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Basis of the 1:1 stoichiometry of the high affinity receptor FcεRI-IgE complex

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Abstract A soluble fragment of the high-affinity IgE receptor FcεRI α-chain (sFcεRIα) binds to the Fc fragment of IgE (IgE-Fc) as a 1:1 complex. IgE-Fc consists of a dimer of the Cε2, Cε3 and Cε4 domains of the ε-heavy chain of IgE. This region of IgE has been modelled on the crystal structure of the Fc region of IgG_1, which exhibits twofold rotational symmetry. This implies that IgE should be divalent with respect to its ligands. X-ray scattering studies reveal however that the twofold rotational symmetry of IgE-Fc is perturbed by a bend in the linker region between the Cε2 and Cε3 domains. The 1:1 stoichiometry could then arise from the conformational asymmetry or from steric occlusion of one of the sites by the overhanging Cε2 domains. To test this hypothesis we have expressed a recombinant ε-chain fragment containing Cε3 and Cε4. This product, Fcε3–4, is secreted from cells as a disulphide linked dimer and binds with higher affinity than either IgE or IgE-Fc to cell surface FcεRI. Titration experiments, together with molecular mass measurements of the Fcε3–4/sFcεRIα complex, reveal that Fcε3–4 binds only a single receptor molecule. This excludes the possibility that steric hindrance by Cε2 accounts for the unexpected stoichiometry.

Key words IgE · FcεRI · Stoichiometry · Analytical centrifugation

Abbreviations PBS phosphate buffered saline · SDS sodium dodecyl sulphate · HPLC high pressure liquid chromatography

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Introduction

The effector function of antibodies depends on their ability to sensitise cells for antigen-induced activation by binding to surface Fc receptors. They must not trigger cell activation in the absence of antigen. In the case of IgE and its high-affinity receptor, FcεRI, pre-formed complexes reside at the exterior of mast cells and basophils in various tissues, particularly on mucosal surfaces, which are exposed to the environment, and thus allow an immediate response to environmental antigens. The advantage conferred by IgE effector functions in humans is alleged to be immune protection from parasites (reviewed in Sutton and Gould 1993). However in susceptible individuals they lead to allergic reactions against otherwise innocuous antigens (allergens).

The attachment of multivalent antigens to receptor-bound antibodies triggers cell activation by crosslinking the receptors in the cell membrane. The reason that antibodies alone are insufficient to trigger cell activation appears to be that they are monovalent with respect to the receptor. We have shown that Fc fragments of both IgG and IgE form 1:1 complexes with soluble class-specific receptors, IgG_1-Fc with sFcγRIII (Ghirlando et al. 1995) and IgE-Fc with sFcεRI (Keown et al. 1995). This stoichiometry is a functional requirement, but is not easily rationalized in terms of antibody structure. The IgG_1-Fc crystal structure (Deisenhofer 1986) is an approximately oblate ellipsoid, consisting of a homodimer of the Cγ2 and Cγ3 portions of the γ-heavy chains. It has twofold rotational symmetry about the longitudinal axis, implying the presence of identical binding sites on opposite sides of the molecule.

The IgE-Fc contains an additional domain, Cε2, in place of the hinge in IgG, while the two C-terminal domains, Cε3 and Cε4, are homologous to the Cγ2 and Cγ3 domains in IgG. The structure of IgE-Fc has been modelled (Padlan and Davies 1986; Helm et al. 1991) on the crystal structure of the Fc region of IgG_1 (Deisenhofer 1986). Owing to the absence of a domain homologous to Cε2 in IgG_1-Fc

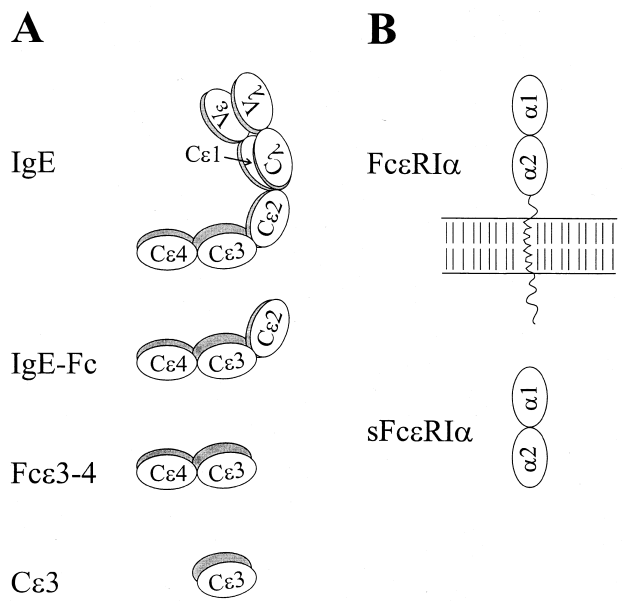

Fig. 1A, B Fragments of IgE and FcεRIα considered in this work: **A** IgE and fragments of IgE. The shapes of IgE and its fragments are adapted from Zheng et al. (1991), although the work of Beavil et al. (1995) suggests that the bend may be concentrated in the linker region between Cε2 and Cε3. **B** The cell-associated and soluble forms of the α-chain of FcεRI

however, the disposition of the Cε2 domains relative to the Cε3 and Cε4 domains remains uncertain. X-ray scattering studies suggest that there is a pronounced bend in the linker region between Cε2 and Cε3 (Beavil et al. 1995; see Fig. 1). The FcεRI binding site in IgE has been mapped to the Cε3 domain of IgE (reviewed in Sutton and Gould 1993). The bend between Cε2 and Cε3 could therefore explain the 1:1 stoichiometry of the IgE-Fc/sFcεRIα complex: we have suggested that the Cε2 domains might obstruct binding of FcεRI to a site located on the concave side of IgE-Fc (Keown et al. 1995). To test this hypothesis, we have expressed a recombinant fragment of the human ε-chain containing only the last two amino acids of Cε2 in addition to the Cε3 and Cε4 domains, and examined the formation of complexes with soluble and cell-associated FcεRI (Fig. 1).

Materials and methods

Protein preparations. The preparations of sFcεRIα and Fcε3–4 are described in detail elsewhere (Shi et al. 1997, Cook and Gould, manuscript in preparation). Purity was assessed by HPLC on a Superdex HR 75 10/30 column in 50 mM sodium phosphate buffer, containing 150 mM NaCl, 0.5 M arginine hydrochloride, 0.05% sodium azide, pH 6.0, and electrophoresis in 15% w/v polyacrylamide gels in SDS under reducing and non-reducing conditions (Laemmli 1970). The proteins were stained with ISS Problue (NBS Biologicals) and the apparent molecular masses of the products were determined with reference to coloured protein markers (MW range 14,300–220,000, Amersham). The concentrations of Fcε3–4 and sFcεRIα were determined spectrophotometrically using calculated molar absorbitivities at 280 nm of 64,520 and 54,700 $M^{-1}\,cm^{-1}$ respectively (Perkins 1986). Purified myeloma IgE was provided by Dr. A. J. Beavil (Beavil 1996).

Binding of Fcε3–4 to FcεRI. Fcε3–4 was iodinated and the kinetics of binding to recombinant FcεRI expressed on Chinese hamster ovary cells was examined as described earlier (Young et al. 1995). The specific activity of the radioiodinated protein was 3.5×10^8 cpm mg^{-1} and 30% of the preparation was active in binding after iodination.

Titration studies. Titrations were carried out by mixing 10^{-6} M solutions of the two proteins in various proportions, thus keeping the total molar protein concentration constant. The Fcε3–4/sFcεRIα complex was separated from the unbound components by HPLC as described above and the concentration of the complex measured from the relative absorbance at the different mixing ratios. This was possible owing to the high affinity of the interaction (see Results below). Titrations were also carried out at constant Fcε3–4 concentration, varying sFcεRIα up to a molar excess of three.

Isolation of the Fcε3–4/sFcεRIα complex. Equimolar amounts of sFcεRIα and Fcε3–4 were mixed and the complex separated from any unbound material by HPLC using the conditions described above. Samples were concentrated by ultrafiltration through Diaflo YM10 membranes (Amicon) and dialysed into PBS/0.05% sodium azide.

Sedimentation equilibrium studies. Sedimentation equilibrium experiments were performed in a Beckman XL-A analytical ultracentrifuge with UV absorption optics. Data were acquired as an average of 25 absorbance measurements at a wavelength of 280 nm and a radial spacing of 0.001 cm. The solvent was PBS/0.05% sodium azide throughout. Solvent densities were measured at 20 °C (Anton Parr DMA58 densimeter) and corrected to 4 °C using standard tables. Sedimentation equilibrium experiments on Fcε3–4 were conducted at various rotor speeds between 16,000 and 20,000 rpm in double sector charcoal filled epon centrepieces with column lengths of about 4 mm at 4 °C. Sedimentation equilibrium experiments on the complex between Fcε3–4 and sFcεRIα were conducted to 4 °C and speeds to 12,000 to 16,000 rpm. In all cases protein concentrations corresponded to a measured A_{280} of approximately 0.5. The distribution was judged to have reached equilibrium when successive scans at 12 hour intervals could be superimposed. Data were analysed to obtain the buoyant molecular mass, $M(1-\bar{\nu}\rho)$ using the Optima XL-A data analysis software (Version 2.0, Beckman) running under MicroCal Origin 2.8, by fitting data from each scan to:

$$A_r = \exp(\ln(A_0) + HM(1-\bar{\nu}\rho)\,(r^2-r_0^2)) + E \qquad (1)$$

where A_0 is the absorbance at a reference point r_0, A_r is the absorbance at radial position, r, H is $\omega^2/2RT$ with ω the angular velocity in rad sec^{-1}, $\bar{\nu}$ is the partial specific volume of the protein, R is the gas constant, T the absolute temperature (K) and E is a small baseline correction term, determined by increased the speed to deplete the meniscus of protein. Residuals were calculated by subtracting the best fit of Eq. (1) from the experimental data. Monodisperse fits gave random residuals in all cases.

Determination of diffusion coefficient ratios

Diffusion coefficient ($D_{20,w}$) ratios for Fcε3–4 and IgE-Fc or IgE were determined using the relation between the sedimentation coefficient $s_{20,w}$ and $D_{20,w}$:

$$s_{20,w}/D_{20,w} = M(1-\bar{\nu}\rho)/RT \quad (2)$$

which yields:

$$D_A/D_B = (s_A/S_B)\,(M(1-\bar{\nu}\rho))_B/M(1-\bar{\nu}\rho)_A) \quad (3)$$

where A represents Fcε3–4 and B IgE-Fc or IgE. The ratios were calculated based on the s_{20w} and buoyant molecular mass data for Fcε3–4 (Beavil and Beavil 1997), the IgE-Fc (Keown et al. 1995) and a myeloma IgE (Ghirlando, unpublished data). $M(1-\bar{\nu}\rho)$ values were corrected for solvent effects.

Results

Polyacrylamide gel electrophoresis was used to examine the purity of Fcε3–4 and sFcεRIα and the formation of the inter-chain disulphide bound in Fcε3–4 (Fig. 2). The sFcεRIα preparation was monodisperse and monomeric under both non-reducing and reducing conditions, as reported in an earlier study (Keown et al. 1995). Under reducing conditions Fcε3–4 migrated as a single species with the apparent molecular mass of 30,000 kDa, corresponding to the monomeric Fcε3–4 polypeptide chain. Under non-reducing conditions, the bulk of the material migrated in the position expected for a dimer (apparent molecular mass 55,000 kDa), with a small proportion (<5%) of residual monomer. This fraction is nevertheless evidently a dimer in the native state, since there was no evidence of monomers in HPLC nor heterogeneity in the sedimentation equilibrium profile (see below). These observations are consistent with those of Basu et al. (1993), who studied a similar fragment of IgE, which however lacked C328 and could not therefore form an inter-chain covalent bond. This fragment exhibited a reversible monomer-dimer equilibrium, with dimers predominanting at the concentrations used for analytical centrifugation.

The kinetics of binding of Fcε3–4 to recombinant FcεRI expressed in Chinese hamster ovary cells was determined and the results are compared with our previously published data on IgE and IgE-Fc (Young et al. 1995) in Table 1. The rate of association of FcεRI with Fcε3–4

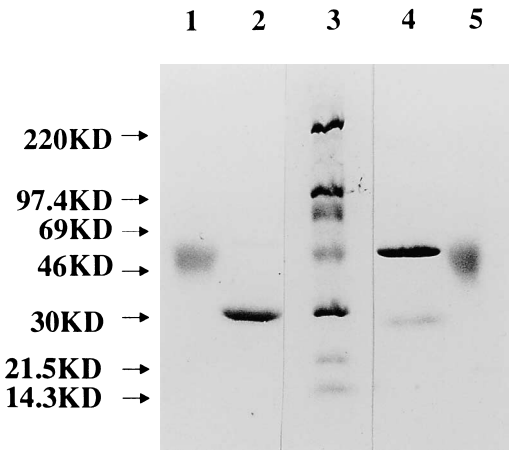

Fig. 2 Polyacrylamide gel electrophoresis profiles of Fcε3–4 and sFcεRIα. Fcε3–4 (lanes *2* and *4*) and sFcεRIα (lanes *1* and *5*) were subjected to electrophoresis in 15% polyacrylamide gels under reducing (lanes *1* and *2*) and non-reducing (lanes *4* and *5*) conditions and compared with molecular weight markers (lane *3*)

Table 1 Summary of the kinetic data obtained for the interaction of Fcε3–4 with FcεRI. To enable comparative assessment of the affinity measurements, results are shown alongside data previously obtained for IgE (WT) and IgE-Fc (Young et al. 1995)

Protein assayed	k_{+1} ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_a (M^{-1})[a]
Fcε3–4	$4.0\pm0.9\times10^6$ (5)	$3.7\pm0.2\times10^{-5}$ (6)	1.1×10^{11}
IgE (WT)	$3.1\pm0.3\times10^5$ (3)	$1.0\pm0.1\times10^{-5}$ (6)	3.4×10^{10}
IgE-Fc	$9.9\pm1.1\times10^5$ (4)	$2.0\pm0.2\times10^{-5}$ (6)	5.0×10^{10}

The number of experiments performed is given in parentheses
[a] Calculated as k_{+1}/k_{-1}

($k_{+1} = 4.0\pm0.9\times10^6\ M^{-1}s^{-1}$) is significantly faster than with IgE or IgE-Fc, whereas the dissociation rate ($k_{-1} = 3.7\pm0.2\times10^{-5}\ s^{-1}$) is only slightly increased. The resulting association constant ($K_a = 1.1\times10^{11}\ M^{-1}$) is therefore greater than the values measured for IgE or IgE-Fc. Our results differ from those of Basu et al. (1993), obtained with Fcε3–4 lacking C328, which exhibited a slightly lower K_a than IgE. The greater conformational freedom of the Cε3 domains in this fragment, together with the presence of monomeric Fcε3–4 chains, may account for the difference. The high affinity justified the use of Fcε3–4 in characterising the interaction between IgE and its high affinity receptor. The remainder of the IgE molecule is not required for the high-affinity interaction. The activity of sFcεRIα is well documented (Hakimi et al. 1990; Blank et al. 1991; Keown et al. 1995).

Titrations were carried out to determine the stoichiometry of the Fcε3–4/sFcεRIα complex. A continuous variation, in which the total molar protein concentration was kept constant and the proportions of the two components were varied, demonstrates that equivalence is reached at a mole ratio of 1:1 (Fig. 3). The results also imply that both

fragments are fully active in respect of binding, since no free fraction of either fragment could be detected in the presence of an excess of the other. A titration of Fcε3–4 at constant concentration with increasing sFcεRIα similarly led to saturation at 1:1 (results not shown).

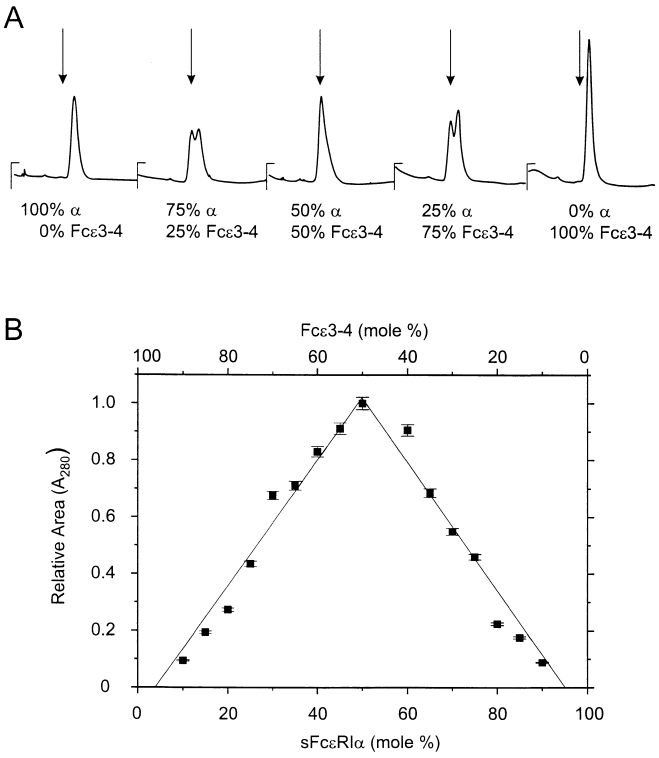

Fig. 3A, B Stoichiometry of the Fcε3–4/sFcεRIα complex. **A** Gel filtration profiles at selected molar ratios were examined on Superdex HR75 10/30 at 0.6 ml min^{-1}. The *arrows* denote the expected position of the complex. **B** relative abundance of the complex as a function of the molar ratio of Fcε3–4 and sFcεRIα. *Straight lines* represent the best linear fit for the data between 0 and 50% and 50–100% mol fractions of sFcεRIα

Sedimentation equilibrium was used to determine the molecular weights of free Fcε3–4 and its complex with sFcεRIα (Fig. 4); studies on sFcεRIα were reported in our earlier work (Keown et al. 1995). The equilibrium distribution for Fcε3–4 showed that the protein is monodisperse within the experimental precision of the method. As this recombinant Fcε3–4 preparation contains two N-glycosylation sites, the molecular mass was calculated using the method described previously, assuming the additivities of the partial specific volumes of the protein and carbohydrate components (Ghirlando et al. 1995). A molecular mass of 52,000 ± 900 g mol^{-1} is obtained, which is identical within experimental error to the value calculated for a dimer of the two Fcε3–4 sequences with a full occupancy of the potential N-glycosylation sites (Table 2). This established that all the preparation was dimeric.

Similar examination of the complex showed that the sample was also monodisperse within experimental error, corresponding to equimolar proportions of the constituents. The buoyant molecular mass was found to be 22,600, whereas those of Fcε3–4 and sFcεRIα were previously determined as 14,000 and 10,200 g mol^{-1} (Keown et al. 1995), respectively (Table 2). The complex thus contains one of each of the two components with an inferred molecular mass of 82,000 ± 2100 g mol^{-1}. Sedimentation velocity experiments indicated that the complex sedimented as a single symmetrical boundary with a sedimentation coefficient of 4.95s ± 0.05. Hydrodynamic modelling of the complex, based on this data is reported elsewhere in this volume (Beavil and Beavil 1997).

Table 2 Data collected from analytical centrifugation of the Fcε3–4 and the complex formed with sFcεRIα

Sample	M $(1-\bar{\nu}\rho)$ (g mol^{-1})	Experimental M (g mol^{-1})	Calculated M (g mol^{-1})
Fcε3–4	14,000 ± 300	52,200 ± 900	52,797
Fcε3–4/sFcεRIα	22,600 ± 700	82,100 ± 2,100	86,579

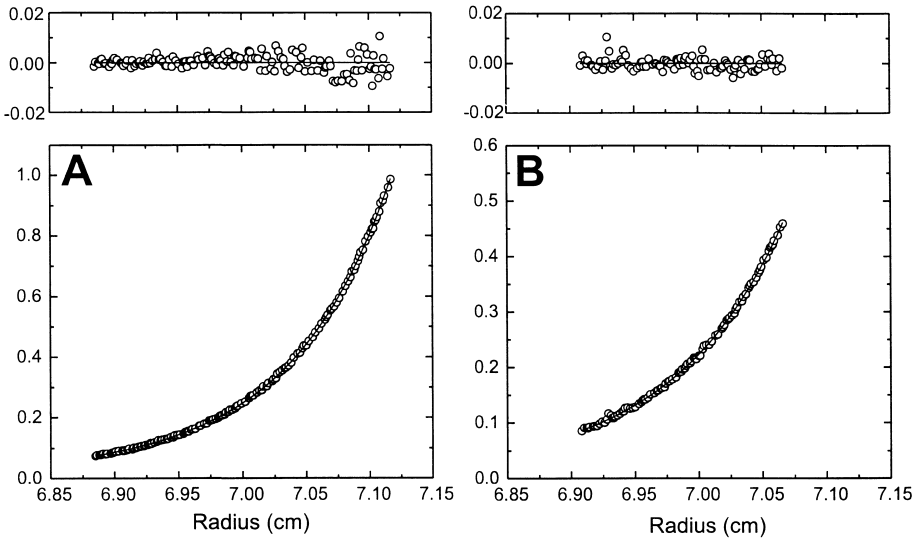

Fig. 4 Sedimentation equilibria for (**A**) Fcε3–4 and (**B**) the Fcε3–4/sFcεRIα complex. Centrifugation was performed at (**A**) 16,000 and (**B**) 12,000 rpm under the conditions described in Materials and methods. Data were analysed for the best single component $M(1-\bar{\nu}\rho)$ fit, shown as a line through the experimental points. Corresponding distributions of the residuals are shown above each plot

Discussion

IgE contains two ε-heavy chains, each possessing one variable and four constant (Cε1–Cε4) domains. In the Fc region (Cε2–Cε4), the ε-chains are thought to be aligned in a parallel fashion and associated through two disulphide bonds (C241–C241 and C328–C328) between the two Cε2 domains and by non-covalent interactions between the two Cε4 domains. The two Cε3 domains on opposite sides of the longitudinal axis are separated from each other (Padlan and Davies 1986; Helm et al. 1991). Our Fcε3–4 sequence contains the two C-terminal residues of Cε2 (C328 and A329) followed by the Cε3 and Cε4 domains. C328 was retained in order to allow the formation of the interchain disulphide bond near the N-terminal end of Cε3, thus constraining the disposition of Cε3 domains in the native structure of IgE.

We have prepared domain deletion mutants of IgE (Fig. 1) to study the kinetics of the interaction and composition of the complexes with FcεRI. Intact IgE (Robertson 1993) and IgE-Fc (Keown et al. 1995) have been studied previously and Fcε3–4 is examined in the present work. The isolated Cε3 domain could not be studied by the same methods because of its much weaker interaction with FcεRI (Henry et al., manuscript in preparation).

In the present work we have found sFcεRIα binds to Fcε3–4 even more strongly than to IgE or IgE-Fc (Table 1). We can account for this in terms of the diffusion constants, accessibility and symmetry of these ligands. The larger value of k_{+1} for Fcε3–4 compared wtih IgE and IgE-Fc (Table 1) must contain a contribution from the larger diffusion coefficient of the smaller fragment and the increased surface area available for productive collisions. If the removal of Cε2 results in the expression of twofold symmetry, but only one site can be occupied, the rate would increase by a further factor of two. Relative diffusion rates were calculated from the values of the molecular masses and sedimentation coefficients, as described in Materials and methods. The calculated diffusion coefficient ratio for Fcε3–4 and IgE is 1.85. Similarly a comparison of IgE-Fc and Fcε3–4 leads to a ratio of 1.15. The corresponding ratios of k_{+1} depend on the sum of the diffusion coefficients of the reactant species; the diffusion coefficient of the cell associated receptor is zero. Multiplying the ratios of the diffusion coefficients by a factor of two to include the symmetrical binding site leads to the calculation of maximum values for the k_{+1} ratios of 3.9 (compared with IgE) and 2.3 (compared with IgE-Fc). The observed ratios are much larger, 13 for FcεRI:IgE and 4 for FcεRI:IgE-Fc. This is most probably a consequence of steric hindrance by the Fab region and Cε2 domains of IgE and the Cε2 domains of IgE-Fc.

The dissociation rates differ by factors of only four (Fcε3–4 vs IgE) and two (Fcε3–4 vs IgE–Fc). This must also reflect the diffusion coefficients and putative doubling of the number of FcεRI binding sites on Fcε3–4 compared with IgE and IgE-Fc. In addition, the Fab region of IgE and Cε2 domain of IgE-Fc could facilitate re-capture of receptor molecules after dissociation.

The present study was undertaken to examine whether the bent structure of IgE and IgE-Fc could account for the paradoxical 1:1 stoichiometry of the IgE-Fc/sFcεRIα complex (Keown et al. 1995). In the preparation of Fcε3–4 we have generated a fragment analogous to IgG_1-Fc, which has an oblate ellipsoidal shape in the crystal structure. Despite this, we observe a 1:1 stoichiometry for the Fcε3–4/sFcεRIα complex. Considering that this IgE fragment binds two molecules of both the low-affinity receptor FcεRII/CD23 (Shi et al., 1997) and anti-IgE antibodies against epitopes in Cε3 (Keown, 1997), we conclude that at least parts of both Cε3 domains of Fcε3–4 are available for binding. Furthermore, the observation that Fcε3–4 binds to FcεRI with affinities similar to those of IgE and IgE-Fc confirms that this fragment retains the full activity with respect to FcεRI binding.

Our results therefore indicate that other factors besides the bent structure prevent the binding of two FcεRI molecules to IgE. We propose two alternative explanations: (1) The location of the binding site may be such that binding of the first receptor occludes the second site. (2) Binding of the first receptor molecule may induce a conformational change in IgE that dramatically weakens the second binding site. Further studies are in progress to distinguish between these mechanisms. Since we have also found that sFcγRIII binds to IgG_1-Fc in a 1:1 complex (Ghirlando et al. 1995), we suggest that this contributes to the general mechanism that prevents the crosslinking of the Fc receptors by antibodies in the absence of antigens.

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