e-09 ± 7.2e-10 ^f	1
γγ	3+	0.5	1	4.0e-09 ± 8.0e-10 ^f	0.6
γ(AA'Be)εγ	— ^g	—	0.3	—	—
γ(A'Be)εγ	—	—	0.2	—	—
γ(Aε)εRIII	3+	0.3	0.5	2.9e-09 ± 4.9e-10	0.7
ε(A'By)εγ	—	—	0.6	—	—
F17A	1+	0.15	0.2	4.1e-08 ± 3.0e-08	0.05
R15A	—	—	0.2	1.0e-06	<0.01
R15L	—	—	0.2	—	—
N14A	1+	0.2 ^h	0.2	ND ⁱ	—
γ(Gε)εγ	3+	0.4	0.3	3.3e-09 ± 9.5e-10	0.65

^a Binding of immune complexes. mIgE-EA rosetted cells were scored on a scale of + to 3+, with 3+ indicating the highest number of EA associated with a cell.

^b The average expression of a receptor determined by the maximum binding of IgE in equilibrium binding assays, and compared to that of γγ, where γγ = 1.

^c Average cell surface expression of the chimera as detected by anti-FcεRI mAb47 in flow cytometry and compared with expression of γγ, where γγ = 1.

^d The equilibrium binding dissociation constant (average taken from three experiments) with the standard deviation indicated.

^e Relative affinity of chimeric receptors determined by K_D (γγ = 1), all other chimeras had a lower apparent affinity.

^f Statistical significance of difference between the K_D of γγ and γγ was determined by Student's *t* test to be *p* = 0.01.

^g Not detected.

^h Expression of N14A relative to γγ was determined by IgE binding and detected by flow cytometry.

ⁱ ND, not determined.

consistent with their failure to bind mIgE complexes (Table II). Thus, despite the fact that these interface sequences were derived from FcεRI, the chimeras were not able to bind IgE.

TABLE III
Expression and integrity of chimeric and mutant receptors of the A strand-A'B loop region of D1

	mAb47 (D2)	mAb15-1(D2)	mAb54(D1)	mAb3B4(D1)
εεγ	4 ^a	4	4	4
γεγ	4	4	—	— ^b
γ(AA'Be)εγ	4	—	—	—
γ(Aε)εRIII	4	4	—	—
ε(A'Bγ)εγ	4	—	4	ND ^c
F17A	4	—	2	1
R15A	4	—	3	2
R15L	4	—	3	2
N14A	4	4	4	ND

^a Receptor expression was determined by flow cytometry using mAbs. Expression was scored on a scale of 1–4 with maximum expression (4) determined after subtraction of background values (fluorescein isothiocyanate-labeled Fab'₂ sheep anti-mouse Ig). 3 = 60–80% of maximum, 2 = 40–60% of maximum, and 1 = less than 40% of maximum.

^b Not detected.

^c ND, not determined.

The γ(AA'Be)εγ (FcεRI residues 1–21) and γ(A'Be)εγ (FcεRI residues 14–21) chimeras differ only by the inclusion or absence of the FcεRI A strand of D1 (FcεRI residues 1–10), and although these chimeras were detected on the cell surface by mAb47 (which maps to an epitope in D2 near the transmembrane region), they were not detected by mAb15-1, an antibody previously reported to detect an epitope in D2 within the IgE-binding site (22). Thus, although the receptors were expressed, they were incapable of binding monomeric hIgE or mIgE complexes, implying significant disruption to the IgE-binding site. This disruption is most probably caused by the segment that is common to both receptor chimeras, that of the A' strand, A'B loop (FcεRI residues 14–21), rather than the A strand, which is present only in the γ(AA'Be)εγ chimera. In the structure of FcγRIIa and the model of FcεRI, the cis-Pro¹¹ at the start of the A' strand, is essential for maintaining the conformation of this part of the interdomain interface. This would imply that the A strand segment up until the cis-Pro has little impact on the interdomain interface and was probably not involved in the disruption to IgE binding, as would be expected from the structure. This was confirmed by testing a chimera with only the A strand segment from the N terminus to the cis-Pro¹¹ from FcεRI in D1 FcγRIIa, namely γ(Aε)εRIII. This A strand chimera bound hIgE with an affinity approaching that of εεγ (Table II) and is discussed below.

Evidently the loss of IgE binding function was related to the alteration of sequences in the interdomain interface, which implies a major role in IgE binding by domain 1. Furthermore, even though these sequences are derived from the same receptor as the IgE binding second domain, they do not provide the correct interactions unless in the context of an autologous first domain. Thus, it may be expected that IgE binding is dependent upon the A' strand-A'B loop segment. To confirm that this change of function was directly related to the A' strand-A'B segment and its impact on the D1/D2 interface, an additional chimera was created. This new chimera was constructed using FcεRI-α (εεγ) as the template, (rather than γεγ) in which the A'B strands and loop of FcγRIIa D1 were inserted into the corresponding position in FcεRI. This ε(A'Bγ)εγ chimera was expressed on the cell surface as measured by mAb47, but mAb15-1 again failed to detect its epitope in the IgE-binding site (Tables II and III). Moreover, the chimera did not bind monomeric hIgE or complexed mIgE, confirming that the interdomain interface has an essential role in the interaction of receptor with IgE.

The inability of the ε(A'Bγ)εγ chimera to bind IgE is not an effect of distortion of D1, because a separate monoclonal antibody (mAb54), which binds within the BC strand region of

FcεRI D1, also binds to this chimera. Thus, on the basis that the D2 mAb47 and D1 mAb54 bind the receptor, and the loss of the mAb15-1 epitope, the effect of the ε(A'Bγ)εγ mutation on IgE binding is related directly to an impact of the D1/D2 interface on the IgE-binding site.

Identification of Crucial Residues within the D1 Interface of FcεRI-α—To further define the role of the interdomain interface, two residues of the A' strand FcεRI, Arg¹⁵ and Phe¹⁷, that have substantial interactions within the interface, were mutated to alanine (R15A and F17A). In addition, Asn¹⁴, with backbone-backbone interactions across the interface was mutated, also to alanine. Both the R15A and F17A mutants were recognized by mAb47 and also by two mAbs with epitopes in FcεRI D1, mAb54 and mAb3B4 (Table III), and neither R15A nor F17A were detected by mAb15-1 the hIgE-binding site specific antibody 15-1. Both point mutants displayed a dramatic reduction in IgE binding, implying that these mutants had altered IgE binding characteristics. The R15A mutant failed to bind monomeric mouse (data not shown) or hIgE or mIgE complexes (Table II). However, the second point mutant, F17A, was able to bind mIgE complexes but showed a substantial reduction in affinity when binding monomeric hIgE (Table II).

The alanine mutants were modeled and compared with the homology model of FcεRI to determine the possible effects of mutation. In the FcεRI model, Arg¹⁵ extends outward toward solvent, whereas in the FcγRII crystal structure (FcγRII, Asn¹⁵) it is constrained within the interface and oriented more toward D2. Asn¹⁵ also forms a hydrogen bond with the Leu⁹⁰ backbone carbonyl in the FcγRII crystal structure. No such hydrogen bond is formed in the FcεRI model with the distance between Leu⁹⁰:c and Arg¹⁵:cγ being 4.75 Å. Arg¹⁵ participates in hydrophobic (van der Waals') contacts with Leu⁸⁹, Phe⁸⁴, and Leu¹⁶⁵ (Figs. 3 and 4) in both the x-ray structure (8) as well as the model, but the interactions with Leu¹⁶⁵ are lost, whereas those with Phe⁸⁴ are severely reduced in the R15A mutant model structure. Furthermore, in the FcεRI model, the Glu⁸² carboxylate is parallel to the guanidinium of Arg¹⁵, and the Arg¹⁵:cζ and Glu⁸²:cδ are 4.2 Å apart. If Arg¹⁵ and Glu⁸² exist in ionized forms in FcεRI, this would lead to substantial loss of coulombic stabilization in the R15A mutant. The loss of fundamental interactions in the R15A mutant would result in destabilization of the interface and consequently the IgE-binding site above. This is consistent with the analysis of an R15L point mutant, which removes the positive charge of arginine while maintaining a similar size and displays a total loss of both hIgE and mIgE binding.

There is hydrophobic or van der Waals' contact between Phe¹⁷ and Trp¹¹⁰ in the FcεRI model, which is consistent with the published structure (8). This is significant because Trp¹¹⁰ is a principal residue in the B/C loop previously defined as a major contributor to the IgE-binding site (1, 11, 25). There are also hydrophobic contacts between Phe¹⁷ and Leu⁸⁸, Leu⁸⁹, Asp⁸⁶, and His¹⁰⁸ in FcεRI. All of these contacts are lacking in the F17A mutant, and it is feasible that their loss would cause considerable distortion of the D1/D2 interface, as well as the binding site. The A'B region is sensitive to change, and the presence of Arg¹⁵ as well as Phe¹⁷ is insufficient to allow IgE binding. This is indicated by the analysis of the γ(A'Be)εγ chimera where the A'B sequence of FcεRI (NRIFKGEN) placed in γεγ, that is D1 FcγRIIa but D2 of FcεRI surprisingly failed to bind IgE (Table I). Thus, the interface clearly maintains a series of complex interactions that work collectively to allow binding of IgE.

From the model structure and the contacts listed above, Phe¹⁷ appears to lie beneath the IgE-binding site and has a

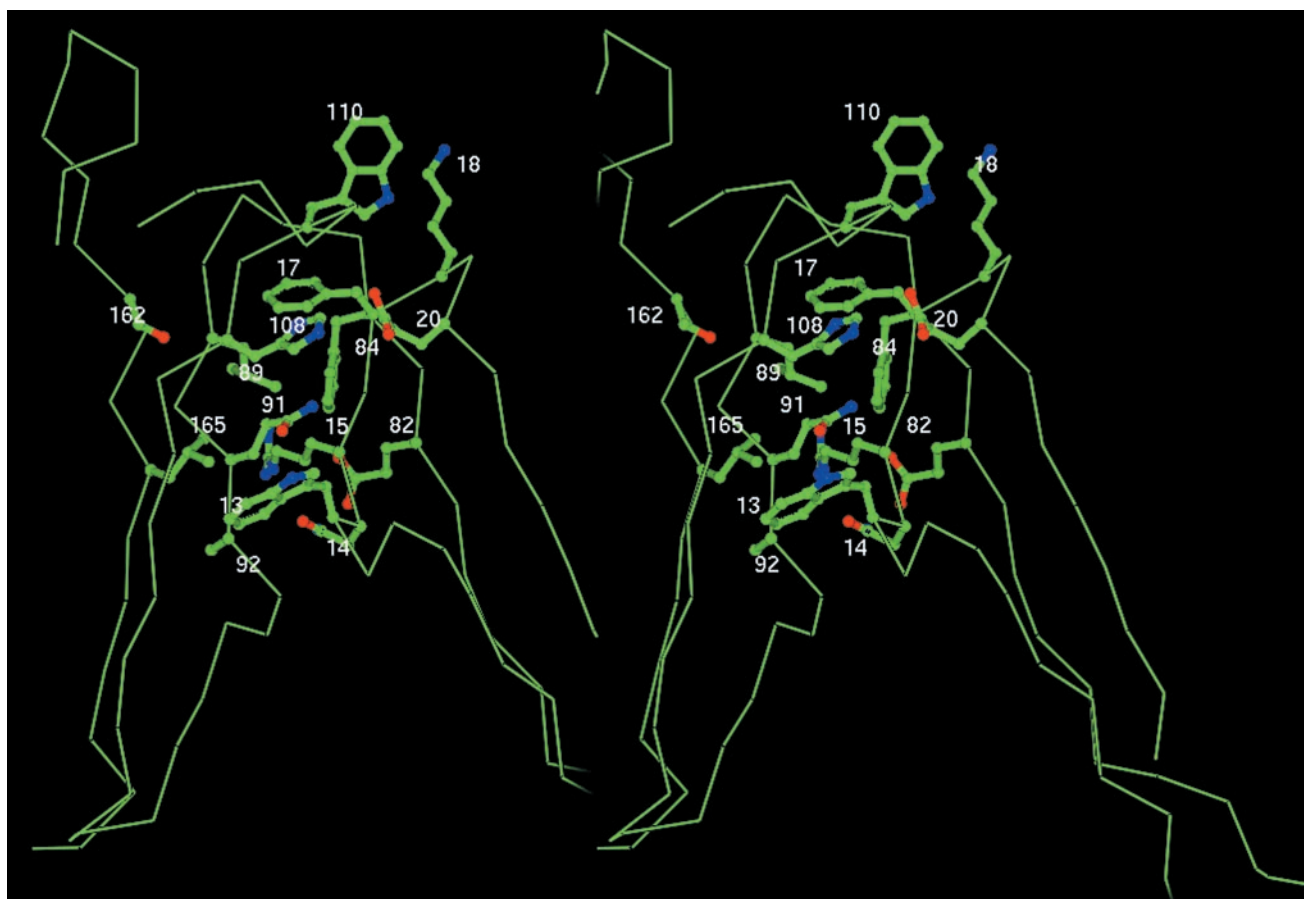


FIG. 3. **Homology model of the FcεRI-α domain 1/domain 2 interface.** Stereo view of the FcεRI-α domain 1/domain 2 interface, with the protein backbone depicted in a green wire format and amino acids involved in transdomain interactions displayed in ball-and-stick form and numbered. Nitrogen atoms are displayed in blue, and oxygen atoms are in red.

critical function in maintaining organization of the linker region between the D1 G strand and D2 A strand. The linker, at the membrane distal portion of the interface, effects the display of the two domains and the ligand-binding region. Arg¹⁵, which plays a more crucial role in maintaining IgE binding, lies closer to the membrane. To test the possibility that distance from the linker may be a factor in determining the magnitude of the effect of mutation, Asn¹⁴ was mutated to Ala (Table II). The N14A mutation has less effect on the binding of hIgE or mIgE, as the FcεRI model suggests by the single backbone interaction of Asn¹⁴ with Ala⁹² across the interface. The analysis of these point mutants would imply that maintenance of the presentation of the ligand-binding site in FcεRI is dependent upon the structure of the D1/D2 interface, which lies below the binding site, and that Arg¹⁵ and Phe¹⁷ are critical residues in this interaction.

Is Arg¹⁵ a Contact Residue Involved in IgE Binding?—The loss of binding by the ε(A'βγ) chimera and the R15A and R15L point mutants could also suggest a possible hIgE contact role for the Arg¹⁵ residue. However it is more unlikely that Arg¹⁵ is not a contact residue because firstly, it is distant from the ligand-binding region, which is exposed to solvent on the superficial surface of the receptor (Fig. 1). Secondly, peptide inhibition² and mutagenesis analysis (23) have separately placed the mAb15-1 epitope close to the IgE-binding site, and mutations within, or expressed within, the D1/D2 interface have caused loss of binding of both IgE and mAb15-1 independently (23). This would confirm that the D1/D2 interface is structurally important in the presentation of the IgE-binding site and that mutations within the interface are sufficient to destroy the structure of this region. Thus, the exchange to

alanine causes distortion of the receptor and not necessarily removal of a critical binding residue. Thirdly, the complete first domain of FcγRIIIa can be substituted for the first domain of FcεRI (which replaces Arg¹⁵ with Asn) while maintaining IgE binding, although with a 2-fold loss of apparent affinity.

Substitution of the complete first domain of FcγRIIIa for the first domain of FcεRI maintains the Arg¹⁵ residue, and this chimera retains the ability to bind both human and mouse IgE with an equivalent affinity to that of the wild-type receptor (4). The presence of the entire FcγRIIIa D1 may stabilize the interface region in the γγ chimera and compensate to some extent for the loss of the Arg¹⁵ residue. The presence or absence of this critical Arg¹⁵ residue may also resolve previously unexplained discrepancies between studies using FcγRIIIa D1/FcεRI D2 and FcγRIIIa D1/FcεRI D2 chimeras.

A recently reported S162A mutant in FcεRI D2 (23) causes destruction of IgE binding. Ser¹⁶² is highly conserved within Fc receptors, and in the FcεRI homology model Ser¹⁶² interacts with Leu⁸⁹ of the D2 A strand, which in turn interacts with Arg¹⁵ of the D1 A' strand (Figs. 3 and 4). The Arg¹⁵ residue has been shown above to be of importance in maintaining the D1/D2 interface, and thus it is possible that the ablation of ligand binding is caused by changes in this linkage. The ability of a point mutation distant from the IgE-binding site to effect sufficient distortion of the receptor to destroy IgE binding further defines the importance and sensitivity of the D1/D2 interface structure in relation to IgE binding.

The A and G Strands of D1 FcεRI Play a Critical Supportive Role in the Maintenance of the D1/D2 Interface—Chimeras containing either the A strand (γ(Aε)RIII) or G strand (γ(Gε)εγ) alone of D1 FcεRI-α in the γγ template chimera were

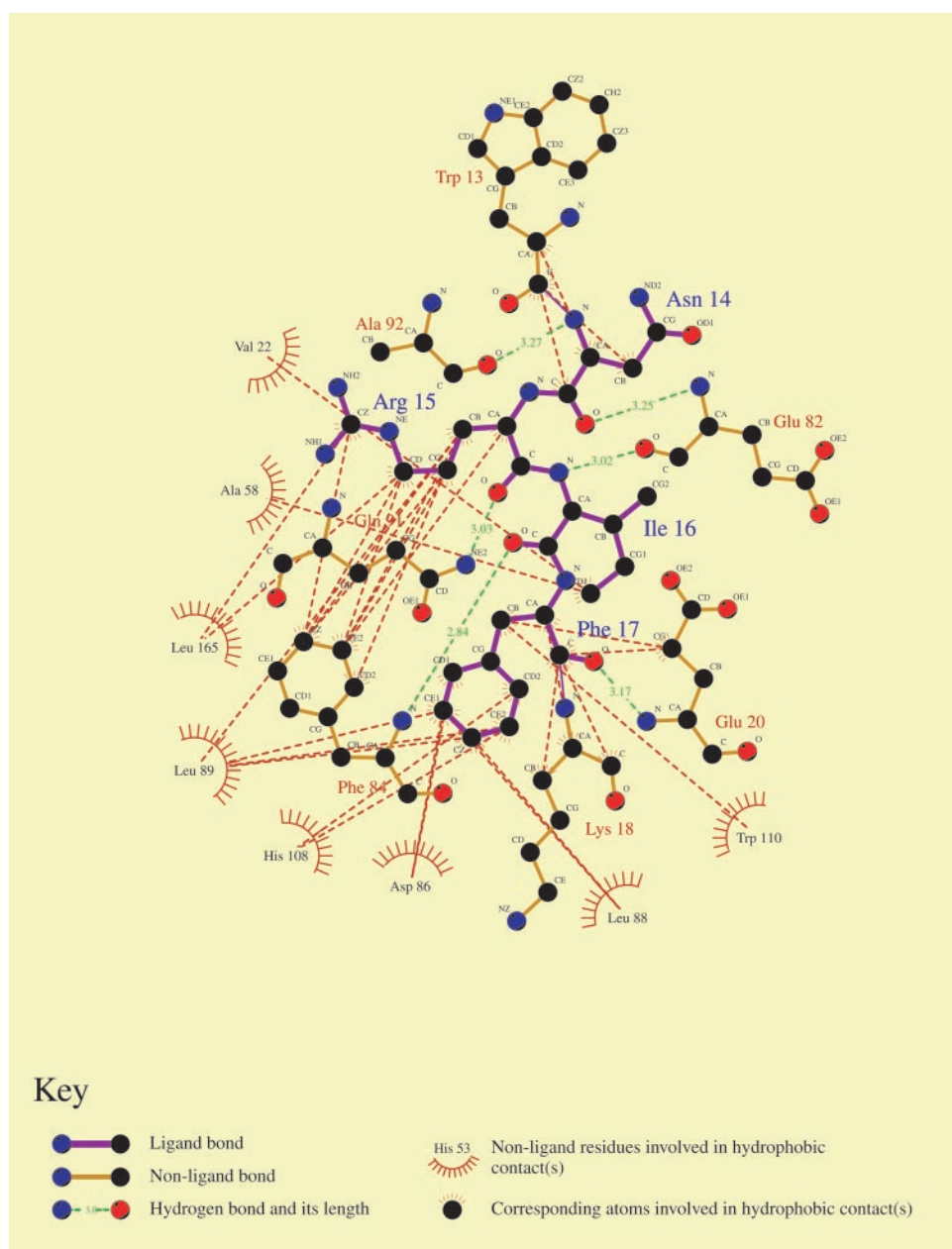


FIG. 4. **Two-dimensional depiction of the domain 1/domain 2 interface displaying distances of interactions between amino acids.** The core amino acids Asn¹⁴, Arg¹⁵, Ile¹⁶, and Phe¹⁷ are shown in *blue*, amino acids hydrogen bonded with the core amino acids are shown in *brown* with the bond length shown in *green*, and other amino acids involved in hydrophobic interactions with the core amino acids are labeled in *black* with a *red* ray. All hydrophobic interactions are displayed in *red* with the atom(s) involved in contact(s) shown in *black* with a *red* ray.

expressed, bound mIgE with an avidity similar to that of $\epsilon\epsilon\gamma$, and showed a small but reproducible increase in affinity for hIgE compared with $\gamma\epsilon\gamma$ (Table II). Thus, although the high affinity of the wild-type $\epsilon\epsilon\gamma$ receptor was not totally restored by these chimeras, the increase in affinity would suggest a foundation role in the presentation of the IgE-binding site.

In the case of the A strand, this role is most likely to be the structural support of the interface. The N terminus of the A strand, and indeed the ϵ receptor, is probably located close to the cell membrane. The A strand interacts with other residues within D1 via hydrogen bonds, both in backbone interactions with the B strand and Asn⁷⁴ of the FG loop. Conformation of the A strand would assist the display of the A' strand in the interface so that crucial residues, such as Arg¹⁵ and Phe¹⁷, are appropriately presented.

The G strand of domain 1 abuts domain 2 directly via the G strand-A strand linker and across the D1/D2 interface; it is also

involved in interactions with the A' strand within D1. The G strand of D1 is highly conserved between FcεRI and FcγRIIIa, with few differences between the interactions of the conserved amino acids. It is therefore surprising that introducing the G strand of FcεRI into the $\gamma\epsilon\gamma$ chimera results in alterations to IgE binding. The residues in the G strand that are not conserved between FcεRI and FcγRIIIa may contribute specifically to IgE binding affinity. Glu⁸² and Phe⁸⁴ interact with D1 A' strand residues Asn¹⁴, Ile¹⁶, and Arg¹⁵, and Asp⁸⁶ with Phe¹⁷; these latter two interactions are with residues shown above to be critical in maintaining the D1/D2 interface. It is therefore probable that interactions of the A and G strands as well as the A' strand of D1 effect a role in maintaining the interface between D1 and D2 and therefore have an indirect effect on IgE binding.

In both the FcεRI model and FcγRIIIa structure, Trp⁸⁷ at the D1/D2 junction interacts with Trp¹¹⁰, which is contained

within the BC loop of D2, a crucial IgE-binding region (1, 11). Residues adjacent to Trp¹¹⁰, (Arg¹⁰⁶ and His¹⁰⁸), also interact with amino acids of the D1/D2 interface, the A' strand of D1, and the A strand D2, thus maintaining links between the interface and the IgE-binding region. The conservation of these residues within the FcR probably contributes to the ability to substitute the D1 of FcγRIIa or FcγRIII for the D1 of FcεRI and retain IgE binding.

The Homology Model of FcεRI—A homology model of FcεRI (Fig. 1) based on the recently solved structure of FcγRIIa (6) was employed to determine the structural basis of alterations in IgE binding by the FcεRI-α chimeras. The two structures are, therefore, very similar with some small variation at the point of sequence disparity in the region of the C'E loop of D1 (Table I). The pattern of β strands is the same in both FcγRIIa and the FcεRI-α homology model, (as stated under "Experimental Procedures"); however, the arrangement of the loops appears to depend more on the positioning of amino acids such as proline in FcγRIIa, whereas in FcεRI-α there are supplementary interactions between amino acids to preserve the loop structure. In this model of FcεRI-α, one face of the molecular surface, largely comprising the juxtaposed C/F/G strands of each domain, has an overwhelmingly negative electrostatic potential (Fig. 2), unlike the opposite face of the molecule. This marked disparity in the electrostatic potential between the two faces is not observed in the case of the FcγRIIa molecule (results not shown) and may be of biological significance. The negative surface of FcεRI-α would tend to sit away from the cell membrane, and as a consequence maintain the binding sites in a membrane distal position, on the upper surface of the molecule. This supports the cell surface data (detailed in Refs. 12, 24, and 25) asserting that the domains are aligned with the membrane along the long axis as shown in Fig. 1 and do not project vertically from the membrane. The γεγ chimera (see "Experimental Procedures") was also modeled and, as expected, was shown to have a similar structure to FcγRIIa and the FcεRI-α homology model.

The FcεRI-α homology model has the features described for the x-ray crystallographic structure of Garman and co-workers (8). First, all the N-linked glycosylation sites, including the three described in the crystal structure, are solvent exposed. Second, the interchain hydrogen bonds correlate well with those disclosed in Fig. 1D of the published FcεRI structure. Third, the two domains are bent relative to each other, and the model has an interdomain angle of 52°, which is similar to that depicted by Garman *et al.* (8). Fourth, the IgE-binding loops of

domain 2 defined by Hulett *et al.* (1, 11) are in close proximity, distal to the membrane and exposed to solvent.

By utilizing the FcεRI-α homology model and the models of the chimeras and mutants, the authenticity of the interactions within the D1/D2 interface and the effects of the mutations on IgE binding could be defined with greater precision and fidelity. In conclusion, these data suggest that the second domain of FcεRI-α is the only domain involved in direct contact with the IgE ligand and that domain one has a structural function of great importance in maintaining the integrity of the domain interface and, through it, the ligand-binding site.

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