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Structure of the Human IgE-Fc Cϵ3-Cϵ4 Reveals Conformational Flexibility in the Antibody Effector Domains

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Summary

IgE antibodies mediate antiparasitic immune responses and the inflammatory reactions of allergy and asthma. We have solved the crystal structure of the human IgE-Fc Cϵ3-Cϵ4 domains to 2.3 Å resolution. The structure reveals a large rearrangement of the N-terminal Cϵ3 domains when compared to related IgG-Fc structures and to the IgE-Fc bound to its high-affinity receptor, FcϵRI. The IgE-Fc adopts a more compact, closed configuration that places the two Cϵ3 domains in close proximity, decreases the size of the interdomain cavity, and obscures part of the FcϵRI binding site. IgE-Fc conformational flexibility may be required for interactions with two distinct IgE receptors, and the structure suggests strategies for the design of therapeutic compounds for the treatment of IgE-mediated diseases.

Introduction

The functional diversity of the antibody repertoire involves both the creation of antigen-specific binding sites and the coupling of these specific binding sites to different effector mechanisms of the immune system. Within the antibody, these two functional roles are found on separable parts of the protein, the Fab and Fc regions. Two antigen binding sites are contained within the Fab regions of antibodies, which are covalently linked through the antibody heavy chain to Fc effector domains (Padlan, 1994; Harris et al., 1999). The Fc domains provide specificity for the activation of downstream effector functions and are derived solely from constant domains of the antibody heavy chain. Isotype switching after B cell activation produces immunoglobulins with identical antigenic specificity connected to different heavy chain constant regions that fall into five major classes or isotypes: IgA, IgD, IgE, IgG, and IgM. Different Fc isotypes interact with distinct sets of cellular receptors or soluble proteins to initiate specific defense mechanisms. Effector mechanisms are adapted for specific pathogens, for the physical location of an infection, and for different stages of the immune response. Fc-associated effector mechanisms include phagocytosis, the initiation of cellular cytotoxicity and inflammation pathways, the activation of complement, and the feedback regulation of antibody production (Daeron, 1997; Ravetch and Clynes, 1998; Kinet, 1999).

IgE antibodies interact through their Fc domains with two cellular receptors of the immune system, FcϵRI and FcϵRII (CD23). IgE antibodies bind to the high-affinity receptor, FcϵRI, on the surface of mast cells, basophils, and eosinophils (Metzger, 1992; Kinet, 1999). Binding of polyvalent antigen by the receptor-bound IgE causes receptor aggregation, triggering cellular activation. On mast cells, this leads to the release of histamine, inflammatory mediators, and vasodilators. Mast cell reactions to environmental allergens are associated with the pathologies of allergy, asthma, and anaphylaxis (Turner and Kinet, 1999). Activation of eosinophils by FcϵRI provides defense mechanisms against parasitic infection (Gounni et al., 1994; Kinet, 1999), while FcϵRI on dendritic cells can deliver IgE-bound antigen into the MHC class II antigen-presentation pathway (Maurer et al., 1998). IgE antibodies also interact with a lower affinity receptor, FcϵRII, involved in antigen presentation, cellular cytotoxicity, and the regulation of IgE production (Sutton and Gould, 1993). While FcϵRI is homologous to a family of antibody receptors specific for IgE, IgG, and IgA antibodies, FcϵRII belongs to a different structural class of proteins and is uniquely associated with the IgE system.

IgE is the target of recent therapeutic approaches for asthma using humanized anti-IgE monoclonal antibodies (Jardieu and Fick, 1999; Chang, 2000). Antibodies directed against the IgE-Fc block receptor binding, leading to a decrease in receptor activation and expression levels and triggering a decrease in IgE serum levels. Structural studies of the IgE-Fc may provide new routes to improving anti-IgE therapies and to designing inhibitors for the treatment of a wide variety of atopic diseases.

IgE contains two antibody light chains associated with two heavy chains of the ϵ isotype. The three C-terminal constant domains of the heavy chain (Cϵ2, Cϵ3, and Cϵ4) dimerize to form the Fc effector domains. Compared to IgG, IgE antibodies have an additional constant domain, Cϵ2 (Figure 1A). The Cϵ3 and Cϵ4 domains are homologous to the IgG-Fc Cγ2 and Cγ3 domains, respectively, with 32% sequence identity between human IgE and IgG1 (Figure 1B). Both intact IgE and IgE-Fc fragments (Cϵ2-Cϵ4, Cϵ3-Cϵ4) bind with high affinity (K_D ~10^{-9}-10^{-10}M) to FcϵRI, and mutagenesis studies have implicated Cϵ3-domain residues in mediating this interaction (Weetall et al., 1990; Nissim et al., 1991; Basu et al., 1993; Presta et al., 1994; Henry et al., 1997), as well as the binding to FcϵRII (Sutton and Gould, 1993; Shi et al., 1997). IgE-Fc Cϵ3-Cϵ4 retains binding to both FcϵRI (Basu et al., 1993; Young et al., 1995; Henry et al., 1997) and FcϵRII (Shi et al., 1997), suggesting a minimal construct for structural studies.

Crystallographic studies of antibody Fc domains have been limited to the IgG class, leaving open many questions regarding the role of sequence and structural diversity in Fc-effector functions. We have solved the crystal structure of the human IgE-Fc Cϵ3-Cϵ4 that is associated with immune responses in allergic reactions, asthma, and parasitic infections (Metzger, 1992; Sutton and Gould, 1993; Kinet, 1999). The IgE-Fc crystal structure reveals a novel, closed conformation for Fc do-

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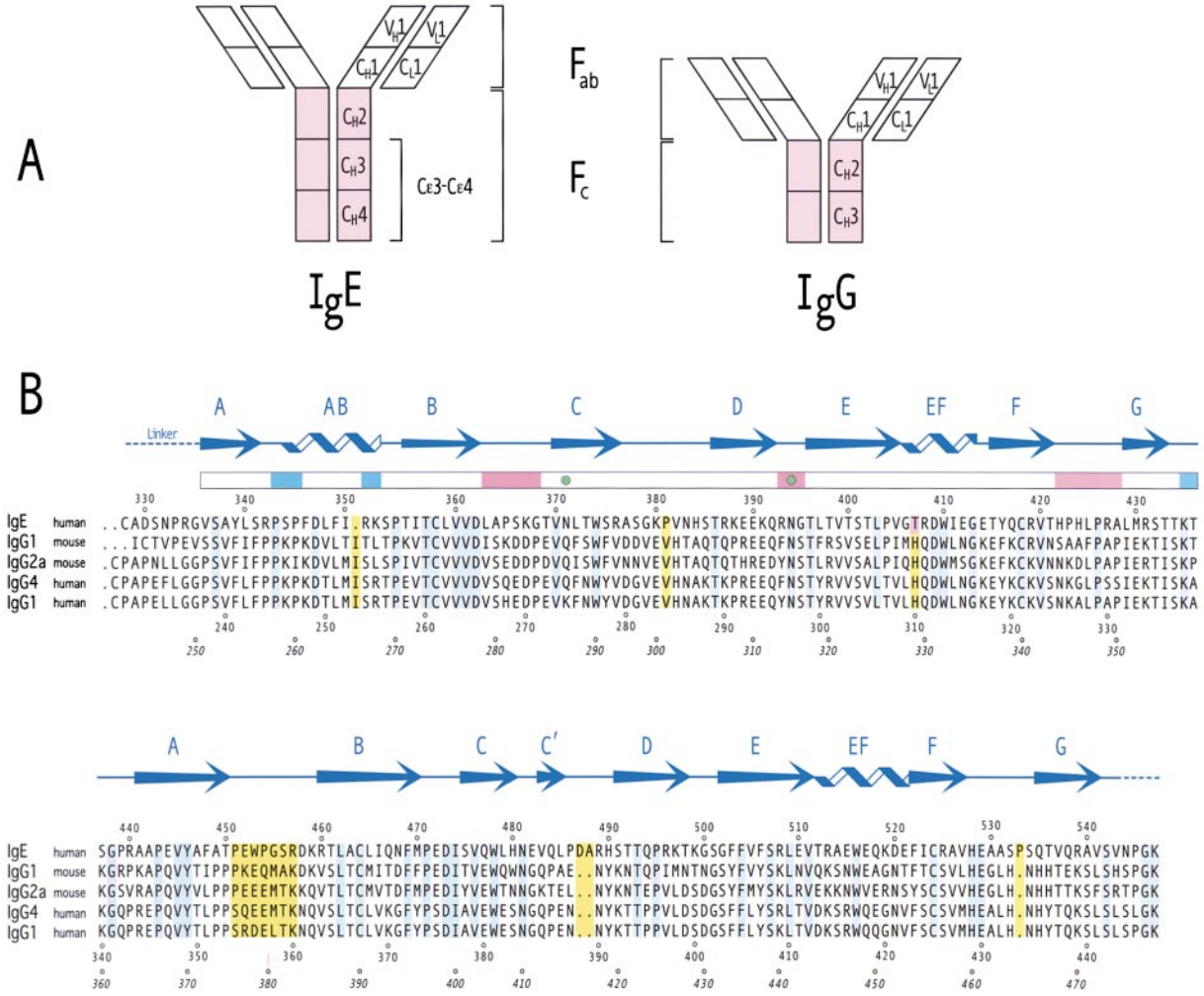

Figure 1. Structure and Sequence Alignments of IgE and IgG

(A) A schematic of the IgG and IgE antibodies. Both antibodies contain two isotype-specific heavy chains and two light chains (H_2L_2). The Fab domains contain both heavy chain and light chain components while the Fc domains are derived exclusively from the heavy chain. The IgE-Fc contains an extra domain pair (Cε2) compared to the IgG-Fc. The IgE Cε3-Cε4 domains are homologous to the IgG Cγ2-Cγ3 domains. (B) Structure-based sequence alignment of human IgE-Fc Cε3-Cε4 with the sequences of four IgG-Fcs for which crystal structures have been solved. IgE secondary structure is indicated using arrows for β strands and ribbons for α helices. Color bars indicate hinge residues (cyan) and FcεRI binding loops (pink). Green dots indicate carbohydrate attachment sites. Conserved residues are indicated in light blue. Structural differences (insertions, deletions, change in secondary structure) between the IgE and IgG are highlighted in yellow. A residue insertion in IgG (I253) is accommodated as a bulge after the AB helix. An insertion in Cε4 (D487-A488) is accommodated in the C′D loop, and a second insertion (P533) is accommodated as a *cis*-proline in a tight turn. In addition, the completely conserved Cγ2 AB helix histidine residue (H310 in IgG1, H329 in IgG2a) and the corresponding residue in IgE, threonine 409, are indicated in yellow and pink, respectively. The IgE numbering (above the sequence) is according to Dorrington and Bennich (1978). The numbering of human IgG1 is given directly below the sequence. The PDB numbering of murine IgG2a (1IGT) is shown in italics at the bottom (note that there are deletions in this numbering system).

mains. Comparison of the structure to the IgE-Fc bound to its high-affinity receptor (Garman et al., 2000) reveals a large conformational rearrangement in the IgE-Fc. Unique structural features of the Cε3-Cε4 interdomain interfaces are identified that may enable this conformational flexibility. Fc domain flexibility may allow IgE to form optimal interactions with both of its receptors, FcεRI and FcεRII. The structure of the IgE-Fc suggests novel strategies for antiallergy treatments including the design of molecules that act allosterically to block receptor binding.

Results and Discussion

Structure Determination

The C-terminal domains of human IgE-Fc, Cε3-Cε4 (Figure 1), were expressed in insect cells as described in the Experimental Procedures. The IgE-Fc Cε3-Cε4 protein contains three potential N-linked carbohydrate attachment sites, but only two are glycosylated in vivo, N371 and N394 (Basu et al., 1993; Young et al., 1995; B. A. W., unpublished data). Characterization of the Fc carbohydrate by endoglycosidase digestion, mass spectros-

Table 1. Data Collection and Refinement

	wtcu3 (pH 6.5)	wtcu1 (pH 7.5)
Data Collection Statistics		
Source	APS DND 5-ID	SSRL 7-1
Wavelength (Å)	0.906	1.08
Resolution (Å)	30.0–2.30 (2.38–2.30)[a]	30.0–2.60 (2.69–2.60)[a]
Completeness	99.8% (100%)[a]	99.9% (99.0%)[a]
Unique reflections (Total)	12,340 (106,855)	8,586 (83,604)
Average redundancy	8.7 (>7.1)[a]	9.7 (>7.4)[a]
$<I/\sigma_I>$	33.9 (10.6)[a]	13.9 (8.6)[a]
R_{merge}	5.4% (25.7%)[a]	6.6% (24.5%)[a]
Refinement (wtcu3)		
No. of Reflections (free)	11053 (1269)	
R_{work}/R_{free}	24.2/27.0	
Atoms (Total)	1,763	
Protein Atoms	1,618	
Water Molecules	145	
Average B factor		
Protein	51.8 Å^2	
Water	59.0 Å^2	
RMS Deviations from Ideality		
Bond angles	1.77°	
Bond lengths	0.007 Å	
Ramachandran (ϕ,ψ)		
Favored	87.3%	
Allowed	12.2%	
Generous	0.0%	
Disallowed	0.6%	

[a] Values for the highest resolution shell are shown in parentheses.
$R_{merge} = \Sigma|I_i - <I>|/\Sigma|I|$, where I_i is the intensity of an individual reflection and $<I>$ is the average intensity of that reflection. $R_{work/free} = \Sigma||F_p| - |F_c||/\Sigma|F_p|$, where F_c is the calculated and F_p is the observed structure factor amplitude. R_{work} and R_{free} were calculated using the working set and test set reflections, respectively.

copy of tryptic peptides, and mutational analysis (B. A. W., unpublished data) shows that high-mannose carbohydrate is attached to N394, which is a conserved glycosylation site in Fc domains. Although both deglycosylated IgE-Fc (Basu et al., 1993) and high-mannose IgE (Granato and Neeser, 1987) retain high binding affinity for FcεRI, deglycosylated IgE-Fc has a tendency to aggregate, making it a poor candidate for crystallographic studies (Basu et al., 1993).

The IgE-Fc was purified to homogeneity and crystallized. Crystals belong to space group $P42_12$ with cell dimensions a = b = 105.6 Å, c = 47.1 Å. The crystals contain a single IgE-Fc chain (half of the dimeric molecule) in the asymmetric unit, with the molecular dimer axis lying along a crystallographic dyad. The crystals diffract X rays to 2.0 Å using synchrotron X-ray sources. Molecular replacement searches using a variety of IgG-Fc models were unsuccessful, as were heavy atom searches. The IgE-Fc Cε3-Cε4 structure was solved by an automated molecular replacement search using ~12,000 distinct conformational variants of core models for the two Ig domains, systematically varying the angles relating the Cε3 and Cε4 domain models. Data collection and refinement statistics are shown in Table 1. The current R_{free} and R_{work} are 27.0% and 24.2%, respectively, to 2.3 Å resolution. There is no density for the ten amino-terminal residues of the protein (including the interchain disulfide) and the four C-terminal residues. In addition, the density for the Cε4 AB loop is poor.

Description of the IgE Structure

The Cε3 and Cε4 domains of IgE belong to the C1 set of Ig constant domains (Murzin et al., 1995). While the IgE Cε3 and Cε4 domains are individually similar to IgG Cγ2 and Cγ3 domains, respectively, a structure-based sequence alignment of the IgE-Fc and IgG-Fcs reveals several changes in secondary structure (Figure 1B, yellow). Compared to IgE, the IgG contains a single residue insertion (I253 in IgG1) that forms a bulge just after the Cγ2 AB helix. The IgE Cε3 domain lacks a C′ strand, and the Cε4 domain has a poorly ordered AB loop in place of the AB helix in Cγ3. Two prolines (P381 and P454) may contribute to the disruption of these secondary structures by altering hydrogen bond capabilities.

A ribbon diagram of the IgE-Fc is shown in Figure 2. As in IgG-Fcs, the upper domains (Cε3) of the IgE-Fc do not form any direct protein:protein contacts. The conserved carbohydrate attachment site (N394) faces the cavity between the Cε3 domains. While the observed electron density is consistent with glycosylation at this site, the poor quality of the density precludes modeling of carbohydrate. Inclusion of carbohydrate residues in the model did not decrease the R_{free} or improve the electron density maps. However, the electron density suggests that carbohydrate residues contact each other near the Fc dimer axis, forming the bottom of a narrow cleft between the Cε3 domains. The Cε4 domains form extensive contacts across the dimer interface, burying ~1,860 Å^2. Fourteen atomic contacts (<4 Å) formed between the Cε3 and Cε4 domains of a single chain bury 872 Å^2 and so bury a total of 1,744 Å^2 in the dimer.

No electron density for residues N-terminal to V336 (the Cε2-Cε3 linker region) is observed, despite the formation of the interchain disulfide in this region (B. A. W., unpublished data). These Cε2-Cε3 linker residues are ordered in the complex with FcεRI (Garman et al., 2000),

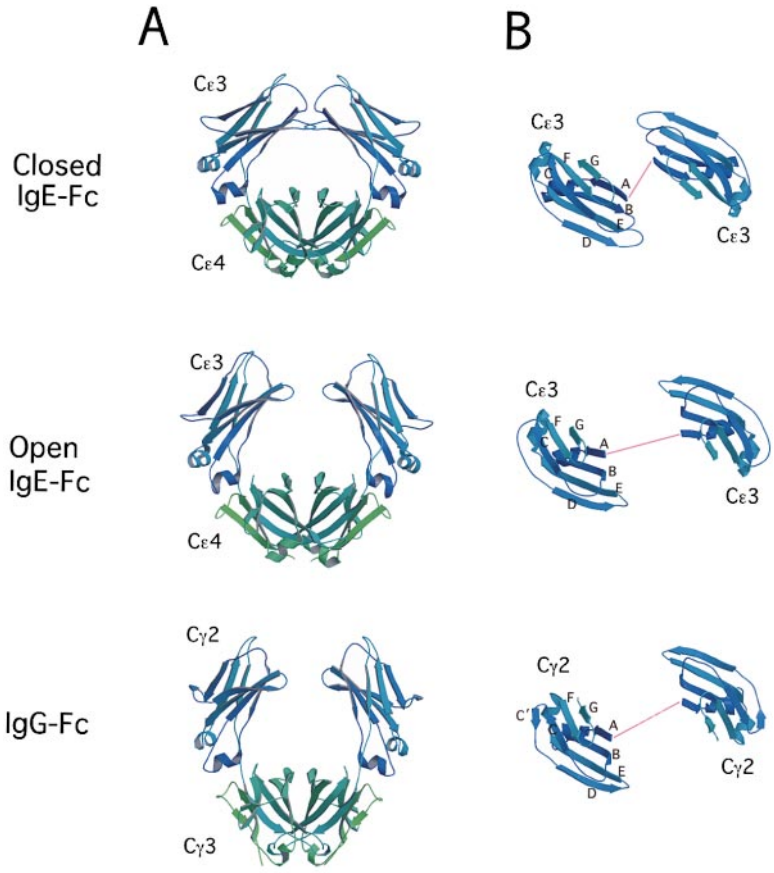

Figure 2. Comparison of the Free IgE-Fc, FcεRI-Bound IgE-Fc, and IgG-Fc Structures

The conformation of free IgE-Fc is referred to as the "closed" form and the conformation of the FcεRI-bound IgE-Fc is referred to as the "open" form. (A) Side views. (B) Top views (N-terminal domains). β strands are labeled (A–G), and a line is drawn between the first residue of the A strands for each Fc structure. In the closed IgE conformation, this distance is 13 Å, in the open form it is 23 Å, and in the IgG-Fc structure it is 22 Å.

and several of these residues interact with the receptor. While the absence of the Cε2 domains may contribute to the disorder of the Cε2-Cε3 linker in the free Fc, the asymmetric binding of linker residues to FcεRI suggests that flexibility is functionally important.

The IgE-Fc Adopts a Novel Conformation

The crystal structure of the IgE-Fc Cε3-Cε4 domains reveals a novel, closed conformation for antibody effector domains (Figure 2). In the free IgE-Fc, the Cε3-Cε4 interdomain angle is more acute than that observed between homologous IgG-Fc domains (Deisenhofer et al., 1976; Harris et al., 1999) or in the FcεRI-bound IgE-Fc (open conformation, Garman et al., 2000). Both the relative dispositions of the two Cε3 domains with respect to each other and to the Cε4 domains is altered. In the closed structure, the IgE-Fc Cε3 domains are closer together and slightly rotated with respect to each other. A top view of the Cε3 and Cγ2 domains illustrates differences in the interdomain gap (Figure 2B). In the IgE-Fc, the distance between the first residue of the Cε3 A strands is only 13 Å. The distance increases to 23 Å in the receptor-bound IgE-Fc, which is similar to the 22 Å observed between the Cγ2 domains in IgG2a-Fc (Harris et al., 1997). The Cε3 domains not only approach each other more closely, but they also lie closer to the Cε4 domains. For example, the top of the Cε3 domain (residue T396 in the DE loop) is 23 Å from the top of the Cε4 domain (residue S491). The distance between the corresponding residues in IgG2a is 33 Å, and in the receptor-bound IgE-Fc (open form) the distance is 31 Å. Thus, in the change between the open and closed forms, the top of each Cε3 domain moves 10 Å toward the other Cε3 domain across the dimer axis and 8 Å toward the Cε4 domain of the same chain. The closer approach of the upper domains of IgE (Cε3) to the lower domains (Cε4) decreases the overall height of the IgE-Fc by ~7 Å compared to the IgG-Fc.

The IgE-Fc conformational change is much greater than any differences observed among IgG-Fc crystal structures. Six crystal structures of the IgG-Fc provide nine different observations of a single chain of the IgG-Fc (in three structures, the two chains are constrained by crystallographic symmetry to be identical). These nine IgG-Fc chains, aligned via their Cγ3 domains, reveal IgG-Fc conformational variability as a family of Cγ2 positions (Figure 3A). In the closed structure, the IgE Cε3 domain lies far outside the range of observed IgG-Fc conformations. When bound to FcεRI, the angle between the Cε3 and Cε4 domains increases, and the Cε3 domains approach the observed positions for IgG Cγ2 domains. Some of the structural variation in the IgG-Fcs may be attributable to sequence differences. While the human IgG structures share ~95% sequence identity and the mouse structures have ~67% identity, the largest difference in IgG Cγ2 positions occurs between the human and mouse structures that share ~64% identity (Harris et al., 1999). However, the largest conformational change occurs between the open and closed forms of the IgE-Fc, which are identical in sequence, demonstrating the inherent flexibility of the IgE-Fc.

Analysis of IgE- and IgG-Fc Conformational Flexibility

The IgE-Fc conformational change can be described by an axis relating the two Ig domains in the open and

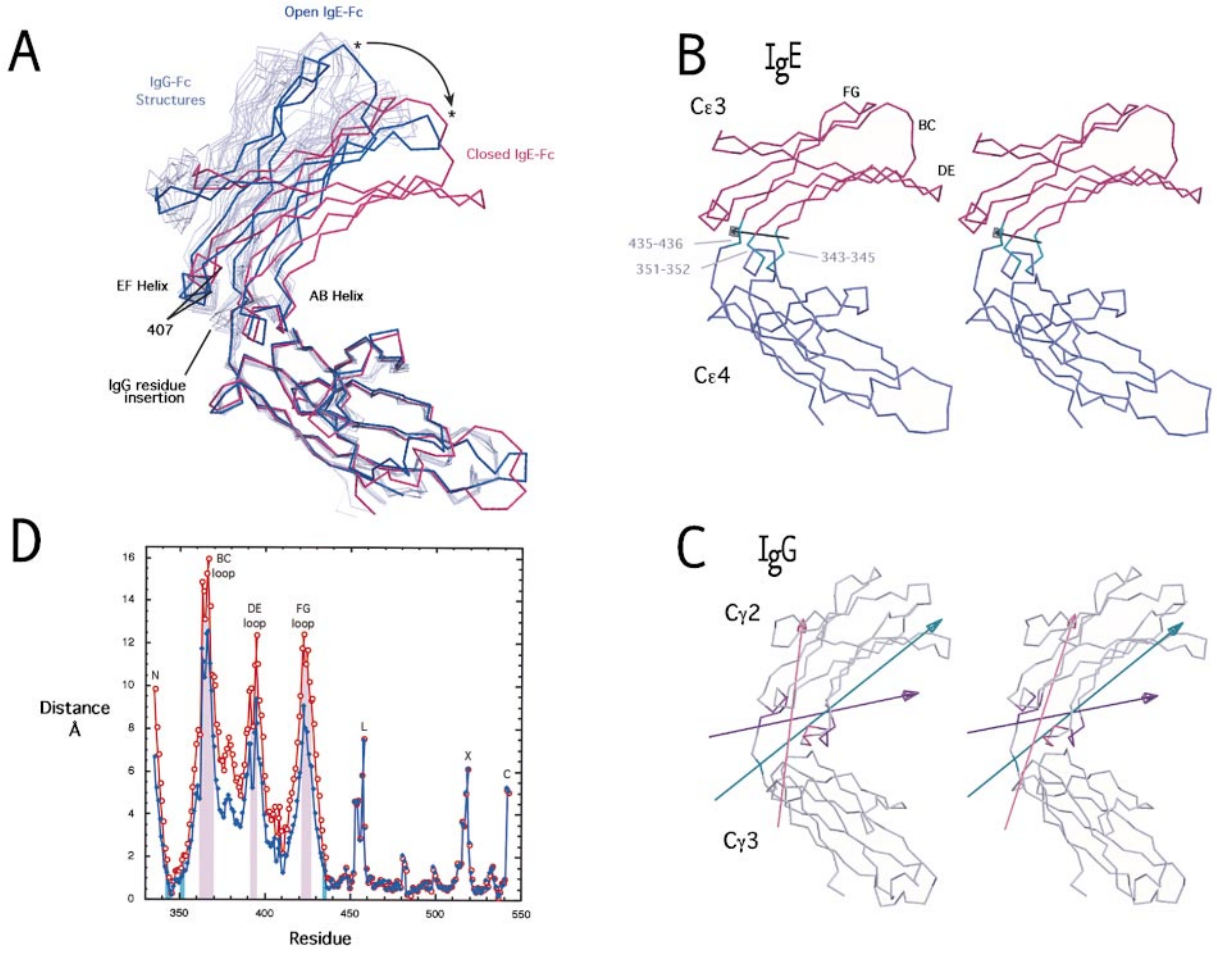

Figure 3. Analysis of the Structural Flexibility in IgE- and IgG-Fcs

(A) Superposition of nine IgG-Fc chains (light blue) with the open (dark blue) and closed (red) IgE-Fc structures. IgG-Fc structures are from the PDB files 1IGT (Harris et al., 1997), 1FC1 (Deisenhofer, 1981), 1FC2 (Deisenhofer, 1981), 1FCC (Sauer-Eriksson et al., 1995), 1IGY (Harris et al., 1998), and 1ADQ (Corper et al., 1997). The open IgE-Fc structure will be reported elsewhere (Garman et al., 2000). An asterisk is placed next to residue 366 in the BC loop in the open and closed IgE-Fc. Note the displacement of the IgE-Fc AB helix away from the interdomain interface, the movement of the IgE-Fc EF helix in the closed conformation, and the close approach of the IgG-Fc AB and EF helices at the site of the IgG residue insertion.

(B) DynDom analysis of the IgE-Fc. A stereo view of one chain of the Fc (closed conformation) is shown with the rotation axis indicated by an arrow. Hinge residues (343–345, 351–352, and 435–436) are highlighted in cyan. Cε3 domain residues that move as a semirigid domain are shown in red. Residues that remain relatively fixed in both the closed and open forms of the Fc are shown in blue and include the entire Cε4 domain, the interdomain linker, and the AB helix of Cε3.

(C) DynDom analysis of three IgG-Fc structures. A stereo view showing the rotation axes and hinge residues for murine IgG1 (1IGY) in cyan, for murine IgG2a (1IGT) in purple, and for human IgG1 (1FC1) in pink on the Cα trace of the IgG2a structure.

(D) The change in Cα coordinates between the closed and open conformations of the IgE-Fc. One chain is shown with red circles, the other with blue diamonds. Receptor binding loops are indicated and highlighted in pink; hinge residues are shown in cyan. "N" indicates Cε3 A strand residues, "C" is the carboxy terminus, "L" identifies the poorly ordered Cε4 AB loop, and "X" identifies a difference due to crystal contacts.

closed conformations. The program DynDom (Hayward and Berendsen, 1998) defines a rotation of ~13° and a translation of 1 Å about this axis (arrow in Figure 3B). Surprisingly, the axis does not lie in the Cε3-Cε4 linker region (436–440) but rather within the Cε3 domain itself, near the Cε3-Cε4 domain interface. Hinge residues that mediate the conformational change lie at both ends of the Cε3 AB helix (residues 343–345 and 351–352) and adjacent to the Cε3-Cε4 linker (residues 435–436).

None of the observed IgG-Fc structures exhibit such a large degree of flexibility. Three IgG-Fc structures have been solved in which the two Cγ2 domains of the same Fc exhibit different orientations with respect to their Cγ3 domains (1FC1 [Deisenhofer, 1981], 1IGY [Harris et al., 1998], and 1IGT [Harris et al., 1997]). Since the structural variation occurs within the same Fc, differences due to sequence variation are eliminated. For each structure, DynDom analysis identifies an axis near the Cγ2-Cγ3 interface that describes a motion of 6°–7° between the two conformers (Figure 3C). The orientations of the axes are different from each other and from that of the IgE-Fc, and they describe distinct movements (e.g., side-to-side) of Cγ2. However, none of the IgG motions match the open-to-closed conformational change seen in the IgE-Fc. The different location of the hinge axes and the much smaller range of motion displayed by the IgG-Fc

suggest that the flexibility of Ig domains involves multiple factors.

The change in Cα coordinates between the closed and open conformations of the IgE-Fc is plotted in Figure 3D. The changes are slightly different for the two Fc chains that bind asymmetrically to the FcεRI (Garman et al., 2000). The Cε3 AB helix (344–352) and the interdomain linker (436–440) remain relatively fixed with respect to the Cε4 domain, while the Cε3 EF helix residues (406–413) show Cα movements of up to 4 Å (Figure 3A). Positional changes become greater further from the hinge, with the greatest displacement of Cε3 residues observed at the top of the Fc in the BC (363–368), DE (393–395), and FG (422–428) loops that bind to FcεRI (Figure 3B). Residues in these loops move 7–16 Å between the open and closed conformations (Figure 3C). Large differences are also observed in the A strand adjacent to the Cε2-Cε3 linker.

IgE-Fc Carbohydrate

In both IgE and IgG, a conserved carbohydrate attachment site faces the cavity between the upper domains (Cε3 and Cγ2, respectively). Carbohydrate residues have not been included in the structure, but partial electron density for ~5 carbohydrate moieties can be observed at the conserved N394 site, branching after the core (–$GlcNAc_2Man$) into two arms. As in IgG, electron density for carbohydrate lies along the inner face of the protein, shielding hydrophobic residues from solvent (IgE residues Y339, L359, and V361). The carbohydrate is not sequestered from solvent, however. In IgE, the carbohydrate attached to N394 can be removed by endoglycosidases under native conditions, suggesting that this region is at least transiently accessible in solution (Basu et al., 1993; B. A. W., unpublished data).

Carbohydrate is not required for high-affinity binding to FcεRI, suggesting that it does not affect the conformation of the IgE-Fc significantly. IgA glycosylation is similarly not required for Fc-receptor binding (Mattu et al., 1998). In contrast, the presence of carbohydrate at a conserved N-linked attachment site in IgG (N297 in IgG1) is critical for maintaining Fc receptor binding activities (Jefferis et al., 1998). Core glycosylation (−$GlcNAc_2Man_3$) of IgG, produced in mammalian, yeast and insect cells, is likely sufficient for this carbohydrate function (Jefferis et al., 1998). Functional and biophysical studies of IgG indicate that the carbohydrate moiety has only a limited and local effect on the Fc structure (Jefferis et al., 1998). A comparison of glycosylated and aglycosylated IgG-Fc with a panel of monoclonal antibodies showed no detectable epitope differences, suggesting that global structural changes were not occurring (Walker et al., 1989). ^{1}H-NMR has been used to study the influence of glycosylation on the structure of IgG-Fc. Histidine resonances were monitored in glycosylated and nonglycosylated IgG-Fc (Lund et al., 1990; Matsuda et al., 1990). Of the five histidines monitored, only one near the conserved glycosylation site (H268 in the Cγ2 BC loop) reported any change in local environment. Histidines at the Cγ2-Cγ3 domain interface did not report any structural differences. Based on the IgE-Fc:FcεRI crystal structure, the Cγ2 BC loop and DE loop containing the conserved glycosylation site are predicted to participate directly in FcγR interactions (Garman et al., 2000). Local structural changes in these loops could affect receptor binding.

Structural Changes at the Interdomain Interface

The interdomain interfaces of both IgG-Fc (Cγ2-Cγ3) and IgE-Fc (Cε3-Cε4) are important for Fc function, and structural differences in the interface may influence Fc domain flexibility. Four proteins bind to this region in IgG: neonatal Fc receptor (Burmeister et al., 1994), rheumatoid factor (Corper et al., 1997), Protein A (Deisenhofer, 1981), and Protein G (Sauer-Eriksson et al., 1995). Direct binding of proteins to this region in IgE has not been demonstrated. The binding site for FcεRII has been broadly mapped to the outer surface of Cε3 (Sutton and Gould, 1993; Shi et al., 1997), while the FcεRI binding site is distal to this interface and encompasses the Cε3 BC, DE, and FG loops as well as the Cε2-Cε3 linker (Presta et al., 1994; Henry et al., 1997; Garman et al., 2000). However, despite the fact that residues at the Cε3-Cε4 domain interface do not form direct contacts to the FcεRI, mutations in this region can have a profound effect on FcεRI binding. For example, substitution of IgE Cε3 AB helix residues with IgG Cγ2 AB helix residues disrupts binding, as does a single amino acid mutation, F329A (Presta et al., 1994), suggesting that interactions at the Cε3-Cε4 domain interface are important in maintaining a functional Fc.

The AB helix (in Cε3 or Cγ2) mediates the majority of atomic contacts (atoms within 4 Å) between the Fc domains in both IgE and IgG (Figure 4A). The AB helix contacts adjacent residues and residues in the EF helix of the upper domain. The AB helix also contacts residues in the C, F and G strands and FG loop of the lower Ig domain (Cε4 or Cγ3). Despite the central role of the AB helix in mediating interdomain contacts, AB helix residues are not conserved between IgE and IgG. Only one residue of the helix (εD347, γD262) is invariant (Figure 1B). In addition, most of the residues that contact the AB helix are not conserved between IgE and IgG.

The pattern of contacts made by AB helix residues is different in IgE and IgG. In IgE, most of the contacts made by the AB helix are to residues of the lower (Cε4) domain (15/21 in the open conformation, Figure 4A). Only one contact is made to the EF helix in the closed form and two additional contacts are formed in the open conformation (dashed lines). In IgG, the majority of the AB helix contacts (12/21) are to other residues within the same Cγ2 domain, with nine contacts to the lower Cγ3 domain. The Cγ2 AB helix forms extensive contacts to the EF helix. Two residues in particular, V263 and H329, form a network of nine contacts within the Cγ2 domain.

There are two striking structural features unique to the IgG interdomain interface (Figures 3A and 4D). A single residue insertion after the IgG Cγ2 AB helix, isoleucine 266, forms a distinct bulge at the end of the helix. This isoleucine, together with adjacent residues, forms part of a shallow pocket on the surface of the IgG (Figure 4D). The second difference in IgG is the presence of a conserved histidine (H329) on the EF helix facing the AB helix. This histidine is completely conserved in IgGs across species and subtypes but is not found in other Ig isotypes. Histidine 329 forms five atomic contacts to the pocket formed by I266 and neighboring residues (Figure 4D). The contacts made by H329 are maintained in all IgG-Fc structures, including a highly distorted Fab-Fc hinge-deleted IgG in which the AB helix no longer contacts the lower Ig domain and has shifted away from the Cγ2-Cγ3 domain interface (Guddat et al., 1993). In nonglycosylated IgG-Fc, the ^{1}H resonances of

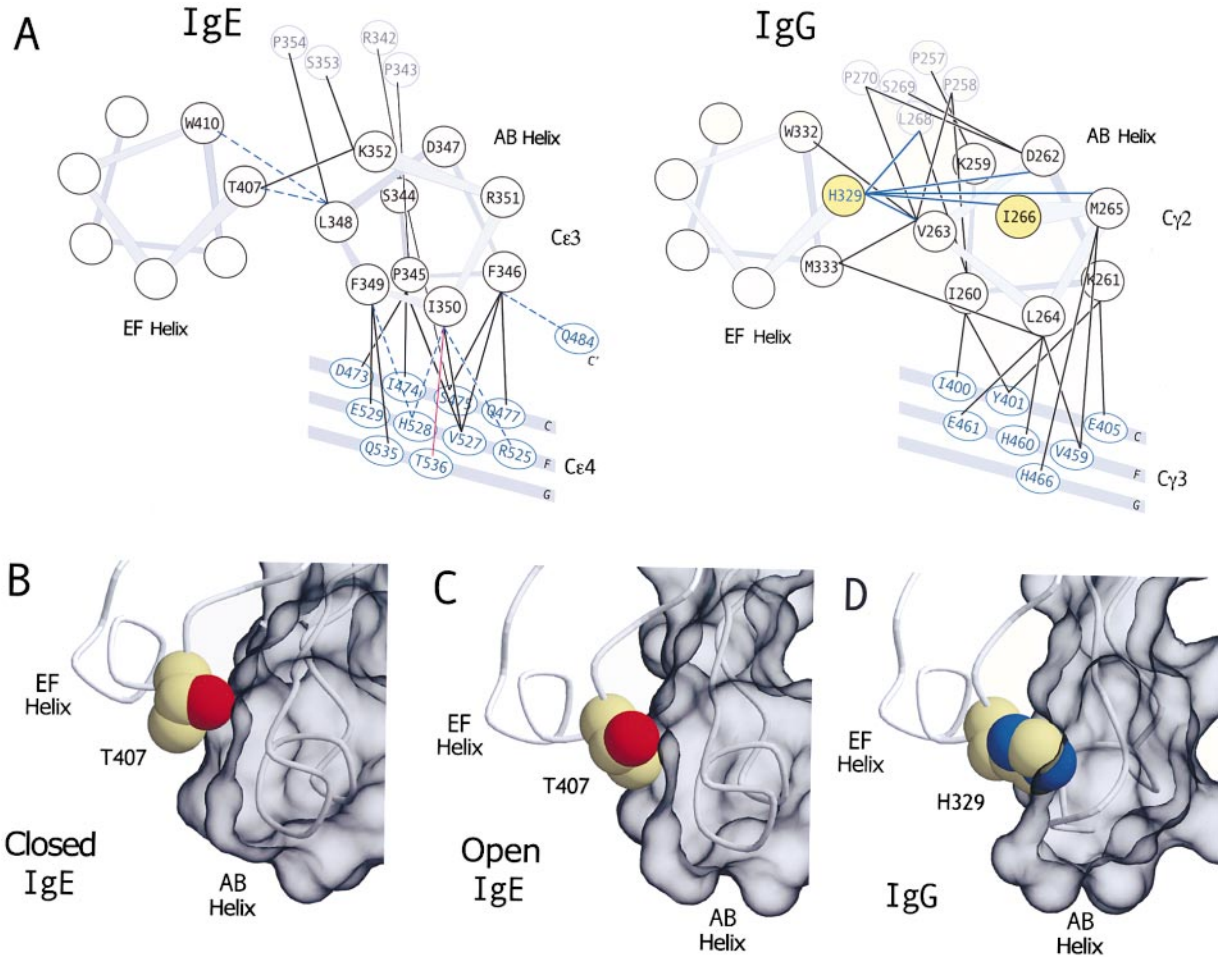

Figure 4. Interactions at the IgE-Fc and IgG-Fc (IgG2a) Domain Interfaces

(A) Schematic diagrams of the contacts made by AB helix residues (IgE Cε3 or IgG Cγ2). Residues of the AB and EF helices are shown on helical wheels, and residues of the lower domain (Cε4 or Cγ3) are shown below. Atomic contacts (<4 Å) are indicated by lines. Upper domain contacts (to Cε3 or Cγ2) involve residues in the EF helix and residues immediately adjacent to the AB helix. Lower domain contacts (to Cε4 or Cγ3) involve residues from the C, C′, F, and G β sheet strands and the FG loop. Contacts formed only in the open form of the IgE-Fc are indicated by dashed blue lines; the single contact formed only in the closed form is indicated by a red line. Contacts made by the conserved H329 in IgG are indicated by solid blue lines. Two IgG residues are shown in yellow: the completely conserved EF helix H329 and the insertion residue I266 that forms a bulge just after the AB helix.

(B) A surface representation of the interactions of EF helix residue T407 with the AB helix in the closed IgE-Fc.

(C) A surface representation of the interactions of EF helix residue T407 with the AB helix in the open IgE-Fc.

(D) A surface representation of the packing interactions of the corresponding residue in IgG-Fc, the conserved H329, with the bulge at the C terminus of the Cγ2 AB helix.

IgG H329 do not change (Lund et al., 1990; Matsuda et al., 1990), suggesting the preservation of these interactions.

In contrast, the corresponding residue in IgE, T407, makes two contacts to the AB helix in the open form (Figures 4A and 4C) and makes only one contact and moves away from the AB helix in the closed form (Figures 3A and 4A–4C). In rat and mouse IgE sequences, T407 is replaced by alanine, suggesting that the conservation of these side chain interactions is not important.

The extensive contacts formed by the IgG AB helix to other Cγ2 domain residues and the close packing of EF helix residue H329 to the AB helix distinguish the IgG Cγ2-Cγ3 interface. In IgG, the AB helix is more closely associated with the upper (Cγ2) domain than the lower (Cγ3) domain. In contrast, the IgE interface is characterized by extensive interactions of the Cε3 AB helix residues with the lower Cε4 domain residues (Figure 4A), and contacts with the EF helix are limited. In both IgE Cε3 and IgG Cγ2, the A and B strands separate and do not form hydrogen bonds on either side of the AB helix, allowing for some flexibility in the positioning of the AB helix. However, the flexibility may be limited in IgG by the extensive interactions of the Cγ2 AB and EF helices. In IgE, the limited contacts made between the Cε3 AB and EF helices may allow the helices to move independently.

Effect of the Conformational Change on the FcεRI Binding Site

The large conformational change of the IgE-Fc structure reorients loops in the Cε3 domains that interact with the

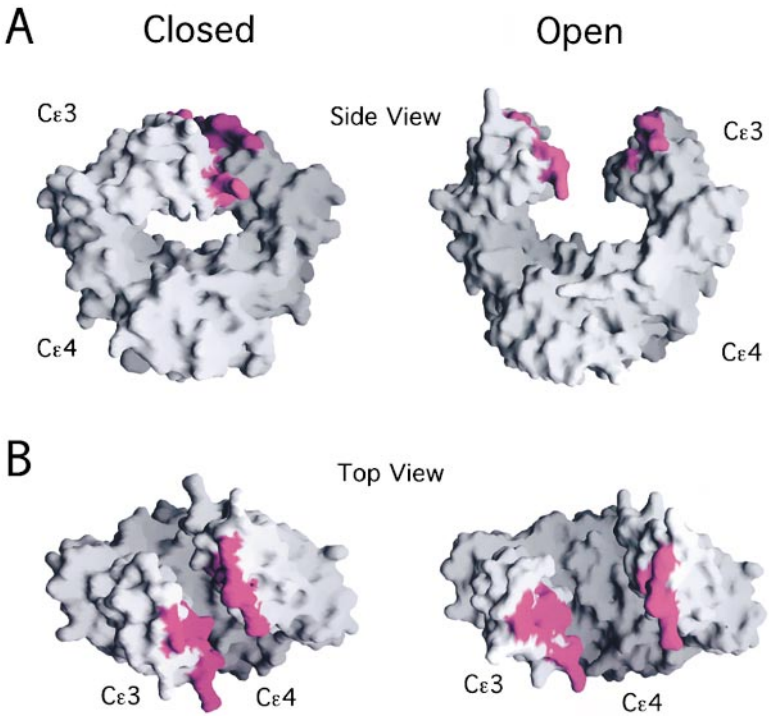

Figure 5. Molecular Surface Representation of the Closed and Open IgE-Fc Structures

Receptor binding residues are shown in magenta and are from the Cε3 BC, DE, and FG loops. (A) A side view of the closed and open IgE-Fc structures. (B) A top view of the closed and open IgE-Fc structures.

high-affinity receptor, FcεRI. The large movement of the FcεRI binding loops suggests that they would be poorly positioned in the closed IgE-Fc structure to interact with the receptor. Figure 5 shows a molecular surface representation of the open and closed Fc structures, with the receptor binding residues highlighted in magenta. In the open form, the receptor binding loops are exposed, and the binding residues display a large concave surface that is available to interact with FcεRI. In the closed form, these loops are partially obscured and point toward each other across the IgE-Fc dyad axis, leaving only a narrow gap between the two Cε3 domains that cannot accommodate the binding of the receptor. While the Cε3 BC, DE, and FG loops are largely inaccessible in the closed conformation, the disordered Cε2-Cε3 linker residues N-terminal to V336 could form an initial interaction with the receptor even in the closed IgE-Fc structure. Binding of the receptor to linker residues might shift the conformation of the Fc toward the open form, exposing the binding loops.

Conclusions

Structural Basis for the IgE-Fc Conformational Flexibility

The IgE-Fc structure reveals an unprecedented conformation for antibody effector domains with implications for Fc-receptor binding and therapeutic intervention in human disease. The structure of the closed IgE-Fc suggests that the effector domains of antibody isotypes may have evolved structural characteristics that are associated with isotype-specific biological functions.

Structural features that could influence the flexibility of the IgE-Fc include the location and packing of hinge residues and the specific interactions at the Cε3-Cε4 domain interface, such as the position and contacts of the Cε3 AB helix. Other factors that could potentially effect a change in conformation have been considered, such as the specific crystal-packing environment, the presence of high-mannose instead of complex carbohydrate, or the lack of the Cε2 domains. We have recently solved a second crystal form of the IgE-Fc containing two IgE-Fc molecules in the asymmetric unit, both in the closed form. These five IgE-Fc chains all adopt a similar conformation, indicating that the closed conformation is not dictated by specific crystal-packing forces.

It remains to be established whether different carbohydrate structures at the conserved attachment site could influence the extent of the observed IgE-Fc conformational change. Biochemical studies of IgG suggest a limited structural role for the conserved carbohydrate in maintaining the overall three-dimensional arrangement of Fc domains, as discussed above. While functional studies of the IgE-Fc (FcεRIα binding) argue against a significant role for the conserved carbohydrate, structural studies of different IgE-Fc glycoforms may resolve this issue.

Biochemical and biophysical studies indicate that the IgE-Fc Cε2 domains form a separate structural unit from the Cε3-Cε4 structure solved here. The Cε2-Cε3 linker is susceptible to proteolytic digestion (Perez-Montfort and Metzger, 1982) and adopts an asymmetric conformation upon binding FcεRI (Garman et al., 2000), suggesting that it is accessible and flexible. The presence or absence of the Cε2 domains in the IgE-Fc does not significantly alter the binding constants or thermodynamic parameters (ΔG°, ΔH°, ΔS°, and ΔCp°) of FcεRI binding (Keown et al., 1998). Therefore, the mode of binding to the receptor is likely to be similar for intact IgE-Fc and IgE-Fc Cε3-Cε4. Together, these results suggest that the Cε2 domains have little influence on the structure or conformation of the Cε3 domains.

Structural characteristics of the IgE Cε3-Cε4 domain interface, as compared to the IgG Cγ2-Cγ3 domain interface, likely enable the conformational flexibility of the

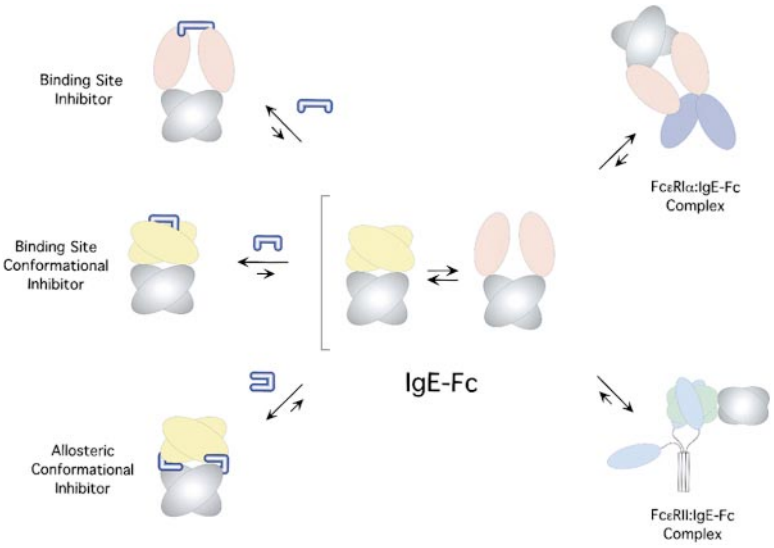

Figure 6. Roles for IgE Flexibility in Fc Receptor Binding and Structure-Based Inhibitor Design

Cε3 domains are colored to correspond to different conformational states, and Cε4 domains are shown in gray. The open (pink) and closed (yellow) IgE-Fc structures are shown in the center. Open forms of the IgE molecule can bind to the high-affinity receptor, FcεRI (Garman et al., 2000). The low-affinity receptor, FcεRII, is a trimeric C-type lectin (Shi et al., 1997) that binds to an unidentified conformation of the IgE-Fc (green). Three potential classes of inhibitors of the IgE:FcεRI interaction are shown: binding site competitive inhibitor, binding site conformational inhibitor, and allosteric conformational inhibitor.

IgE-Fc. The AB helix of the first domain (Cε3 or Cγ2) mediates most of the interdomain contacts in the Fc structure and is not conserved in sequence across the five different antibody classes. Packing contacts of the AB helix with the two Fc Ig domains may differ significantly across antibody isotypes, potentially influencing Fc conformation, flexibility, and function. The range of conformational flexibility of the Fc domains of different antibody classes could be linked to the evolution of isotype-specific effector functions. The more limited flexibility of IgG structures may reflect similarities in the structural requirements for FcγR and complement (C1q) interactions.

Other experimental evidence has suggested that IgE adopts a bent configuration in solution and that conformational changes may occur upon binding to FcεRI. The design and interpretation of these experiments could not have anticipated the specific IgE-Fc conformational change presented here. Binding of IgE-Fc to FcεRI (Keown et al., 1998) is characterized by a relatively large change in heat capacity ($\Delta Cp^\circ = -815$ cal/mol K), which could be in part be caused by IgE-Fc conformational changes. In contrast, binding of IgG-Fc to its homologous low-affinity receptor, FcγRIII, exhibits a smaller change in heat capacity ($\Delta Cp^\circ = -360$ cal/mol K). Fluorescence energy transfer experiments have shown that the average distance between the N and C termini of the IgE is only ~70 Å, a distance that is possible only if the IgE bends significantly out of the plane of the typical antibody Y or T shape (Zheng et al., 1991). Neutron scattering studies have shown that the intact IgE-Fc (Cε2-Cε4) has a significantly more compact shape than a linear arrangement of the domains would allow (Beavil et al., 1995), suggesting that a bend occurs within the IgE-Fc region. The IgE-Fc crystal structure supports the interpretation of bending of the intact IgE at the Cε2-Cε3 linker region and may provide a better model for the analysis of the neutron scattering data. Experimental tests of IgE flexibility can now be developed based on the structure.

Biological and Therapeutic Implications for IgE-Fc Conformational Flexibility

Conformational flexibility in the IgE-Fc may be important for unique aspects of IgE biological function. IgE-Fc flexibility may allow induced-fit interactions with FcεRI, contributing to the high-affinity binding, and may be important for interactions with the low-affinity IgE receptor, FcεRII (Figure 6). FcεRII is a trimeric C-type lectin that is thought to interact with the IgE-Fc through two of its three lectin domains (Shi et al., 1997). IgG antibodies do not have a corresponding lectin-like receptor, suggesting that the conformational flexibility of the IgE-Fc may be important for this unique antibody-receptor interaction.

The existence of a closed conformation for the IgE-Fc and the demonstration that the open form binds to the high-affinity receptor (Garman et al., 2000) suggest strategies for the design of inhibitors of the IgE:FcεRI interaction (Figure 6). Monoclonal antibodies that bind the IgE-Fc and block interactions with FcεRI have demonstrated the therapeutic potential of this approach for the treatment of allergy and asthma (Jardieu and Fick, 1999; Chang, 2000). Stabilization of the closed IgE-Fc conformation by the binding of molecules, either at directly competitive or indirect allosteric sites, could block receptor association, leading to a novel class of therapeutic inhibitors for the treatment of IgE-mediated allergic diseases. The IgE Cε3-Cε4 domain interface may provide a target for the binding of small in vitro-selected ligands, as shown for the IgG-Fc (DeLano et al., 2000), that have the potential to act as allosteric inhibitors of receptor binding. The closed conformation of the IgE-Fc provides the foundation for exploring novel routes to alleviating atopic disease and exploring the functional role of Fc domain flexibility in biological effector mechanisms.

Experimental Procedures

Expression and Purification of the Human IgE-Fc

The expression, purification and characterization of the IgE-Fc from insect cells will be described in detail elsewhere (B. A. W., unpublished data). In brief, DNA coding for the human IgE heavy chain residues 330–547 (Cε3-Cε4 domains) plus an additional N-terminal cysteine residue was subcloned into a baculovirus transfer vector (pAcGP67A, Pharmingen) 3′ to the baculovirus GP67 signal sequence. Recombinant virus was generated using the Baculogold system (Pharmingen). The Fc was expressed and secreted from

insect cells (*Trichoplusia ni*). The mature Fc has an N-terminal sequence (ADPC*DSN...) that includes four amino acids (ADPC) upstream of the native sequence beginning with residue *D330 (Bennich numbering [Dorrington and Bennich, 1978]). The cysteine is displaced by one residue from the native IgE-Fc C328 (...CA*DSN-) but forms the interchain disulfide in ~95% of the protein. IgE-Fc Cε3-Cε4 was expressed at ~1 mg/L of culture and was functionally active (bound to soluble FcεRIα in titration experiments and competitive ELISAs). The IgE-Fc was purified from concentrated cell culture supernatants by cation exchange, gel filtration, and hydroxyapatite chromatography. The Fc was glycosylated at two of the three potential N-linked glycosylation sites (N371 and N394) to a limited and discrete extent, as shown by mass spectroscopy (B. A. W., unpublished data).

Crystallization and Treatment of Crystals

Purified IgE-Fc was concentrated to 10 mg/ml in 10 mM Tris (pH 8.0), using an ϵ_{280nm} of 1.32 cm^{-1} $(mg/ml)^{-1}$. Crystals were grown in hanging drops using the vapor diffusion method by mixing 0.5 μl protein and 0.5 μl of precipitant (25 mM sodium acetate [pH 4.6], and 33% polyethylene glycol [PEG] 4000). Crystals grew at 22°C in 1–3 days and were sensitive to small changes in salt or PEG concentration and temperature. Crystals were harvested into 25 mM sodium acetate (pH 4.6), and 37% PEG 4000, transferred briefly (< 30s) to cryoprotectant solution (harvest solution plus 15% (v/v) ethylene glycol), and cooled rapidly in liquid nitrogen. Crystals belong to the space group *P*42,2 (a = b = 105.6 Å, c = 47.1 Å) and contain one Cε3-Cε4 chain per asymmetric unit. Crystals were transferred serially to higher pH harvest solutions to facilitate metal binding and redox chemistry. Heavy atom screening (~100 conditions) with a wide range of compounds (27), concentrations (0.1–20 mM), and pH ranges (4.6–8.5) did not yield an isomorphous or anomalous derivative. However, mercury- or platinum-treated crystals diffracted better than native crystals. Based on these observations, crystals were treated with 1 mM copper (II) chloride prior to cooling and data collection. Native crystals diffracted to ~2.8 Å resolution using a synchrotron source and displayed strong anisotropy and, occasionally, split lattices. Copper-treated crystals diffracted to at least 2.0 Å resolution, with little or no anisotropy. We and others (Basu et al., 1993) have observed that a small fraction of the IgE-Fc does not form the interchain disulfide. The copper II may have oxidized the remaining free cysteines to the disulfide.

Data Collection, Molecular Replacement, and Refinement

Data sets were collected at –160°C from copper-treated crystals at SSRL beamline 7–1 (wtcu1) using a Mar300 imaging plate system and at the Advanced Photon Source DND-CAT 5ID beamline (wtcu3) using a MarCCD detector. The data were processed and integrated using the HKL suite of programs (Otwinowski and Minor, 1997). Initial molecular replacement (MR) searches with AMoRe (CCP4, 1994), CNS/XPLOR (Brünger et al., 1998) and EPMR (Kissinger et al., 1999) failed, using a variety of models of IgE based on IgG-Fc structures, including individual Ig domains and a composite model incorporating seven IgG structures. A systematic exploration of the bend, twist, and rotation angles of Cε3 relative to Cε4 was then undertaken. A model was constructed from the Cγ2-Cγ3 domains derived from an intact IgG structure (murine IgG2a, PDB entry 1IGT [Harris et al., 1997]), by truncating loops and nonhomologous sidechains, resulting in a 144 residue model for the 222 residue IgE-Fc. The Cγ2-Cγ3 linker region of the model was placed at the origin, with Cγ2 and Cγ3 oriented to allow bending to occur about the Z-axis. Rotations around X, Y, and Z were applied to the Cγ2 domain (3° steps), while leaving the Cγ3 fixed. Approximately 12,000 models were generated automatically with the program lsqkab (CCP4, 1994) and used in complete AMoRe (CCP4, 1994) searches with the 15–4 Å data from crystal wtcu1 (Table 1). The models covered an angular range of –30 to +40 degrees around the starting IgG2a structure. A single solution, ~17° from the starting structure, was found and improved by a local search using 1.0° rotational increments. The finer search yielded a solution with a correlation coefficient of 38% and an R_{factor} of 48.9%. Cycles of model building into simulated-annealing composite-omit electron density maps and refinement were continued with the higher resolution data from crystal wtcu3 using the programs O (Jones et al., 1991) and CNS (Brünger et al., 1998). Refinement was performed against all data from 30–2.3 Å using $|F|>0$ and an anisotropic bulk solvent correction. Only refinement steps that decreased the R_{free} were accepted. The model includes residues 336–543 and lacks 10 N-terminal and 4 C-terminal residues present in the construct. The receptor binding loops (Cε3 BC, DE, and FG loops) have weaker density and higher B-factors than most of the other residues. Density for the Cε4 AB loop is particularly poor, and this loop was modeled sterically. All of the residues lie within the accepted regions of the Ramachandran plot, with the exception of N481. There is good density for this residue, however, which is in a tight turn that lacks a Gly, Ala, or Ser residue. While some density for carbohydrate was observed at the N394 site, attempts to build carbohydrate did not improve the R_{free} or the electron density maps, and so it was not included in the model. There is no electron density for the carbohydrate attached to the N371 site. The current refinement statistics are summarized in Table 1. Figures were made using the programs MOLSCRIPT (Kraulis, 1991), GRASP (Nicholls et al., 1991), and Raster3D (Merritt and Bacon, 1997).

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Protein Data Bank ID Codes

Coordinates have been deposited in the PDB with accession number 1FP5.