



The structure of the IgE Cε2 domain and its role in stabilizing the complex with its high-affinity receptor FcεRIα

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The stability of the complex between IgE and its high-affinity receptor, FcεRI, on mast cells is a critical factor in the allergic response. The long half-life of the complex of IgE bound to this receptor *in situ* (~2 weeks, compared with only hours for the comparable IgG complex) contributes to the permanent sensitization of these cells and, hence, to the immediate response to allergens. Here we show that the second constant domain of IgE, Cε2, which takes the place of the flexible hinge in IgG, contributes to this long half-life. When the Cε2 domain is deleted from the IgE Fc fragment, leaving only the Cε3 and Cε4 domains (Cε3–4 fragment), the rate of dissociation from the receptor is increased by greater than 1 order of magnitude. We report the structure of the Cε2 domain by heteronuclear NMR spectroscopy and show by chemical shift perturbation that it interacts with FcεRIα. By sedimentation equilibrium we show that the Cε2 domain binds to the Cε3–4 fragment of IgE. These interactions of Cε2 with both FcεRIα and Cε3–4 provide a structural explanation for the exceptionally slow dissociation of the IgE–FcεRIα complex.

Allergic reactions are characterized by immediate hypersensitivity, manifested by the occurrence of adverse immunological reactions within minutes of allergen provocation. When IgE bound to its high-affinity receptor (FcεRIα) on the surface of mast cells and basophils is cross-linked by multivalent allergen, an activation signal causes the release of inflammatory mediators, resulting in the type I immediate hypersensitivity reaction. This response depends on the unique high affinity of the interaction between IgE and FcεRIα. This affinity is at least four orders of magnitude higher than that of IgG for either of the receptors FcγRII (CD32) or FcγRIII (CD16) and two orders of magnitude greater than IgG for FcγRI (CD64) or IgE for FcεRII (CD23)^{1,2}. The high affinity of the IgE–FcεRIα complex is largely due to the slow rate of dissociation of IgE from the receptor³. The half-life of this complex on cells in suspension is ~16 h but is considerably longer in tissues because of restricted diffusion and the rebinding of IgE. In human tissue, the half-life of the IgE–receptor complex is ~2 weeks⁴ compared with only hours for IgG (H.J.G., unpublished data). The structural basis for both the extraordinary stability of this complex and for the slow dissociation of IgE from FcεRIα has eluded explanation. In addition, this basis has not been resolved by the recently determined crystal structures of the complexes of IgE–Fc(3–4), a homodimer of the Cε3 and Cε4 domains, with FcεRIα⁵, and IgG–Fc with FcγRIII⁶, which exhibit remarkably similar surface areas of interaction and similar numbers of hydrogen bonds and salt bridges.

One notable difference between IgE and IgG, however, is the presence of an extra immunoglobulin domain pair, Cε2, which replaces the flexible hinge region found in IgG. The IgE–Fc fragment that was cocrystallized with FcεRIα contained only the Cε3 and Cε4 domains. Because of this, it was concluded that the Cε2 domain plays no role in the interaction with receptor⁵. However, we show here, using IgE–Fc fragments with and without the Cε2 domains (hereafter termed Cε2–4 and Cε3–4, respectively), that Cε2 has a profound effect on the kinetics of the receptor interaction and, in particular, the dissociation rate constant (k_d).

Cε2 stabilizes the interaction of IgE with FcεRIα

We monitored the kinetics of IgE, Cε2–4 or Cε3–4 binding to FcεRIα by surface plasmon resonance with the receptor immobilized as a fusion protein with IgG4–Fc⁷. IgE and Cε2–4 displayed identical kinetics, whereas Cε3–4 showed a somewhat faster on-rate (~4-fold) and a markedly faster off-rate (~20-fold)

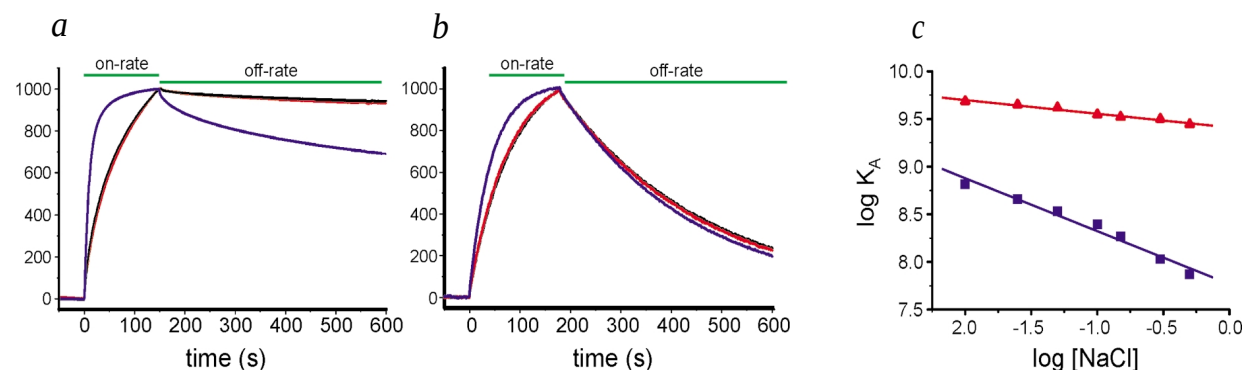


Fig. 1 The Cε2 domain affects IgE binding to the high affinity receptor FcεRIα. **a**, A comparison of the kinetics of binding by surface plasmon resonance of IgE (black), Cε2–4 (red), and Cε3–4 (blue) to immobilized FcεRIα and the lectin domain of FcεRII. For clarity, only a single concentration is shown for each ligand (62.5 nM for binding to FcεRIα, 1.0 μM for FcεRII). Each has been normalized to give a maximal response of 1000 RU. The calculated rates for the interactions are as follows. **a**, Kinetics of binding to immobilized FcεRIα: IgE ($k_{on} = 2.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 1.8 \times 10^{-4} \text{ s}^{-1}$), Cε2–4 ($k_{on} = 3.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 1.9 \times 10^{-4} \text{ s}^{-1}$), Cε3–4 ($k_{on} = 9.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 3.4 \times 10^{-3} \text{ s}^{-1}$). **b**, Kinetics of binding to immobilized FcεRII: IgE ($k_{on} = 4.6 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 2.2 \times 10^{-3} \text{ s}^{-1}$), Cε2–4 ($k_{on} = 4.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 2.3 \times 10^{-3} \text{ s}^{-1}$), Cε3–4 ($k_{on} = 1.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 3.2 \times 10^{-3} \text{ s}^{-1}$). **c**, A comparison of the effects of ionic strength on the binding of Cε2–4 (red) and Cε3–4 (blue) to FcεRIα. The slope of the Cε2–4 line is -0.13 ± 0.01 and Cε3–4 has a slope of -0.55 ± 0.04 .

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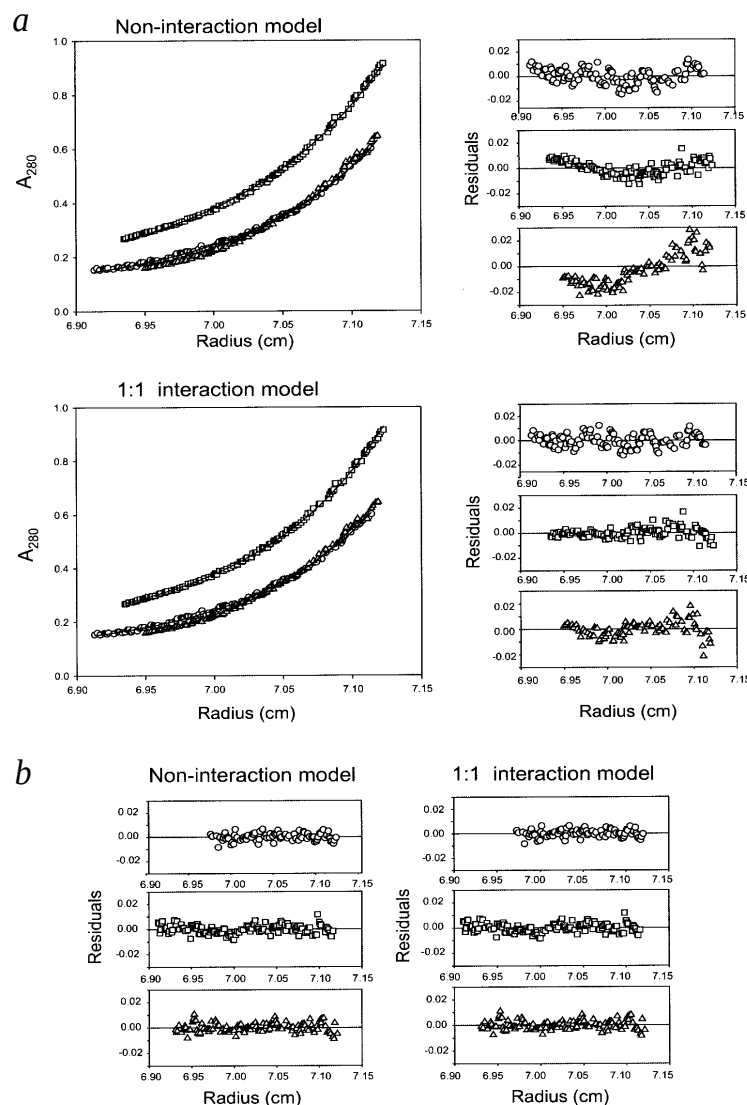


Fig. 2 Analytical ultracentrifugation of Cε2 in complex with Cε3-4. **a**, Curves and residuals for samples measured in PBS-azide. The three curves correspond to different ratios of Cε2 and Cε3-4 in the mixtures (squares, 2:1; circles, 1:1; triangles, 1:2). Data were fit to two models: one for two noninteracting species and another for a 1:1 interaction. Randomly distributed residuals are only obtained using the 1:1 interaction model. **b**, Residuals only are shown for samples measured in PBS-azide with 1 M NaCl added. Two models, as described in (a), are fitted to the data, and randomly distributed residuals are obtained for both; the 1:1 model returns an affinity constant of zero.

showed a ~10-fold decrease (Fig. 1c). Thus, the interaction surface that Cε2 contributes to the IgE-FcεRIα complex is likely to be principally hydrophobic in nature, and this additional set of interactions significantly alters the overall thermodynamic property of the complex.

Cε2 interacts with Cε3-4

To investigate the structural basis for the effect of Cε2 on the kinetics of the IgE-FcεRIα interaction, we constructed a monomeric Cε2 domain, in which the two Cys residues (Cys 241 and 328) forming the disulfides⁹ between the heavy chains of Fc were mutated to Ser. Equilibrium sedimentation was used to study the binding of the mutated Cε2 domain to its neighboring Cε3-4 in IgE. The experimental molecular weight and hydrodynamic behavior of Cε2 was consistent with a monomeric species (experimental *M_r* 11,470; calculated *M_r* 11,471.6). The dimeric Cε3-4 construct has an apparent molecular weight of 52,200, as determined elsewhere⁶. Under physiological conditions, the Cε2 and Cε3-4 fragments mixed in various molar ratios (2:1, 1:1 and 1:2) by equilibrium centrifugation gave a dissociation constant (*K_d*) of 4.5 μM and a stoichiometry of one Cε2 molecule to one Cε3-4 molecule (Fig. 2). This affinity is comparable to that between IgG-Fc and its receptors FcγRII or FcγRIII¹⁰. This is the first evidence for a direct interaction between Cε2

(Fig. 1a). Since IgE (~190 kDa) and Cε2-4 (~75 kDa) show very similar on-rates despite their very different sizes and hydrodynamic properties, the faster on-rate for Cε3-4 (~50 kDa) cannot be simply attributed to diffusion rate limitations. It is likely therefore that the presence of the Cε2 domains and local structure at the Cε2-3 interface restricts the initial interaction and thus reduces the on-rate for fragments containing the Cε2 domain (see below). The much slower off-rate for IgE and Cε2-4 compared with Cε3-4 implies that Cε2 plays a role in enhancing the stability of bound IgE. The binding of IgE, Cε2-4 or Cε3-4 to a soluble, monomeric fragment of FcεRII⁸ (CD23) was also performed. As with FcεRIα, IgE and Cε2-4 showed identical kinetics of binding to FcεRII, and Cε3-4 showed a faster on-rate (~3-fold). In contrast to FcεRIα, however, the off-rate for Cε3-4 was the same as the other fragments (Fig. 1b), suggesting that Cε2 plays no role in the interaction between IgE and receptor FcεRII.

In addition to changes in the rates of interactions, the ionic strength dependencies of binding of Cε2-4 and Cε3-4 to FcεRIα differed significantly over a concentration range of NaCl from 10–500 mM. Cε2-4 showed a <2-fold decrease, but Cε3-4

and Cε3-4 and suggests a structural basis for the compact, bent models of IgE-Fc derived from X-ray and neutron scattering¹¹ and fluorescence resonance energy transfer experiments with IgE¹². The observed stoichiometry of 1 module Cε2 : 1 dimer Cε3-4 could be due to either steric interference or a conformational change following the binding of the first Cε2 domain. Our model (see below) does not predict steric interference but there is some evidence suggesting potential conformational change. The crystal structures of the Cε3-4 fragment, free⁵ and in complex with FcεRIα¹³, show that the Cε3 domains in Cε3-4 are readily distorted, and we have shown that the isolated Cε3 domain is inherently labile¹⁴. The interaction between Cε2 and Cε3-4 was highly sensitive to ionic strength, and we could detect no interaction at 1.0 M NaCl (Fig. 2), indicating that it may be predominantly electrostatic in nature.

The structure of Cε2

To provide a basis for studying the interaction between Cε2 and FcεRIα, we have determined the three dimensional structure of the Cε2 domain by heteronuclear NMR spectroscopy. The Cε2 domain was entirely monomeric under the conditions used for

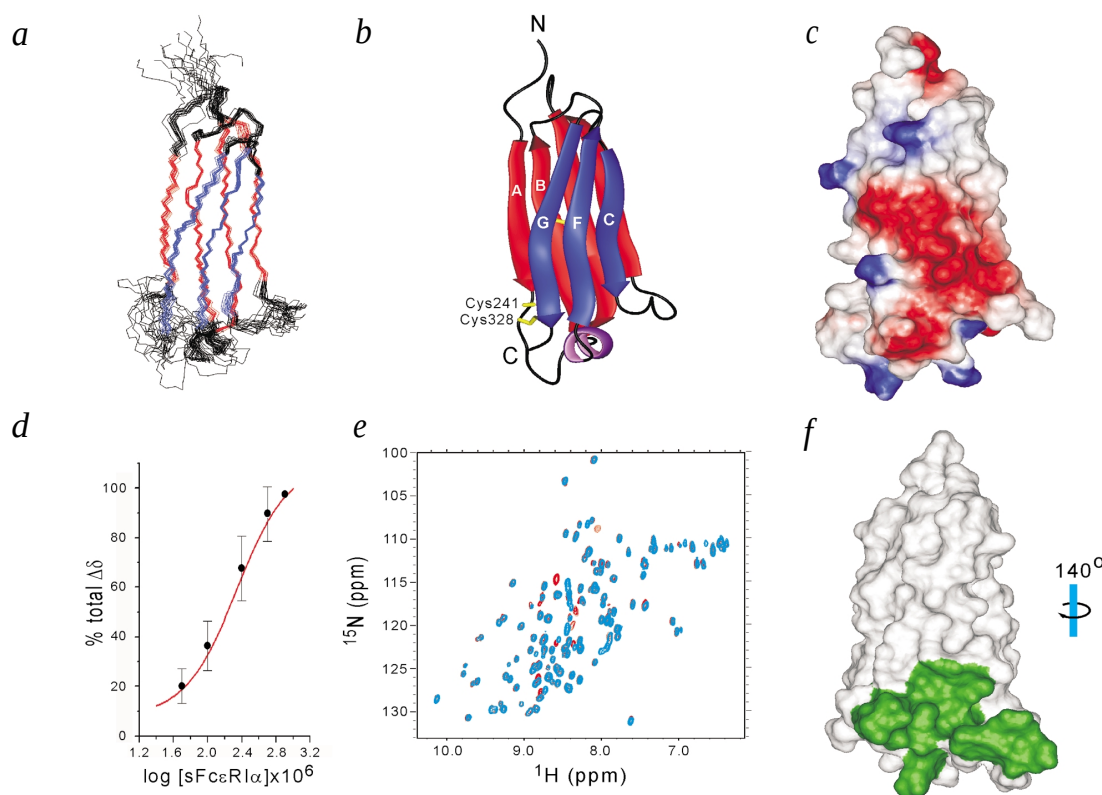


Fig. 3 The solution structure of Cε2 and interaction surface with FcεRIα. **a**, View of the backbone (N, Cα, C) of 15 superimposed NMR-derived structures of Cε2; β-strands are colored in blue and red, and loops in black. **b**, A ribbon diagram of Cε2 with termini, β-strands and location of the intermolecular cysteine residues identified. **c**, A surface representation of Cε2 colored according to electrostatic potential, colored such that regions with potential < -8 k_BT are red, whereas those > +8 k_BT are blue (k_B = Boltzmann constant; T = absolute temperature). **d**, For each titration point of sFcεRIα, the percentage change in chemical shift position (Δδ) was averaged for five Cε2 ¹H-¹⁵N HSQC peaks. The K_d of Cε2 for sFcεRIα is estimated from the concentration of sFcεRIα required for a 50% change in chemical shift position. **e**, A comparison of the ¹H-¹⁵N HSQC maps of Cε2 in the absence (blue) and the presence of sFcεRIα. **f**, Residues that show significant chemical shift changes upon binding to FcεRIα are colored in green. Please note that this structure is rotated 140° relative to the representations shown in (a), (b) and (c). The figures were generated using MOLMOL³⁵.

these studies. No evidence for dimerization was observed over a range of protein concentrations from 40 μM to 3 mM, monitored by hydrodynamic properties (translation diffusion rates and estimates of T₂ relaxation times) of the sample¹⁵. Cε2 is a disulfide-linked dimer¹⁶ within the complete IgE molecule, but in the absence of these disulfide bridges we find, surprisingly, that the Cε2 domains have no measurable affinity for each other. This is consistent with the sedimentation data described above and suggests that previous models of the Cε2 domain pair showing a close association of domains are inaccurate¹⁷. The three-dimensional structure of Cε2 (Fig. 3) consists of seven β-strands arranged in two β-sheets composed of three and four strands. The topology is that of an immunoglobulin domain (C1 set). A structural similarity search using DALI¹⁸ found several immunoglobulin constant domains that showed root mean square (r.m.s.) deviations of <1.5 Å for backbone atoms in secondary structural elements.

The interaction surface of Cε2 with FcεRIα

We then mapped its ligand binding surfaces by titrating the ¹⁵N-labeled Cε2 domain with unlabeled sFcεRIα (and with the monomeric 16 kDa fragment of CD23 as a control, which does not bind to Cε2; ref. 8). The results indicated that Cε2 displays an intrinsic affinity for FcεRIα, even in the absence of the other IgE domains. The estimated affinity (K_D ~200 μM, Fig. 3d) is

weaker than that of the larger — that is, Cε2-4 and Cε3-4 — fragments. The chemical shift perturbation data reveal a single continuous interaction surface on the Cε2 monomer in a region close to the linker region to Cε3 (Fig. 3f). Residues from four regions of the primary sequence of Cε2 form one contiguous surface and make up the interaction site. Residues in β-strand B and loops A–B, C–D and E–F show significant changes in chemical shift position in a ¹H-¹⁵N HSQC experiment (residues Ser 240, Gly 244, His 246, Ile 251, Gln 252, Met 275, Asp 276, Val 277, Asn 278, Thr 298, Leu 299 and Lys 302) (Fig. 3e). We could detect no interaction between Cε2 and CD23 in a parallel analysis. This implies that the CD23 binding site of IgE has no contribution from Cε2, which is fully consistent with the SPR kinetic data that showed identical off-rates for IgE Cε2-4 and Cε3-4.

Modeling Cε2 in the interaction of IgE with FcεRIα

In order to visualize the interactions of Cε2 with both Cε3-4 and FcεRIα within the complex, we first derived a model for the dimer of Cε2 by incorporating the pair of interdomain disulfide bridges^{16,19,20} as distance constraints in structure calculations using the program DYANA²¹. The resulting family of structures differs mainly in the angle between the two domains, but all involve only a small area of intermonomer interaction. Using this model of the Cε2 dimer together with the recently solved

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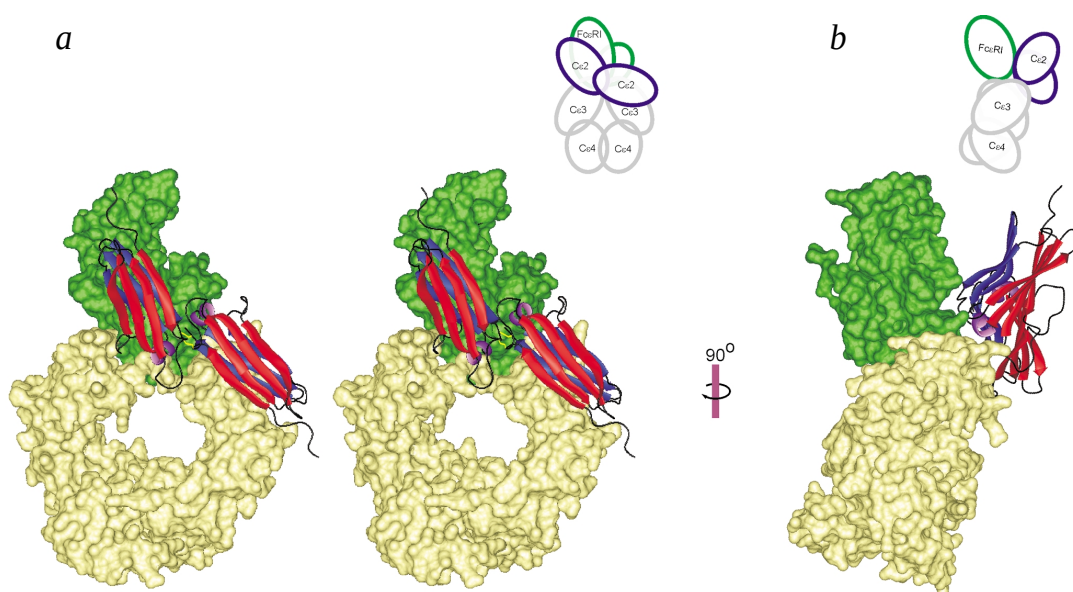


Fig. 4 The overall architecture of Cε2-4 complexed with FcεRIα. **a**, Stereo view and **b**, 90° rotation of Cε2 modeled onto the complex of Cε3-4 with FcεRIα³. Cε2 is shown as a ribbon structure (colored as in Fig. 3b) with intermolecular disulfide bonds colored in yellow; Cε3-4 (gold) and FcεRIα (green) are shown as surface representations. Figure was generated in MOLMOL³⁵.

Table 1 Summary of restraints and structural statistics

Restraints	
NOEs	
Intraresidue	222
Sequential	335
Medium range ($l < 5$)	230
Long range	764
Total NOEs	1,551
Dihedral angles (ϕ , ψ , θ)	
Hydrogen bonds	48
Total number of restraints	1,820
Number of violations ¹	
Distance restraints (>0.5 Å)	0
Dihedral angle restraints ($>5^\circ$)	0
Precision ^{1,2}	
Backbone heavy atoms (Å)	0.46 ± 0.11
All heavy atoms (Å)	1.19 ± 0.16
Structure quality ³	
Residues in most favored regions	81.8%
Residues in additional allowed regions	14.8%
Residues in generously allowed regions	2.1%
Residues in disallowed regions	1.3%

¹Values are reported as the average values over 15 of the lowest target value structures.

²The average r.m.s. deviation for the coordinate set was calculated by superimposing each of the 15 structures onto the mean coordinate set. This superposition was over backbone C α , C, N, and O atoms of residues 7–14, 25–33, 41–47, 55–62, 67–76, 84–92 and 95–103.

³By Procheck analysis³⁶.

structure of Cε3-4 in complex with FcεRIα³, we modeled the complex of Cε2-4 with FcεRIα (Fig. 4a,b) as follows. The C-termini of the Cε2 domains were positioned to be consistent with their covalent link to the Cε3 N-termini, and conformers were identified that permitted the Cε2 surface defined from the chemical shift perturbation experiment to interact with FcεRIα. Only a closely related set of Cε2 conformers was sterically allowed. We illustrate a representative structure with one Cε2 domain interacting with FcεRIα through a largely hydrophobic interface. In this structure, the second Cε2 domain is positioned such that a prominent electronegative patch of Cε2 (Fig. 3c) interacts with a large electropositive patch on the Cε3 domain (not shown). This is consistent with the ionic strength dependence observed for this interaction and suggests that specific contacts between one Cε2 domain of the dimer pair and Cε3 may help orient the other Cε2 domain for its interaction with FcεRIα. The Cε2-FcεRIα interaction site is predominantly hydrophobic. This is consistent with the observed low salt sensitivity of binding and with data from site-directed mutagenesis studies²², in particular the fact that mutation of Trp 87 in FcεRIα differentially affects the kinetics of binding to Cε2-4 compared to Cε3-4²³.

Role of the Cε2 domain in allergic reactions

The data presented here demonstrate that the Cε2 domain interacts directly with both FcεRIα and the Cε3 domain within the IgE molecule and that the presence of Cε2 markedly reduces the dissociation rate of IgE from the complex. This consequent enhancement of the half-life of IgE bound to mast cells and basophils results in the long-term sensitization of these cells toward activation by allergens. The presence of the Cε2 domain in IgE results in different immune response kinetics mediated by IgE versus those mediated by IgG — that is, the more stable IgE-FcεRIα complexes support the ‘immediate hypersensitivity’ reactions characteristic of allergic responses. The realization that



the off-rate is the critical parameter for IgE suggests that disruption of the interaction between Cε2 and FcεRIα, or possibly between Cε2 and Cε3 within the IgE molecule itself, may profoundly enhance the off-rate, decrease the half-life and, thus, reduce the ability of IgE to sensitize mast cells. This may be a promising approach for the design of therapeutic agents to combat allergic disease.

Methods

Protein expression and purification. Using the numbering of Dorrington and Bennich⁹, the Cε2 domain is defined by intron/exon boundaries and comprises Ser 226–Asp 330. The Cε2 construct, with C241S and C328S mutations, was produced by PCR using an IgE-Fc cDNA clone isolated from U266 cells²⁴. Recombinant Cε2 was expressed in the *E. coli* host strain BL21(DE3). Recombinant Cε2 was extracted from the cell pellets using a procedure adapted from Bohmann and Tjian²⁵ and refolded according to the procedure of Taylor *et al.*²⁶. Unlabeled, ¹⁵N- and ¹³C,¹⁵N-labeled Cε2 samples were prepared on minimal media or with the addition of ¹⁵NH₄Cl or ¹³C-glucose/¹⁵NH₄Cl to the media, respectively. The production and purification of Cε3–4, sFcεRIα and FcεRII samples have been described in detail^{8,23,27}.

Surface plasmon resonance. All experiments were performed at 25 °C on a Pharmacia BIAcore2000 instrument (Pharmacia Biosensor AB). A specific binding surface was prepared by coupling the IgG₄-Fc-(sFcεRIα)₂ fusion protein to a CM5 sensor chip as described⁷. Coupling density was limited to 400 resonance units (RU). A parallel surface on which IgG₄-Fc was coupled to the same density as the IgG₄-Fc-(sFcεRIα)₂ was also prepared in order to assess nonspecific binding. Samples of IgE, Cε2–4 or Cε3–4 were diluted in HBS (10 mM Hepes, pH 7.4, 3.4 mM EDTA, 0.005% (v/v) surfactant P-20, NaCl at 10–500 mM) and injected over the sensor chip at 10 μl min⁻¹. A 3 min association phase was followed by a 7 min dissociation phase. The sensor surface was regenerated by three 60 s pulses of 0.2 M Glycine-HCl, pH 2.5. Data were analyzed using the BIAevaluation software version 3.1.

Analytical ultracentrifuge. Equilibrium sedimentation experiments were performed on Cε2, Cε3–4 and mixtures of the two using a Beckman Optima XL-A analytical ultracentrifuge as described⁸. Samples were dialyzed into phosphate buffered saline (PBS), 140 mM NaCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4, + 0.05% sodium azide, and data were acquired as an average of 25 absorbance measurements at a wavelength of 280 nm and a radial spacing of 0.001 cm. Sedimentation equilibrium experiments were performed at 4 °C and rotor speeds of 27,000, 23,000 and 19,000 rpm for Cε2 alone, and 12,000, 14,000, and 16,000 rpm for both Cε3–4 alone and mixed with Cε2. For the interaction study, the two proteins were mixed in 1:1, 2:1 and 1:2 molar ratios with the overall protein loading concentrations corresponding to a measured A₂₈₀ of ~0.6. Data for Cε2 and Cε3–4 alone at equilibrium were analyzed in terms of a single ideal solute to obtain the buoyant molecular mass M(1 – $\bar{v}$ ρ) as described¹⁰. The molecular mass was calculated using the determined solvent density¹⁰ and a calculated value of $\bar{v}$ based on the amino acid composition²⁸. The equilibrium data for the 1:1, 2:1 and 1:2 mixtures of Cε2 and Cε3–4 were simultaneously fit to a range of models using the experimental molecular weights determined for the individual components as described⁸. These included 1:1, 2:1, 1:2 models, and a two-component noninteracting model. Samples were also dialyzed into PBS with 1.0 M NaCl to investigate the effect of ionic strength. Values of ρ were corrected using standard tables²⁹, as were M(1 – $\bar{v}$ ρ) values for each component.

NMR spectroscopy. The assignment and structure derivation of Cε2 were determined using standard heteronuclear NMR methods, as in McDonnell *et al.*³⁰. Assignment made use of the following NMR experiments: HNCA, HN(CO)CA, NHCACB, CBCA(CO)NH, HNCO, HN(CA)CO, HNHA, CC(CO)NH, HC(CO)NH and HCCH-TOCSY (mixing time = 20 ms)^{31–34}. Analyses of the ³J_{HN-Hα} coupling constants were performed using HNHA and HMQC-J experiments. NOE correlations were measured using ¹⁵N- and ¹³C-edited NOESY experiments (mixing times = 100 ms). Translational diffusion coefficients were estimated using pulsed field-gradient NMR methods, as described¹⁵. Automated structure calculations were performed using the program DYANA²¹. From 1,000 calculated structures, the 15 with the lowest target functions were selected. Statistics for these structures are listed in Table 1.

Coordinates. The coordinates have been deposited in the Protein Data Bank (accession code 1G84).

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