Conformation of the Isolated Cε3 Domain of IgE and Its Complex with the High-Affinity Receptor, FcεRI†

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ABSTRACT: Immunoglobulin E (IgE) exhibits a uniquely high affinity for its receptor, FcεRI, on the surface of mast cells and basophils. Previous work has implicated the third domain of the constant region of the ε-heavy chain (Cε3) in binding to FcεRI, but the smallest fragment of IgE that is known to bind with full affinity is a covalent dimer of the Cε3 and Cε4 domains. We have expressed the isolated Cε3 in *Escherichia coli*, measured its affinity for FcεRI, and examined its conformation alone and in the complex with FcεRI. Sedimentation equilibrium in the analytical centrifuge reveals that this product is a monomer. The kinetics of binding to an immobilized fragment of the FcεRI α-chain, measured by surface plasmon resonance, yields an affinity constant $K_a = 5 \times 10^6$ M^{-1}, as compared with 4×10^9 M^{-1} for IgE. The circular dichroism spectrum and measurements of fluorescence as a function of the concentration of a denaturant do not reveal any recognizable secondary structure or hydrophobic core. On binding to the FcεRI α-chain fragment, there is no change in the circular dichroism spectrum, indicating that the conformation of Cε3 is unchanged in the complex. Thus the isolated Cε3 domain is sufficient for binding to FcεRI, but with lower affinity than IgE. This may be due to the loss of its native immunoglobulin domain structure or to the requirement for two Cε3 domains to constitute the complete binding site for FcεRI or to a combination of these factors.

Allergic reactions are triggered by multivalent antigen (allergen) binding to IgE[1] attached to the high-affinity receptor FcεRI on mast cells and basophils (*1*). The nature of this complex is of fundamental interest. We and others have attempted to map the binding site for FcεRI in the sequence of IgE. The existing evidence suggests that this site lies exclusively within the third constant domain of the ε heavy chain (Cε3). If this is so, the isolated domain should exhibit full activity, but this has never been demonstrated.

Most previous studies have employed either fragments of IgE containing all or parts of other domains (*2*−*6*), or chimeric immunoglobulins (*7*−*12*). More recently, single amino acid substitutions in IgE (*13*−*15*) have been used to locate regions of contact between FcεRI and IgE. All of the amino acids that have been implicated in the binding to FcεRI lie in the Cε3 domain of IgE. While Vangelista et al. (*16*) have demonstrated that the isolated Cε3 domain bound to the α-chain of FcεRI in a nitrocellulose blot and that it inhibited binding of IgE in this assay, their results provided no information about the relative affinities of Cε3 and IgE. To date, the smallest fragment of IgE that is known to retain full affinity for FcεRI is a dimer of the Cε3 and Cε4 domains (*5*, *17*), confirming that Cε2 is not involved.

Work with chimeric immunoglobulins showed that Cε4 is unnecessary for recognition by FcεRI, since Cε4 in IgE can be replaced by the homologous Cγ3 domain of IgG without significant loss of affinity for FcεRI (*7*, *8*). Furthermore, substitution of human Cε3 for Cγ2 in IgG converts IgG into IgE in respect of its recognition by the class-specific receptors (*12*). Human IgE does not bind to the rodent IgE receptor, and it was therefore possible to locate the domain recognized by rodent and human receptors by replacing individual domains from mouse IgE into human IgE. Mouse Cε3 was both necessary and sufficient to obtain high-affinity binding of the chimeric IgE to rat FcεRI (*9*−*11*). Although these experiments demonstrate that Cε4 is not recognized as a part of the FcεRI-binding site, per se, the evidence falls short of excluding Cε4 from an indirect role in the IgE−receptor interaction. This requires the examination of Cε3 in isolation from Cε4.

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[1] Abbreviations: CD, circular dichroism; cDNA, complementary DNA; Cε2, Cε3, Cε4, the three constant domains of IgE−Fc; ELISA, enzyme-linked immunosorbent assay; Fcε3−4, disulide-linked dimer of Cε3−Cε4; FcεRI, high-affinity receptor for IgE; FcεRII, low-affinity receptor for IgE, CD23; FcγRI, IgG Fc-receptor type I, CD64; GuHCl, guanidine hydrochloride; HBS, Hepes-buffered saline; HPLC, high-pressure liquid chromatography; IgE, immunoglobulin E; IgE−Fc, the Fc portion of IgE, Cε2-Cε4; IgG, immunoglobulin G; IgG_4-Fc-$(sFc\varepsilon RI\alpha)_2$, fusion of IgG_4-Fc with sFcεRIα in place of the two Fab arms; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RU, resonance unit; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; sFcεRIα, the extracellular domains of the α-chain of FcεRI; SPR, surface plasmon resonance.

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We have therefore expressed the Cε3 sequence in *Escherichia coli* and characterized the purified product by sedimentation equilibrium, circular dichroism, and fluorescence spectroscopy. The affinity of Cε3 for FcεRI has been measured by competitive ELISA and from the kinetics of binding to the immobilized receptor by surface plasmon resonance. Further, the conformation of Cε3 in the complex with soluble FcεRI α-chain was examined by circular dichroism.

EXPERIMENTAL PROCEDURES

(a) Preparation of Recombinant Ce3. Using the numbering of Dorrington and Bennich (*18*), the Cε3 domain is defined by intron/exon boundaries and comprises Asp330−Ser437. The Cε3 construct includes Cys328 and Ala329, with a Cys328Ser mutation, was produced by PCR using an IgE−Fc cDNA clone isolated from U266 cells (*19*), and was presented in pSC213 (*20*). The PCR fragment was subcloned into the *Nde*I and *Bam*HI cloning sites in the pET5a (Novagen) *E. coli* expression vector at the *Nde*I and *Bam*HI cloning sites, and the integrity of the clone was confirmed by DNA sequencing (*21*). This cloning strategy adds a methionine to the N-terminus of the expressed protein. Thus, the construct expresses MS328−S437 of the human myeloma ND ε-chain.

Recombinant Cε3 was expressed in the *E. coli* host strain BL21(DE3). Transformants were grown at 37 °C in complete M9 medium (*22*) containing 100 μg/mL ampicillin. Overnight cultures were diluted 40-fold into fresh M9 medium and grown with aeration at 37 °C. The culture was induced at mid-log phase (A_{600} ca. 0.7) for 5 h with isopropyl β-D-thiogalactoside (IPTG) (final concentration of 0.4 mM). Cells were harvested by centrifugation, and the cell pellets stored at −70 °C.

Recombinant Cε3 was extracted from the cell pellets using a procedure adapted from Bohmann and Tjian (*23*). The cell pellet from a 1 L culture was resuspended in 72 mL of solution A [10 mM Tris-HCl, pH 7.9, 25% sucrose, 100 mM KCl, 2 mM DTT, 2 mM phenylmethanesulfonyl fluoride (PMSF)]. A total of 18 mL of solution B (300 mM Tris-HCl, pH 7.9, 100 mM EDTA, 4 mg/mL lysozyme) was added, and the suspension incubated for 10 min on ice. A total of 90 mL of solution C (1 M LiCl, 20 mM EDTA, and 0.5% NP-40) was added and the suspension sonicated on ice (MSE Soniprep) for 5 min to disrupt the cells. Insoluble material was pelleted by centrifugation for 10 min at 16000*g*. The pellet was resuspended in 200 mL of solution D (10 mM Tris-HCl, pH 7.9, 100 μM EDTA, 500 mM LiCl, 0.5% NP-40, 1 mM DTT, and 1 mM PMSF) and sonicated and pelleted as before. Finally, the pellet was resuspended in 200 mL of solution E (10 mM Tris-HCl, pH 7.9, 100 μM EDTA, 0.5% NP-40, 1 mM DTT, and 1 mM PMSF), sonicated, and pelleted as before, and stored at −70 °C.

The extracted pellet was solubilized in 5 mL of 6 M GuHCl, 10 mM DTT, and 20% sucrose and incubated for 1 h at room temperature. The solution was clarified by centrifugation for 20 min at 12000*g*, and purified Cε3 was obtained by size-exclusion chromatography on a Sephacryl S-300 (Pharmacia) 30 × 100 cm gel filtration column, equilibrated with 6 M GuHCl.

Recombinant Cε3 was refolded according to the procedure of Taylor et al. (*24*). Briefly, the extracted Cε3 was solubilizd at 10 mg/mL in 6 M GuHCl, and the intramolecular disulfide bond was formed by diluting the protein to 1 mg/mL in 6 M GuHCl, 100 mM Tris-acetate, pH 8.6, containing 0.1 M oxidized glutathione. The denaturant was removed by further dilution to 10 μg/mL in 100 mM Tris-acetate, 3 mM L-cysteine, pH 8.6. After removal of the denaturant, the protein was concentrated to 5 mg/mL. The covalently monomeric Cε3 was purified by gel filtration in 6 M urea in PBS on a Superdex HR-75 (Pharmacia) 1 × 30 cm gel filtration column. Fractions containing covalently monomeric Cε3 were identified by electrophoresis of the protein in nonreducing SDS−PAGE, pooled, and dialized against PBS. The yield of monomers was 30% of the total protein.

Biologically active recombinant Cε3 was further purified by passage down a FcεRI affinity column. The extracellular portion of the α-chain of FcεRI was presented in the form of a fusion protein with IgG_4-Fc ([gG_4-Fc-(sFcεRIα)$_2$ (*25*)] and coupled to CNBr activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Cε3 was bound to the matrix and washed in PBS. The bound material was eluted with 0.2 M glycine-HCl, pH 2.5, and immediately neutralized with 1 M Tris-HCl, pH 8.0. Eluted material was concentrated, dialyzed against PBS containing 0.05% sodium azide, and stored at 4 °C until further use. The material was quantified spectrophotometrically using a calculated molar extinction coefficient of 14,120 (*26*).

(b) Competition of IgE Binding to sFcεRIα (ELISA). At all stages, the well volume was 100 μL. All incubation times were 60 min at 37 °C, and the wells were washed with PBS and 0.05% Tween-20 (PBS/T). ELISA plates (Maxisorb, Nunc) were coated with 100 ng/well of the extracellular portion of the soluble α-chain of human FcεRI (sFcεRIα, described in ref 27) in 0.2 M carbonate buffer, pH 9.8. Unreacted sites on the plastic were blocked with 2% milk protein in PBS. Nonspecific binding was determined by preincubating wells with rat IgE (Serotec) at 200 μg/mL for 45 min before the addition of 100 ng biotinylated human IgE. Maximum binding was determined by incubating the wells with 100 ng of biotinylated human IgE in the absence of any competitor. Samples for analysis were serially diluted in 1% milk protein in PBS/T and added to the wells in the presence of 100 ng of biotinylated human IgE. The binding of the biotinylated IgE was detected with streptavidin-HRP (Amersham), diluted 1:1000 in 1% milk protein in PBS/T. The captured streptavidin-HRP was visualized by incubating the wells with 100 μL of tetramethyl benzidine dihydrochloride (10 mg/mL stock in DMSO diluted 100-fold in 0.1 M sodium acetate containing 0.005% H_2O_2). The color reaction was stopped by the addition of 100 μL of 3 M HCl and the A_{450} read on a Titertek ELISA reader. Percentage binding of the biotinylated human IgE was calculated as a fraction of the total specific binding and IC_{50} values were calculated.

(c) Kinetics of Binding to sFcεRIα Measured by Surface Plasmon Resonance. All experiments were performed at 24 °C on a Pharmacia BIAcore instrument (Pharmacia Biosensor AB, Uppsala, Sweden). A specific binding surface was prepared by coupling the IgG_4-Fc-(sFcεRIα)$_2$ fusion protein to a CM5 sensor chip using the amine coupling kit according to the manufacturer's instructions. Coupling density was restricted to 1000 RU. A parallel surface on which IgG_4-Fc was coupled to the same density as the IgG_4-Fc-(sFcεRIα)$_2$

was also prepared in order to assess nonspecific binding. Samples of IgE-WT or Cϵ3 were diluted in HBS [supplied by Biosensor, being 10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) Surfactant P-20] and injected over the sensor chip at 5 μL/min. A 300 s association phase was followed by a 300 s dissociation phase. The sensor surface was regenerated by three 60 s pulses of 0.2 M glycine-HCl, pH 2.5. The data were analyzed using the BIA evaluation analysis package (version 2.1, Pharmacia Biosensor). Nonspecific binding was subtracted from the specifc binding prior to kinetic analysis. The data were analyzed as previously described (*14*).

(d) Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge. Data were acquired as an average of 25 absorbance measurements at a wavelength of 280 nm and a radial spacing of 0.001 cm. Experiments were run in PBS and 0.05% sodium azide using charcoal-filled Epon double-sector centerpieces with column lengths of about 4 mm. Sedimentation equilibrium experiments were performed at 4 °C and rotor speeds of 30 000, 35 000, and 40 000 rpm with Cϵ3 loading concentrations corresponding to a measured A_{280} of about 0.6. Data at equilibrium were analyzed in terms of a single ideal solute to obtain the buoyant molecular mass $M(1 - \bar{\nu})$ as previously described (*28*). The molecular mass was calculated using the previously determined solvent density (*28*) and a calculated value of $\bar{\nu}$ based on the amino acid composition (*29*).

(e) Circular Dichroism. Circular dichroism (CD) measurements were performed on Jobin-Yvon (Longjumeau, France) CD6 spectrophotometer using cylindrical quartz cells of 0.05 cm path length. The spectrophotometer was calibrated for wavelength and ellipticity using *d*-10-camphorsulfonic acid. Samples were measured in the concentration range 100−500 μg/mL in 20 mM sodium phosphate, pH 7.4, at constant temperature in a thermostated cell holder.

Spectra were recorded in 0.2 nm steps with an integration time of 4 s and corrected by subtraction of the solvent spectrum obtained under identical conditions. The units of $\Delta\epsilon$ are M^{-1} cm^{-1} per backbone amide.

The ellipticity of different proportional mixtures of Cϵ3 (prepared as described above) and sFcϵRIα (prepared as described in ref *27*) was measured at a series of defined wavelengths. The spectrophotometer was set to collect data at the desired wavelength for 3 min with a 30 s integration time for each data point. Six separate data points were taken, and the mean ± the standard deviation was plotted against the changing composition of the mixture. The CD curves were plotted and analyzed with MicroCal Origin (Version 3, MicroCal Software, MA).

(f) Fluorescence Emission Spectroscopy. The quenching of the intrinsic tryptophan fluorescence of recombinant proteins was measured as a function of stepwise GuHCl denaturation. Fluorescence measurements were made on a Fluoromax fluorimeter (Spex Industries Inc., Edison, NJ), with a thermostated cell mounting using 4 × 10 mm quartz cuvettes. Excitation was at 290 nm, and the wavelength at which maximum emission occurred was determined by scanning from 300 to 400 nm in 0.5 nm steps, with a 0.1 s integration time.

Samples were measured at 2 μg/mL in 20 mM sodium phosphate buffer (pH 7.4) at a constant temperature of 20 °C. The desired GuHCl concentration was obtained from a 6 M stock solution. Background emission and the water Raman band were subtracted using blank solutions corresponding to each GuHCl concentration. Repeated measurements at the wavelength of maximum emission for each GuHCl concentration were obtained by increasing the integration time to 10 s and collecting data for 70 s. Six separate data points were averaged and the data plotted as the mean fluorescence ± standard deviation as a function of GuHCl concentration.

(g) Carboxymethylation of Cϵ3. Cϵ3 was carboxymethylated according to the procedure of Glazer et al. (*30*). After carboxymethylation, the material was dialyzed against PBS with 0.05% sodium azide.

RESULTS

Expression, Refolding, and Purification of the Cϵ3 Domain. Cϵ3 was expressed in *E. coli*, as described in the Experimental Section. Cys328 was replaced by serine to prevent dimerization and incorrect pairing with either of the remaining two cysteine residues that form the conserved intradomain disulfide bridge characteristic of Ig domains. Monomers were purified by HPLC in 6 M urea under nonreducing conditions at a yield of 70%. Over 90% of this product bound to a sFcϵRIα affinity column, indicating that it was biologically active. Analytical HPLC and SDS−PAGE confirmed that the preparation was monodisperse, covalently monomeric and essentially free of detectable contaminating protein (Figure 1).

The molecular mass was determined by equilibrium sedimentation in the analytical centrifuge at three different rotor speeds. Analysis of each data set in terms of a single ideal solute returned excellent fits with values of $M(1 - \bar{\nu})$ that did not, within the experimental precision of the method, depend on the rotor speed. An average buoyant molecular mass of 3120 ± 150 g/mol is returned experimentally, corresponding to a molecular mass M of 11 950 ± 600 g/mol. The calculated value of M is 12 283 g/mol; the sample is therefore monodisperse and monomeric in solution.

Competition with IgE for Binding to FcϵRI. The affinities of Cϵ3 and native IgE (WT) for sFcϵRIα were determined by competitive ELISA. The IC_{50} values were 1.5 ± 1.4 μM and 12.5 ± 10.4 nM, respectively.

Kinetics of Binding of Cϵ3 to FcϵRI. The kinetics of Cϵ3 binding to sFcϵRIα were measured by surface plasmon resonance (SPR), as described in the Experimental Section. The IgG_4-Fc-$(sFc\epsilon RI\alpha)_2$ fusion protein was immobilized via primary amine groups to a carboxylated dextran-coated CM5 sensor chip at a receptor density low enough to avoid conditions of mass transport limited binding (*31*). Nonspecific binding effects were examined by coupling parallel CM5 sensor surfaces with IgG_4-Fc or FcγRII (as a fusion with human serum albumin) or simply exposing the sensor surface to the coupling chemistry in the absence of any protein. There was no difference in Cϵ3-binding capacity between the three nonspecific surfaces and this total nonspecifc binding did not exceed 30% of the total binding to the sFcϵRIα surface (data not shown).

The binding and dissociation of Cϵ3 and IgE were recorded for a range of dilutions (Figure 2), and the data

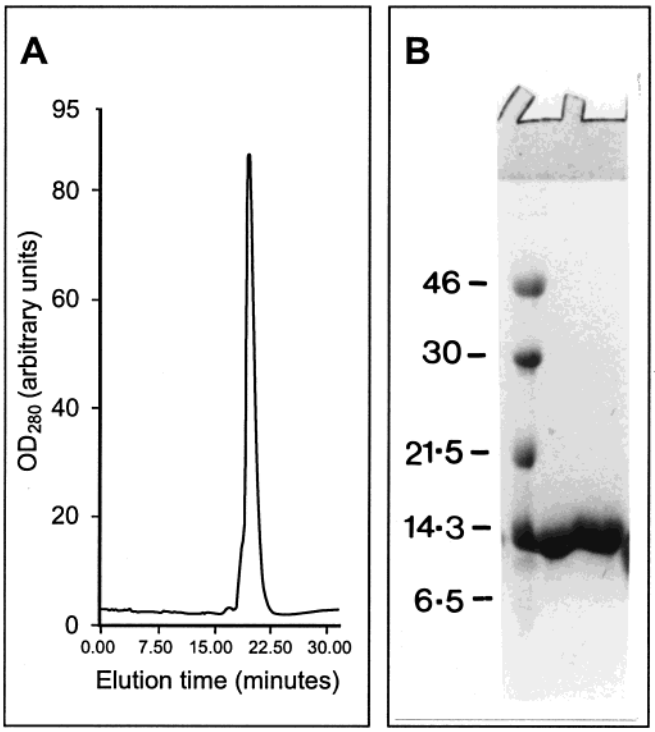

FIGURE 1: Characterization of refolded monomeric Cε3. Cε3 was refolded and the monomeric fraction obtained by gel filtration, and the biologically active fraction obtained by passage over a sFcεRIα affinity column. The active fraction was analyzed by gel filtration and SDS−PAGE. (A) Analytical gel filtration chromatography of Cε3 at 0.5 mL/min on a Superdex 75HR (1 × 30 cm) column. (B) Coomassie stained 20% nonreducing SDS−PAGE gel of Cε3. (Lane 1) molecular mass markers, with corresponding sizes indicated in kilodaltons; (lane 2) 30 μg of Cε3.

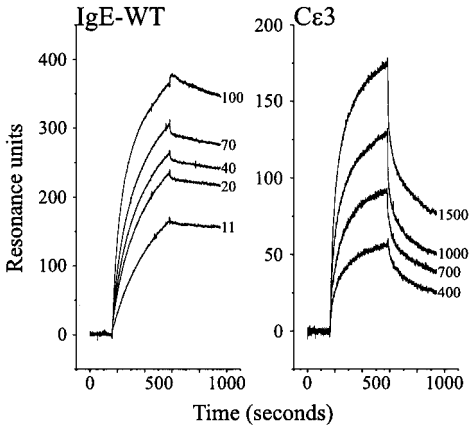

FIGURE 2: Analysis of the binding of Cε3 and IgE-WT to FcεRIα by surface plasmon resonance. The binding of Cε3 and IgE-WT to immobilized sFcεRIα was determined at several different ligand concentrations as indicated; values shown are nM. sFcεRIα was presented as a fusion with IgG_4-Fc [IgG_4-Fc-$(sFc\epsilon RI\alpha)_2$]. Nonspecific binding was determined by using a sensor surface coated with IgG_4-Fc at the same density as the fusion protein. 300 s of association phase was followed by 300 s of dissociation phase when HBS buffer was passed over the sensor surface at 5 μL/min. Nonspecific binding was subtracted from the specific binding data prior to data analysis.

were first fitted to a first-order monophasic rate equation to determine the rate constants k_a and k_d (*32*). It could be seen from the poor curve fits and nonrandom distribution of large residuals that neither association nor dissociation can be adequately described by this model. When fitted to a biphasic

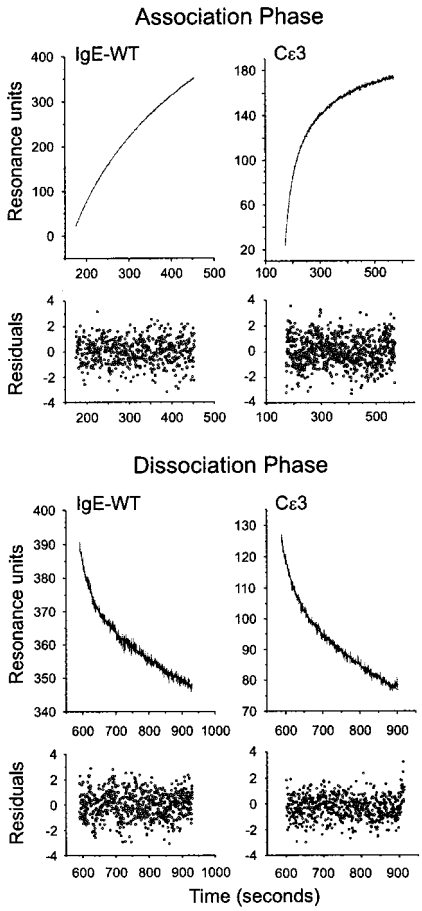

FIGURE 3: Fitting of the SPR data for IgE-WT and Cε3 binding to immobilized IgG_4-Fc-$(sFc\epsilon RI\alpha)_2$. The association and dissociation phases were fitted to a biphasic model of binding. Representative fits are shown for the highest concentration of each ligand assayed. The fitted curves are overlaid with the experimental data for each plot. Residual plots (calculated by subtracting the calculated fit from the experimental data) are shown for each fit.

Table 1: Kinetics of IgE−Fc (*27*, *34*) and Cε3 Binding to SFcεRIα (*27*) as Determined by Surface Plasmon Resonance[a]

	protein assayed	
constant	IgE-Fc	Cε3
k_{a1} (M^{-1} s^{-1})	$(2.5 \pm 0.7) \times 10^5$	$(2.0 \pm 1.9) \times 10^4$
k_{a2} (M^{-1} s^{-1})	$(7.9 \pm 5.9) \times 10^4$	$(5.6 \pm 4.2) \times 10^3$
k_{d1} (s^{-1})	$(6.9 \pm 1.8) \times 10^{-3}$	$(2.4 \pm 0.4) \times 10^{-2}$
k_{d2} (s^{-1})	$(1.9 \pm 0.7) \times 10^{-5}$	$(1.1 \pm 0.2) \times 10^{-3}$
K_{a1} (M^{-1})[b]	3.6×10^7	8.6×10^5
K_{a2} (M^{-1})[c]	4.2×10^9	5.0×10^6
R_1/R_0[d]	0.0095 ± 0.0047	0.25 ± 0.022

[a] Results are expressed as the mean ± standard deviation for five separate determinations for IgE-Fc in the concentration range 11−100 nM and for eight separate determinations for Cε3 in the concentration range 400−3000 nM. [b] K_{a1} calculated as k_{a1}/k_{d1}. [c] K_{a2} calculated as k_{a2}/k_{d2}. [d] Determined from the fitting of the dissociation phase data. The quoted figure gives the proportional contribution of component 1.

model, however, excellent curve fits are obtained, and the residuals are both considerably smaller and randomly distributed (Figure 3). The rate constants derived from these fits are given in Table 1, where k_{a1} and k_{a2} are the two components of the association rate, and k_{d1} and k_{d2} are the two dissociation rate constants. K_a is calculated as k_a/k_d. The ratio of the contributions that each rate component makes to the total binding is given by R_1/R_0.

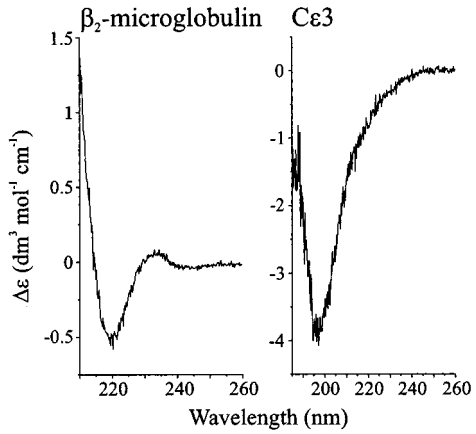

FIGURE 4: Circular dichroism spectra of refolded Cϵ3 compared with purified human β_2-microglobulin. The CD spectra were measured in a 0.5 mm path length cylindrical cell at 0.2 nm steps with a 4 s integration time. Both samples were at 300 μg/mL in 20 mM sodium phosphate buffer, pH 7.4.

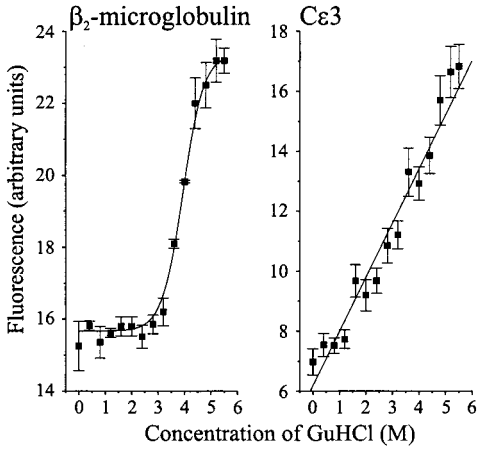

FIGURE 5: Denaturation of Cϵ3 and β_2-microglobulin monitored by fluorescence emission spectroscopy. Samples of Cϵ3 and β_2-microglobulin at 2 μg/mL in 20 mM sodium phosphate buffer, pH 7.4, were excited at 290 nm and the wavelength of maximum emission was determined. Fluorescence at the wavelength of maximum emission was followed as a function of guanidine hydrochloride concentration. Data are plotted as the mean ± sd of five repeated readings of the fluorescence emission at each guanidine hydrochloride concentration.

For IgE, the R_1/R_0 ratio indicates that the dominant component (99%) of the interaction is that described by the slower association and dissociation rates ($k_{a2} = 7.9 \times 10^4$ M^{-1} s^{-1} and $k_{d2} = 1.9 \times 10^{-5}$ s^{-1}). For Cϵ3, the slower component also dominates, but to a lesser extent (75%), and for this component the on-rate is slower for Cϵ3 than IgE and the off-rate is faster ($k_{a2} = 5.6 \times 10^3$ M^{-1} s^{-1} and $k_{d2} = 1.1 \times 10^{-3}$ s^{-1}; see Table 1). These yield K_{a2} values for IgE and Cϵ3 of 4.2×10^9 M^{-1} and 5.0×10^6 M^{-1} for IgE and Cϵ3, respectively. Thus, the higher affinity component of the Cϵ3 interaction with sFcϵRIα is ~800-fold lower than that of IgE. Comparison between the lower affinity components of each interaction reveals a 40-fold difference ($K_{a1} = 3.6 \times 10^7$ M^{-1} and 8.6×10^5 M^{-1} for IgE and Cϵ3, respectively), again resulting from a slower on-rate and a faster off-rate for Cϵ3 compared with IgE. These differences between the K_a values are broadly in agreement with the difference detected by the competition ELISA assay, and the K_{a2} value for IgE binding to sFcϵRIα is only slightly less than values previously determined for the affinity of the IgE−FcϵRI interaction by cell binding methods (*5*, *33*, *34*), which yielded values of $K_a \approx 10^{10}$ M^{-1}.

Circular Dichroism Spectroscopy of Cϵ3. CD spectra of the Cϵ3 peptide and β2-microglobulin are shown in Figure 4. The spectrum of β2-microglobulin is typical of the β-sheet structure expected for a folded Ig-like domain, with a negative band near 217 nm returning to positive ellipticity as it approaches 200 nm (*35*), and is in good accord with previously published spectra (*36*). The spectrum of Cϵ3 is typical of a random coil, having a strong negative feature near 200 nm, and no evidence of β-structure (*37*, *38*). The Cϵ3 spectrum was also recorded at 4, 20, and 70 °C, but there were no significant changes, and all were virtually indistinguishable from the spectrum of β2-microglobulin after denaturation by heating to 70 °C (data not shown). It thus appears that the active Cϵ3 peptide has little or no (α or β) secondary structure observable by CD in solution, and that temperature reduction to 4 °C does not encourage the formation of secondary structure.

Fluorescence Spectroscopy of Cϵ3. To determine whether the hydrophobic core of the domain had formed in the absence of defined secondary structure, the intrinsic fluorescence of the tryptophan residues of Cϵ3 was recorded as a function of the concentration of a denaturant, GuHCl, and compared with that of β2-microglobulin. Both Cϵ3 and β2-microglobulin contain two tryptophan residues. The conserved core tryptophan is buried in the interior of the domain and located close to the intrachain disulfide bond (*39*), and its fluorescence is greatly quenched by the disulfide bond (*40*). The titration for β2-microglobulin shows a clear transition characteristic of the loss of quenching of the buried tryptophan residue in the core as the protein is progressively unfolded (Figure 5). In contrast, over the same concentration range, no such unfolding transition is seen to occur in Cϵ3.

Carboxymethylation of Cysteine Residues in Cϵ3. Formation of the intrachain disulfide bond between Cys358 and Cys418 was confirmed by Ellman assay. Reduction and carboxymethylation resulted in a product that was inactive in binding to sFcϵRIα, as measured by SPR (results not shown). This may imply that the structural constraint of a disulfide bond facilitates complex formation.

Circular Dichroism Spectroscopy of the Cϵ3:sFcϵRIα Complex. To discover whether a conformational change occurs in Cϵ3 upon formation of the Cϵ3−sFcϵRIα complex, CD spectra were measured for the individual components and a 1:1 mixture of the two. In addition, the ellipticity was recorded at four wavelengths for various ratios of the two components. The total protein concentration was kept at 15 μM, which was 75 times higher than the measured K_d value for the interactions (200 nM, determined by SPR, Table 1), and the mixtures were incubated for several hours to ensure that equilibrium had been reached. The results are shown in Figure 6. The spectrum of the 1:1 complex was found to be the sum of those of the free components (Figure 6A), and this is confirmed by the linear variation of ellipticity with molar ratio at all the four wavelengths monitored throughout the titration (Figure 6B). There is therefore no evidence for a conformational change upon receptor binding detectable by CD spectroscopy. The sensitivity of this technique for detecting conformational changes is at least 0.2 mdeg, as indicated by the error bars in Figure 6B. This is equivalent

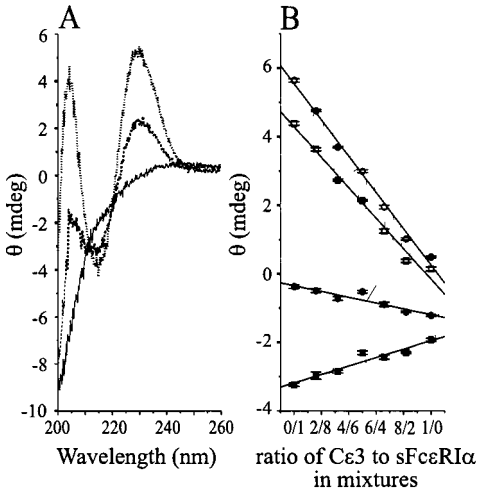

FIGURE 6: Analysis by CD of the secondary structure of Cε3 and sFcεRIα as a consequence of complex formation. Cε3 and sFcεRIα were mixed together at different ratios, maintaining the protein concentration at 15 μM. (A) CD spectra of Cε3 (—), sFcεRIα (•• ••), and the 1:1 mixture of Cε3 and sFcεRIα (- - -). (B) The ellipticity of the different mixtures was determined at 215 (■), 220 (●), 225 (□), and 230 (○) nm. Data are shown as mean ± sd for seven separate determinations at each wavelength. Linear regression analysis was used to fit the best straight line to the data.

to a 6% change in the β-structure of the complex, assuming that a fully folded Cε3 domain would have ~40% β-structure (*41*).

DISCUSSION

We have cloned and expressed in *E. coli* a recombinant peptide corresponding to the Cε3 domain of IgE and purified and characterized the product and studied its interaction with sFcεRIα. This peptide binds with substantial affinity to sFcεRIα, as shown by its retention on a receptor affinity column, and by quantitative measurements of the binding affinity relative to IgE. However, as judged by CD and fluorescence spectroscopy, the isolated Cε3 domain appears to be unfolded and devoid of secondary structure. In contrast, the single Ig-like domain of β-microglobulin, analyzed under the same conditions, was fully folded, as judged by these same criteria.

We have employed two binding assays, one a competition ELISA, another an assay of direct binding of the Cε3 sequence to immobilized sFcεRIα using surface plasmon resonance. The K_a deduced from the IC_{50} value in the ELISA assay is around 10 times lower than the K_a calculated from the kinetic (SPR) data, but this is an observation that many previous authors have noted on comparing the results of equilibrium binding assays and kinetic assays of IgE binding to FcεRI. The kinetics of IgE binding to immobilized sFcεRIα by SPR also gives a somewhat lower value for K_a than cell-binding assays [4.2×10^9 vs 3.4×10^{10} M^{-1} (*34*)]. These differences in the K_a values may reflect a requirement for membrane components, in addition to the α-chain, to confer high-affinity binding to FcεRI. It has been reported that the γ-chain dimer in the IgG receptor, FcγRI, is required for high affinity IgG binding (*42*). FcεRI utilizes the same γ-chain dimer (*43*), and it is therefore possible that it is essential for IgE to bind to its receptor with native affinity. The biphasic kinetics observed in the SPR assay has been observed for other IgE fragments binding to FcεRIα, as discussed elsewhere (*14*).

Kinetic analysis of the interaction of the isolated Cε3 domain with sFcεRIα leads to an association constant (K_a) of 5×10^6 M^{-1}. While this is 800 times lower than that of IgE, it is of the same order of magnitude as that observed for the binding of IgG to FcγRII and FcγRIII [the low-affinity IgG receptors (*44*)]. Previous authors have shown that supernatants from insect cells expressing recombinant Cε3 contained material that was active in binding to immobilized FcεRIα and inhibiting IgE binding to immobilized FcεRIα; however, these results were only qualitative (*16*). A number of partial Cε3 sequences have been shown to display significant affinity for FcεRI in cell-binding assays (*3*, *4*, *6*), but that of the entire Cε3 domain has not been measured quantitatively before.

Why is the affinity of the Cε3 domain lower than that of IgE, if no amino acids in other domains are directly involved in the binding of FcεRI? The lack of glycosylation in the bacterially expressed Cε3 can probably be ruled out as the main reason, because the IgE−Fc (*2*, *3*, *20*, *45*, *46*) and the Cε3-Cε4 dimer (*47*) synthesized in *E. coli* bind with essentially full affinity to FcεRI. We and others have refolded isolated immunoglobulin domains, starting with reduced and denatured material (*36*, *48*, *49*, and our unpublished results on Cε2 and Cε4). In the available models of IgE, Cε3 resembles its homologue Cγ2 in IgG, which is distinguished from all other Ig domains in IgG in that it does not associate "laterally", Cγ2 to Cγ2. From the homology with IgG-Fc, it is also expected in IgE−Fc that there will be "longitudinal" contacts between Cε3 and Cε4, as there are between Cγ2 and Cγ3 (*50*); thus the lack of contact of Cε3 with Cε4 may well be significant.

Consistent with this interpretation is the observation that Cγ2 released from IgG by proteolytic digestion appears to be partially (*51*) if not completely (*52*) unfolded. The former peptide was, nevertheless, functionally active in complement binding (*53*, *54*), although at a reduced level when compared with intact IgG. Three key residues that constitute a part of the complement binding site in Cγ2 (*55*) form a linear array within a short stretch of sequence (residues 318, 320, and 322), accounting for this activity. We infer that FcεRI similarly recognizes one or more linear epitopes within the sequence of Cε3. It is not uncommon for antibodies to recognize unstructured peptides representing linear epitopes within globular proteins. The affinity of Cε3 for FcεRI is well within the range of affinities displayed by antibodies for such peptides (*56*).

It must be remembered, however, that in IgE there are two Cε3 domains, and both the IgE−Fc and the Cε3-Cε4 dimer bind to FcεRI with similar affinities and identical thermodynamic parameters (*57*). Although the Cε3 domains are predicted to be separated from each other in the model structure (*58*), as are the homologous Cγ2 domains in the crystal structure of IgG (*50*), the two Ig-like domains (α1 and α2) in the extracellular region of the FcεRI α-chain would be able to span the distance between the two Cε3 domains. Such a model of the IgE−FcεRI complex was indeed suggested on the basis of site-directed mutagenesis in the IgE−Fc (*14*, *59*). For such a complex, folding of the Cε3 domains may also be required for full affinity binding of FcεRI. Dissection of the contributions of dimerization and

folding of Cε3 to the formation of the high-affinity binding site in IgE will require further experiments.

Since the Cε3 domain in an unfolded state binds to FcεRIα, we postulated that it might undergo a conformational change to a more ordered state upon complex formation. This proved not to be the case at least as judged by CD spectroscopy. The CD spectra recorded for mixtures of Cε3 and sFcεRIα in various proportions are identical to the sum of the spectra of the free components. However, some structural requirement may be indicated by the effect of reduction of the disulfide bond between Cys358 and Cys414 and carboxymethylation of the sulfhydryl groups.

In summary, we have demonstrated that Cε3 binds to sFcεRIα the isolated Cε3 domain peptide with an affinity of the order of 10^6 M^{-1}, a result that is consistent with the identification of several critical residues within this domain. The attainment of full binding affinity may presumably require a dimerized and/or fully folded Cε3 domain. Determination of the requirements for folding of this domain will help us understand the structural basis for the uniquely high affinity of IgE for its receptor FcεRI.

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