

The Analysis of the Human High Affinity IgE Receptor FcεRIα from Multiple Crystal Forms

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We have solved the structure of the human high affinity IgE receptor, FcεRIα, in six different crystal forms, showing the structure in 15 different chemical environments. This database of structures shows no change in the overall shape of the molecule, as the angle between domains 1 and 2 (D1 and D2) varies little across the ensemble. However, the receptor has local conformational variability in the C' strand of D2 and in the BC loop of D1. In every crystal form, a residue inserts between tryptophan residues 87 and 110, mimicking the position of a proline from the IgE ligand. The different crystal forms reveal a distribution of carbohydrates lining the front and back surfaces of the structure. An analysis of crystal contacts in the different forms indicates regions where the molecule interacts with other proteins, and reveals a potential new binding site distal to the IgE binding site. The results of this study point to new directions for the design of molecules to inhibit the interaction of FcεRIα with its natural ligand and thus to prevent a primary step in the allergic response.

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Introduction

Fc receptors (FcRs) are found on the surface of a broad range of hematopoietic cells and bind to the crystallizable fragment (Fc) portion of antibody molecules. FcRs pair the antigenic specificity of antibodies to the diverse effector functions of different immune system cells. By binding an antibody specific for a given antigen, FcRs enable an immune system cell to respond to the antigen. This mechanism is used by a broad range of cells including mast cells, basophils, eosinophils, macro-

phages, neutrophils, and natural killer cells.¹ Fc receptors fall into three families based on homology. One family contains two or three immunoglobulin (Ig) domains in tandem, and includes FcεRI, FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64).² A second family has homology to the major histocompatibility complex (MHC) class I molecule and includes the neonatal Fc receptor, FcRn.^{3,4} The third family contains members homologous to C type lectins and includes the low affinity IgE receptor, CD23 or FcεRII.⁵

The human high affinity Fc receptor, FcεRI, is found on the surface of mast cells, basophils, dendritic cells, platelets, and eosinophils.^{6–10} This receptor mediates anti-parasitic immune responses,⁹ but when inappropriate activation of this pathway occurs, the resulting disease pathologies include allergies, anaphylaxis, and asthma.^{11–13}

Abbreviations used: FcR, Fc receptor; MHC, major histocompatibility complex; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; NCS, non-crystallographic symmetry.

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Human FcεRI contains four distinct polypeptide chains: an α chain, a β chain, and a dimer of γ chains. The extracellular portion of the α chain binds with high affinity to the Fc region of the IgE antibody ($K_D \approx 10^{-9}$ – 10^{-10} M).^{14–18} Both the β and γ chains participate in the early stages of the intracellular signal transduction cascade through phosphorylation of their immunoreceptor tyrosine based activation motifs (ITAMs).^{19,20} The β chain amplifies the signal produced by the complex of proteins,²¹ while the γ chain has homology to the ζ chain of the T cell receptor.²² In the usual FcεRI signaling complex, a large aggregate of proteins assembles: a polyvalent antigen and at least two IgE antibodies (with four polypeptide chains each) bound to two FcεRI complexes (with four chains each). This complex of at least 17 chains then recruits the src family kinase Lyn and the kinase Syk to initiate the intracellular signal.^{23,24} In mast cells, this signal cascade ultimately leads to degranulation and the release of histamine, cytokines, and other inflammatory mediators.¹⁰

The interaction between FcεRIα and IgE is one target for the treatment of allergies and asthma,²⁵ based upon clinical studies of the monoclonal antibody rhuMAb-E25 against the IgE molecule.²⁶ Further understanding of the IgE-FcεRIα interaction may allow for the development of small molecules to disrupt binding of the antibody to the receptor. The analysis presented here points to features of the FcεRIα molecule that may be exploited by inhibitors of the interaction between FcεRIα and IgE.

We have previously solved the structure of the extracellular portion of the FcεRI α chain, both alone²⁷ and in complex with its ligand, the Fc portion of the human IgE antibody.²⁸ Those studies revealed two distinct binding sites on the surface of the receptor upon formation of the complex with the IgE-Fc. Binding site 1 in the complex centers around Tyr131 on the C' strand in D2 of the receptor. Binding site 2 in the complex includes four exposed tryptophan residues on the top surface of the receptor. Two of these tryptophan residues participate in a proline sandwich, with Trp87 and Trp110 from the receptor packing against each side of Pro426 from the antibody.

The paper presented here extends the study of the FcεRIα molecule, by showing the structure of the FcεRIα extracellular domains from six different crystal forms and from four different glycoforms. The receptor shows surprising conformational adaptability depending on its chemical context. The comparison of the structure in different crystal forms reveals sites on the molecule favored for protein-protein interactions. This analysis identifies regions of FcεRIα known to be involved in the biological signaling aggregate and points to additional surfaces that may be involved.

Results

Overall description of FcεRIα structures

The structure of the FcεRIα molecule has been solved in six different crystal forms, five of the receptor by itself, and one of the receptor in complex with the Fc portion of the IgE antibody. The receptor alone crystallizes in two monoclinic crystal forms (forms M1 and M2), two tetragonal crystal forms (forms T1 and T2), and a hexagonal crystal form (form H1). The receptor in a complex with its Fc ligand forms a rhombohedral crystal (form Complex1). In the crystallographic asymmetric unit, forms M1, H1 and Complex1 contain a single copy of the receptor, form M2 contains two copies, and forms T1 and T2 contain five copies. Taken as a group, the six crystal forms show the receptor in 15 different chemical environments, and thus provide a database for understanding the biochemical properties of the receptor. Table 1 summarizes the crystallographic, X-ray data collection, and refinement statistics for the six crystal forms.

Figure 1(a) shows a superposition of 11 representative structures. The receptor shows little overall change in shape, as the angle superimposing D2 on D1 ranges from 158° to 163° across all the structures (Table 2). When superimposed against the highest resolution form M1, the other structures have deviations ranging from 0.76 to 1.12 Å for 153 to 157 C^α atoms (Table 2). Two regions of the structure do not superimpose well, the C' strand in D2 and the BC loop in D1. In addition, the structures differ at the termini of the polypeptide chain. Figure 1(b) plots the average deviation from the mean C^α position for each residue, indicating the regions with the greatest deviations.

Differences among the crystal forms

The C' strand in D2, central to binding site 1 when FcεRIα binds to the IgE molecule, varies across the structures in the ensemble. Canonical Ig domains contain seven β strands forming two β sheets, with hydrogen bonds between adjacent antiparallel strands within a sheet. In canonical Ig domains, strands A, B, E, and D form one sheet, and strands C, F, and G the other. C' strands (found in the V, C2, and I subsets of Ig domains) are hydrogen bonded to the C strand, while D strands occur within the other β sheet, hydrogen bonded to the E strand.^{29,30} Based upon sequence homology, FcεRIα D2 belongs to the C2 subset of the Ig fold family, predicted to have a C' strand and lack a D strand.³¹ In the ensemble of FcεRIα structures presented here, the stretch of residues in D2 from 125 to 131 forms a C' strand, or a D strand, or falls in locations between these two extremes. Twelve of the 15 copies of the FcεRIα molecule described in this study contain a C' strand for this stretch of residues. However, the structures in crystal forms H1, M1, and copy A of

Table 1. Crystallographic, X-ray data collection, and refinement statistics

Crystal name	M1	M2	T1	T2	H1	Complex 1
<i>A. Crystal summary</i>						
Space group	C2	C2	$P4_3$	$P4_3$	$P6_122$	$R32$
Cell lengths (Å)	88.6, 69.7, 49.4	136.9, 73.8, 79.4	145.1, 145.1, 62.7	150.5, 150.5, 74.2	58.7, 58.7, 232.0	192.8, 192.8, 302.4
Cell angles (deg.)	90, 116.7, 90	90, 117.7, 90	90, 90, 90	90, 90, 90	90, 90, 120	90, 90, 120
No. per asymmetric unit	1	2	5	5	1	1
Residues in construct	1-176	1-176	1-172	1-172	1-172(I170C)	1-176
Cell line	High Five	High Five	Lec1 CHO	Lec1 CHO	Sf9	High Five
<i>B. Data collection statistics</i>						
Beamline	SSRL 7-1	APS 5ID	CHESS A1	CHESS A1	APS 5ID	APS 5ID
Wavelength (Å)	1.08	1.005	0.914	0.92	1.004	1.034
Resolution (last shell) (Å)	40-2.4 (2.49-2.4)	40-3.2 (3.31-3.2)	40-3.2 (3.31-3.2)	40-4.1 (4.25-4.1)	20-3.2 (3.31-3.2)	40-3.5 (3.63-3.5)
R_{sym} (last shell) (%) ^a	5.7 (22.6)	9.7 (43.6)	10.5 (36.1)	5.8 (30.0)	10.3 (51.6)	9.1 (46.1)
$\langle I/\sigma_i \rangle$ (last shell)	9.5 (4.5)	9.2 (3.0)	7.7 (2.7)	7.3 (1.9)	11.8 (5.3)	16.5 (2.7)
Completeness (last shell) (%)	96.9 (92.5)	99.3 (100.0)	97.5 (87.2)	86.8 (83.2)	93.2 (95.4)	99.8 (99.7)
Measured/Unique	40,243/10,248	47,238/11,641	159,490/21,353	25,057/11,510	32,661/4076	84,873/27,484
<i>C. Refinement statistics</i>						
$R_{\text{work}}/R_{\text{free}}$ (%) ^b	22.6/25.4	24.5/27.8	26.2/30.9	27.3/29.1	26.4/30.6	25.4/27.3
Atoms: total	1536	3131	7606	7660	1536	5251
Protein	1371	2843	6905	6905	1381	4821
Carbohydrate	92	288	701	755	155	259
Water	73	0	0	0	0	0
Ramachandran plot						
Favored (%)	89.8	82.6	81.2	76.7	81.1	77.0
Allowed (%)	7.5	16.5	16.7	22.6	17.6	21.5
Generous (%)	2.0	1.0	1.5	0.0	0.7	1.5
Disallowed (%)	0.7	0.0	0.7	0.7	0.7	0.0
RMS deviations from ideal						
Bonds (Å)	0.0072	0.0079	0.0089	0.0086	0.0116	0.0100
Angles (deg.)	1.51	1.33	1.44	1.41	1.72	1.55
Dihedrals (deg.)	27.0	24.7	26.1	26.5	26.2	25.3
Impropers (deg.)	0.81	0.87	0.79	0.81	0.94	1.23
PDB code	1F2Q	1J86	1J88	1J89	1J87	1F6A

^a $R_{\text{sym}} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$, where $I_{h,i}$ is the i th intensity measurement of reflection h and $\langle I_h \rangle$ is the average intensity of that reflection.

^b $R_{\text{work}}/R_{\text{free}} = \sum_h |F_P - F_C| / \sum_h |F_P|$, where F_C is the calculated and F_P is the observed structure factor amplitude of reflection h for the working/free set, respectively.

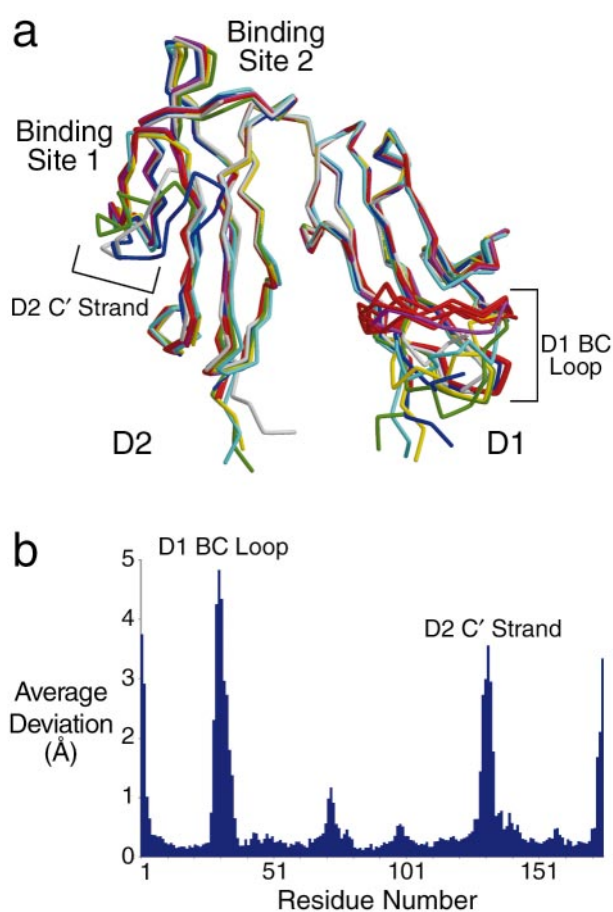


Figure 1. The ensemble of structures. (a) Eleven different copies of the receptor are superimposed. C α traces are colored according to the scheme: form M1, white; form M2 (copy A), green; form M2 (copy B), yellow; form H1, blue; form Complex1, cyan; form T1 (copies A-E), red; form T2 (copy A), magenta. Form T2 contains four other copies not shown in the superposition, as they are identical (due to tightly restrained NCS) to the one shown. The objects were superimposed with the LSQ options in the program O using all C α atoms in the receptor. Binding site 1 includes differences in the C' strand of form M1, form M2A, and form H1. The BC loop in D1 and the termini also show variability among the different crystal forms. (b) A plot of the average deviation for each residue. This Figure graphs the average deviation from the mean position of each C α atom in the 15 structure ensemble. The labeled regions identify the segments of the structure with the largest deviations.

form M2 (M2A) show differences for this stretch: it is in a D strand in H1 and an intermediate conformation in the other two. Figure 2 shows an expanded view of this region of the receptor, with the positions of the C' and D strands indicated with arrows. Figure 3 shows simulated-annealing composite omit electron density^{32,33} for this region from four representative structures. Table 2 summarizes the conformation of this region for the molecules in the six crystal forms.

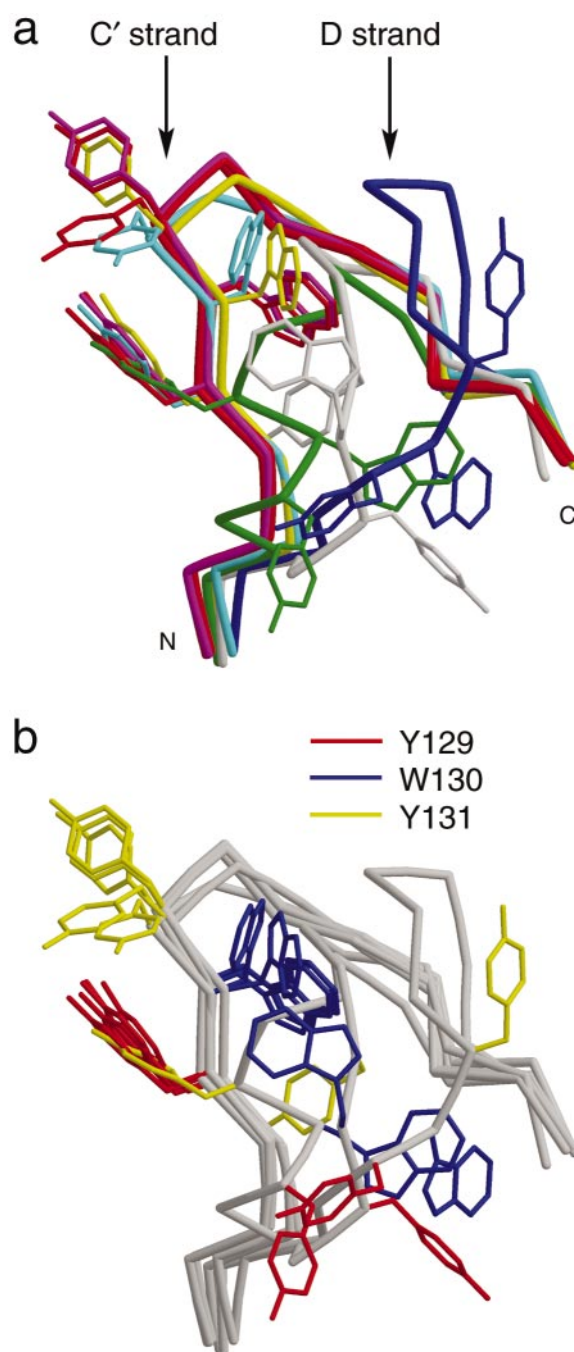


Figure 2. Close-up of binding site 1 in eight different structures. C α positions for residues 125-138 are shown, along with side-chain atoms for residues Tyr129, Trp130, and Tyr131. (a) Chains are colored according to the scheme in Figure 1: form M1, white; form M2 (copy A), green; form M2 (copy B), yellow; form H1, blue; form Complex1, cyan; form T1 (copies A and B), red; form T2 (copy A), magenta. Other copies of the tetragonal forms are identical; only one representative structure is shown. In form M1, form M2A, and form H1, the coordinates differ from canonical C' structure. (b) The same Figure is shown with a different coloring scheme, showing the variation in location of three aromatic residues. Side-chain atoms for Tyr129 appear red, for Trp130 appear blue, and for Tyr131 appear yellow. Tyr131 in form M2A falls in nearly the same location as Tyr129 in most crystal forms.

Table 2. Summary of FcεRIα models

Crystal and chain	D1-D2 angle (deg.)	RMS deviation <i>versus</i> M1		Binding site 1:	Binding site 2:
		(Å)	C α atoms	130 Loop conformation	Sandwich contents
M1	161	0	171	Intermediate	Val143
M2 A	161	0.758	153	Intermediate, helix	Pro173
M2 B	162	0.774	154	C' strand	Phe32
T1 A	159	0.834	153	C' strand	Phe32
T1 B	158	0.868	153	C' strand	Phe32
T1 C	160	0.856	154	C' strand	Phe32
T1 D	159	0.832	154	C' strand	Phe32
T1 E	159	0.817	153	C' strand	Phe32
T2 (all 5) ^a	159	0.858	154	C' strand	Phe31, Phe32, Asp33
H1	162	0.853	153	D strand	Tyr131
Complex 1	163	1.124	157	C' strand	Pro426 (from Fc)

^a With the NCS symmetry imposed on form T2, all five copies are identical. Given the low resolution for form T2, the residues inserting into the sandwich are uncertain, but residues 31-33 are in the immediate vicinity.

The BC loop in D1 (residues 27-38) shows the greatest variability across the ensemble. The conformation of this loop depends upon the chemical environment, as it moves as much as 16 Å for the C α position of Phe31 between structures T1A and M2A. The electron density for this loop is often the poorest protein density in a map, suggesting that the loop varies in conformation even within a single crystal. Forms M1 and H1 have four and three residues absent, respectively, from the model due to disorder in this loop and resultant weak electron density. In the T1 and T2 structures, this loop is higher than the others (when the molecule is viewed in the orientation of Figure 1, with the FG loop of D2 at the top and the cleft between the domains at the bottom). In the tetragonal cells, the C α atom of residue Glu33 in the BC loop of D1 passes within 5.5 Å of the C α atom of Ser51. In the two copies of the receptor in M2, this loop shifts down due to crystallographic contacts, and this distance increases to 12 Å for M2A. In these two copies, this region shows good density for the loop, which moves away from the rest of the molecule to produce a small empty pocket inside. The location of these loops in H1 and M1 is intermediate to those of the tetragonal cells and M2.

There are minor variations in surface-exposed loops among the different structures. In particular, the FG loop in D1 (residues 69 to 74) shows a 5 Å shift between form M1 and form H1, with the other forms found between these two structures. This movement is typical for flexible loops in protein structures, and this loop may have increased mobility due to the carbohydrate attached to Asn74.

The density at the termini tends to be poorly ordered. While the Complex1, H1, and M2 crystal forms show density for the protein's N terminus, the other models begin at amino acid 4. At the C terminus, the M1 and M2A models extend to residue 174 out of 176, the M2B model to residue 173 out of 176, the H1 model to the C-terminal residue 172, and the T1 and T2 tetragonal models to residue 171 out of 172. Figure 1(b) identifies those

regions on the protein with the greatest variability in the ensemble.

IgE binding residues in the crystal forms

In the complex of the receptor with the Fc portion of the IgE molecule, tryptophan 87 and 110 from the receptor surround proline 426 from the IgE-Fc, forming a "proline sandwich." In the other crystal forms, Trp87 and Trp110 participate in variants of this proline sandwich where the tryptophan residues also enclose a hydrophobic group. In the other crystal forms, these tryptophan residues pack around a phenylalanine, a tyrosine, and a valine as well as a proline. Tryptophan-proline pairing is strongly preferred in protein-protein interfaces, while tryptophan-phenylalanine, tryptophan-tyrosine, and tryptophan-valine pairings are also favored.³⁴ Figure 4 shows a panel of representative structures from the ensemble, highlighting the two tryptophan residues and the interior of the sandwich. Table 2 lists the contents of the proline sandwich in the different structures.

Upon the binding of IgE-Fc, the FcεRIα molecule undergoes no major conformational rearrangements, as the interdomain angle remains virtually identical (Table 2). In the two crystal forms previously described, (forms M1 and Complex1), the receptor binding site 1 region shows minor changes, with residues 127 to 131 moving. The C' strand in D2 is longer in the complex compared to form M1, as this strand extends to residue 131 in the complex, but in form M1 the strand stops at residue 127 (with random coil continuing to residue 131). However, these differences are not specific to IgE-Fc binding, because the conformation seen in form Complex1 is the predominant one in the ensemble of unliganded forms. Eleven structures of the receptor by itself (including M2B and all copies in T1 and T2) show the same C' strand conformation as in Complex1. While the three structures deviating from the C' norm (M1, H1, and M2A) have crystal contacts in this region, the 12 other structures also participate in crystal

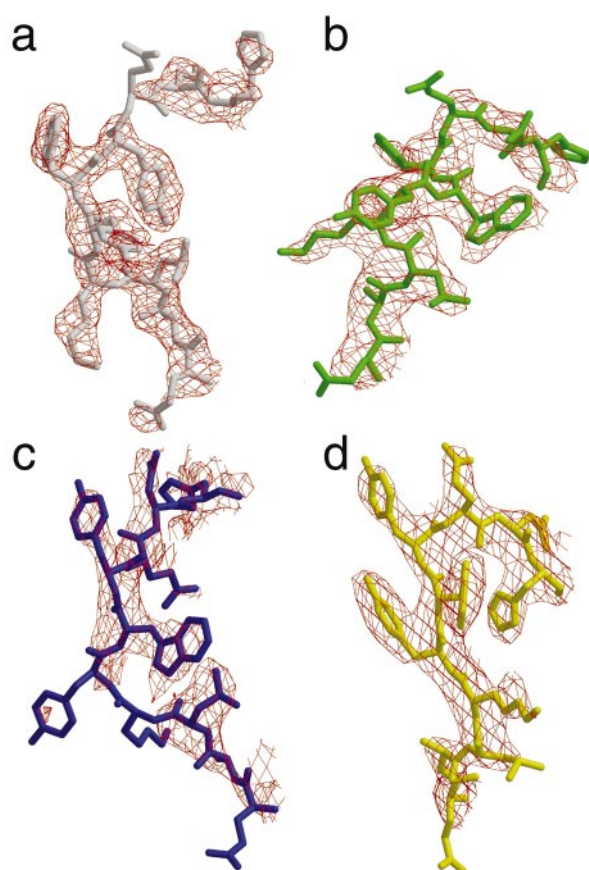


Figure 3. Electron density from four structures. Electron density maps were calculated using σ_a -weighted simulated annealing composite omit map protocols in the program CNS. Atoms are drawn from residue 125 to 134, near binding site 1 in domain D2, and the maps are drawn around the atoms. (a) Form M1. The 2.4 Å map is contoured at 1.2σ . The strand is in a location intermediate between C' and D strands. (b) Form M2, copy A. The 3.2 Å map is contoured at 1.2σ . The atoms form a single turn of α -helix surrounded by random coil and are in found in an intermediate location between C' and D strands. (c) Form H1. The 3.2 Å map is contoured at 0.9σ . The atoms have crossed over to the opposite sheet of the Ig domain; they form β strand D hydrogen-bonded to the E strand. (d) Form M2, copy B. The 3.2 Å map is contoured at 1.2σ . This is the most common conformation for the FcεRIα chain, a C' strand hydrogen-bonded to the C strand in D2, seen in 12 of the 15 structures.

contacts in this region. The conformation of the C' strand in the complex reflects the majority of the receptor models in the ensemble. However, the distinct conformations seen in different packing environments suggests this strand contains some unusual flexibility.

Carbohydrate ensemble

Three different expression systems produced glycoprotein used in this study, Lec-1 CHO cells,

and baculovirus-infected Sf9 and High Five insect cells. The Lec-1 CHO cells attach only high mannose carbohydrate to N-linked sites,³⁵ while the High Five and Sf9 cells can attach more highly processed carbohydrate.^{36,37} High Five cells produce FcεRIα glycoprotein less sensitive to PNGase F digestion than the Sf9 material (B. A. Wurzburg, personal communication), because the two types of insect cells can produce different carbohydrate glycoforms at N-linked sites.^{38,39}

The FcεRIα molecule contains seven potential N-linked carbohydrate sites, all seven of which have attached carbohydrate.⁴⁰ When carbohydrate is in an identical conformation in each copy within the crystal, electron density will appear in a map. Three of the seven carbohydrate sites on the FcεRIα molecule (Asn21, Asn42, and Asn166) show density in all of the crystal forms, implying that they are rigid relative to the protein in all variant glycoforms and crystal forms. The other four carbohydrate attachment sites (Asn50, Asn74, Asn135, and Asn140) show no density in some of the structures in the ensemble. This suggests that these carbohydrates are not well ordered within their crystal lattice, but are flexibly attached to the protein.

For each of the seven carbohydrate attachment sites, density for carbohydrate appears in at least two of the 15 molecules in the ensemble. The structures comprise a database showing where the carbohydrate can appear on the surface of the FcεRIα molecule. Figure 5(a) and (b) shows the carbohydrate ensemble produced by superposition of the C α positions of all 15 structures. Carbohydrate attached to Asn21, Asn42, and Asn166 in different crystal forms tends to superimpose, while the carbohydrate attached to the other sites varies more in location across the ensemble. The main-chain atoms of these three residues fall in well-ordered regions of the structure, potentially limiting the motion of the attached carbohydrate. Although the protein was produced in three different cell lines attaching unique carbohydrate glycoforms to the asparagine residues, the core carbohydrate superimposes well for three of the seven attachment sites.

Taken as a group, these carbohydrates cover the front and back faces of the receptor. Figure 5(c) and (d) shows the residues buried by the carbohydrate ensemble. The two binding sites for the IgE-Fc remain clear of carbohydrate, while much of the rest of the surface of the molecule contains carbohydrate. The crystal structures show only well-ordered carbohydrate, so Figure 5 represents a minimal subset of the carbohydrate covering the receptor. When found on the granulocyte membrane, the receptor is even more heavily decorated with carbohydrate, as the extracellular portion of the α chain contains nearly 50% carbohydrate by weight.⁴¹

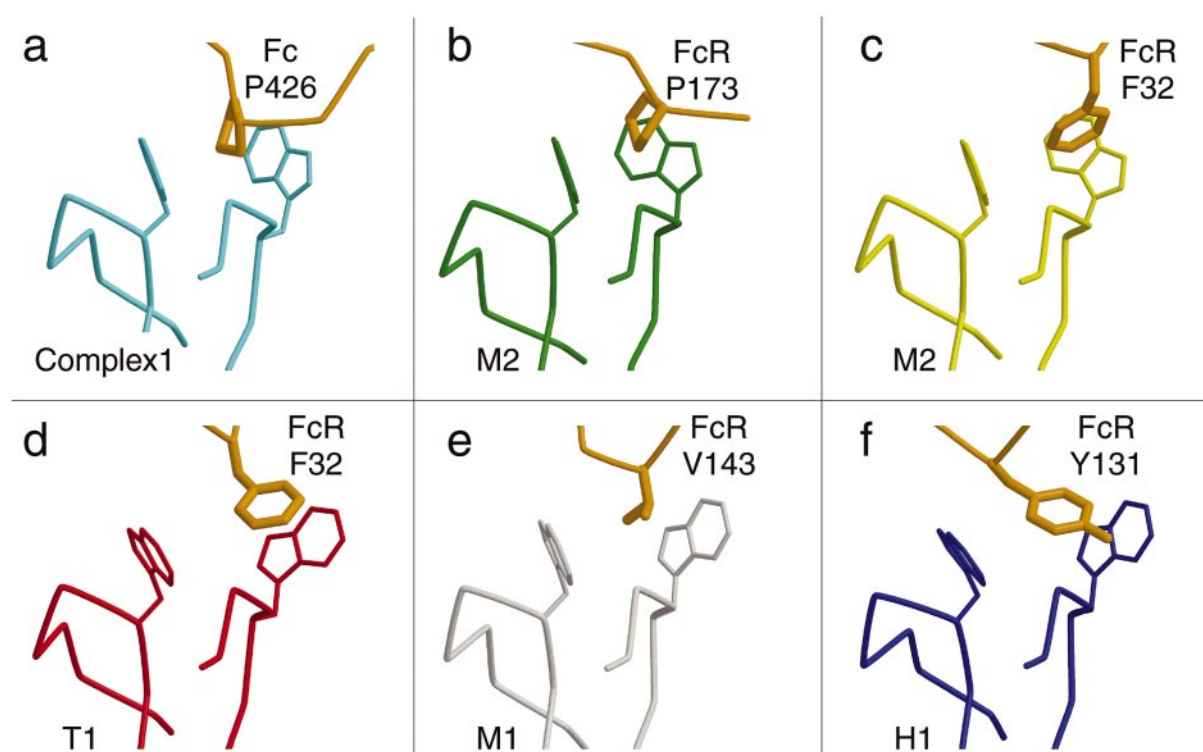


Figure 4. Contents of the proline sandwich in binding site 2. This Figure shows the contents of the proline sandwich in six representative structures. In FcεRIα, C^α atoms are shown for residues 83-89 and 108-116, side-chain atoms are shown for Trp87 (on the right) and Trp110. The bound molecule is shown in orange, with side-chain atoms drawn for the residue captured between the two tryptophan residues. The first panel shows the structure of the complex with IgE-Fc; all other panels are structures of the receptor by itself. (a) The wild-type interaction from form Complex1. Pro426 from the Fc is bound between the two tryptophan residues on the surface of the receptor. (b) Form M2, copy A. Pro173 from the receptor mimics the interaction seen in the complex. This interaction is found only when the receptor construct extends to residue 176 at the C terminus. (c) Form M2, copy B. Phe32 from a symmetry copy of the receptor inserts between the two tryptophan residues. (d) Form T1, copy A. Phe32 from a symmetry copy of the receptor inserts between the two tryptophan residues. This interaction is similar to the rest of the copies in crystal form T1, and represents that sample. (e) Form M1. Val143 from a symmetry copy of the receptor inserts between the two tryptophan residues. (f) Form H1. Tyr131 from a symmetry copy of the receptor inserts between the two tryptophan residues. In this form, binding site 1 and binding site 2 from a second molecule come together. This crystal form has the greatest change in the location of the C' strand (containing Tyr131) in D2.

Crystal contact map

Crystal contact sites contain protein-protein interactions, including potentially biologically relevant ones. By mapping the sites on the molecule participating in multiple crystal contacts, important binding sites may appear.⁴² Figure 6(a) maps known protein binding sites on the receptor by coloring those receptor residues buried upon formation of a complex with the IgE-Fc. Figure 6(b) shows the frequency of each residue appearing in a crystal contact in the six different crystal forms of the FcεRIα molecule. For each residue, the number of times it forms a crystal contact (defined as an atom less than 3.5 Å away from a residue in a neighboring molecule) is counted. The colors range from white for the absence of crystal contacts to dark blue for the two residues (Trp87 and Tyr131) used in all six crystal forms. Figure 6(b) details the hot spots for protein-protein contacts, or the areas on the molecule most often participating in crystal

lattice contacts. In the case of FcεRIα, the sites on the molecule most often found in crystal contacts correspond to the known binding sites for the IgE ligand. Thus, the database of crystal contacts correlates with the biologically relevant areas on the molecule. When a molecule crystallizes in a number of crystal forms, this method may be included with other techniques for identifying protein binding surfaces.⁴³⁻⁴⁷ The crystal contacts may map regions of the structure with favorable protein binding properties, analogous to mapping protein binding sites using phage display selection.⁴⁷

In the case of FcεRIα, two residues make crystal contacts in all six crystal forms in this study, and they are central to the biological function of the receptor. Tyr131 and Trp87 are key residues in binding site 1 and binding site 2 of the IgE complex, respectively. Tyr131 buries 130 Å² of surface area upon complex formation, more than any other receptor residue. This tyrosine, the central receptor residue in binding site 1, makes contacts with five

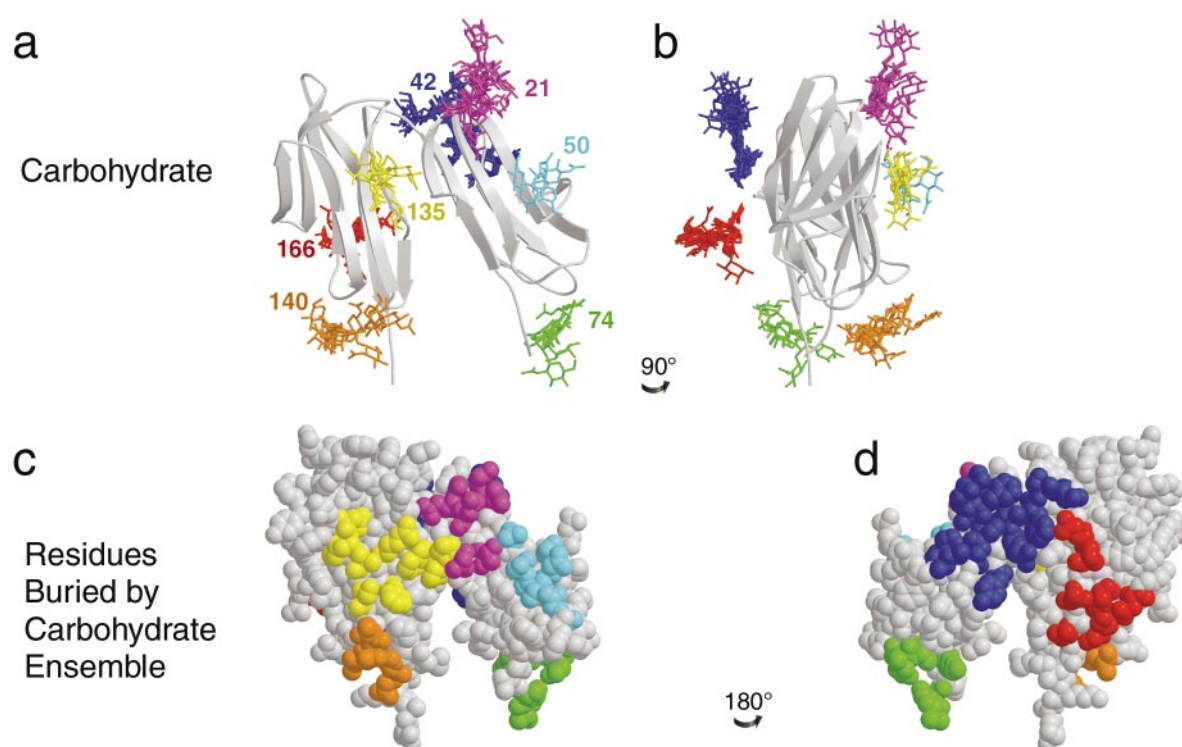


Figure 5. The carbohydrate ensemble and residues buried by carbohydrate. (a) and (b) The receptor is shown as a grey ribbon with carbohydrates as colored bonds. The 15 receptor molecules in the study were superimposed using C α atoms for the receptor, and the transformations were applied to the carbohydrate atoms. Carbohydrates are modeled where there was electron density in a composite omit $2F_o - F_c$ map and/or a $F_o - F_c$ map. Carbohydrate atoms are colored according to the attached asparagine residue: magenta, attached to Asn21; blue, attached to Asn42; cyan, attached to Asn50; green, attached to Asn74; yellow, attached to Asn135; orange, attached to Asn140; red, attached to Asn166. Some sites show little density for carbohydrate; this is likely due to crystallographic disorder, as mutational studies have shown that carbohydrate is attached to all seven N-linked sites. (a) Front view of the receptor, as in Figure 1. (b) Side view rotated 90° from (a). The C' strand in D2 is towards the viewer in this panel. (c) and (d) A CPK representation of the receptor protein atoms highlighting those residues buried by the carbohydrate ensemble. Residues are colored when more than 5 Å² of surface area of the residue is buried by a member of the carbohydrate ensemble. The coloring scheme used in (a) depicts which member of the carbohydrate ensemble buries the residue. (c) The same orientation as (a), while (d) is rotated 180° from that orientation.

residues from the IgE-Fc. This residue is likely important for determining the specificity of the interaction between FcεRIα and the IgE molecule, as it varies across other members of the FcR family.²⁸ In binding site 2, Trp87 buries 65 Å² of surface area upon complex formation. Trp87 forms part of the pair of tryptophan residues on the receptor that enclose a proline from the antibody. The two tryptophan residues and the proline in this sandwich are strictly conserved across homologous members of the family, and form a key interaction in binding site 2 for this family of Fc-FcR complexes.

One region of the receptor distal to the binding site for the IgE molecule makes crystal contacts in many forms. This region, the BC loop in D1, is far from the binding site for the Fc part of the IgE antibody, and is unlikely to be involved in interactions with the intact antibody. Since the FcεRI receptor is comprised of an α, a β, and two γ chains, this region of the α chain may contact the β and/or γ chains. Alternatively, this region may interact with

another as yet unknown molecule, or with the α chain of a second FcεRI receptor in the signaling aggregate, or with the cell membrane.

Discussion

The structure of this receptor from multiple crystal forms changes our understanding of how the receptor binds to the IgE-Fc. Had we seen only one crystal form each of the receptor and of the complex, we would conclude that the C' strand of D2 on the receptor changes conformation in response to ligand binding and is a switch sensitive to ligand binding. However, the ensemble of structures shows that this strand can adopt different conformations in different crystal forms, independent of the binding of IgE. This region does not contact carbohydrate (Figure 5), so the conformational variability is not likely due to the different glycoforms used in the study. This strand shows some unusual conformational adaptability, perhaps due to exposed aromatics. Three large aro-

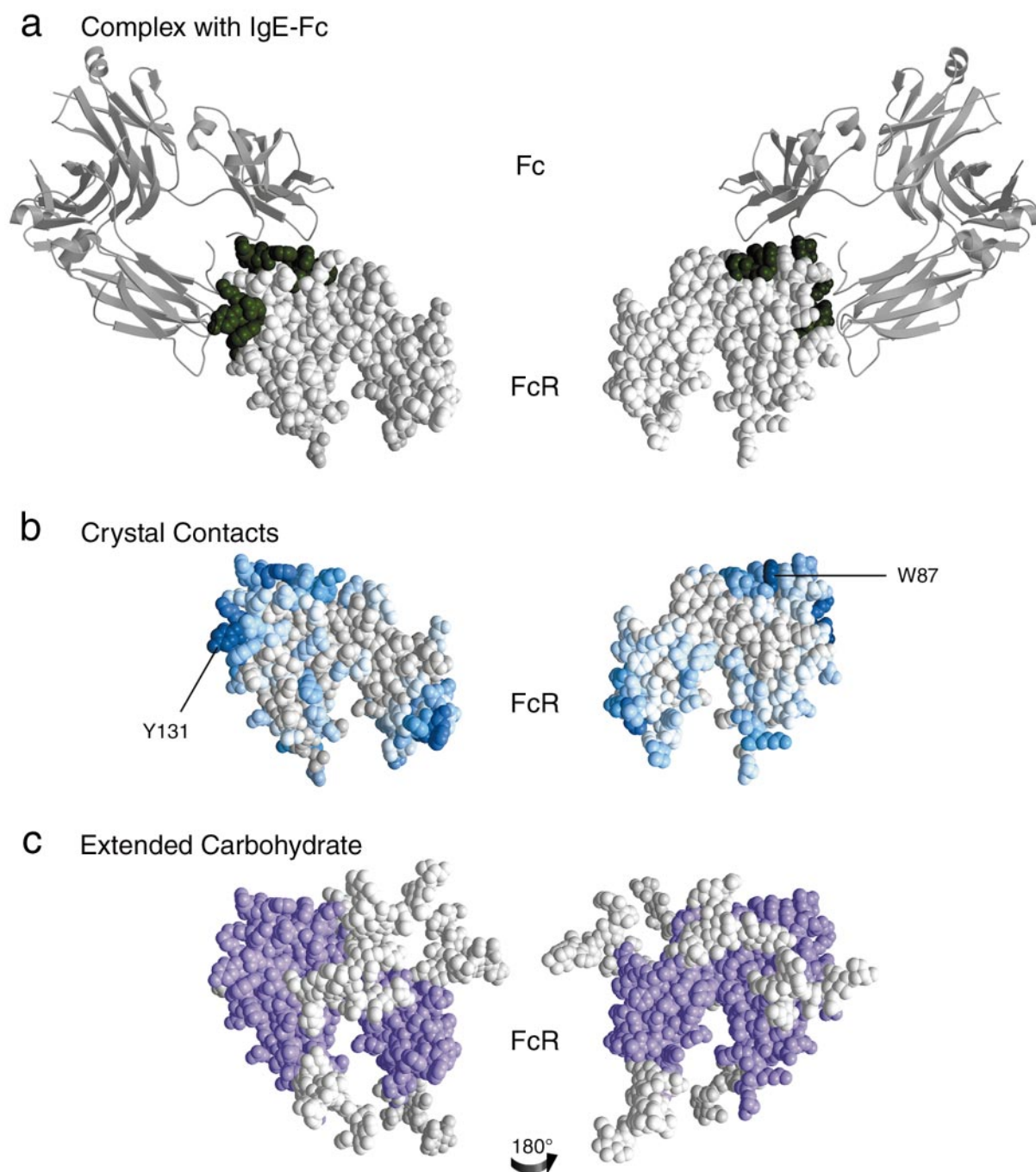


Figure 6. FcR complex with IgE-Fc, crystallographic contact map, and extended carbohydrate model. The receptor appears in CPK representation with different coloring schemes. The receptor orientation is identical in each panel with two views rotated by 180° within a panel. (a) The structure of the complex between IgE-Fc and Fc ϵ RI α . The Fc is shown in ribbon format and the receptor as CPK atoms, with carbohydrate omitted for clarity. The receptor residues buried in the complex appear green. (b) Residues are colored according to the number of crystal forms where the residue appears in a crystal contact. White residues are not used in crystal contacts in any of the six crystal forms, while dark blue residues are in crystal contacts in all six crystal forms. Other residues are colored intermediate for one to five crystal contacts. Tyr131 and Trp87, important residues in binding of the receptor to its Fc ligand, are the two residues in crystal contacts in all six crystal forms and are labeled. (c) Starting with core carbohydrate seen in electron density maps, carbohydrate was extended to nine residues using a model from the antibody 1IGT. The carbohydrate atoms are white and the receptor atoms purple. This approximates the glycosylation state of the receptor when on the surface of an immune cell. The carbohydrate protects large portions of the receptor surface, with only small patches available for protein-protein interactions. Note the correlation between the receptor residues buried in the complex in (a), those participating in crystal contacts in the ensemble in (b), and those sites on the protein free from carbohydrate in (c).

matics (Tyr129, Trp130, and Tyr131) in this stretch typically point outward, and do not generally pack within the protein's hydrophobic core. The presence of these surface-exposed aromatics may contribute to the flexibility of this strand, as it is not anchored by hydrophobic interactions to the interior of the protein.

The human FcεRIα molecule has a surprising ability to adapt to different chemical environments. The receptor has evolved to bind with high affinity to a single specific target, the Fc portion of the human IgE antibody. Despite this, the receptor shows the ability to adjust conformation locally in response to a different chemical environment. Flexibility in the receptor may allow optimal interactions with the flexible IgE-Fc Cε3 loops,⁴⁸ as these rearrangements are commonly seen in protein-protein recognition sites.⁴⁶ The protean adaptability of this protein could be exploited by those interested in inhibiting the interaction between FcεRIα and IgE.

The FcεRIα molecule has an unusually hydrophobic surface, with 1660 Å² out of 9870 Å² total surface area due to the exposed aromatics phenylalanine, tyrosine, and tryptophan. Exposed hydrophobic residues contribute free energy when buried in protein-protein interfaces.^{45,49–54} On the FcεRIα molecule, these three hydrophobic residues form much of the binding site for the IgE molecule. IgE-Fc binds very tightly to FcεRIα ($K_D \approx 10^{-10}$ M), yet the 1850 Å² buried in the complex is typical for protein-protein interfaces.⁴⁶ On the FcεRIα molecule, binding of IgE-Fc buries 16 residues and 930 Å² of surface area, of which eight residues and 460 Å² of surface area derive from exposed phenylalanine, tyrosine, and tryptophan residues. The large number of hydrophobic residues buried in the interface contribute to the high affinity of the FcεRIα molecule for the IgE-Fc.

A small molecule inhibitor of the interaction between FcεRIα and IgE might prove to be a promising drug candidate. Given the high affinity of the receptor for IgE, an optimal inhibitor should contact both binding site 1 and binding site 2 of the complex. An inhibitor targeted at the receptor should meet two requirements. First, it should contact Tyr131, the principal residue in binding site 1 of the native complex. Upon complex formation, this residue buries more surface area than any other on the FcεRIα molecule, so preventing the insertion of Tyr131 into its pocket on the Fc would significantly disrupt binding site 1. Second, an inhibitor should fill the proline sandwich site by intercalating between Trp87 and Trp110. This will disrupt the interactions at binding site 2 in the complex with IgE-Fc. Trp87 and Tyr131 are the only two residues found in protein-protein interactions in all of the structures of the FcεRIα molecule so far. Mutation of Trp87 to Asp decreases IgE affinity sevenfold,⁵⁵ while mutation of Tyr131 to Ala reduces IgE binding on transfected cells to 30 % of the normal value.⁵⁶ Disruption of these two

residues is one path toward rationally designed inhibition of the complex between FcεRIα and IgE.

The FcεRIα molecule is heavily glycosylated, with seven carbohydrate attachment sites in two Ig domains. Carbohydrate covers much of the surface of the receptor, and accounts for approximately 45 % of the mass of the full-length FcεRIα glycoprotein.⁴¹ Thus, *in vivo*, a polysaccharide averaging more than ten saccharide residues long attaches to each of the seven N-linked carbohydrate sites. Figure 6(c) shows a model for the receptor as it might appear on the surface of a mast cell. Based upon the core carbohydrate seen in electron density maps, carbohydrate was modeled to extend to nine saccharide residues,⁵⁷ using a model from the antibody 11GT.⁵⁸ The seven carbohydrate polymers protect large portions of the receptor surface, with isolated patches available for protein-protein interactions. Why is this molecule so heavily glycosylated? On a mast cell, cross-linking of FcεRI complexes initiates the signaling pathway that leads ultimately to the release of histamines, leukotrienes, prostaglandins, and cytokines. Carbohydrate on the surface of FcεRIα may help orient aggregated receptors for optimal signal transduction. Additionally, the carbohydrate may prevent premature aggregation in the absence of antigen by covering the extracellular surface of FcεRIα and obscuring potentially sticky surfaces. The cost of accidental aggregation of the receptors is inappropriate signaling, and the extensive carbohydrate coat on the receptor surface lowers the probability of a signaling error.

In the minimal FcεRI functional assembly, the α chain interacts with two γ chains on the surface of the granulocyte.⁵⁹ Given that the BC loop in D1 appears in crystal contacts in many of the structures used in this study (Figure 6(b)), this region shows some preference for participating in protein contacts. Distal to the IgE binding site at the top surface of FcεRIα, the BC loop in D1 is unlikely to be involved in antibody binding. Figure 6(c) indicates that this region is free from carbohydrate. The BC loop in D1 varies in location among the crystal forms (Figure 1), and this flexible loop could be stabilized by binding to another protein, a common occurrence upon formation of protein-protein interfaces.⁴⁶ However, it is also possible that the flexibility in this region allows the loop to adopt whatever conformation is stabilized by the crystal environment. This loop is a promising candidate for a site where the FcεRI α chain could interact with another protein on the extracellular side of the granulocyte membrane. This site may confer the α chain the ability to interact with another α chain, or the β or γ chains of the FcεRI tetramer.

The BC loop in D1 of FcεRIα has highly variable sequence across the members of the FcR family, and homologous members of the FcR family have different quaternary structures. In humans, for example, FcεRI is an αβγ₂ tetramer or an αγ₂ trimer, FcγRI and FcγRIIA are αγ₂ trimers, FcγRIIA

and FcγRIIC are single chains with ITAMs, while FcγRIIB1 and FcγRIIB2 are single chains with immunoreceptor tyrosine-based inhibitory motifs (ITIMs).¹ In addition to the sequence variability, there are structural differences in D1: when D1 of FcεRIα is superimposed on the homologous domain of FcγRIIA,⁶⁰ FcγRIIB,⁶¹ or FcγRIIIA,^{62–64} the main-chain path differs significantly in the BC loop, with minor differences at the N terminus and the FG loop. The sequence and structural variability in the BC loop of D1 could help each homologue bind different components of its unique signaling apparatus.

Materials and Methods

Six crystal forms contributed to the structural analysis described in this paper, five of the FcεRIα extracellular domains by themselves, and one of the FcεRIα extracellular region in complex with the Cε3 and Cε4 domains of the Fc portion of the human IgE antibody. The crystallographic statistics are summarized in Table 1.

Protein for this study came from expression in Chinese hamster ovary (CHO) cells and in baculovirus-infected lepidopteran cells. The CHO cell line Lec1, used for production of glycoprotein in crystal forms T1 and T2, is deficient in carbohydrate processing, attaching only high-mannose carbohydrates at N-linked sites.³⁵ Expression and purification of the Lec1 CHO glycoprotein was done as described.^{40,65} Two different species of insect cells were infected with baculovirus to produce glycoprotein for this study: *Trichoplusia ni* (High Five cells, Invitrogen) cells for crystal forms M1, M2, and Complex1, and *Spodoptera frugiperda* (Sf9) cells for form H1. Expression and purification of the insect cell protein was done as reported.²⁷

Crystal forms of the human high affinity IgE receptor α chain

Form M1

This monoclinic crystal form was solved and refined as described.²⁷ The model has been refined using all the observed data $|F| > 0$ to 2.4 Å. The model comprises residues 4–174 of the mature polypeptide, except the missing loop 32–35, and includes carbohydrate attached to Asn21, Asn42, and Asn166.

Form T1

This tetragonal crystal form was grown *via* vapor diffusion using a well solution of 20–32% (w/v) polyethylene glycol (PEG) 10000, 0.1 M ammonium citrate (pH 5.6), 0.1 M sodium chloride, and a protein starting concentration of 5–10 mg/ml. A variety of PEGs, buffers, and salts were also substituted, including PEG 4000, PEG 20000, sodium citrate (pH 5.6), sodium acetate, and ammonium sulfate. The free set represents 5% of the observed reflections selected using resolution shells. This crystal form was solved by molecular replacement using the program AMoRe,^{66,67} using a model from form M1. In the asymmetric unit, five copies of the receptor pack to form an approximate 5₄ screw axis. Fivefold non-crystallographic symmetry (NCS) averaging, as implemented in the programs DM^{66,68} and SPANCSI,⁶⁹ dramatically improved the electron density maps. The model has

been refined using all the observed data $|F| > 0$ to 3.2 Å. Non-crystallographic symmetry (NCS) restraint constants were applied: 300 kcal/mol per Å² on positions and 2 Å² on temperature factors to all protein atoms except flexible loops, loops in crystal contacts, and carbohydrate-attachment asparagine residues. The model includes five copies of residues 4–171 of the mature polypeptide plus attached carbohydrate. This crystal is a less-solvated variant of form T2, below, with approximately 22% less volume in the unit cell, shifted crystal contacts, additional crystal contacts, and a higher resolution limit. Forms T1 and T2 merge poorly, with a 55.5% overall R_{factor} value on Fs in the 20–3.8 Å resolution range.

Form T2

This tetragonal crystal form grew under similar conditions as crystal form T1, above. The free reflection set was chosen to match that of form T1. Given the low resolution diffraction limit from this form and its relation to form T1 above, the model from T1 was used as the starting model for this crystal. There are five copies of the receptor in the crystallographic asymmetric unit, forming a non-crystallographic 5₄ screw axis as in form T1. Fivefold NCS averaging, using the programs DM^{66,68} and SPANCSI,⁶⁹ was essential for the generation of interpretable maps. This form was refined conservatively, with tight NCS restraints (300 kcal/mol per Å²) on every atom. This crystal form was refined using all the observed data $|F| > 0$ to 3.8 Å; the resolution limit was later reduced to 4.1 Å. The model includes five identical copies of residues 4–171 of the mature polypeptide with attached carbohydrate.

Form M2

This monoclinic crystal form was grown *via* vapor diffusion using a well solution of 12–20% PEG 4000, 0.1 M Hepes (or Tris) (pH 7.5), 0 to 10% (v/v) isopropanol, and a starting protein concentration of 5–30 mg/ml. The free reflection set represents 5.3% of the observed reflections chosen using thin resolution shells. Initial phases came from molecular replacement using the program AMoRe^{66,67} with a model from form M1. This crystal form has been refined using all the observed data $|F| > 0$ to 3.2 Å. An NCS restraint constant of 300 kcal/mol per Å² has been imposed for all atoms except selected ones in loops and crystal contacts (residues 1–3, 27–38, 41–43, 69–75, 87, 98, 111–117, 125–135, 144, 152–158) and the N-linked carbohydrate atoms. The two molecules in the asymmetric unit include residues 1–174 (M2A) and residues 1–173 (M2B) of the mature polypeptide, and each molecule contains carbohydrate attached to five of the seven sites.

Form H1

These hexagonal crystals came from protein expressed as amino acid residues 1–172 of the mature human sequence with a cysteine substituted at position 170 in place of the wild-type isoleucine. The crystals were grown *via* vapor diffusion using a well solution of 20–30% PEG 4000, 0.1 M sodium citrate (pH 5.6), 0.1 M sodium chloride, and 5–40 mM methyl-6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside (HECAMEG), and a protein starting concentration of 10 mg/ml. The free set represents 10% of the observed reflections selected randomly. Molecular replacement with the program

AMoRe^{66,67} produced initial phases, using a model from form M1. This crystal form has been refined using all the observed data $|F| > 0$ to 3.2 Å. The model includes residues 1-171 of the mature polypeptide, except the missing loop 33-35, with carbohydrate attached to Asn21, Asn42, Asn140, and Asn166.

Crystal form of the human high affinity IgE receptor α chain in complex with its ligand, the Cε3 and Cε4 domains of the heavy chain of the IgE molecule

Form Complex1

The purification, crystallization, and structure determination of this complex has been described.²⁸ The receptor in the crystal contains mutations that prevent N-linked carbohydrate attachment at the wild-type sites 74, 135 and 140. Rhombohedral crystals grow with one copy of the FcεRIα:IgE-Fc complex in the crystallographic asymmetric unit. The model has been refined using all the observed data $|F| > 0$ to 3.5 Å. The FcεRIα portion of model includes residues 1-173 of the mature polypeptide, with carbohydrate attached to Asn21, Asn42, and Asn166.

For all structures

X-ray data were processed using the programs DENZO,⁷⁰ SCALEPACK,⁷⁰ and TRUNCATE.⁶⁶ Coordinates were built into electron density maps using the program O⁷¹ and refined using the program CNS.⁷² Model building was followed by rigid body refinement, simulated annealing, conjugate gradient minimization, and/or temperature factor refinement prior to map calculation and subsequent model building. The models were refined against all observed data with $|F| > 0$, using a bulk solvent model applied to the calculated structure factors below 8 Å resolution. Calculated structure factors were scaled to the observed set using an overall anisotropic temperature factor. Non-crystallographic symmetry restraints were used when available, and kept tight at 300 kcal/mol per Å². Refinement was performed using a maximum likelihood target of Hendrickson-Lattman coefficients, when available, otherwise using a maximum likelihood structure factor amplitude target.⁷³ Refinement steps were only accepted when they resulted in a decrease in R_{free} . Models were built into simulated annealing composite omit maps,³³ $2F_o - F_c$ maps, and $F_o - F_c$ maps, all calculated using σ_a -weighting.⁷⁴ Ramachandran plots were made using PROCHECK.⁷⁵ Superpositions were done in the program O,⁷¹ and average deviations calculated from these superpositions. Figures were drawn using the programs GRASP⁷⁶ and MOLSCRIPT.⁷⁷

PDB accession codes

Coordinates have been deposited in the RCSB under the following codes: form M1, 1F2Q; form M2, 1J86; form T1, 1J88; form T2, 1J89; form H1, 1J87; form Complex1, 1F6A.

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