

Conformational changes in IgE contribute to its uniquely slow dissociation rate from receptor FcεRI

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Among antibody classes, IgE has a uniquely slow dissociation rate from, and high affinity for, its cell surface receptor FcεRI. We show the structural basis for these key determinants of the ability of IgE to mediate allergic hypersensitivity through the 3.4-Å-resolution crystal structure of human IgE-Fc (consisting of the Cε2, Cε3 and Cε4 domains) bound to the extracellular domains of the FcεRI α chain. Comparison with the structure of free IgE-Fc (reported here at a resolution of 1.9 Å) shows that the antibody, which has a compact, bent structure before receptor engagement, becomes even more acutely bent in the complex. Thermodynamic analysis indicates that the interaction is entropically driven, which explains how the noncontacting Cε2 domains, in place of the flexible hinge region of IgG antibodies, contribute together with the conformational changes to the unique binding properties of IgE.

The global incidence of allergic disease has increased markedly in recent years and continues to rise. Asthma currently affects 22.2 million people in North America and 5.4 million in the UK, where the rate of incidence, especially in children, is among the highest in the world¹. Anaphylactic reactions to foods such as nuts, virtually unknown 30 years ago, are now relatively common. The reason for this increase is debated, but these and other allergic conditions (rhinitis, eczema and so on) are all mediated by IgE, and the viability of targeting its interaction with FcεRI therapeutically has been demonstrated by the recent success of the anti-IgE monoclonal antibody omalizumab in treating severe asthma².

IgE binds to FcεRI through its Fc region, and this tight binding ($K_a \approx 10^{10} \text{ M}^{-1}$) sensitizes mast cells and basophils for immediate activation upon cross-linking by multivalent allergen³. The markedly slow dissociation rate ($k_{\text{off}} \approx 10^{-5} \text{ s}^{-1}$) of this binding reaction, which is several orders of magnitude slower than that of IgG from any of its receptors⁴, contributes to the persistence of sensitization, and together with local IgE synthesis ensures that the receptors in tissue of allergic individuals are saturated with IgE⁵. The kinetics of IgE and IgE-Fc binding to FcεRI on cells, and to a soluble fragment comprising the two extracellular domains of the α chain, sFcεRIα, which are responsible for IgE-binding activity, have been studied extensively^{6–9}. Particular interest has centered on the role of the Cε2 domains, which contribute to the slow dissociation rate¹⁰, by comparing the binding kinetics of the complete IgE-Fc (dimeric Cε2, Cε3 and Cε4 domains) with a subfragment lacking the Cε2 domains, here referred to as

Fcε3-4. The Cε2 domains have no counterpart in IgG, in which all four subclasses have in their place hinge regions of various lengths and degrees of flexibility.

Early fluorescence resonance energy transfer (FRET) studies of labeled chimeric IgE indicate a more compact and bent structure than the extended, flexible Y-shaped IgG¹¹, and later solution scattering studies of IgE-Fc are consistent with such a structure¹². The crystal structure of IgE-Fc showed for the first time the extent and nature of this bend¹³, which is made possible by the presence of the Cε2 domains. Notably, the molecule is so acutely and asymmetrically bent, with the Cε2 domain pair folded back across the Cε3 domains, that one Cε2 domain contacted the Cε4 domain of the other chain. The first view of the receptor interaction comes from the structure of a complex between sFcεRIα and an Fcε3-4 fragment lacking the Cε2 domains (ref. 14). Both Cε3 domains are involved, with a distinct subsite on each domain, thus explaining the observed 1:1 stoichiometry despite the presence of two identical ε chains. However, when compared with the unliganded IgE-Fc structure containing the Cε2 domains, only one of these two Cε3 subsites is fully accessible, suggesting that conformational changes must occur, involving not only the Cε3 but also the Cε2 domains. We have thus proposed an opening up of the bent structure¹³ and involvement of the Cε2 domains¹⁰ upon receptor engagement.

The structure of the human IgE-Fc-sFcεRIα complex reported here, together with that of free IgE-Fc at higher resolution, reveals the conformational changes that occur in both IgE and receptor upon

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Table 1 Data collection and refinement statistics

	IgE-Fc-sFcER1 α	IgE-Fc
Data collection		
Space group	$P2_12_12_1$	$P2_12_12$
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	99.49, 103.34, 110.12	130.50, 75.28, 79.14
Resolution (Å)	3.4 (3.49–3.40)	1.9 (1.97–1.90)
R_{merge}	0.112 (0.522) ^a	0.074 (0.533)
<i>I</i> / σ <i>I</i>	8.5 (1.6)	26.3 (2.9)
Completeness (%)	99.9 (99.9)	99.9 (100)
Redundancy	5.9 (6.1)	7.3 (7.4)
Refinement		
Resolution (Å)	37–3.4	36–1.9
No. reflections	16,204 (1,174)	62,035 (6,147)
R_{work} / R_{free}	0.245 / 0.292	0.193 / 0.234
No. atoms		
Protein	5,263	5,017
Nonprotein	92 ^b	175 ^c
Water	0	533
<i>B</i> -factors (Å ²)		
Protein	77	48
Nonprotein	76 ^b	77 ^c
Water	–	45
R.m.s. deviations		
Bond lengths (Å)	0.006	0.005
Bond angles (°)	1.104	1.001

Values in parentheses are for highest-resolution shell. Data for each structure were collected from a single crystal.

^a R_{pim} precision-indicating R_{merge} . ^bCarbohydrate. ^cCarbohydrate, SO₄²⁻, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), polyethylene glycol (PEG).

binding, and shows how the slow dissociation rate is achieved despite the notable lack of direct contact between the receptor and the C ϵ 2 domains. Our thermodynamic analysis of the interaction indicates how the noncontacting C ϵ 2 domains nevertheless contribute to the unique receptor-binding properties of IgE. Knowledge of these structures and their conformational changes also suggests ways in which small molecules might allosterically prevent high-affinity binding; inhibition of protein-protein interactions such as this have hitherto been considered intractable¹⁵.

RESULTS

Structure of IgE-Fc bound to sFcER1 α

We determined the crystal structure of the complete IgE-Fc bound to sFcER1 α at a resolution of 3.4 Å, and also extended the resolution of the free IgE-Fc structure to 1.9 Å (see Online Methods and Table 1). Although crystallized under different conditions, the free IgE-Fc structure was essentially identical to that reported earlier at a resolution of 2.6 Å¹³; however, features such as the carbohydrate and edge strands in the C ϵ 2 domains (see below) were more clearly defined. The structure of the complex (Fig. 1 and Supplementary Video 1) shows that the bound IgE molecule is oriented with its antigen-binding (Fab) arms pointing directly away from the cell membrane. Conformational changes occur in both the receptor and the C ϵ 2 and C ϵ 3 domains of IgE upon receptor engagement, but rather than ‘unbending’, IgE-Fc becomes more compact and bent, with no direct contact between the C ϵ 2 domains and receptor. This bend in the IgE molecule can be described in terms of the acute angle between the local two-fold axes of symmetry of the (C ϵ 2)₂ and (C ϵ 4)₂ domain pairs, which in free IgE-Fc is 62° but in the complex is 54°. Despite the more compact structure, the C ϵ 3 domains move apart upon

formation of the complex, allowing interaction at both subsites, designated 1 (in C ϵ 3A) and 2 (in C ϵ 3B) according to earlier nomenclature^{13,14} (Fig. 1a). In free IgE-Fc, only subsite 2 (chain B) is fully accessible to receptor binding, and access to subsite 1 is achieved by rotation of C ϵ 3A, which takes both C ϵ 2 domains with it, relative to the C ϵ 4 domain pair, and away from C ϵ 3B. (Supplementary Fig. 1 and Supplementary Videos 2 and 3).

This conformational change affects several interdomain interactions within IgE-Fc. The extent of contact between C ϵ 2A and C ϵ 3B is reduced substantially (from 23 van der Waals contacts in free IgE-Fc to only 3 in the complex), and contact between C ϵ 2B and C ϵ 4A is lost entirely upon receptor binding. Although the molecule becomes increasingly bent, C ϵ 2B swings away from C ϵ 4A, with the loss of two salt bridges (Glu270B-Lys497A and Lys302B-Glu472A) and associated hydrogen bonds. But as C ϵ 2B and C ϵ 4A move apart, C ϵ 2B moves closer to C ϵ 3B (Fig. 2), forming two potential hydrogen bonds (Asp271 in C ϵ 2B to the first and only ordered carbohydrate unit N-linked at Asn394 in C ϵ 3B; Fig. 2b) and van der Waals contacts (between Thr309 and Asn394, hidden behind Arg308 and the carbohydrate unit in Figure 2b). These contacts prevent any further domain movement in this direction. This structural change, however, presents an opportunity for allosteric inhibition. Occupation of this cleft between C ϵ 2B and C ϵ 3B in free IgE (Fig. 2a) by a small molecule could prevent its closure, thereby blocking the conformational change, access to subsite 1 on C ϵ 3A and consequently high-affinity receptor binding.

The relative orientation of the two extracellular domains of FcER1 α observed in the free state^{16,17} is unchanged upon formation of complex, but, as has been observed in the earlier Fc ϵ 3-4-sFcER1 α complex¹⁴, the loop and β -strand following the C strand in the α 2 domain adopts a C' conformation alongside the C strand, rather than forming a D strand adjacent to the E strand on the other face of the domain. Notably, a similar strand shift occurs in both C ϵ 2 domains of the IgE-Fc upon binding (Supplementary Fig. 2).

IgE contains carbohydrate chains N-linked to Asn394 in each C ϵ 3 domain, and these are high-mannose glycans, in contrast to the complex-type carbohydrate attached at the homologous location in the C γ 2 domains of IgG¹⁸. The first five units, (N-acetyl-glucosamine)₂-(mannose)₃ ((GlcNAc)₂-(Man)₃), are well defined on both chains in the 1.9-Å-resolution free IgE-Fc structure, but only the first GlcNAc

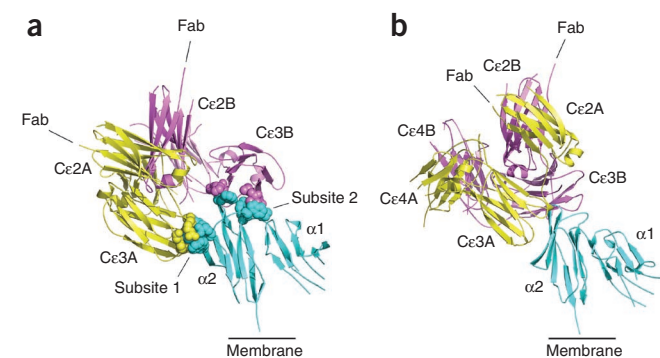
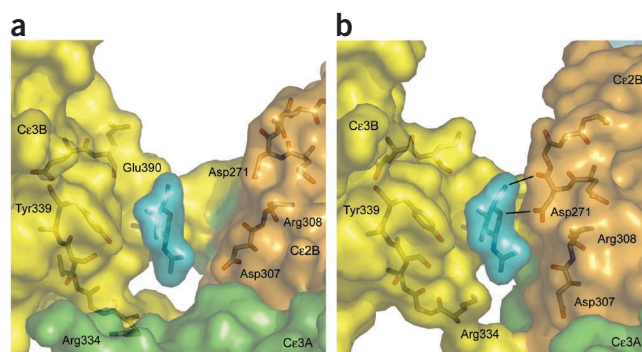


Figure 1 Overall structure of the IgE-Fc-sFcER1 α complex, in two approximately orthogonal views. sFcER1 α is blue, IgE-Fc chain A is yellow and chain B is purple. (a) The extensive interface with two distinct subsites, one on each C ϵ 3 domain, in a space-filled representation of the interacting side chains. The C ϵ 4 domains are hidden in this orientation. (b) The acute bend, with the pair of C ϵ 2 domains packed against the C ϵ 3 and C ϵ 4 domains, can be seen. The connections to the Fab regions are indicated, and the bend in the IgE molecule ensures that they are oriented away from the membrane (see also Supplementary Video 1).

Figure 2 Closure of an interdomain cleft in IgE-Fc upon receptor binding. (a) In the free IgE-Fc structure, there is a cleft between the first *N*-acetylglucosamine carbohydrate unit (blue), *N*-linked to Asn394 (behind) in Cε3B (yellow), and residues Asp271 and Asp307 in Cε2B (orange). (b) In the complex, the movement of Cε3A (green), Cε2B (orange) and Cε2A (not shown) as a rigid unit relative to Cε3B (yellow) and the Cε4 domains (not shown) closes the cleft, and Asp271 and carbohydrate make contact, with the formation of two potential hydrogen bonds (black lines). Both figures are centered on the first *N*-acetylglucosamine unit. For clarity, other carbohydrate residues are not shown for the unbound form (a); all carbohydrate units except for the first *N*-acetylglucosamine were disordered in the complex (b).



unit is visible on each chain in the complex; the rest are disordered and mobile (**Supplementary Fig. 3**). The single ordered unit on Cε3B is contacted by residues of Cε2B after the conformational changes associated with receptor binding (**Fig. 2b**), and this contact may affect the rest of the carbohydrate chain because in the Fcε3-4-sFcεRIα complex¹⁴ (determined at a similar resolution), three- and six-carbohydrate units can be seen on the two Cε3 domains. No Cε2 contact exists in the Fcε3-4 complex, and so disorder of the IgE-Fc carbohydrate may be caused by complex formation, and occur also in IgE.

Nature of the IgE-receptor interface

Only the Cε3 domains of IgE make direct contact with the receptor, through subsites 1 (Cε3A) and 2 (Cε3B) (**Fig. 1a**). The former contacts the C-C' loop region of the receptor α2 domain, whereas the latter interacts with the linker region between α1 and α2 and the B-C and F-G loops of α2, as in the earlier Fcε3-4-sFcεRIα structure¹⁴. Given their effect on the binding kinetics, it is notable that there is no direct contact between the Cε2 domains and the receptor. On the basis of our earlier report of an interaction in solution by NMR¹⁰, we have suggested that unbending of the IgE might allow such contact¹³. In fact, the conformational change involving the Cε2 domains enhances contact between the two proteins but indirectly. Superposition of the structures of IgE-Fc and Fcε3-4 receptor complex shows that relative to the receptor, the two IgE fragments differ by a rotation of ~5° (**Supplementary Fig. 3**), leading to tighter contact and many more van der Waals interactions in the IgE-Fc complex. This difference in the angle of engagement seems to be caused by the immediate proximity of the Cε2 domains, which affects the conformations of the linker from Cε2 leading into strand A and the adjacent B-C and F-G loops of Cε3. These regions present the contact residues, and thus the Cε2 domains have an indirect role in enhancing receptor binding.

Subsite 1 on Cε3A includes two salt bridges between IgE-Fc and receptor (Arg334-Glu132 and Asp362-Lys117; **Fig. 3a**) and the former forms a closer interaction and an additional hydrogen bond in the IgE-Fc complex than in the Fcε3-4 complex. Tyr129 and Trp130 (**Fig. 3a**) also form closer and more extensive contacts in the IgE-Fc complex. (The electron density in this region is shown in **Supplementary Fig. 4a**.) The dominant feature of subsite 2 on Cε3B, and one that contributes a substantial fraction of the buried hydrophobic surface area, is the 'proline sandwich,' in which Pro426 of Cε3B packs between Trp87 and Trp110 of the receptor α2 domain (**Fig. 3b**), a feature conserved in IgG-FcγR interactions. However, there is a notable difference in the present structure: the orientation of the Trp87 side chain in the IgE-Fc-receptor complex is 'flipped' by precisely 180° compared with the Fcε3-4-receptor complex (and both of the known IgG1-Fc-FcγRIII complexes^{19,20}), and its six-membered ring, rather than the five-membered ring, thus contributes additional van der Waals contacts to the proline sandwich (**Fig. 3b**). Both Trp87

rotamers have been observed in free receptor structures^{16,17}, and IgE-Fc may initially 'sample' both conformations, but the electron density (**Supplementary Fig. 4b**) indicates only a single conformation in the complex. Two other tryptophan residues in the receptor α2 domain (Trp113 and Trp156) contribute to the hydrophobic interaction and also make tighter contacts with IgE-Fc than Fcε3-4; Trp156, for example, makes 14 rather than 5 van der Waals contacts. Overall, the interaction is dominated by hydrophobic interactions, and the total buried surface area of interaction is 1,840 Å² (subsite 1, 930 Å²; subsite 2, 910 Å²).

Thermodynamics of the interaction and role of Cε2 domains

The presence of the Cε2 domains thus indirectly enhances stabilization of the bound complex, and this is supported by kinetic data showing that their effect is principally to decrease the dissociation rate¹⁰. We also investigated the thermodynamics of the interaction with and without the Cε2 domains, and found another marked difference that further clarifies their role in the mechanism of binding. We determined the temperature dependence of the binding kinetics and equilibrium binding affinities by surface plasmon resonance (SPR; **Fig. 4** and **Supplementary Figs. 5** and **6**), from which we derived thermodynamic parameters (**Table 2**) for IgE-Fc and Fcε3-4 binding to both wild-type receptor and a point mutant designed to assess the contribution of Trp87, part of the proline sandwich in subsite 2 described above. The differences between the temperature dependence of the IgE-Fc and Fcε3-4 traces and the gradients of the van't Hoff plots indicate that the balance between the enthalpic and entropic contributions to the free energies of interaction depends on whether the Cε2 domains are present. Whereas formation of the Fcε3-4-sFcεRIα

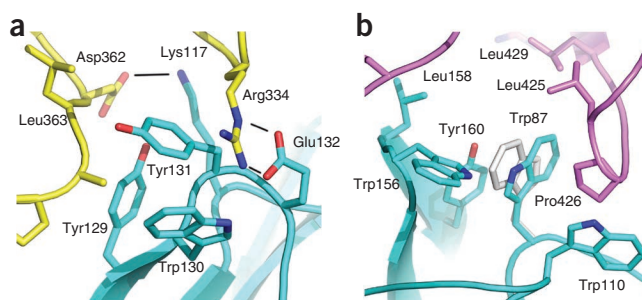


Figure 3 Interactions at the two subsites. (a) Two salt bridges (Arg334-Glu132 and Asp362-Lys117) with three hydrogen bonds (black lines) between residues of the Cε3A domain of IgE-Fc (yellow) and the receptor (blue) contribute to subsite 1. (b) The proline sandwich at subsite 2, with Pro426 in the Cε3B domain of IgE-Fc (purple) packed between Trp87 and Trp110 of the receptor (blue). The alternative orientation of Trp87 observed in the Fcε3-4-sFcεRIα complex (light gray) makes fewer contacts and a weaker interaction with Pro426.

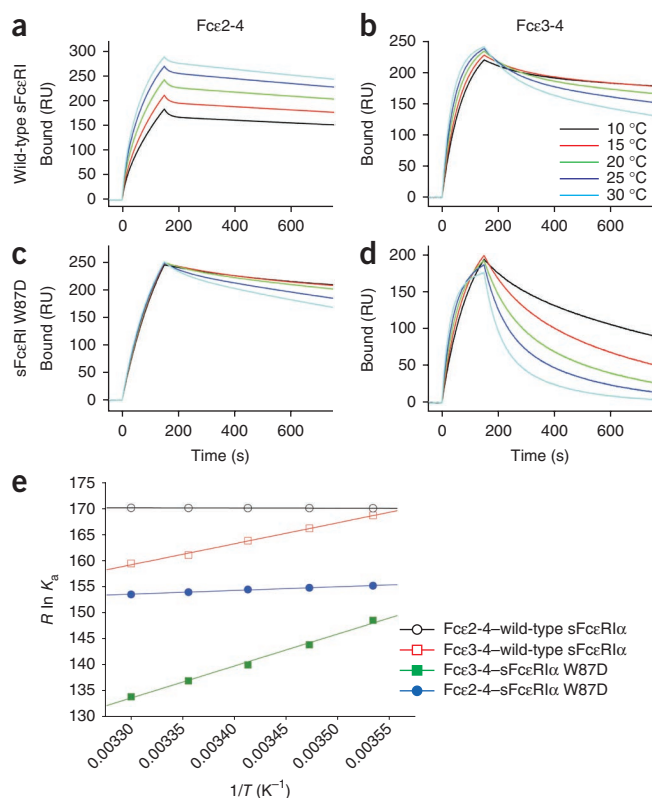


Figure 4 Thermodynamics of the IgE-FcεRI interaction. (a–d) SPR sensorgrams of Fcε2-4 (IgE-Fc) and Fcε3-4 binding to immobilized wild-type sFcεRIα (a,b) and to immobilized sFcεRIα W87D (c,d), over a range of temperatures. A series of analyte concentrations were tested; sensorgrams for a single concentration point (125 nM) are shown for each temperature (see **Supplementary Figs. 5 and 6** for full range of concentration data). (e) van't Hoff plot of temperature dependence of equilibrium binding affinities for Fcε2-4–wild-type sFcεRIα, Fcε2-4–sFcεRIα W87D, Fcε3-4–wild-type sFcεRIα, and Fcε3-4–sFcεRIα W87D. The fits for both Fcε2-4 interactions are linear ($R > 0.99$), whereas the Fcε3-4 interactions show small deviations from linearity ($R = 0.96$ – 0.98), consistent with a minor contribution from ΔC_p . The derived thermodynamic parameters are summarized in **Table 2**.

DISCUSSION

The discovery that the bend in the IgE molecule is even more pronounced in the complex is consistent with the original FRET studies reporting increased rigidity of a chimeric murine IgE upon receptor binding¹¹. This conformation orients the Fab arms of IgE away from the cell surface (**Fig. 1**) and, although there is some uncertainty in the orientation of the two extracellular receptor domains, this might place structural constraints on the ability of IgE to recognize and be cross-linked by particular allergens and their epitopes. An acutely bent structure must also impose restrictions on allergen recognition by the membrane-anchored form of IgE (mIgE) expressed on B cells, if the Fab arms are in that case oriented toward the cell surface. Recent crystal structures of allergen Fab complexes have provided some initial insights into this issue^{21–25}: a feature of two of these complexes is the dimeric nature of the allergen^{23,24}, which exhibits the epitopes in a defined (dyad-related) manner. Indeed, dimerization may be critical for allergens with a single epitope specificity to promote cross-linking^{23,24,26}, and epitope location together with the orientation of the IgE Fab relative to each allergen monomer may determine whether it can be recognized by mIgE or trigger mast cells sensitized with receptor-bound IgE. A key question in our structural understanding of IgE-Fc and Fab–allergen complexes is the degree of Fab flexibility relative to Fc in the IgE molecule; currently only limited information is available about the segmental flexibility of IgE through FRET¹¹ or EM²⁷ studies.

The adoption of this more acutely bent IgE structure does not lead to any direct receptor contact with either Cε2 domain. How, therefore, do the Cε2 domains influence the kinetics of the interaction? Their effect is seen in the slower off-rates for IgE-Fc (Fcε2-4) compared with Fcε3-4 (**Fig. 4**), which are even more marked for the W87D mutant, and the notably different temperature dependences and derived thermodynamic parameters (**Table 2**). IgE-Fc binding is exclusively driven by a large entropic component, in contrast to the enthalpically driven Fcε3-4; this provides a clue to the stabilizing contribution of the Cε2 domains.

The Cε3 domain in isolation adopts a partially unfolded ‘molten globule’ state^{28,29} and becomes more structured upon receptor binding²⁹. In the context of IgE-Fc, Cε3 is more sensitive than Cε2 to thermal denaturation (refs. 30,31) and becomes more resistant to thermal denaturation when bound to receptor³¹. This loss of conformational entropy of the two Cε3 domains will be considerable for the Fcε3-4 fragment, which lacks any stabilizing interactions of the Cε2 domains packing against them in the bent IgE structure; indeed, the several crystal structures of Fcε3-4 show substantial conformational flexibility^{32,33}. This large entropic penalty upon binding counteracts the favorable (solvent) entropic contribution from the hydrophobic nature of the interaction, leading to the much smaller net entropic term for Fcε3-4 ($T\Delta S = +11.0$ kJ mol^{−1}) than for IgE-Fc ($T\Delta S = +51.0$ kJ mol^{−1}).

complex is enthalpically driven ($\Delta H = -37.0$ kJ mol^{−1}) with a small favorable entropic component ($T\Delta S = +11.0$ kJ mol^{−1}), formation of the IgE-Fc–sFcεRIα complex is strongly entropically driven ($T\Delta S = +51.0$ kJ mol^{−1}), with virtually no overall enthalpic component ($\Delta H = +0.3$ kJ mol^{−1}). Because the binding interfaces of both fragments with FcεRI are generally similar, this marked difference in the thermodynamic parameters can only be due to the presence or absence of the Cε2 domains.

One might expect that the strongly hydrophobic nature of the IgE-Fc–FcεRIα interface would lead to a large and favorable (solvent) entropic contribution to the free energy of interaction, as we observed with wild-type receptor. When we measured the binding of IgE-Fc to the W87D receptor mutant, the $T\Delta S$ term fell from +51.0 kJ mol^{−1} to +38.4 kJ mol^{−1} (with an even larger reduction for Fcε3-4 binding), consistent with this interpretation. The disruption of this key hydrophobic interaction leads to greatly destabilized complexes; the W87D receptor mutant with Fcε3-4 has kinetic rate constants that are more typical of IgG–receptor interactions⁴, and demonstrates the contribution that a single residue can make to an interaction. However, hydrophobicity differences cannot explain the effect of the Cε2 domains on the thermodynamics of the interaction, which we discuss below.

Table 2 Comparative thermodynamics of the interaction of sFcεRIα (wild-type and W87D mutant) with IgE-Fc and Fcε3-4

	ΔH (kJ mol ^{−1})	$T\Delta S$ (kJ mol ^{−1})	ΔG (kJ mol ^{−1})
IgE-Fc (Fcε2-4)			
sFcεRIα WT	0.3	51.0	−50.7
sFcεRIα W87D	−7.5	38.4	−45.9
Fcε3-4			
sFcεRIα WT	−37.0	11.0	−48.0
sFcεRIα W87D	−63.6	−22.6	−41.0

Derived from the data presented in **Figure 4**. WT, wild type.

This ordering of the Cε3 domains in Fcε3-4 upon receptor binding is reflected in the favorable enthalpy term ($\Delta H = -37.0 \text{ kJ mol}^{-1}$ compared with $\Delta H = +0.3 \text{ kJ mol}^{-1}$ for IgE-Fc), as more stable intradomain hydrogen bonds and optimal van der Waals interactions form; this is a typical example of entropy-enthalpy compensation. In summary, the Cε2 domains in IgE-Fc (and IgE) 'preorder' the Cε3 domains, thereby minimizing the loss of conformational entropy upon binding (or gain of entropy upon dissociation) for this intrinsically unstable domain. The full benefit of the hydrophobic interaction can thus be achieved.

The kinetic and thermodynamic parameters for IgE binding to FcεRIα are very similar to those of IgE-Fc (also derived by SPR; data not shown), and thus the Cε2 domains have the same ordering role in the intact antibody. IgG has no counterpart to the Cε2 domains, only the flexible hinge region, and the thermodynamics of its binding to Fc receptors⁴ is more like that of Fcε3-4, enthalpically driven and with lower affinities and faster dissociation rates. The unique binding properties of IgE, high affinity and slow dissociation rate, thus result from several factors: (i) the IgE-FcεRI interface contains a greater fraction of buried hydrophobic surface area than that of the structurally homologous IgG-FcγR interactions³⁴, and other differences in detail between the interfaces may also contribute; (ii) the Cε2 domains are part of a conformational change in the IgE-Fc that involves movement of three of the six domains relative to the other three, making new interdomain contacts while breaking others, whereas the conformational change in IgG-Fc involves only the loosely tethered Cγ2 domains moving apart; (iii) the presence of the Cε2 domains leads to a closer engagement with the receptor of the Cε3 domains in IgE-Fc (with the formation of more van der Waals interactions and hydrogen bonds compared with Fcε3-4); and (iv) the Cε2 domains stabilize the interacting Cε3 domains and give rise to a bent and less flexible antibody molecule than IgG, thus reducing the entropic penalty of binding through these intrinsically less-ordered domains. All these factors together contribute to creating a larger energy barrier against dissociation, and thus a slower off-rate for IgE compared with IgG. It is notable that the Cε2 domains, with no direct receptor contact, contribute so markedly to the defining functional characteristics of the IgE molecule.

Knowledge of these structures and their conformational changes offers a basis for the design of small-molecule inhibitors to target this extensive, high-affinity protein-protein interaction. Earlier studies with receptor-derived peptides³⁵ or IgE-Fc, including the intact Cε3 domain³⁶, show inhibition by direct blocking with K_i values in the micromolar range, but none of these have been developed into a small, nonpeptide analog. However, there are several locations in IgE-Fc at which the binding of a small molecule might prevent the conformational change, access to both subsites and the formation of a long-lived, stable complex. One example has already been highlighted (Fig. 2); a small molecule bound in the cleft between the Cε2B and Cε3B domains in free IgE could 'lock' the antibody in a conformation incapable of fully engaging the receptor using both subsites. We have previously determined the affinity for receptor binding to a single Cε3 domain in the context of an Fcε3-4 fragment and found that this was reduced by at least three orders of magnitude ($K_a \approx 10^6 \text{ M}^{-1}$), owing principally to a faster off-rate³⁷. An attenuation of two orders of magnitude in the affinity of IgE binding, again with a faster off-rate, leads to a 75% reduction of passive cutaneous anaphylaxis *in vivo* in a transgenic mouse model³⁸, and thus partial inhibition can achieve a physiological effect. An allosteric approach may be the key to inhibiting this protein-protein interaction, and could herald a new generation of anti-IgE therapeutics for intervention in allergic disease.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Accession codes. Protein Data Bank: Coordinates and structure factors have been deposited with accession codes 2WQR (IgE-Fc) and 2Y7Q (complex).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

M.D.H. and A.M.D. carried out the crystallographic analysis of the complex, and B.D. the crystallographic analysis of IgE-Fc; M.D.H., J.E.N., J.H., A.J.B. and R.J.O. produced the proteins; S.C.B. and J.M.M. carried out the thermodynamic analysis; E.G. contributed to the analysis of the conformational changes; H.J.G., A.J.B. and B.J.S. planned and directed the project; M.D.H., A.M.D., J.M.M. and B.J.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. The human IgE-Fc (Fcε2-4) construct has been reported¹³ and, as for Fcε3-4, was expressed in mammalian NS0 cells and purified as described⁷. The sFcεRIα construct coding for residues 1–176 of a triple glycosylation mutant (N74A N135A T142A)¹⁴ with C-terminal Lys-His₆ tag was amplified and subcloned into a pHLSec vector⁴⁰—produced by transient transfection in HEK293S cells, which restrict protein glycosylation to (GlcNAc)₂-(Man)₅—and purified as described⁴⁰. The complex was formed by mixing IgE-Fc and sFcεRIα in a 1:1 molar ratio at 4 °C overnight, purified by size exclusion (Superdex 200 column), eluted in 20 mM Tris, pH 7.5, 200 mM NaCl, concentrated by centrifugation at 4 °C ($A_{280} = 20$) and stored at 4 °C.

Crystallization. The complex was crystallized by vapor diffusion. The reservoir contained 95 μl of 2.8 M sodium acetate, pH 7.0, and the drop consisted of 100 nl of the concentrated protein solution ($A_{280} = 20$) with an equal volume of reservoir solution. A single crystal suitable for data collection, measuring ~110 × 40 × 30 μm, grew after 2 months at 21 °C. IgE-Fc was buffer-exchanged to 25 mM Tris-HCl, pH 7.5, 20 mM NaCl and 0.05% (w/v) sodium azide, and concentrated to 2 mg ml⁻¹. Crystals were grown by vapor diffusion, with a 500-μl reservoir of 100 mM Tris-HCl, pH 8.5, 14% (w/v) PEG 8000 and 200 mM lithium sulfate, and a drop containing 1 μl of protein solution and 0.5 μl of the reservoir solution. Crystals, typically ~50 × 50 × 10 μm, grew after 1–2 weeks at 18 °C.

Data collection, structure determination and refinement. One crystal of the complex was cryoprotected in perfluoropolyether oil and flash-cooled to 100 K. X-ray data were collected at beamline ID29 at the European Synchrotron Radiation Facility, Grenoble, France, integrated with MOSFLM⁴¹ and further processed with the CCP4 program suite⁴². The space group was $P2_12_12_1$, and the Matthews coefficient indicated one complex (one sFcεRIα and one IgE-Fc dimer) in the asymmetric unit with 48% solvent content. The structure was solved using MOLREP⁴³ from the CCP4 suite. Fcε3-4 and sFcεRIα from the structure of the Fcε3-4-sFcεRIα complex¹⁴ (PDB 1F6A) and (Ceε)₂ from the structure of IgE-Fc¹³ (PDB 1O0V) were used as search models for the first stage of molecular replacement. Each refinement round was alternated with one round of manual model building with Coot⁴⁴. Early rounds were carried out with CNS⁴⁵, and the sFcεRIα chain and each of the six Fc domains were refined as rigid units with one overall *B*-factor per domain. Electron density maps were calculated omitting each domain turn to check for and prevent any bias. Later rounds were carried out with PHENIX⁴⁶. Noncrystallographic symmetry restraints were applied for Ce3 and Ce4 domain residues with grouped *B*-factor refinement (one per residue). Carbohydrate atoms were subsequently incorporated into the structure but no water or other small molecules. The coordinate error was 0.47 Å, and the final model was analyzed with SFCHECK⁴⁷ and MolProbity⁴⁸; Ramachandran analysis revealed 90.9% (favored) and 97.7% (allowed).

X-ray data for free IgE-Fc were collected at the Diamond Light Source, Harwell, UK, beamline I04, from a crystal flash cooled to 100 K in 100 mM Tris-HCl, pH 8.5, 30% (w/v) PEG 8000 and 200 mM lithium sulfate. The structure was solved by molecular replacement using the reported structure as a search model¹³. Although the crystallization conditions are different, the space groups are the same, and cell dimensions differ only slightly. Data processing was carried out using HKL2000 (ref. 49), and a molecular replacement solution was found using MOLREP⁴³. Iterative cycles of refinement using PHENIX⁴⁶ (including bulk-solvent scaling, conjugate gradient minimization, individual *B*-factor refinement and TLS refinement) alternated with manual model building with Coot⁴⁴. To minimize bias, the model was built into $2F_o - F_c$ composite omit, $2F_o - F_c$ and $F_o - F_c$ electron density maps. TLS groups were generated using the TLSMD web server⁵⁰. Polyethylene glycol molecules were identified using AFITT⁵¹. The coordinate error was 0.26 Å, and the final model evaluated with MolProbity⁴⁸; Ramachandran analysis revealed 97.0% (favored) and 100% (allowed).

Molecular superpositions were carried out using CCP4MG⁴², and interfaces were determined using the Protein Interfaces Surfaces and Assemblies (PISA) service at the European Bioinformatics Institute⁵². Buried surface areas were

calculated using CNS⁴⁵. Contact residues were defined using CCP4 CONTACT⁴² (with a cutoff of 4.0 Å) and confirmed by manual inspection. Secondary structures were assigned using KSDSSP implemented in CHIMERA⁵³. Protein domain movements were analyzed with DynDom^{54,55} using PDB 1O0V, 1FP5 and 1F6A and the structures described here. Planar and dihedral angles were measured by defining vectors corresponding to the local two-fold axes of the (Ce4)₂ and (Ce2)₂ domain pairs and calculating the dot product between them or by direct measurement in PyMOL (DeLano Scientific). Figures were produced with PyMOL and videos were created using the eMovie plugin⁵⁶ for PyMOL. Morphs were created using RigiMOL in iPyMOL.

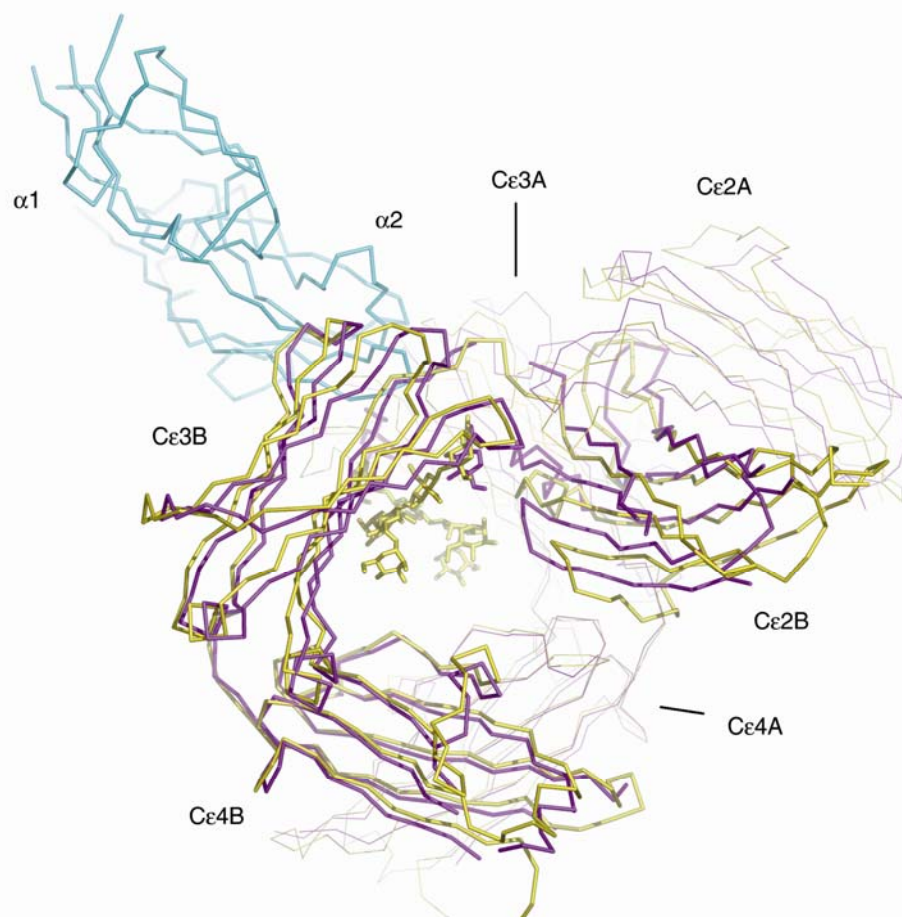
Surface plasmon resonance analysis. Experiments were carried out on a Biacore 2000 instrument (GE Healthcare). Specific binding surfaces were prepared by coupling sFcεRIα through carbohydrate to a CM5 sensor chip via aldehydes as described⁹. Coupling density was limited to <400 RU. Samples of IgE, Fcε2-4 (IgE-Fc) or Fcε3-4 were dialyzed into HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.01% (v/v) Surfactant P-20) and injected over the sensor chip at a flow rate of 25 μl min⁻¹ at concentrations of 125, 62.5, 31.3, 15.6, 7.7 and 3.9 nM. Each data set was collected at 10, 15, 20, 25 and 30 °C. Analyte concentrations were injected in duplicate, a titration series of 'low-to-high' analyte concentration followed by a 'high-to-low' series. A 2-min association phase was followed by a 15-min dissociation phase. The sensor surface was regenerated by three 60-s pulses of 0.2 M glycine-HCl, pH 2.5. Standard double-referencing data subtraction methods were applied⁵⁷ before evaluation of rate constants and equilibrium binding. Association and dissociation curves were fitted using a biphasic interaction model, as described⁸. Data were analyzed using curve-fitting software from Microcal Origin (OriginLab).

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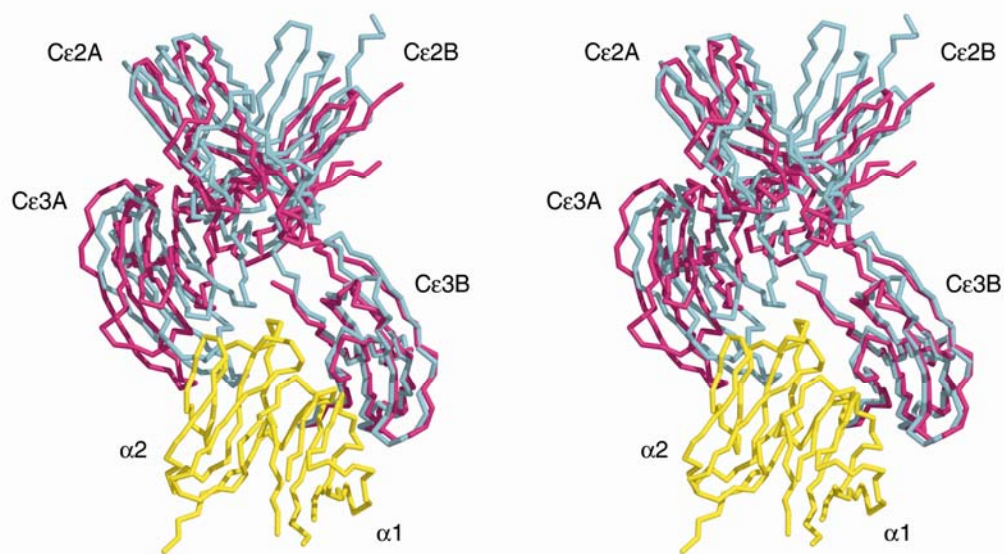
**Conformational changes in IgE contribute to its
uniquely slow dissociation rate from receptor FcεRI**

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E. Girardi, J. Hunt, H.J. Gould, A.J. Beavil, J.M. McDonnell, R.J. Owens
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A



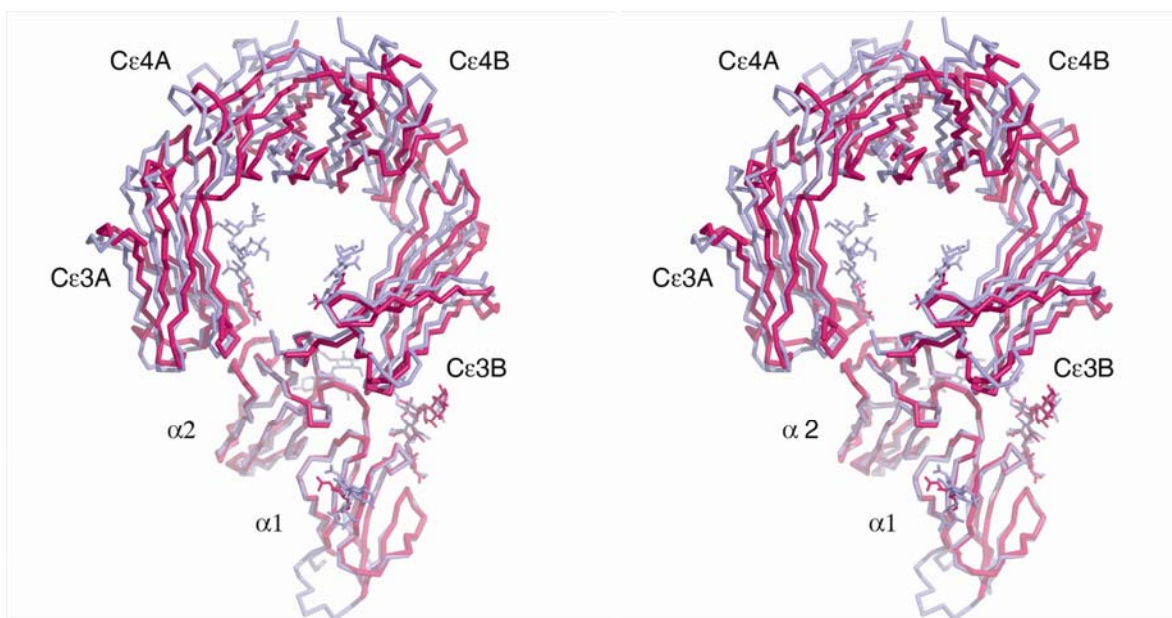
B



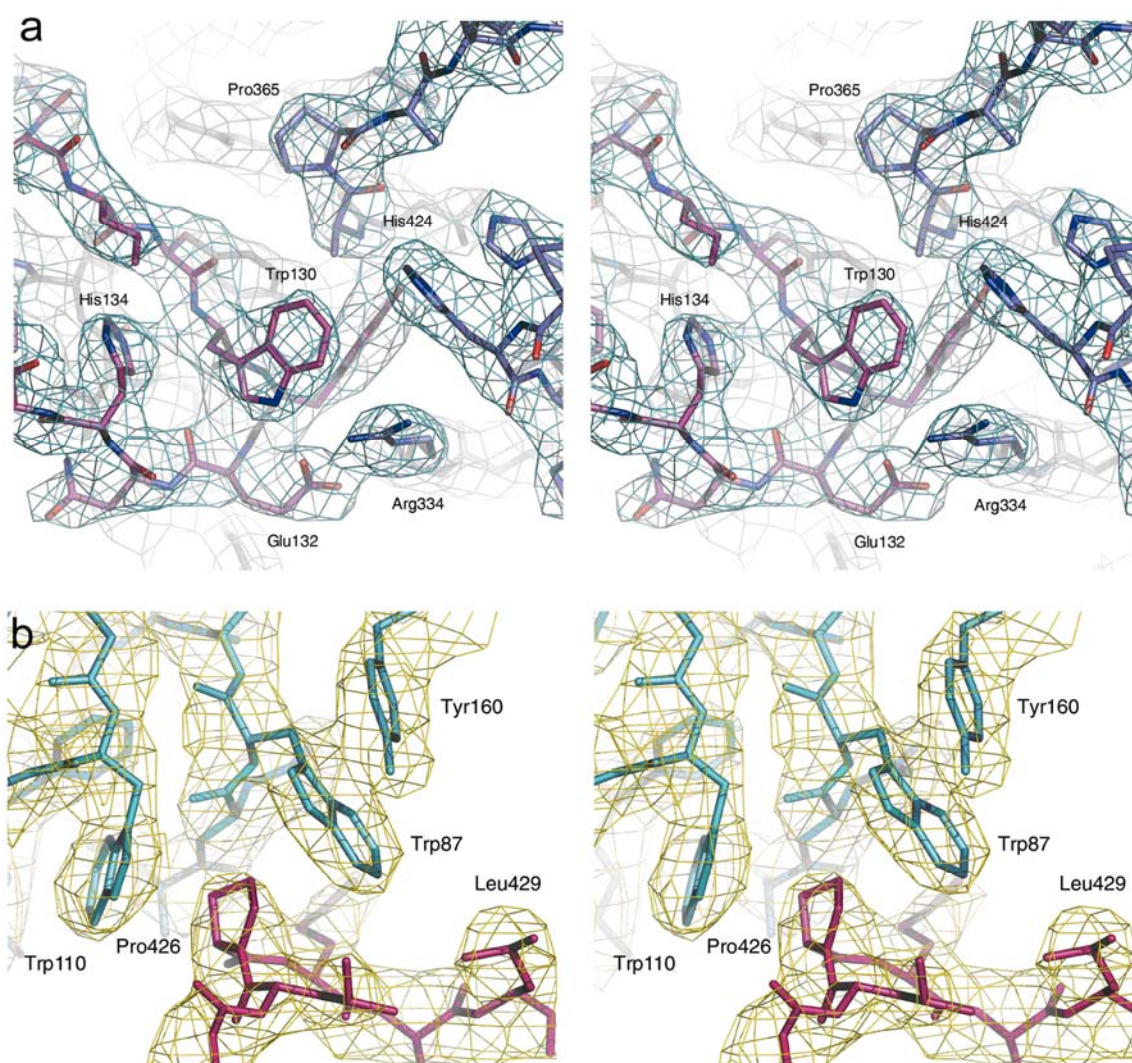
Supplementary Figure 1. (A) Conformational change between free IgE-Fc (yellow) and complexed (purple) with sFcεRIα (blue). The figure was generated by superposing the Cε4 domain pair, and in both IgE-Fc structures, chain B is shown in thicker lines. Cε3B moves only slightly, whereas Cε3A (just visible at the back) and the two Cε2 domains move substantially as a rigid unit. If the (Cε4)₂ dimer is taken as a fixed point of reference between the free and bound IgE-Fc structures, the movement of the three domains (Cε3A, Cε2A and Cε2B) is a rotation of 4.5° about the local two-fold axis of the (Cε4)₂ pair, followed by a rotation of 8° towards this axis, leading to the more compact and bent structure. Carbohydrate is shown only for IgE-Fc. **(B)** Illustration of the conformational change in the Cε2 and Cε3 domains upon receptor binding (stereo pair). sFcεRIα (yellow) and the Cε2 and Cε3 domains (pink) from the structure of the complex, together with the Cε2 and Cε3 domains of uncomplexed IgE-Fc (light blue), are superposed on the Cε3B domain. The conformational change in IgE-Fc consists of the three domains Cε2A, Cε2B and Cε3A moving as a rigid unit relative to the Cε3B and the two Cε4 domains (latter not shown).



Supplementary Figure 2. Schematic illustration of a β -strand shift in both C ϵ 2 domains upon complex formation. Shown on the left (blue) is the classical immunoglobulin fold as seen in the C ϵ 2 domains of uncomplexed IgE-Fc, and on the right (pink) is the alternative conformation with the C' strand instead of the D strand, as seen in both C ϵ 2 domains of the complexed IgE-Fc structure. The two are superposed in the center panel. This structural change in the edge β -strand affects residues 272 to 281. The D strands in both C ϵ 2 domains are involved in inter-molecular packing contacts in the free IgE-Fc crystal structure. The same conformation is observed in the NMR structure of isolated C ϵ 2 in solution¹⁰, suggesting that this is the preferred structure in the free molecule. In the complex, in C ϵ 2B (with no crystal packing contacts), the D to C' strand shift is stabilized by new *intra*-chain contacts made between the immediately preceding CC' loop and residues of C ϵ 3B. The strand shift appears to be promoted by the quaternary structural changes involved in receptor binding. The same strand shift occurs in C ϵ 2A, but crystal packing contacts in the complex preclude a D strand location.



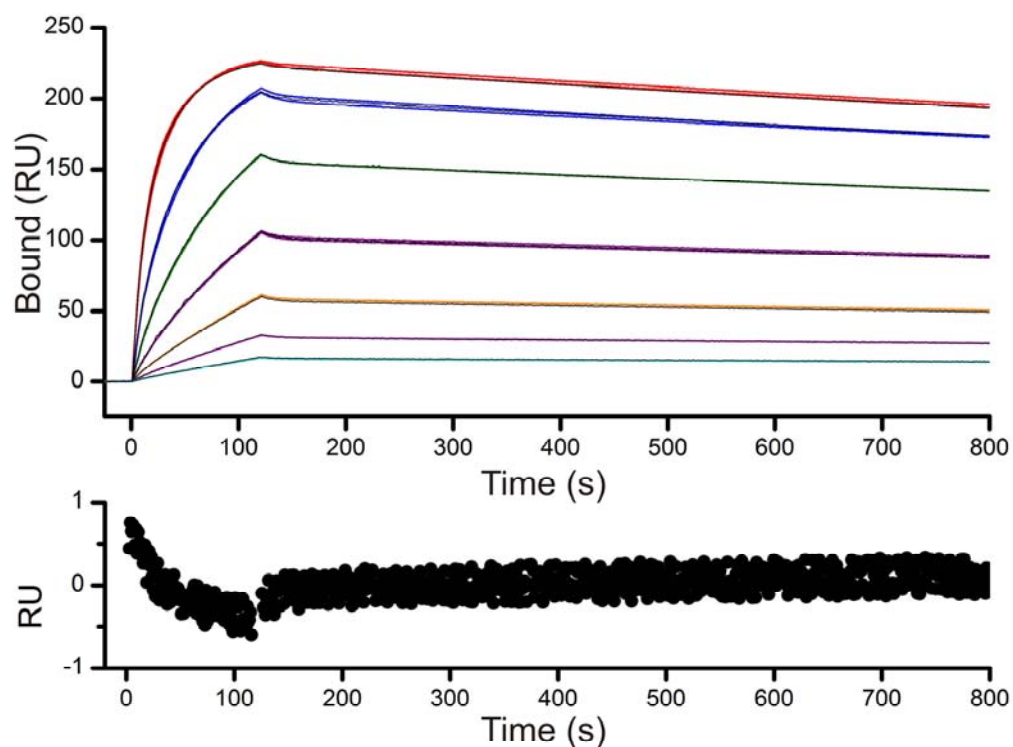
Supplementary Figure 3. Stereo view of the C α traces of the structures of the IgE-Fc/sFc ϵ RI α complex (C ϵ 3 and C ϵ 4 domains only) and Fc ϵ 3-4/sFc ϵ RI α complex (pink and light blue respectively), superposed on the receptor. The approximately 5° difference in the orientation of the antibody Fc domains relative to the receptor can be seen. N-linked carbohydrate chains in both the Fc and receptor are also shown in all-atom representation. In IgE-Fc only a single GlcNAc residue is visible on each chain; in Fc ϵ 3-4¹⁴, (GlcNAc)₂-(Man)₄ and (GlcNAc)₂-(Man)₁ were observed. Of the seven potential N-glycosylation sites on the receptor in the IgE-Fc complex, three were removed by mutagenesis, and carbohydrate chains are visible at only two, Asn21 and Asn42, where (GlcNAc)₁ and (GlcNAc)₂-(Man)₂ respectively were built; both are stabilized by crystal packing contacts. No density was visible at either of the two remaining sites, Asn50 and Asn166, where there are no such crystal packing contacts.



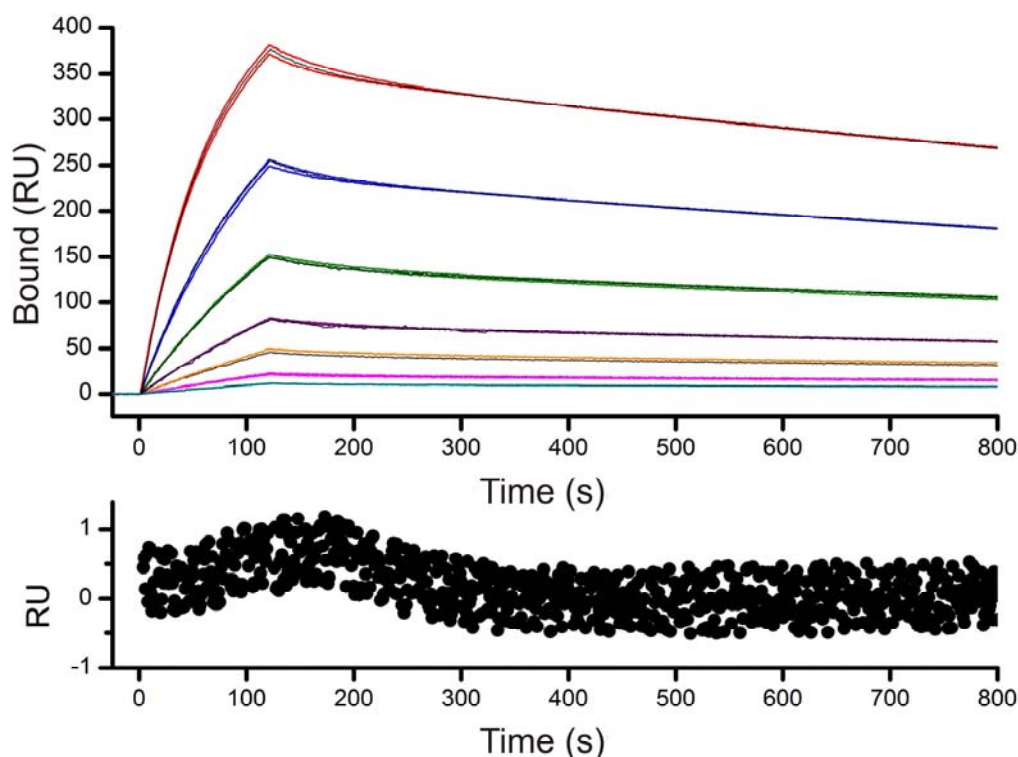
Supplementary Figure 4. Electron density (stereo views) for interface regions of the complex.

a) Sub-site 1: the hydrophobic interactions of Trp130 (sFcεRIα, purple carbon atoms) and the salt bridge between Glu132 (sFcεRIα) and Arg334 (IgE-Fc, light blue carbon atoms). Oxygen atoms are colored red, nitrogen atoms dark blue. Density is contoured at 1.0σ in this $2F_{obs}-F_{calc}$ map.

b) Sub-site 2: the "proline sandwich" in which Pro426 (IgE-Fc, in purple) is flanked by Trp87 and Trp110 (sFcεRIα, in blue). Density is contoured at 1.0σ in this $2F_{obs}-F_{calc}$ map.



Supplementary Figure 5. SPR sensorgrams of Fcε2-4 binding to sFcεRIα wildtype at 25°C. sFcεRIα samples were immobilized through carbohydrate moieties *via* aldehyde coupling. The following range of analyte concentrations was injected at a flow rate of 25 μl min⁻¹: 250 nM (red), 125 nM (blue), 62.5 nM (green), 31.25 nM (purple), 15.63 nM (orange), 7.8 nM (magenta), and 3.9 nM (cyan). Concentrations were tested in duplicate using a low-to-high titration series, followed by a high-to-low series; the duplicate sensorgrams are shown for each concentration. Binding curves were fitted to a biphasic interaction model, as described previously⁸; black lines indicate the global fits obtained for the binding and residuals of the fit for the highest concentration are shown as black circles below. The rate constants for the principal kinetic component are: $k_{on} = 1.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$; $k_{off} = 1.5 \times 10^{-4} \text{ s}^{-1}$.



Supplementary Figure 6. SPR sensorgrams of Fcε2-4 binding to sFcεRIα W87D at 25°C. sFcεRIα samples were immobilized through carbohydrate moieties *via* aldehyde coupling. The following range of analyte concentrations was injected at a flow rate of 25 μl min⁻¹: 250 nM (red), 125 nM (blue), 62.5 nM (green), 31.25 nM (purple), 15.63 nM (orange), 7.8 nM (magenta), and 3.9 nM (cyan). Concentrations were tested in duplicate using a low-to-high titration series, followed by a high-to-low series; the duplicate sensorgrams are shown for each concentration. Binding curves were fitted to a biphasic interaction model, as described previously⁸; black lines indicate the global fits obtained for the binding and residuals of the fit for the highest concentration are shown as black circles below. The rate constants for the principal kinetic component are: $k_{on} = 5.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; $k_{off} = 5.3 \times 10^{-4} \text{ s}^{-1}$.

Supplementary Video 1: Overall structure of the IgE-Fc/sFcεRIα complex. This video shows the proposed orientation of the complex in relation to the cell membrane and highlights the acute bend in IgE-Fc, with the Cε2 domains packed against the Cε3 and Cε4 domains. The complex is rotated by 90° clockwise, 180° anti-clockwise, and 90° clockwise about an axis orthogonal to the cell membrane. IgE-Fc chains A and B are colored in green and purple respectively, and sFcεRIα in yellow.

Supplementary Video 2: Conformational change in IgE-Fc. This video demonstrates the conformational change that takes place in IgE-Fc upon sFcεRIα binding. The video shows the same conformational change from four views, each 90° apart. The change is demonstrated by morphing the free structure of IgE-Fc into that of the receptor-bound form, and then back to the free form. IgE-Fc chains A and B are colored in green and purple, respectively.

Supplementary Video 3: Conformational change in Cε2 and Cε3 domains on receptor binding. This video demonstrates the conformational change that takes place in IgE-Fc upon sFcεRIα binding (with emphasis on the Cε2 domains), and shows how the Cε2A, Cε2B and Cε3A domains move together as a rigid unit. The video also shows the small conformational change within Cε3B. The free structure of IgE-Fc is first shown, and then morphed into the receptor-bound form. The receptor is briefly displayed, after which IgE-Fc is morphed back to the free form. IgE-Fc chains A and B are colored in green and purple respectively, and sFcεRIα in yellow.