Disulphide linkage controls the affinity and stoichiometry of IgE Fcε3-4 binding to FcεRI

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IgE antibodies cause long-term sensitisation of tissue mast cells and blood basophils towards allergen-induced cross-linking and triggering of allergic inflammation. This persistence of IgE binding is due to its uniquely high affinity for the receptor FcεRI, and in particular its slow rate of dissociation once bound. The binding interface consists of two sub-sites, one contributed by each Cε3 domain of IgE Fc in a 1:1 complex. We have investigated the contributions of Cε3 disulphide linkage and glycosylation to the kinetics and affinity of binding of an Fc sub-fragment (Fcε3-4) to a soluble receptor fragment (sFcεRIα). In contrast to IgG Fc, where deglycosylation abrogates receptor-binding activity, removal of the N-linked carbohydrate at Asn 394 in Fcε3-4 only reduces binding affinity by a factor of 4, due principally to a faster off-rate. Removal of the inter-heavy chain disulphide bond unexpectedly resulted in a fragment with a much faster off-rate and the potential to form a complex with a 2:1 stoichiometry (sFcεRIα:Fcε3-4). This permitted determination of the stoichiometry of a single, natively folded, Cε3 domain for the first time. The low affinity, \( K_a \approx 10^2-10^6 \) M\(^{-1}\), similar to that determined previously for an isolated Cε3 domain, expressed in E. coli and lacking carbohydrate. Recent structural data indicate that conformational change in IgE is required to allow both Cε3 domains to bind, and thus an allosteric inhibitor that prevents access to the second Cε3 has potential to reduce the ability of IgE to sensitise allergic effector cells.

Immunoglobulin E (IgE) is the antibody class that plays a central role in the allergic response [1]. Mast cells and basophils express a high-affinity receptor for IgE, FcεRI, and it is the cross-linking of receptor-bound IgE on these cells by multivalent allergens that triggers the immediate release of pre-formed inflammatory mediators. The affinity of IgE for FcεRI is uniquely high among Ig-receptor complexes, two to five orders of magnitude higher than that of IgG for its receptors FcγRI-III [2]. This is due principally to a very slow off-rate; the half-life of IgE bound to FcεRI is hours [3], compared with only minutes for IgG bound to its receptors [4]. Inhibition of IgE binding to FcεRI, or at least modulation of its binding kinetics, is a potential therapeutic strategy. IgE Fc binds to the receptor with a 1:1 stoichiometry [5] using both Fc heavy chains [6]. The crystal structure of the complex between the IgE fragment, Fcε3-4 (a homodimer of epsilon chains consisting of the Cε3 and Cε4 domains), and soluble fragment of the IgE-binding α-chain of the receptor, sFcεRIα [6], revealed the precise details of the involvement of the two Cε3 domains in the complex. The extensive binding interface consists of two sub-sites, one contributed by each Cε3 domain, and thus maintenance of the dimeric site might be expected to be important for high-affinity binding. The Cε3 domain also contains an attachment site (Asn394) for N-linked carbohydrate that is conserved in other antibody classes (e.g. Asn297 in the Cγ2 domain of IgG Fc). Although the crystal structure did not reveal any contact with carbohydrate [6], an indirect effect of glycosylation upon receptor binding via stabilisation of the polypeptide conformation cannot be ruled out. Indeed, an isolated Cε3 domain, expressed in E. coli and lacking
carbohydrate, was found to be only partially folded [7], and NMR analysis of the same fragment indicates that it may have a molten-globule-like structure [8].

The aim of this study was to determine the contributions of these two factors, dimerisation and glycosylation of the Cε3 domains, to the kinetics and affinity of FceRI binding. We have analysed these effects using four variants of the Fce3-4 fragment: i) disulphide-linked and glycosylated (Fce3-4), ii) lacking only the disulphide link (redFce3-4), iii) lacking only glycosylation (deglyFce3-4), iv) lacking both structural features (Fce3-4ΔC). Fce3-4 lacks the Cε2 domains of the complete IgE Fc, but we have shown previously that not only does this fragment display a 1:1 stoichiometry of binding [9], but it also retains full binding affinity [10], although the kinetics of binding are affected [3]. It has been reported previously that deglycosylation of IgE has little effect upon receptor binding [11], and that E. coli expressed fragments retain high affinity receptor binding activity [12]. This is in contrast to the behaviour of IgG, where loss of carbohydrate from the Fc destroys receptor binding altogether [13]. Here we report the first full kinetic characterisation of a fully folded, carbohydrate-free IgE Fc sub-fragment. We show that glycosylation makes a minor contribution to its affinity for FceRI. Furthermore, irrespective of glycosylation, we show loss of the inter-heavy-chain disulphide bond has a more profound effect, generating, remarkably, a fragment that can bind two molecules of receptor. This unexpected stoichiometry of complex formation enabled us to determine, for the first time, the affinity of a single folded Cε3 domain for receptor. The result, an affinity 10^3 to 10^4 times lower than native and comparable to that of the isolated and partially unfolded Cε3 domain [7,8], has implications for the development of inhibitors of the IgE:FceRI interaction.

**Experimental Procedures**

(a) Protein expression and purification. The production and purification of recombinant human Fce3-4 (residues 328-547), sFceRIα (residues 1-176) and IgG4-Fc(sFceRIα)_2 fusion protein from NS-0 cells has been described previously [14,15].

A further recombinant Fce3-4 fragment with the Cys328Ser mutation was made in E. coli. This construct, Fce3-4ΔC, was produced by PCR using an IgE Fc cDNA clone isolated from U266 cells [16] and was presented in pSC213 [17]. The PCR fragment was sub-cloned into the Ndel and BamHI sites of the pET5a (EMD Biosciences Inc., Madison, WI) E. coli expression vector. The integrity of the clone was verified by DNA sequencing. Recombinant Fce3-4ΔC was expressed in the E. coli host strain BL21 (DE3). Inclusion body protein was extracted from cell pellets using a procedure adapted from Bohmann and Tjian [18] and refolded according to the method of Taylor et al [19]. We have previously described in detail the production and purification of an IgE Fc domain using these methods [7].

(b) Deglycosylation of Fce3-4. Native deglycosylation of mammalian Fce3-4 was carried out using PNGaseF according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). Material was then repurified by size exclusion chromatography on a Superdex S75 gel filtration column (Amersham Biosciences, Piscataway, NJ). Deglycosylation was assessed by SDS-PAGE and a combination of Coomassie and Schiff’s staining. Complete deglycosylation of Fce3-4 was also confirmed by MS.

(c) CD Spectroscopy. The folded status of each protein was examined by CD, as described previously [14]. Briefly, CD measurements were performed on a Jobin-Yvon CD6 spectrophotometer (Longjumeau, France) calibrated for wavelength and ellipticity using d-10-camphorsulfonic acid. Samples were analysed in cylindrical quartz cells of 0.05cm path length in the concentration range 100-500µg/mL in 20mM sodium phosphate, 50mM sodium fluoride, pH 7.4, at 20°C in a thermostated cell holder. Spectra were recorded in 0.2nm steps with an integration time of 4s and corrected by subtraction of the solvent spectrum obtained under identical conditions. The units of ∆ε are M⁻¹ cm⁻¹ per backbone amide unit.

(d) Kinetics of Fce3-4 fragments binding to FceRI. The binding of all fragments to IgG4-Fc(sFceRIα)_2 fusion protein was measured by SPR. All experiments were performed at 24°C on
either a Biacore 1000, 2000 or 3000 instrument (Biacore Int. SA, Switzerland). Methods and kinetic analysis have been described previously [3,15,20]. In these experiments, coupling density was typically restricted to 500RU, flow rate 20μL/min, and exposure time to analyte 360s.

(e) Analytical ultracentrifugation. The interaction between sFcεRIα and the various Fcε3-4 fragments was studied by sedimentation equilibrium in an Optima XL-A analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) as described previously [14]. Samples were dialysed into PBS with 0.05% azide and mixed in molar ratios of 1:1, 2:1 and 1:2 sFcεRIα:Fcε3-4, with overall loading corresponding to an absorbance A280 ~0.6. The mixtures were spun at 4°C at rotor speeds of 8000, 11000 and 14000 r.p.m. until equilibrium was reached. Data were collected as an average of 25 A280 measurements at a radial spacing of 0.001cm. The equilibrium data for the mixtures were then simultaneously fitted to a range of models using the experimentally determined buoyant molecular masses of Fcε3-4 (14000 ± 300), Fcε3-4αC (14115 ± 480) and sFcεRIα (10200 ± 400), from which the association constants were derived. To study the reduced Fcε3-4 species, the minimum level of DTT required to reduce the inter-chain disulphide bond was determined by observing the change in apparent molecular weight by SDS-PAGE for a range of DTT concentrations. The concentration of DTT used (0.4 mM) to break the inter-heavy chain bridge was far lower than that required to break intra-domain bridges (between 2-10 mM) [21]. Nitrogen-purged ultracentrifugation cells containing mixes of Fcε3-4 and sFcεRIα were then prepared as described, with the addition of DTT to the PBS-azide immediately prior to loading. Maintenance of the reduced form of Fcε3-4 was confirmed by non-reducing SDS-PAGE following the centrifuge run.

Results

Expression and purification of Fcε3-4 & Fcε3-4αC.
Fcε3-4, with N-terminal residue Cys328, was expressed in mammalian NS-0 cells [14], and Fcε3-4αC, containing the Cys328Ser mutation, was expressed in E. coli. The former is therefore glycosylated and a covalent dimer (Fig. 1, lanes A & D), while the latter is unglycosylated and unable to form the inter-heavy chain disulphide bridge. The refolded Fcε3-4αC was purified by size exclusion chromatography, and confirmed as monomeric by SDS-PAGE (Fig. 1, lanes C & F), but it behaved as a monodisperse, non-covalent dimer by analytical ultracentrifugation (data not shown) and analytical gel filtration chromatography (Fig. 2). The integrity of the E. coli product was checked by MS, which yielded a molecular weight of 24,559 Da, within 1 Da of the theoretical value (24,558). The slower migration on SDS-PAGE for Fcε3-4αC non-reduced (Fig. 1, lane C) and fully reduced (lane F) was indicative of the formation of the intra-domain disulphide bonds during the refolding process.

Deglycosylation of Fcε3-4
Deglycosylation of the NS-0 expressed Fcε3-4 fragment (deglyFcε3-4) was assessed by Schiff’s staining and the change in apparent molecular weight on SDS-PAGE (Fig. 1, lanes B and E). Complete deglycosylation was confirmed by further PNGaseF digestion under denaturing conditions and the release of no further sugar residues as detected by MS.

Gel filtration chromatography
The three fragments Fcε3-4, deglyFcε3-4 and Fcε3-4αC were run sequentially on a gel filtration column as shown in Figure 2. The two disulphide-linked dimers behaved similarly, but the retention time of Fcε3-4αC was ~ 0.5 min. longer, indicating that although it too behaved as a (non-covalent) dimer, there was a discernable reduction in its effective volume. This suggests that it forms a somewhat more compact structure. Since deglyFcε3-4 ran identically to Fcε3-4, it appears to be the loss of the disulphide bond rather than the carbohydrate that is the cause of this conformational difference.

CD spectroscopy
The CD spectra of Fcε3-4, deglyFcε3-4 and Fcε3-4αC (Fig. 3) were all typical of a folded protein consisting principally of β-structure, and in agreement with data previously published for Fcε3-4 fragments [10,14,20]. This indicates that neither the refolding of the protein expressed in E. coli, nor deglycosylation of the NS-0 material, affected the folding of the fragment.
**Surface plasmon resonance analysis**

Comparison of representative sensorgrams for the three Fcε3-4 species (Fig. 4) shows that there are major differences between their binding properties for receptor (immobilised on the biosensor surface as a fusion protein IgG4-Fc(sFcεRIα)2), especially in their off-rates. deglyFcε3-4 (Fig. 4B) clearly dissociates faster than Fcε3-4 (Fig. 4A), and for Fcε3-4ΔC the off-rate is faster still (Fig. 4C). As we found in our previous studies of Fcε3-4 fragments [15,20], only a biphasic model could satisfactorily fit the observed binding curves for Fcε3-4 and deglyFcε3-4; representative curve fits and residuals are shown in Figs. 4A and 4B, and the resulting kinetic parameters are summarised in Table 1. While the on-rates (k_{a1} and k_{a2}) for Fcε3-4 and deglyFcε3-4 are very similar, the chief component of the off-rate, k_{d2}, is faster for the latter protein, leading to a four-fold lower affinity constant (K_{d2} = 4.92 \times 10^8 M^{-1} for Fcε3-4 c.f. K_{d2} = 1.14 \times 10^8 M^{-1} for deglyFcε3-4) for the principal binding component. This difference is accentuated by the fact that the contribution of the first (lower affinity K_{a1}) component of the fit (given by the R_I/R_0 ratio, Table 1) is higher for deglyFcε3-4 (38%) than for Fcε3-4 (15%).

Kinetic analysis of the Fcε3-4ΔC fragment was possible with a 1:1 model of association. Representative curve fits and residuals are shown in Fig. 4C, and the resulting kinetic parameters are summarised in Table 1. The rate of dissociation of this fragment is over two orders of magnitude faster than that of the major component of the Fcε3-4 biphasic analysis (k_{d2} = 6.26 \times 10^{-4} s^{-1} for Fcε3-4 c.f. k_d = 6.63 \times 10^{-2} s^{-1} for Fcε3-4ΔC). The rate of association is also faster however, leading to only an order of magnitude difference in overall affinity (K_a = 4.92 \times 10^8 M^{-1} for Fcε3-4 c.f. K_a = 4.17 \times 10^7 M^{-1} for Fcε3-4ΔC).

**Sedimentation equilibrium analysis**

We previously studied the binding of sFcεRIα to NS-0 derived Fcε3-4 in the analytical ultracentrifuge, and found that it formed a single species with 1:1 stoichiometry and an affinity too high to measure by this technique [9]. As observed in SPR experiments, Fcε3-4ΔC, lacking both carbohydrate and the inter-chain disulphide bond, behaved very differently under the same conditions (Fig. 5A & B). The data were collected at three different molar ratios (1:1, 2:1 and 1:2 sFcεRIα:Fce3-4ΔC) and each curve was fitted to three different binding models, a simple 1:1 interaction, a mixture of 1:1 and 2:1 (sFcεRIα:Fce3-4ΔC), and a solely 2:1 interaction. It is clear from the curve fits and residual plots (Fig. 5A) that a 1:1 sFcεRIα:Fce3-4ΔC model of association does not fit the data. However, the curves did fit a 1:1 + 2:1 model, residuals for which are shown in Figure 5B. The equilibrium constants for the formation of the 1:1 and then the 2:1 complex, A + B ⇌ AB + B ⇌ AB2 (where A is Fcε3-4ΔC and B is sFcεRIα) are K_{AB} = 1.8 (± 0.4) \times 10^5 M^{-1} and K_{AB2} = 7.2 (± 2.2) \times 10^4 M^{-1}.

In order to determine whether this difference in behaviour was a result of the absence of the carbohydrate or the inter-chain disulphide bond at Cys328, the deglyFcε3-4 and a partially reduced Fcε3-4 preparation (redFcε3-4) were examined in the analytical ultracentrifuge in the same way. The residuals for these two species are shown in Figures 5C and 5D. deglyFcε3-4 simply formed a high-affinity 1:1 complex and behaved in the same way as Fcε3-4 (Fig. 5C); the data for redFcε3-4, however, could not be fitted by a 1:1 model (data not shown) but was instead fitted to a 1:1 + 2:1 model, as for Fcε3-4ΔC (Fig. 5D). Clearly it is the loss of the disulphide bond that leads to the formation of a 2:1 complex with the soluble receptor. The corresponding equilibrium constants for the association of sFcεRIα with redFcε3-4 are K_{AB} = 1.3 (± 0.05) \times 10^6 M^{-1} and K_{AB2} = 1.0 (± 0.16) \times 10^4 M^{-1}. After centrifugation, the partial reduction process was judged from SDS-PAGE analysis (data not shown) to leave a residual amount (less than 5%) of the material in the unreduced state. The presence of this species, which would bind with higher affinity and 1:1 stoichiometry, may explain the higher value for the K_{AB} component. The affinity may have been enhanced further by the presence of glycosylation in redFcε3-4, consistent with the Biacore data presented in the previous section.

**Discussion**

The recent crystal structures of IgE Fc [22], the Fcε3-4 fragment [23] and the latter complexed with sFcεRIα [6], have not only provided a description of the binding interface, but also suggested that engagement of the receptor by IgE involves a substantial conformational change in IgE Fc. The interaction surface is extensive, and
consists of two sub-sites, one on each Ce3 domain. However, in neither the free IgE Fc, nor the Fcε3-4 fragment, are both sub-sites accessible: in IgE Fc the Ce2 domains are acutely and asymmetrically bent back against the Ce3 domains so that one sub-site is accessible while the other is hidden [22]; in Fcε3-4, neither site is accessible and the Ce3 domains adopt a “closed” quaternary conformation [23]. Thus a conformational change involving the Ce3 domains (and probably also Ce2 [3]) must occur upon receptor binding to allow contact at both sites and full affinity to be achieved. We and others have suggested that inhibition of the conformational change, and thus engagement of both sites, may offer an alternative strategy to blocking the binding interaction directly [22]; it was not known however what the individual contributions of the two sub-sites were to the overall affinity, and thus how effective such a strategy of restricting the interaction to only one sub-site (such as the only accessible sub-site in IgE Fc) might be. The present study answers this question.

A further question concerns the importance of glycosylation for IgE binding to FcεRI. The crystal structure of the complex between IgG Fc and FcεRIII [24,25] shows a structurally homologous interaction, yet it is known that glycosylation of the IgG Fc is essential for receptor-binding activity [13], whereas for IgE it is not [12]. We therefore set out to explore in more detail the contribution of glycosylation to the binding kinetics and uniquely high affinity of IgE for its receptor using Fcε3-4, the fragment structurally homologous to IgG Fc.

In order to identify the contributions to folding and receptor binding of Ce3 glycosylation and covalent dimerisation by the inter-chain disulphide, we generated and tested variants of the Fcε3-4 fragment of IgE lacking one or both of these structural features. The native-like protein, Fcε3-4, expressed in mammalian NS-0 cells, contained both features; deglyFcε3-4, prepared from Fcε3-4, lacked glycosylation but retained the disulphide bridge; Fcε3-4ΔC, produced in E.coli, lacked both glycosylation and the disulphide bridge. A fourth preparation, derived from Fcε3-4 by partial reduction and termed redFcε3-4 (glycosylated and with at least 95% of molecules lacking the disulphide bridge) was used only to confirm the conclusions derived from the other three fully isolated and characterised molecules.

The role of glycosylation

The effect of removing the carbohydrate at Asn394 in Fcε3-4 on the kinetics of receptor binding can be seen in the results of the SPR analysis (Fig. 4 and Table 1) with deglyFcε3-4 clearly displaying a faster dissociation rate than Fcε3-4. Although this is a striking result, leading to an overall 4-fold reduction in affinity of the principal binding component, it is very different to the behaviour of IgG Fc, for which removal of the structurally homologous carbohydrate at Asn297 leads to total loss of receptor binding [13,26].

The crystal structures of both the IgG Fc/sFcεRIII [24,25] and the Fcε3-4/sFceRIα complexes [6] reveal that there are only tenuous, if any, direct interactions between Fc carbohydrate and receptor, but clearly indirect effects are possible. Indeed, the effect upon the IgG Fc structure of progressive removal of the carbohydrate has been investigated crystallographically [27], and a gradual “closing” of the Cγ2 domains is found as the carbohydrate chains are shortened. Since the Cγ2 domains must adopt a more “open” conformation to form the complex, and changes in the mobility of loop regions that make contact with receptor can also be detected as carbohydrate is removed [27], a (indirect) structural link between glycosylation and receptor binding in IgG Fc has been established.

In IgE Fc the role of carbohydrate is different. Although the regions of contact with their respective Ce3 and Cγ2 domains are similar, the fact that deglycosylation of Fcε3-4 has a much less profound effect upon receptor binding than in IgG Fc may depend upon other structural differences, such as the more extensive interface between Ce3 and Ce4 compared to Cγ2 and Cγ3, or the different degree of conformational change required for receptor binding [reviewed 28]. Our observation that in analytical gel filtration experiments the Fcε3-4 and deglyFcε3-4 behaved identically also contrasts with published data for native and deglycosylated IgG Fc, which show a difference in retention time that is interpreted as a more compact conformation for the deglycosylated IgG Fc [26]. Thus while the Cγ2 domains of IgG may move closer together upon removal of carbohydrate (consistent with the crystallographic
observations [27]), this appears not to occur for the Ce3 domains of IgE. This observation is supported by examination of the Fce3-4 crystal structure [23], in which it can be seen that the glycosylated Ce3 domains are already in a “closed” conformation. In contrast, we observed that the removal of the inter-chain disulphide bridge led to a marked change in mobility in gel filtration experiments (Fig. 2), indicating that disulphide linkage of the Ce3 domains, rather than glycosylation, is likely to be the major contributor to maintenance of the correct conformation for receptor engagement.

The role of disulphide linkage between the two Ce3 domains

The comparative SPR binding studies showed that Fce3-4AC, lacking both the inter-heavy chain bridge at Cys328 and Ce3 glycosylation at Asn394, displayed an even faster dissociation rate than either Fce3-4 or deglyFce3-4 (Fig. 4). More surprisingly, it fitted a monophasic model. The fitting of this model returned a $K_a$ value of $4.17 \times 10^7$ M$^{-1}$ (Table 1). When studied in the analytical ultracentrifuge, the reason for the change in mode of interaction became apparent, as Fce3-4AC behaved very differently from Fce3-4 and deglyFce3-4. We had earlier reported that Fce3-4 formed a high affinity 1:1 complex with sFceRI$\alpha$ [9], but the Fce3-4AC fragment can form a 2:1 complex, i.e. two molecules of sFceRI$\alpha$ binding to a single Fce3-4AC molecule (Fig. 5A & B). The data were fitted to an equilibrium between the unbound components, a 1:1 and a 2:1 stoichiometric complex, with two approximately equal binding constants for the addition of the first ($K_{AB} = 1.8 \pm 0.4 \times 10^5$ M$^{-1}$) and the second ($K_{AB2} = 7.2 \pm 2.2 \times 10^5$ M$^{-1}$) sFceRI$\alpha$ molecule. These values represent the affinity of a single, folded Ce3 domain for the receptor, i.e. the affinity contributed by each of the two sub-sites.

Confirmation that this stoichiometry and dramatically reduced affinity are the result of removing the inter-heavy chain disulphide bond came from the parallel analysis, under identical conditions, of the mildly reduced preparation of glycosylated Fce3-4, redFce3-4 (Fig. 5D). This species behaved in the same way as Fce3-4AC, fitting only to a 1:1 + 2:1 stoichiometry, yielding values of $K_{AB} = 1.3 \pm 0.05 \times 10^6$ M$^{-1}$ and $K_{AB2} = 1.0 \pm 0.16 \times 10^4$ M$^{-1}$. The analytical ultracentrifugation analysis of the fragment that maintained the disulphide bond at position 328 but lacked carbohydrate (deglyFce3-4), demonstrated a high affinity complex that behaved as a single species (Fig. 5C). This was consistent with the drop in affinity of only a factor of four determined by Biacore analysis, and further confirmed that the disulphide bridge is the key determinant of stoichiometry.

We envisage that this change in stoichiometry upon removal of the disulphide bond occurs because the two sub-sites, one in each Ce3 domain, that together constitute the high-affinity binding site, are now presented in such a way that each can bind independently to an FceRI$\alpha$ molecule. This implies that the Ce3 domains must move sufficiently far relative to each other that there is no steric hindrance between the two FceRI$\alpha$ molecules; the crystal structures of free [23] and complexed Fce3-4 [6], and Fce2-4 [22], demonstrate that there is substantial flexibility in the disposition of the Ce3 domains. The gel filtration profiles (Fig. 2) indicate that this relative movement of the Ce3 domains in Fce3-4AC results in a structure that is considerably more compact even than that seen in the Fce3-4 crystal structure [23], yet one in which the sub-sites of both Ce3 domains are exposed.

The affinities measured for Fce3-4AC by analytical ultracentrifugation ($K_a \approx 10^5 - 10^6$ M$^{-1}$) and the value obtained by Biacore ($K_a = 4.17 \times 10^7$ M$^{-1}$) differ, presumably due to differences between the assay formats. In the latter the receptor is immobilised on the biosensor surface, whereas in the former both species are free to associate in solution. An earlier study in which a cell-binding assay was used to measure the ability of an Fce3-4 fragment lacking Cys328 altogether (residues 329-547) to compete with the binding of IgE to native surface FceRI ($\alpha\beta$), gave an $IC_{50}$ value of $32.9 \pm 25.8$ nM [11]. When Fce3-4AC was tested in a competition ELISA with immobilised receptor we recorded an $IC_{50}$ value of $89.6 \pm 1.5$ nM [29]. The assay format clearly affects the affinity values that are returned, but the Biacore and solution phase analytical ultracentrifugation measurements enabled us to detect differences in stoichiometry and affinity due to glycosylation and disulphide linkage in the Fce3-4 fragments.
**Implications for inhibitor design**

These affinities recorded for a single folded Ce3 domain in Fce3-4ΔC (1.8 x 10^5 M^-1 and 7.2 x 10^5 M^-1) are similar to that measured for an isolated recombinant Ce3 domain (~ 5 x 10^6 M^-1 in a Biacore assay), which we reported in an earlier study [7]. This isolated Ce3 domain is not fully folded, as judged by CD spectroscopy [7] and NMR [8], and it may therefore be inferred that the binding event involves substantial interaction with linear or unstructured regions of the Ce3 domain. The crystal structure of the Fce3-4/sFceRIα complex [6] shows that many of the contacts are indeed made by residues in the extended N-terminal region and loops of Ce3, and this may also explain why short peptides have been reported to have inhibitory activity [12,30]. However, the present study demonstrates that even though complete folding may not be necessary, optimised dimerisation of the Ce3 is essential for full binding affinity, and also that the contribution of glycosylation is not critical for the formation of a high affinity complex. Thus peptides, or non-peptide analogues, if presented as a dimer, may attain sufficiently high inhibitory activity.

Furthermore, these affinity values of 10^5 – 10^6 M^-1 represent the contribution of just one of the two sub-sites that constitute the total binding interface seen in the crystal structure of the Fce3-4/sFceRIα complex [6]. The recently determined crystal structure of the complete IgE Fc including the Ce2 domains [22] revealed that only one of the two sub-sites was accessible in the free IgE Fc, and that a conformational change is required for access to the second site. If therefore an inhibitor can be found that restricts the conformational change, its effect would be to reduce the affinity of the interaction by three to four orders of magnitude with a correspondingly faster dissociation rate. This is very likely to be sufficient to result in a therapeutically beneficial reduction in mast cell sensitisation.

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**Footnotes**

The authors wish to thank Asthma UK, BBSRC (UK), MRC (UK) and The Wellcome Trust for supporting this work.

Abbreviations: DTT – dithiothreitol; SPR - Surface plasmon resonance; CD – circular dichroism; ELISA – enzyme-linked immunosorbent assay; MS – mass spectrometry; Cε2 Cε3 and Cε4 – second, third and fourth constant region domains of the IgE ε heavy chain; Fε3-4 – mammalian expressed homodimer of the third and fourth constant region domains of the IgE ε heavy chain disulphide linked at the N-terminus by Cys328 and glycosylated at Asn 394; deglyFε3-4 – as Fε3-4 but enzymatically deglycosylated; redFε3-4 – as Fε3-4 but with the disulphide linkage at the N-terminus removed by partial reduction with DTT; Fε3-4ΔC - *E. coli* expressed homodimer of the third and fourth constant region domains of the IgE ε heavy chain with a Cys-Ser mutation at position 328; IgE Fc – dimer of the second, third and fourth constant region domains of the IgE ε heavy chain; Cγ2 and Cγ3 - second and third constant region domains of the IgG γ heavy chain; sFεRIα – the soluble fragment of the high affinity IgE receptor FεRI α-chain.

**Figure legends**

**Fig. 1.** SDS-PAGE analysis of Fε3-4 fragments. Non-reducing (left panel) and reducing (right panel) 15% polyacrylamide gels, A & D: Fε3-4 (NS-0); B & E: deglyFε3-4 (NS-0); C & F: Fε3-4ΔC (*E. coli*).
**Fig. 2.** Gel filtration elution profiles of the Fε3-4 fragments. Superdex S200 HR column, run at 0.75 ml/min in Tris pH 7.2 plus 250mM NaCl.

**Fig. 3.** Circular dichroism spectra of the Fε3-4 fragments. Data were acquired at 20°C in a 0.5mm cell at a protein concentration of 0.5 mg/ml.

**Fig. 4.** SPR analysis of Fε3-4 fragments. A) Fε3-4 100nM - 6.25nM, B) deglyFε3-4 100nM - 6.25nM, C) Fε3-4ΔC, 200nM - 12.5nM, all binding to immobilised IgG4-Fc(sFεRIα)_2. Data were recorded using either a Biacore 3000, 2000 or 1000 respectively (flow rate 20µL min⁻¹ in HBS). All traces are baseline corrected for non-specific binding by the subtraction of data obtained from an IgG4 control surface treated in an identical manner. Curves corresponding to the fitted interaction model (Fε3-4 and deglyFε3-4-biphasic; Fε3-4ΔC – monophasic) are plotted as white dots on the same axes as the experimental data, with the residuals shown beneath. All values derived from the fitting procedures are given in Table 1.

**Fig. 5.** Analytical ultracentrifugation of Fε3-4 fragments in complex with sFεRIα. All data shown were collected at 11,000 RPM. A) The three curves and corresponding residuals for fitting to a 1:1 interaction model are for different loading ratios of Fε3-4ΔC and sFεRIα in the mixtures (1:1, 2:1 and 1:2). The residuals are non-randomly distributed, indicating a poor fit. B) Residuals only for a 1:1 + 2:1 model fitted to the same data as A, with random residuals indicating a good fit. C) Residuals only for 3 mixtures of deglyFε3-4ΔC and sFεRIα fitted to a model in which deglyFε3-4ΔC and sFεRIα form a high affinity 1:1 complex which does not then interact with an excess of sFεRIα. D) Residuals only for a 1:1 + 2:1 model fitted to 3 mixtures of redFε3-4 and sFεRIα indicating a good fit.
|                | Fce3-4          | deglyFce3-4     | Fce3-4ΔC       |
|----------------|-----------------|-----------------|----------------|
| $k_{a1}$ (M$^{-1}$s$^{-1}$) | (4.77 ± 1.88) x 10$^5$ | (4.88 ± 1.97) x 10$^5$ | $k_a$ (M$^{-1}$s$^{-1}$) | (2.76 ± 0.04) x 10$^6$ |
| $k_{d1}$ (s$^{-1}$)    | (1.21 ± 0.19) x 10$^2$ | (1.50 ± 0.29) x 10$^2$ | $k_d$ (s$^{-1}$)    | (6.63 ± 0.05) x 10$^2$ |
| $k_{a2}$ (M$^{-1}$s$^{-1}$) | (3.08 ± 0.65) x 10$^5$ | (1.97 ± 0.15) x 10$^5$ | $K_a$ (M$^{-1}$)    | 4.17 x 10$^7$ |
| $k_{d2}$ (s$^{-1}$)    | (6.26 ± 1.29) x 10$^4$ | (1.73 ± 0.23) x 10$^4$ |
| $K_{d1}$ (M$^{-1}$)    | 3.94 x 10$^7$     | 3.25 x 10$^7$     |
| $K_{d2}$ (M$^{-1}$)    | 4.92 x 10$^8$     | 1.14 x 10$^8$     |
| $R_1/R_0$               | 0.15 ± 0.03       | 0.38 ± 0.03       |

Table 1. Kinetic parameters and affinity constants derived from the SPR analyses of different Fce3-4 fragments binding to immobilised IgG4-(sFceRIα)$_2$. Fce3-4 and deglyFce3-4 were analysed using a biphasic interaction model from which association and dissociation constants were derived for each component (shown ± standard deviation for at least ten determinations in the concentration range 6.25–100nM). $R_1/R_0$ describes the fractional contribution of the first component to the overall fit. The Fce3-4ΔC parameters (shown ± standard error) were derived from a global analysis using a 1:1 model of association simultaneously fitted to five different analyte concentrations (12.5nM-200nM).
Figure 1
Figure 2
Figure 4
Figure 5

A

$F_{\alpha3-4\Delta C:sF_{\alpha3}R\alpha}$

1:1 Interaction

Absorbance

Radius

Residuals

Radius

B

$F_{\alpha3-4\Delta C:sF_{\alpha3}R\alpha}$

1:1 + 2:1 Interaction

Residuals

Radius

C

$deglyF_{\alpha3-4\Delta C:sF_{\alpha3}R\alpha}$

Non-interacting model of 1:1 complex + excess $sF_{\alpha3}R\alpha$

Residuals

Radius

D

$redF_{\alpha3-4\Delta C:sF_{\alpha3}R\alpha}$

1:1 + 2:1 interaction

Residuals

Radius
Disulphide linkage controls the affinity and stoichiometry of IgE Fc ε 3-4 binding to Fc ε 3-4 binding to
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J. Biol. Chem. published online March 2, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500965200

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