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

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The high affinity receptor for IgE, FceRI, 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 FceRI, either through a minor contact interaction or in a supporting structural capacity. The results reported here demonstrate that domain one of FceRI 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 FceRI-α based on the solved structure of FcγRIIIa, 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 FceRIα 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 FceRI-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, FceRI, is a tetrameric complex composed of an IgE-binding α subunit associated with a transmembrane β subunit and homodimeric γ subunits and is a key player in IgE-dependent effector mechanisms. The α subunit, FceRI-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 FceRI 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. FceRI, however, is related to FcγRIIa, and the recent description of the three-dimensional structure of FcγRIIIa (6), FcγRIIIb (7), and FcγRII-α (8) may provide a basis for the understanding of the roles of the individual domains in FceRI and other Fc receptors.

In the crystal structure of FcγRIIIa 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 FceRI and FcγRIIa show 40% amino acid identity and considerably higher amino acid homology, it is probable that FceRI has a similar structure to that of FcγRIIa, confirmed by the recent publication of the solved FcγRI structure (8).

In the study described herein we have utilized a model of FcγRII-α (see Fig. 1) based on the solved crystal structure of FcγRIIIa (6) and undertaken a mutagenesis study of domain 1 to define its role in the interaction with IgE. The solved x-ray structure of FcγRII-α (8) strongly resembles that of FcγRIIa and therefore that of the FcγRII-α homology model. Indeed, the FcγRII-α homology model and the x-ray structure of FcγRII-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 FcγRI-α.

EXPERIMENTAL PROCEDURES

Production and Nomenclature of FcγRII-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 chimerar nomenclature are displayed in Table 1. The first template was designated αα and comprised domain one (D1α) and domain two (D2) of FcγRII-α linked with the transmembrane region and cytoplasmic membrane anchor of...
FcγRIIa. The second chimeric template was based on a simple domain exchange and comprised D1 of FcγRIIa and D2 of FcεRI-a, also with the transmembrane region and cytoplasmic sequence of FcγRIIa, and was designated γεγ. Chimeric receptors were generated using the template receptor γεγ or εεγ. Specific loops, strands, or regions of the FcγRIIa D1 were replaced with the equivalent portion of FcεRI (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 FcγRIIIB replacing the FcγRIIa cytoplasmic tail of the geg construct. This chimera was designated γεRIII and was generated by SOE-PCR. Substitution into domain one of the γεRIII template receptor of the A strand of FcεRI D1 produced the γεεεRIII chimeras. The FcεRI D1 A' strand point mutants, R15A and F17A, were made using SOE-PCR and incorporated into εεγ. The FcεRI D1 A' strand point mutants N14A and R15L were constructed using the QuikChange™ 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 0.5M sodium citrate/0.5 M NaCl (pH 2.7) into tubes containing 50 μl 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 μg/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 μl/well, 60 min). rsFcεRII was then added (50 μl/well, 60 min). Bound rsFcεRI was detected using horseradish peroxidase-conjugated 3B4, a nonblocking anti-human FcεRI mAb (1 μg/ml, 50 μl/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 μl 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 (moIgE or moIgG) 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
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 radiiodinated using IODO-GEN (Pierce) according to the manufacturer’s instructions. The ¹²⁵I disintegrations/min were determined separately for the cell pellets (bound IgE) and the supernatant (free IgE) in a WALLAC 1470 WIZARD™ 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,

\[ y = \frac{a \times x}{b + x}; \]

where \( y \) = IgE bound and \( x \) = 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 (\( B_{\text{max}} \)) 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 μg/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 FcεRI 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 Szee Tan for use as an isotype control antibody. The anti-TNP mouse monoclonal antibodies were moIgE anti-TNP (ATCC clone TIB142) and moIgG1 anti-TNP (A3), and the latter was the gift of Dr. A. Lopez.

Modeling of FcεRI, Chimerae, and Mutants—The extracellular regions of the α-chain of the human Fce receptor type I (FcεRI-α) and the human Fcγ Receptor type II a (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
angle between the two domains. However, the cartesian coordinates of
the crystal structure of Fc\(\text{e}\)RI were not available, and therefore we
made use of the homology model of Fc\(\text{e}\)RI built in this work.

\textbf{Fc\(\text{e}\)RI Model—}Modeler (14) as implemented in the InsightII_Ho-
mology software package (Insight II (97.0), Molecular Simulations Inc.,
San Diego, CA) was used to build three-dimensional models of Fc\(\text{e}\)RI using a number of different initial sequence alignments and two struc-
tural templates of Fc\(\text{g}\)RIIa. One of the structural templates was the
three-dimensional coordinates of Fc\(\text{g}\)RIIa 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 se-
lected. In each Modeler run, five structural models of Fc\(\text{e}\)RI were
generated. The following parameter values or options were used: li-
brary_schedule of 1, max_var_iterations of 300, md_level of refine1
9
, repeat_optimization of 3, and max_molpdf of 106. The best model from
these runs had the sequence alignment given in Table I and used the
structural template of Fc\(\text{g}\)RIIa 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 \(2\ln (\text{molecular probability density function})\), 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 ex-
ample, positive plot scores throughout the sequence).

Next, Modeler was used to generate 20 different structural models of
Fc\(\text{e}\)RI using the sequence alignment and template selected above and
using the parameter values and options listed above. From these, the
model with the lowest \(-\ln (\text{molecular probability density function})\)
value was then further improved (as measured by ProsaII, Profiles-3D,
and Procheck (17)) by being selected as the template to generate struc-
tural models of the Fc\(\text{e}\)RI-\(\alpha\) sequence in the next cycle of Modeler runs.
At the end of four such cycles, the best three-dimensional model of the
Fc\(\text{e}\)RI-\(\alpha\) structure (i.e. the model with the lowest value of the Modeler
objective function) was selected as the final structural model of the
Fc\(\text{e}\)RI-\(\alpha\) monomer. Secondary structure prediction performed on
Fc\(\text{e}\)RI-\(\alpha\) sequence confirmed the validity of the alignment given in Table
I and showed the pattern of \(\beta\) strands is the same in both Fc\(\text{e}\)RI-\(\alpha\) and
Fc\(\text{g}\)RIIa. The secondary structure prediction methods used were PHD
(18) and PREDATOR (19). The model is displayed in Fig. 1.

\textbf{Mutant and Chimeric Receptors—}The R15A and the F17A point
mutants of Fc\(\text{e}\)RI-\(\alpha\) were modeled from the above Fc\(\text{e}\)RI-\(\alpha\) model by
mutating the R15 and F17 residues to alanines with InsightII_Homol-
ogy 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 minimi-
zation was done with the conjugate gradients method until the maxi-
mum energy gradient was less than 0.01 kcal/\(A\).

Three chimera structures of Fc\(\text{e}\)RI that were experimentally con-
structed and the binding to IgE investigated were modeled based on the
structural template of Fc\(\text{g}\)RIIa. The sequences of these three chimera,
labeled Fc\(\gamma\gamma\), Fc\(\text{A}B\text{e}\gamma\), and Fc\(\text{A}B\text{g}\gamma\), 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 Fc\(\text{e}\)RI-\(\alpha\) (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.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{continued}
\end{figure}
ever, the cell surface and thus were assumed not to be expressed. How-

the equivalent portion of FcRII cDNA (Table I). The receptors
were assayed to determine which segments conferred a gain of
function. Four chimeric receptors were constructed that encom-

were tested for their ability to bind monomeric hIgE, which is
monitored in an equilibrium binding assay (Table 1).

Finally the electrostatic potential was calculated and mapped onto
the molecular surface (Fig. 2) of the constructed FcRI-a model.
It can be seen that one face of the D1/D2 interface of D1 FcRI
has a considerably more negative electrostatic potential than
the other face.

The co-ordinates of the FcRI-a model are available on request.

RESULTS AND DISCUSSION

Amino Acid Sequences of FcRRIIa, FcRIIb, and Chimeric Receptors

| DOMEIN 1 | A | A' | B | C | C' | E | F | G |
|----------|---|----|---|---|---|---|---|---|
| hFcRIIa  | 1 | 2  | 3 | 4 | 5 | 6 | 7 | 8 |
| hFcRI    | 9 | 10 | 11| 12| 13| 14| 15| 16|
| hFcRI-a  | 17| 18| 19| 20| 21| 22| 23| 24|

a) "Wild-type" receptor sequences with identity indicated between hFcRIIa and hFcRI-a.

b) Domain I aminoacid sequences of chimeric clones

| CLOSED | A | A' | B | C | C' | E | F | G |
|--------|---|----|---|---|---|---|---|---|
| hFcRIIa | 1 | 2  | 3 | 4 | 5 | 6 | 7 | 8 |
| hFcRI   | 9 | 10 | 11| 12| 13| 14| 15| 16|
| hFcRI-a | 17| 18| 19| 20| 21| 22| 23| 24|

c) Aminoacid sequences of membrane proximal (MP), transmembrane (TM), and membrane anchor (MA) regions of chimeric constructs with FcRII or FcRIIb.

Table II: Binding and expression of chimeric and mutant receptors of the A strand/Ab loop or G strand regions of D1

|       | mAbG E rosetting | Relative expression detected by IgE | Kd | Relative affinity |
|-------|------------------|-----------------------------------|----|------------------|
| Aa    | 3                | 1                                 | 1  | 0.2 e-09 - 1.0 e-10 |
| AaBe  | 3                | 0.5                               | 0.5 | 4.0 e-09 - 1.0 e-10 |
| Ab    | 3                | 0.3                               | 0.3 | 4.0 e-09 - 1.0 e-10 |
| AbBe  | 3                | 0.5                               | 0.5 | 4.0 e-09 - 1.0 e-10 |
| AbIII | 3                | 0.3                               | 0.3 | 4.0 e-09 - 1.0 e-10 |
| AbBe  | 3                | 0.5                               | 0.5 | 4.0 e-09 - 1.0 e-10 |
| F17A  | 1                | 0.15                              | 0.2 | 4.0 e-09 - 1.0 e-10 |
| R15A  | 1                | 0.2                               | 0.2 | 4.0 e-09 - 1.0 e-10 |
| N14A  | 1                | 0.2                               | 0.2 | 4.0 e-09 - 1.0 e-10 |
| yG    | 4                | 0.3                               | 0.3 | 4.0 e-09 - 1.0 e-10 |

* Binding of immune complexes. MoIgE-EA rosetted cells were scored on a scale of + to 3+, with 3+ indicating the highest number of EA associated with a cell.
* The average expression of a receptor determined by the maximum binding of IgE in equilibrium binding assays, and compared to that of eeg, where eeg = 1.
* Average cell surface expression of the chimera as detected by anti-FcRII mAb47 in flow cytometry and compared with expression of eeg, where eeg = 1.
* The equilibrium binding dissociation constant (average taken from three experiments) with the standard deviation indicated.

|       | M   | IgG | mAb | Kd | Relative affinity |
|-------|-----|-----|-----|----|------------------|
| eeg   | 3+  | 1   | 4   | 2.1 e-09 - 7.2 e-10 | 1   |
| yeg   | 3+  | 0.5 | 0.5 | 4.0 e-09 - 1.0 e-10 | 0.6 |
| yAAbBe| 3   | 0.3 | 0.3 | 4.0 e-09 - 1.0 e-10 | 0.6 |
| yAb   | 3   | 0.3 | 0.3 | 4.0 e-09 - 1.0 e-10 | 0.6 |
| yAbIII| 3   | 0.3 | 0.3 | 4.0 e-09 - 1.0 e-10 | 0.6 |
| yAbBe | 3  | 0.3 | 0.3 | 4.0 e-09 - 1.0 e-10 | 0.6 |
| F17A  | 1   | 0.15| 0.2 | 4.0 e-09 - 1.0 e-10 | 0.5 |
| R15A  | 1   | 0.2 | 0.2 | 4.0 e-09 - 1.0 e-10 | 0.5 |
| N14A  | 1   | 0.2 | 0.2 | 4.0 e-09 - 1.0 e-10 | 0.5 |
| yG    | 4   | 0.3 | 0.3 | 4.0 e-09 - 1.0 e-10 | 0.5 |

* Relative affinity of chimeric receptors determined by Kd (k = 1), all other chimeras had a lower apparent affinity.

a) Statistical significance of difference between the Kd of eeg and yeg was determined by Student's t test to be p = 0.01.
* Not detected.
* Expression of N14A relative to eeg was determined by IgE binding and detected by flow cytometry.
* ND, not determined.

consistent with their failure to bind moIgE complexes (Table II). Thus, despite the fact that these interface sequences were derived from FcRII, the chimeras were not able to bind IgE.
Domain 1 of FceRI Regulates Binding to IgE

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

| mAb47 (D2) | mAb15 (D2) | mAb54 (D1) | mAb3B4 (D1) |
|------------|------------|------------|-------------|
| IgE        | 4          | 4          | 4           |
| γE         | 4          | 4          | 4           |
| γAABeγ     | 4          | 4          | ND          |
| γAeεRIII  | 4          | 4          | ND          |
| eABγeγ     | 4          | 4          | ND          |
| F17A       | 4          | 2          | 1           |
| R15A       | 4          | 3          | 2           |
| R15L       | 4          | 3          | 2           |
| N14A       | 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 of sheep anti-mouse Ig). 3 = 60–80% of maximum, 2 = 40–60% of maximum, 1 = less than 40% of maximum.

b ND, not determined.

The γ(AA Be)γ (FceRI residues 1–21) and γ(A′ Be)eγ (FceRI residues 14–21) chimeras differ only by the inclusion or absence of the FceRI A strand of D1 (FceRI 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 molIgE complexes, implying significant disruption to the IgE-binding site.

This disruption is most probably caused by the segment that is present only in the γ(AA Be)γ chimeras. 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)εRII. This A′ strand chimera bound hIgE with an affinity approaching that of eγ (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 FceRI-α (eγγ) as the template, (rather than γeγ) in which the A′B strands and loop of FcγRIIa D1 were inserted into the corresponding position in FcεRI. This e(A′B)eγγ 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 molIgE, confirming that the interdomain interface has an essential role in the interaction of receptor with IgE.

The inability of the e(A′B)eγγ 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 FceRI 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 e(A′B)eγγ 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 FceRI—To further define the role of the interdomain interface, two residues of the A′ strand FceRI, 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 molIgE complexes (Table II). However, the second point mutant, F17A, was able to bind molIgE 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 FceRI 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. Asn14 also forms an hydrogen bond with the Leu80 backbone carbonyl in the FcγRII crystal structure. No such hydrogen bond is formed in the FcεRI model with the distance between Leu80:c and Arg15:c 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:ζ and Glu82:ζ 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 molIgE 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, Leu90, Asp96, 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′Beγγ chimera where the A′B sequence of FcεRI (NFIFKGERN) placed in γeγ, 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
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 moIgE, as the Fc\textsubscript{eRI} model suggests by the single backbone interaction of Asn14 with Ala\textsuperscript{92} across the interface. The analysis of these point mutants would imply that maintenance of the presentation of the ligand-binding site in Fc\textsubscript{eRI} is dependent upon the structure of the D1/D2 interface, which lies below the binding site, and that Arg15 and Phe 17 are critical residues in this interaction.

Is Arg\textsuperscript{15} a Contact Residue Involved in IgE Binding?—The loss of binding by the e(A'\textsuperscript{B}g\textsuperscript{e})\textsuperscript{chimera} and the R15A and R15L point mutants could also suggest a possible IgE contact role for the Arg\textsuperscript{15} residue. However it is more unlikely that Arg\textsuperscript{15} 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 inhibition\textsuperscript{2} 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 Fc\textsubscript{RIIIa} can be substituted for the first domain of Fc\textsubscript{eRI} (which replaces Arg\textsuperscript{15} with Asn) while maintaining IgE binding, although with a 2-fold loss of apparent affinity.

Substitution of the complete first domain of Fc\textsubscript{RIIIa} for the first domain of Fc\textsubscript{eRI} maintains the Arg\textsuperscript{15} 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 Fc\textsubscript{RIIIa} D1 may stabilize the interface region in the \(\gamma(e\gamma\gamma)\) chimera and compensate to some extent for the loss of the Arg15 residue. The presence or absence of this critical Arg\textsuperscript{15} residue may also resolve previously unexplained discrepancies between studies using Fc\textsubscript{RIIIa} D1/Fc\textsubscript{eRI} D2 and Fc\textsubscript{RIIIa} D1/Fc\textsubscript{eRI} D2 chimeras.

A recently reported S162A mutant in Fc\textsubscript{eRI} D2 (23) causes destruction of IgE binding. Ser\textsuperscript{162} is highly conserved within Fc receptors, and in the Fc\textsubscript{eRI} homology model Ser\textsuperscript{162} interacts with Leu\textsuperscript{89} of the D2 A strand, which in turn interacts with Arg\textsuperscript{15} of the D1 A\textsubscript{9} strand (Figs. 3 and 4). The Arg\textsuperscript{15} 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 Fc\textsubscript{eRI} Play a Critical Supportive Role in the Maintenance of the D1/D2 Interface—Chimeras containing either the A strand (\(\gamma(Ae)\textsubscript{RIII}\)) or G strand (\(\gamma(Ge)\textsubscript{e}\gamma)\) alone of D1 Fc\textsubscript{eRI}-a in the \(\gamma(e\gamma\gamma)\) template chimera were
expressed, bound mOgE with an avidity similar to that of eγγ, and showed a small but reproducible increase in affinity for hIgE compared with γγ (Table II). Thus, although the high affinity of the wild-type eγγ 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 e 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 FcγRIIa, with few differences between the interactions of the conserved amino acids. It is therefore surprising that introducing the G strand of FcεRI into the γγ chimera results in alterations to IgE binding. The residues in the G strand that are not conserved between FcεRI and FcγRIIa 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 FcγRIIa 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 Trp^{110}, (Arg^{106} and His^{108}), 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 FcγRIIIa or FcγRII for the D1 of FcRI and retain IgE binding.

The Homology Model of FcεRI—A homology model of FcεRI (Fig. 1) was based on the recently solved structure of FcγRIIIa (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 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. 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, and as a consequence maintain the binding sites 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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