

An Engineered Disulfide Bond Reversibly Traps the IgE-Fc₃₋₄ in a Closed, Nonreceptor Binding Conformation*

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Background: IgE antibodies bind the high affinity receptor on mast cells and basophils and trigger allergic diseases.

Results: An engineered disulfide bond in the IgE-Fc traps a closed conformational state, blocking receptor, but not inhibitor, binding.

Conclusion: Disulfide bond trapping reveals different conformational requirements for IgE ligand binding.

Significance: Better understanding of IgE conformational dynamics may lead to novel approaches to treating allergic diseases.

IgE antibodies interact with the high affinity IgE Fc receptor, FcεRI, and activate inflammatory pathways associated with the allergic response. The IgE-Fc region, comprising the C-terminal domains of the IgE heavy chain, binds FcεRI and can adopt different conformations ranging from a closed form incompatible with receptor binding to an open, receptor-bound state. A number of intermediate states are also observed in different IgE-Fc crystal forms. To further explore this apparent IgE-Fc conformational flexibility and to potentially trap a closed, inactive state, we generated a series of disulfide bond mutants. Here we describe the structure and biochemical properties of an IgE-Fc mutant that is trapped in the closed, non-receptor binding state via an engineered disulfide at residue 335 (Cys-335). Reduction of the disulfide at Cys-335 restores the ability of IgE-Fc to bind to its high affinity receptor, FcεRIα. The structure of the Cys-335 mutant shows that its conformation is within the range of previously observed, closed form IgE-Fc structures and that it retains the hydrophobic pocket found in the hinge region of the closed conformation. Locking the IgE-Fc into the closed state with the Cys-335 mutation does not affect binding of two other IgE-Fc ligands, omalizumab and DARPin E2_79, demonstrating selective blocking of the high affinity receptor binding.

IgE antibodies are associated with allergic reactions and asthma, triggering inflammatory responses through interactions with the high affinity IgE-Fc receptor (FcεRI) expressed on mast cells and basophils (1). Cross-linking of FcεRI on these cells by allergen-antibody complexes leads to the immediate release of histamines, followed by the secretion of additional mediators of inflammation such as leukotrienes and cytokines

(2). The interaction of the IgE-Fc region with FcεRI is of high affinity (~1 nM), leading to the stable recruitment of antibody to mast cell surfaces even prior to allergen binding (2, 3). One currently available treatment for allergic asthma is the anti-IgE antibody, omalizumab (Xolair), which interferes with receptor binding (4–6). However, omalizumab is not suitable for treating all allergies, being restricted to children age 12 and older with persistent allergic asthma (Genentech, and Novartis). Other approaches to inhibiting IgE-mediated allergic reactions are needed to complement this therapeutic strategy, and efforts to target mast cell signaling pathways are ongoing (8).

The human high affinity IgE receptor exists as a heterotrimer or tetramer on the surface of mast cells and basophils. The tetramer contains an α-chain, a β-chain, and two γ-chains, whereas the trimer is formed by an α-chain and two γ-chains (2, 9, 10). The β- and γ-chains are signaling subunits of the receptor complex, whereas the α-chain contains two extracellular immunoglobulin domains that bind IgE with high affinity. The IgE contains two antibody light chains in association with two heavy chains of the ε isotype (Fig. 1A). Compared with IgG, IgE antibodies have an additional immunoglobulin constant domain (Ce2) located between the antigen-binding region (Fab) and the C-terminal Ce3-4 domains (Fig. 1A). The Ce3 and Ce4 domains are homologous to the IgG-Fc region, which is formed by two immunoglobulin constant domains (Cγ2 and Cγ3). The human IgE-Fc and IgG-Fc regions exhibit ~32% sequence identity. These Fc domains play analogous structural and functional roles: in IgE the two Ce3 domains interact with the Fc receptor, whereas Ce4 domains mediate heavy chain dimerization (Fig. 1A). Both intact IgE and IgE-Fc fragments (Ce2-4 or Ce3-4) bind with high affinity ($K_D = \sim 10^{-9}$ – 10^{-10} M) to FcεRI. The full IgE-Fc Ce2-4 protein will be referred to here as IgE-Fc₂₋₄, whereas the smaller, IgG-Fc homologous construct Ce3-4 protein will be referred to as IgE-Fc₃₋₄.

Crystal structures of the human IgE-Fc₃₋₄ and IgE-Fc₂₋₄ alone and in complex with FcεRI α-chain extracellular domains (FcεRIα) have provided significant insights into their interactions. We have previously determined the crystal structures of the FcεRIα (11, 12), the IgE-Fc Ce3-4 domains (13), and a complex of the IgE-Fc₃₋₄ bound to the receptor (9, 14). The IgE-

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The atomic coordinates and structure factors (code 4GT7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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Structure of a Covalently Closed IgE-Fc

Fc₃₋₄-FcεRIα complex structure revealed a two-pronged binding interaction between the receptor and the two N-terminal Ce3 domains of the IgE-Fc, explaining the 1:1 receptor-Fc stoichiometry. A recent structure of the IgE-Fc₂₋₄-FcεRIα complex (15) further demonstrated that IgE Ce2 domains do not play a role in receptor engagement but interact with the Ce3-Ce4 domains to influence receptor binding kinetics and complex stability. Comparisons of the free and receptor-bound IgE-Fc₃₋₄ structures revealed that the IgE-Fc Ce3 domains undergo a large conformational rearrangement upon receptor binding (13). The free IgE-Fc₃₋₄ was observed to be in a “closed” conformation incapable of binding the receptor. Similarly, the structure of the IgE-Fc₂₋₄ protein in the absence of receptor shows a partially closed state that would require opening of the Ce3 domains to bind receptor (16, 17). Recently, the structure of the IgE-Fc₃₋₄ in complex with the low affinity IgE receptor (CD23) has been determined, demonstrating that CD23 binds to the Ce3-4 hinge region, favoring a closed conformation for the Fc (18).

These crystallographic observations suggest that conformational dynamics of the IgE affect interactions between IgE and its receptors (9, 13, 16). In addition, these structures indicate that one might be able to regulate IgE conformational dynamics, using protein or small molecules, providing a novel strategy for the development of inhibitors of the IgE-FcεRI interaction (13). To further explore this possibility and to develop new reagents for the identification and isolation of IgE-Fc conformational modulators, we have produced a disulfide bond mutant of the IgE-Fc (Cys-335) that is “trapped” in the closed state. The formation of this disulfide bond in solution is consistent with previous crystallographic snapshots of the IgE dynamics, based on multiple crystal structures. We present the crystal structure of the conformationally trapped IgE-Fc Cys-335 protein, and we demonstrate that the Cys-335 Fc does not bind to FcεRIα unless the disulfide bond is reduced, freeing the IgE to undergo a conformational change required for receptor binding. In contrast to its inability to bind to FcεRI, Cys-335 IgE binds two other inhibitory ligands, omalizumab and DARPin E2_79 (19–21), similarly to wild type IgE, demonstrating a selective block in FcεRI binding.

EXPERIMENTAL PROCEDURES

Mutagenesis—Mutations were introduced into the wild type IgE-Fc₃₋₄ gene to remove the native cysteine residue at position 328 and to introduce a cysteine residue at various positions along the Ce2-Ce3 linker. In each construct, the cysteine residue at position 328 was mutated to alanine (C328A), whereas the wild type residue at a specified residue was mutated to cysteine. The constructs are named by the position of the introduced cysteine. For example, the structure described here is “335” and contains the mutations C328A and G335C. The cysteine series of mutations were generated by PCR using the wild-type IgE-Fc gene as the template. The PCR products and pACgp67A vector were digested with BamHI and NotI and ligated. The mutants were confirmed by DNA sequencing. The N terminus of the resulting secreted protein contains three vector-derived residues (ADP) at the N terminus followed by residue 328 of the Fc.

Expression and Purification of Proteins—Expression and purification of the soluble FcεRI α-chain ectodomain was carried out as previously described (11, 14). Omalizumab (Xolair) was purchased from Novartis. Selection, purification, and characterization of the DARPin E2_79 has been reported elsewhere (20). The IgE-Fc₃₋₄ cysteine mutants were expressed in insect cells, and Cys-335 IgE-Fc was purified as previously described for WT IgE-Fc₃₋₄ protein (16).

Gel Filtration Chromatography of WT and Cys-335 IgE-Fc with FcεRIα—Individual proteins (wild type IgE-Fc₃₋₄, Cys-335 IgE-Fc₃₋₄, and FcεRIα) were diluted to 200 μl with buffer (20 mM Tris, pH 7.5) and injected separately onto a Superdex® 75 gel filtration column (GE Healthcare) equilibrated in 20 mM Tris, pH 7.5, 150 mM sodium chloride. Reduced IgE-Fc samples were treated as follows. 20 μg of Fc was incubated with 5 mM DTT in buffer (20 mM Tris, pH 7.5), in a total volume of 20 μl for 15 min at room temperature. 160 μl of buffer was added to the samples, and then either 20 μl of FcεRIα (10 mg/ml) or 20 μl of buffer was added to the sample and then injected onto the gel filtration column. For the nonreduced IgE-Fc samples (20 μg), either 160 μl of buffer plus 20 μl of FcεRIα (10 mg/ml) or 180 μl of buffer were added to the Fc, and the sample was injected onto the column.

Cys-335 Crystallization—The Cys-335 IgE-Fc was crystallized by vapor diffusion using the hanging drop method. The protein was concentrated to 8 mg/ml (using an $\epsilon_{280\text{ nm}}$ of $1.32\text{ cm}^{-1}(\text{mg/ml})^{-1}$) in 10 mM Tris, pH 8.0. Drops (1 μl) were set up at room temperature by mixing 0.5 μl of buffered detergent solution (20 mM sodium acetate, pH 4.6, 28.8% (w/v) PEG 4000, 2% (w/v) Anapoe®-X-405; final pH ~5.3) with 0.5 μl of the concentrated protein solution and suspending the sample over 700 μl of well solution (25 mM sodium acetate, pH 4.6, 36% (w/v) PEG 4000; final pH ~5.3). Small crystals grew in a variety of morphologies (plates, wedges, and rods) in the presence or absence of detergent. The structure described here was determined from a crystal grown in the presence of detergent. Crystals were moved into harvest buffer (25 mM sodium acetate, pH 4.6, 40% (w/v) PEG 4000, 1% (w/v) Anapoe®-X-405) and treated briefly with copper solution (1 mM copper (II) chloride, 25 mM sodium acetate, pH 4.6, 40% (w/v) PEG 4000, 1% (w/v) Anapoe®-X-405). Treated crystals were moved very briefly into cryoprotectant buffer (25 mM sodium acetate, pH 4.6, 40% (w/v) PEG 4000, 1% (w/v) Anapoe®-X-405, 15% (v/v) ethylene glycol) and flash cooled in liquid nitrogen.

X-ray Data Collection, Molecular Replacement, and Refinement—Data were collected at $-160\text{ }^{\circ}\text{C}$ at the Advanced Photon Source DND-CAT 5ID Beamline using a Mar Mosaic Detector. The data were processed and integrated using XDS (22). The Cys-335 crystal grew in space group $P2_1$, with unit cell dimensions $a = 106.8\text{ }\text{\AA}$, $b = 104.8\text{ }\text{\AA}$, $c = 45.9\text{ }\text{\AA}$, and $\beta = 96.2^{\circ}$. The structure was solved by molecular replacement with Phaser (23), using a closed Fc chain as the starting model (chain D from the protein data bank structure 3H9Z). Because the asymmetric unit contains two Fcs (four chains), reflections for R_{free} test set (5% of data) were taken from thin resolution shells. Initial refinement with CNS (24) was performed against all data from 21 to $2.61\text{ }\text{\AA}$ using $|F| > 0$ and an anisotropic bulk solvent correction, followed by manual model building into composite omit maps using O (25). Several cycles of refinement and model

building yielded a structure with an R_{work} of 22.8% and an R_{free} of 27.0%. The structure was further improved with cycles of refinement using Phenix (26) with loose non-crystallographic symmetry restraints (2.5 Å root mean square) on nonloop regions of the protein, followed by model building, to give a structure with an R_{work} of 19.9% and an R_{free} of 25.8%. The structure was evaluated using WHAT IF (27), PROCHECK (28), pdb-care (29), and Coot (30). The final structure contains residues 334–545 in chain A, 333–545 in chain B, 332–545 in chain C, and 333–545 in chain D. Carbohydrate residues were modeled at each of the Asn-394 attachment sites (five for chain A, five for chain B, five for chain C, and four for chain D). 135 waters were included in the model. The structural model and diffraction data are deposited in the RCSB under Protein Data Bank code 4GT7.

ELISA Binding Assay with FcεRIα—100 μl of purified FcεRIα was incubated in microtiter plates at a concentration of 1 μg/ml in 0.05 M sodium carbonate buffer. The plates were rinsed with Tris/NaCl buffer (50 mM Tris, pH 7.6, 150 mM NaCl) containing 0.1% (v/v) Tween 20 and blocked with the Tris/NaCl buffer with 5% dry milk. WT or Cys-335 IgE-Fc samples (ranging from 0 to 83 nM) were added in duplicate to wells coated with FcεRIα. The binding of IgE-Fc proteins to FcεRIα was monitored using anti-human IgE-Fc AP-conjugated antibody (KPL, 075-1004). The plates were washed and 100 μl of a 1:1000 dilution of the anti-human IgE-Fc AP conjugated antibody (1 mg/ml) in the Tris/NaCl buffer with 5% dry milk was added to the wells and incubated for 1 h at room temperature. The plates were washed and developed using the AP reagent *p*-nitrophenyl phosphate (PNPP, KPL 50–80-00). The plates were read using a Synergy 4 multimode plate reader (BioTek) at 405 nm.

ELISA Binding Assay with E2_79—100 μl of purified WT IgE-Fc or Cys-335 IgE-Fc was incubated in microtiter plates overnight at 4 °C at a concentration of 1 μg/ml in 0.05 M sodium carbonate buffer. The plates were rinsed and blocked as in the FcεRIα ELISA binding assay. His-tagged E2_79 (0 to 714 nM) was added in duplicate to wells coated with WT or Cys-335 IgE-Fc. The binding of E2_79 to plate-bound IgE-Fc was monitored using an anti-His tag antibody (Novagen, 70796-3) as a primary antibody and an anti-mouse IgG HRP-conjugated antibody (R & D Systems, HAF007) as the secondary antibody. The plate was washed, and 100 μl of a 1:1000 dilution of the anti-His tag antibody (200 μg/ml) in Tris/NaCl buffer with 5% dry milk was added to the wells and incubated 1 h at room temperature. The plate was washed, and 100 μl of a 1:1000 dilution of the anti-mouse IgG HRP-conjugated antibody in the Tris/NaCl buffer with 5% (w/v) dry milk was added to the wells and incubated for 1 h at room temperature. The plates were washed and developed using TMB single solution (Invitrogen, 00-2023). Microplates were read using a Synergy 4 multimode plate reader (BioTek) at 650 nm.

ELISA Binding Assay with Omalizumab—100 μl of purified WT or Cys-335 IgE-Fc was incubated in microtiter plates, and plates were rinsed and blocked by the same procedures as in the E2_79 ELISA binding assay. Omalizumab samples (ranging from 0 to 62.5 nM) were added in duplicate to wells coated with the IgE-Fc. The binding of omalizumab to IgE-Fcs was monitored using peroxidase-conjugated anti-human IgG, F(ab)₂ fragment

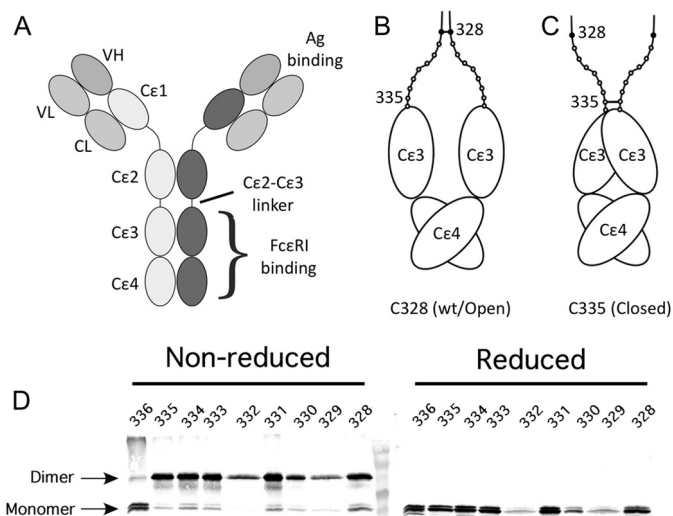


FIGURE 1. IgE-Fc conformational flexibility allows disulfide bond formation at every residue of the Cε2-Cε3 linker region. A, schematic of the IgE antibody with V and C domains indicated to the left. The antigen binding site, Cε2-Cε3 linker region, and minimal FcεRI binding Fc construct are indicated to the right. B, schematic of the open IgE-Fc conformation (Cε3 domains far apart) and the residues in the flexible Cε2-Cε3 linker represented as circles. The native Cys-328 is indicated with a filled circle, and the interchain disulfide bond formed in the IgE-Fc₃₋₄ is represented by the solid bar. C, schematic of the closed IgE-Fc conformation, with Cε3 domains shown in a closed, non-receptor binding conformation. A disulfide bond for the G335C/C328A (C335) double mutant is shown at residue 335. Cysteines at positions 334 and 335 were predicted to form an interchain disulfide bond only in the closed IgE-Fc conformation. D, interchain disulfide bond formation of the cysteine mutants. Nonreducing (left panel) and reducing (right panel) SDS-PAGE of the IgE-Fc cysteine mutation series analyzed by Western blotting. All of the cysteine mutants, except for Cys-336, are able to form the disulfide bond similarly to the wild type protein. Dimer refers to the Fc that has formed the interchain disulfide bond, whereas Monomer chains lack the interchain disulfide.

specific antibody (Jackson ImmunoResearch, 109-036-006). The plates were washed, and 100 μl of a 1:1000 dilution of the anti-human IgG antibody in the Tris/NaCl buffer with 5% dry milk was added to the wells and incubated for 1 h at room temperature. The plates were washed, developed and read by the same procedures described in the E2_79 binding assay.

RESULTS

Disulfide Scanning in the IgE Cε2-Cε3 Linker—Because previous x-ray crystal structures of the IgE-Fc₃₋₄ suggested that the IgE-Fc can adopt multiple conformational states in solution, populating intermediate states between closed and open arrangements of the Cε3 domains (Fig. 1, A–C) (9, 13, 16), we set out to probe these conformations and potentially trap a closed conformational state. In the IgE and the IgE-Fc₂₋₄, two interchain disulfides are formed between the Cε2 domain residue Cys-241 of one chain and the Cε2-Cε3 linker residue Cys-328 of the other chain. However, in the shorter IgE-Fc₃₋₄ protein that lacks residue Cys-241, a single interchain disulfide is instead formed between the linker region Cys-328 residues. To trap the Cε3 domains in the closed conformation, we systematically introduced cysteines into the Cε2-Cε3 linker region (residues 328–335) all the way up to and including the first Cε3 domain residue, 336 (Fig. 1, A–C). In previous free IgE-Fc₃₋₄ structures (9, 13, 16), the linker region was flexible and not observed, but in the complex structure with the high affinity recep-

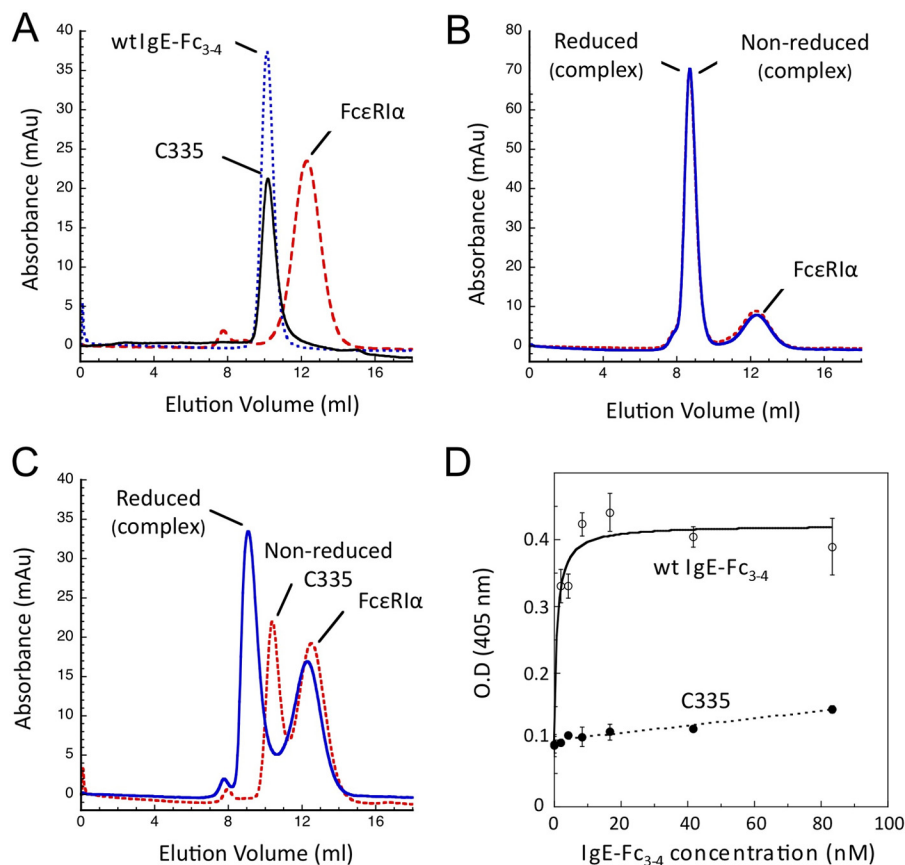


FIGURE 2. The Cys-335 mutant does not bind to FcεRIα unless the interchain disulfide bond is reduced. A–C, gel filtration chromatography analysis of FcεRIα binding to WT and Cys-335 IgE-Fc₃₋₄. A, separate elution profiles of purified FcεRIα (red dashed line), WT IgE-Fc₃₋₄ (blue dotted line), and Cys-335 mutant (black solid line). B, elution profile of mixtures of reduced (blue solid line) and nonreduced (red dotted line) WT IgE-Fc₃₋₄ with FcεRIα. Both reduced and nonreduced WT IgE-Fc₃₋₄ show similar levels of complex formation, with a new peak appearing at (~8.8 ml) upon incubation with excess FcεRIα. C, elution profile of mixtures of reduced (blue solid line) and nonreduced (red dotted line) Cys-335 mutant with FcεRIα. Reduced Cys-335 IgE-Fc shows formation of a complex, similar to that observed with WT IgE-Fc₃₋₄. D, FcεRIα binds only to WT IgE-Fc but not the Cys-335 mutant in an ELISA assay. FcεRIα was plated and incubated with WT IgE-Fc (open circles, solid line) or Cys-335 IgE-Fc (filled circles, dashed line), and IgE-Fc binding was detected with polyclonal anti-IgE antibody.

tor, several linker residues contacted the receptor, and the entire linker region including the interchain disulfide was observed.

For the IgE-Fc₃₋₄ cysteine scanning mutagenesis of residues 329–336, the WT Cys-328 was simultaneously mutated to alanine to remove the only interchain disulfide bond. Interchain disulfide formation via the introduced cysteine mutation could then be assessed by monitoring the molecular weight of the Fc. The series of single cysteine mutants containing the secondary C328A mutation was expressed, and the migration of the mutants in reducing and nonreducing SDS-PAGE was compared (Fig. 1D). Cysteine mutants at residues 329–335 quantitatively formed the interchain disulfide similarly to the wild type IgE-Fc₃₋₄ (Cys-328) protein, indicating that the closed conformational state was dynamically accessible to the majority of the mutants. Notably, the Cys-336 mutant did not form an interchain disulfide bond.

These results are consistent with the expectations based on IgE-Fc crystal structures (16). Because of the flexibility of the linker, most of the cysteine mutants should be able to form an interchain disulfide bond regardless of the IgE-Fc conformation. However, for residues closest to the Cε3 domain (linker residues 334 and 335 and Cε3 residue 336), the interchain distance in the open conformation is too great to allow disulfide

bond formation. These residues could only form an interchain disulfide bond if the IgE-Fc accessed the closed conformation. The efficiency of the Cys-334 and Cys-335 disulfide bond formation was nearly quantitative and comparable with Cys-328, indicating that conformational constraints did not hinder cysteine oxidation. The observation that the Cys-336 mutant did not form the interchain disulfide indicates that there is no additional flexibility in the Cε3-Cε4 hinge or Cε3 domain, allowing closer approach and cross-linking of Cys-336 in the two Fc chains (9).

The Cys-335 Fc Protein Is Locked in a Non-receptor Binding State—The Cys-335 disulfide, located closest to the Cε3 domains, locks the Fc in the closed state (Fig. 1C). We tested the binding of WT and Cys-335 IgE-Fc₃₋₄ to purified soluble FcεRI (FcεRI) using gel filtration chromatography (Fig. 2, A–C). Free WT IgE-Fc₃₋₄ and Cys-335 mutant proteins behaved similarly, eluting with an apparent molecular mass of 50 kDa (Fig. 2A). The soluble receptor α chain (FcεRIα) eluted later, consistent with a molecular mass of ~28 kDa (Fig. 2A). When the WT IgE-Fc₃₋₄ was incubated with an excess of the soluble receptor, a complex peak appeared at the expected molecular mass of ~70 kDa, and the free Fc peak disappeared (Fig. 2B). The WT IgE-Fc₃₋₄ could be subjected to mild reducing conditions, and the complex was still formed (Fig. 2B).

TABLE 1
Crystallographic data and refinement statistics

Crystal	IgE-Fc Cys-335
Space group	$P2_1$
Unit cell dimensions	
a (Å)	106.8
b (Å)	104.8
c (Å)	45.9
α, β, γ (°)	90, 96.2, 90
Molecules/asymmetric unit	
Resolution (Å) ^a	30–2.61 (2.72–2.61)
Completeness ^a	97.1% (93.7%)
Reflections, unique (total)	29,836 (114,148)
Average redundancy	3.82
$\langle I/\sigma_I \rangle$	14.04
R_{sym} (%) ^{a,b}	6.9 (35.4)
Number of reflections (free)	29,836 (1,395)
$R_{\text{cryst}}/R_{\text{free}}$ (%) ^c	19.9/25.8
Atoms, total	
Protein	7,082
Water	6,723
Carbohydrate	126
Average B factor (Å²)	
Protein	60.6
Water	40.3
Carbohydrate	88.2
Root mean square deviations from ideality	
Bond angles (°)	1.24
Bond lengths (Å)	0.009
Ramachandran plot	
Most favored regions (%)	90.4
Allowed regions (%)	9.6
Generous regions (%)	0
Disallowed (%)	0

^a The values for the highest resolution shell are shown in parentheses.

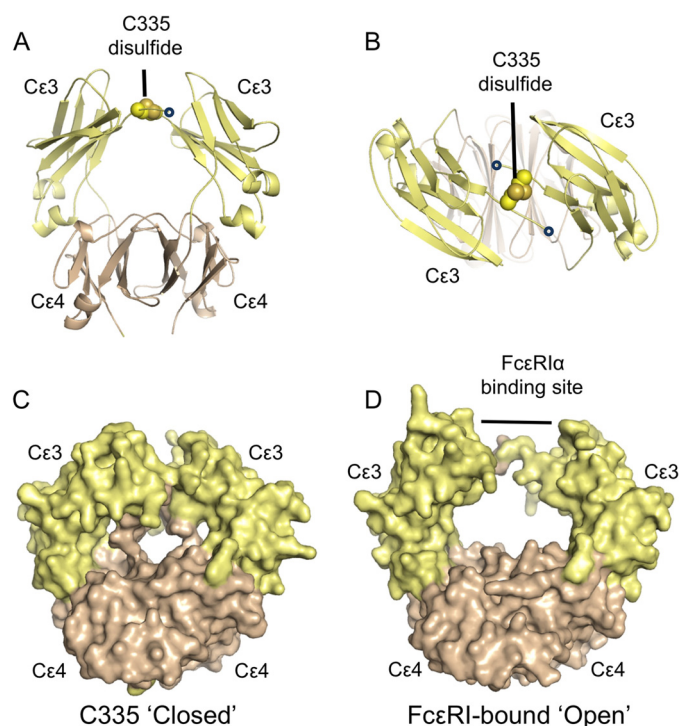
^b $R_{\text{sym}} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ = the observed intensity, and $\langle I(h) \rangle$ = the mean intensity for multiple measurements.

^c R_{cryst} and $R_{\text{free}} = \sum_h |F(h)_{\text{obs}}| - |F(h)_{\text{calc}}| / \sum_h |F(h)_{\text{obs}}|$ for the working and test set reflections, respectively.

In contrast, when the Cys-335 IgE-Fc was incubated with an excess of soluble receptor, no complex peak was formed; only individual peaks corresponding to the free Fc and the receptor were observed (Fig. 2C, red trace). When the Cys-335 Fc was subjected to mild reducing conditions and incubated with receptor, the complex peak was observed (Fig. 2C, blue trace). The covalently closed Cys-335 was unable to bind receptor, but binding could be restored by mild reduction of the disulfide bond, allowing the Cε3 domains to open. Although the native residue Gly-335 is in the FcεRIα binding interface, the cysteine is compatible with receptor binding. Comparison of Cys-335 and WT IgE binding to FcεRIα in ELISA assays further confirmed a lack of receptor binding by the mutant (Fig. 2D).

The Crystal Structure of the Cys-335 Mutant Reveals That the Novel Interchain Disulfide Bond Restrains the Fc in the Closed Conformation—To confirm the presence of the Cys-335 disulfide bond and investigate the conformational state of the Cys-335 mutant, we determined its crystal structure. Crystals of the Cys-335 IgE-Fc grew in space group $P2_1$ and diffracted X-rays to 2.61 Å. The structure was solved by molecular replacement. The data processing and final refinement statistics are collected in Table 1.

Although previous free IgE-Fc_{3–4} structures lacked electron density for residues in the Cε2–Cε3 linker, the Cys-335 IgE-Fc structure reveals the engineered disulfide bond at residue 335 (Fig. 3, A and B) and up to three additional linker residues (residues 332–334). The Cys-335 interchain disulfide bond restricts the Cε3 domains to a closed state, unlike the open form

**FIGURE 3. Structure of the Cys-335 mutant reveals an undistorted closed conformation and the presence of the engineered interchain disulfide.**

A and B, side (A) and top views (B) of one of the Cys-335 IgE-Fcs within the asymmetric unit showing the 335 disulfide bond as spheres. The structure is in the closed conformation and shows additional linker residues not observed in previous free IgE-Fc_{3–4} structures. The Cα atoms of the N-terminal residues (332 in one chain and 333 in the other) are indicated by blue circles. C, surface representation of the Cys-335 mutant, colored as in A. Note the small gap between the Cε3 domains, characteristic of closed IgE-Fc conformations. D, surface representation of the IgE-Fc in the open, receptor-bound conformation, colored as in A, taken from Protein Data Bank entry 1F6A. Note the large gap between Cε3 domains that allows FcεRIα binding.

necessary for FcεRI binding (Fig. 3, C and D). In free IgE-Fc structures (13, 16, 17), the distance across the Fc dimer axis from Val-336 to Val-336 (Cα–Cα) varies from 12.4 to 16.7 Å, whereas in the open, receptor-bound Fc this distance increases to the maximal observed 23.5 Å (Fig. 3D). In the Cys-335 Fc structure, the Val-336–Val-336 distance is decreased to 11.8 Å because of the presence of the disulfide bond.

The IgE-Fc Cε3 domain movements involve a combination of an open-closed motion relative to the Cε4 domain and a swinging motion relative to the dimer axis and the other Cε3 domain (16). Comparison of the Cys-335 IgE-Fc with an ensemble of other IgE-Fc structures shows that the two Cys-335 dimers are among the most closed and the most inwardly swung conformations but fall within the range of previously observed conformations. The overall Cys-335 IgE-Fc conformation is not distorted by the introduced disulfide bond at residue 335, based on these comparisons with previously determined IgE-Fc structures.

The elbow region of the Cys-335 Fc corresponding to the linker between the Cε3 and Cε4 domains most closely resembles those of other closed form Fcs. A salt bridge between Arg-342 and Asp-473 observed in other closed forms is retained, whereas a second salt bridge between Arg-440 and Glu-529 is present in three of four chains observed in the asymmetric unit.

Structure of a Covalently Closed IgE-Fc

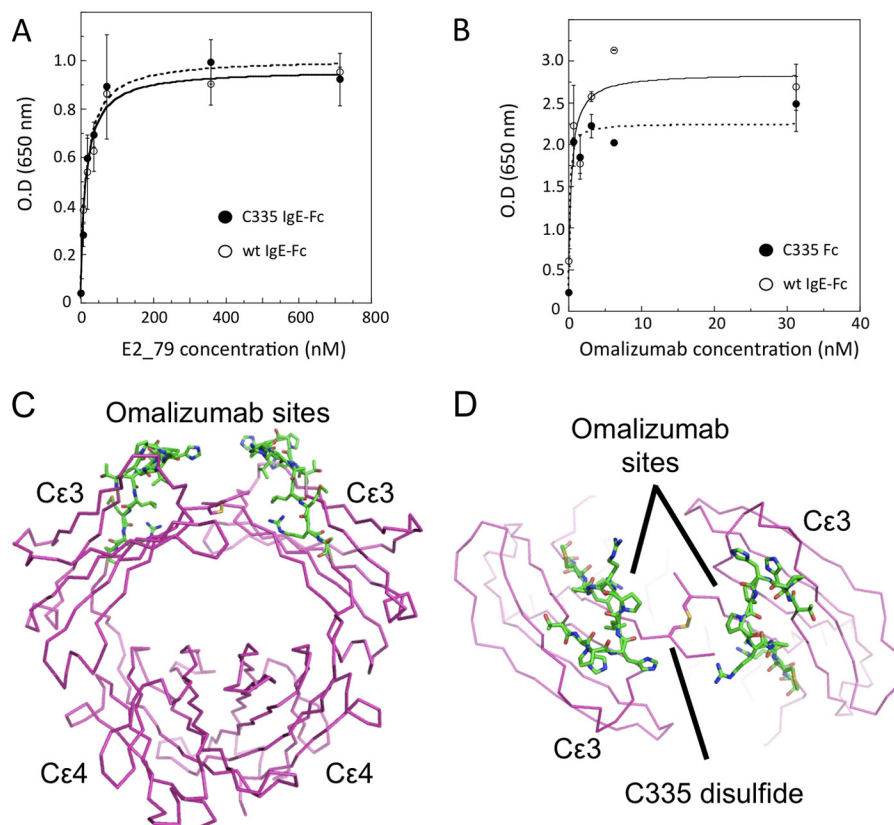


FIGURE 4. E2_79 and omalizumab bind to both WT and Cys-335 IgE-Fc proteins. *A*, E2_79 binds to both WT IgE-Fc (open circles, solid line) and Cys-335 IgE-Fc (filled circles, dashed line) in an ELISA assay. IgE-Fc proteins were plated and incubated with E2_79 at the indicated concentrations, and E2_79 binding was detected with an anti-His tag antibody. *B*, omalizumab binds to both WT IgE-Fc (open circles, solid line) and Cys-335 IgE-Fc (filled circles, dashed line) in an ELISA assay. IgE-Fc proteins were plated and incubated with omalizumab, and omalizumab binding was detected with a polyclonal anti-IgG antibody. *C* and *D*, side (*C*) and top (*D*) views of the omalizumab-binding region on the IgE-Fc Cε3 domain. The main chain conformation of the IgE-Fc Cys-335 structure and the disulfide bond at residue 335 are shown in magenta, whereas residues mapped to the omalizumab binding region are shown in stick representation. Omalizumab binding interactions have been mapped to IgE residues 421–432 including the FG loop residues 424–426 (HLP) that lie within the FcεRIα binding interface (33). Despite the conformational restriction imposed by the Cys-335 disulfide, the omalizumab epitope remains accessible to binding.

Each chain retains a small hydrophobic pocket at the elbow region, found in other closed form IgE-Fc structures (16).

Omalizumab and DARPin E2_79 Bind to Cys-335 IgE-Fc Similarly to WT IgE-Fc—Omalizumab (Xolair) and an engineered protein inhibitor, DARPin E2_79 (19–21), both bind to IgE and inhibit FcεRI binding. Anti-IgE DARPins, or engineered Designed Ankyrin Repeat Proteins (32), have been selected by ribosome display from large libraries of sequence variants. DARPins have been shown to bind IgE and inhibit receptor interactions and allergic reactions (19–21). We conducted binding experiments with the WT and Cys-335 IgE-Fc proteins, using omalizumab and the anti-IgE DARPin E2_79, to test whether the Cys-335 mutant could be recognized by these two IgE inhibitors (Fig. 4). E2_79 was able to bind both proteins equally well, with K_d values of ~20 nM, demonstrating that it does not recognize a site that is blocked by the Cys-335 disulfide bond (Fig. 4A). Omalizumab also exhibited binding to both WT and Cys-335 IgE-Fc proteins (Fig. 4B). Together, these data demonstrated that E2_79 and omalizumab exhibit binding profiles to the IgE-Fc that are different from FcεRI and that both protein inhibitors are not affected by locking the IgE-Fc_{3–4} into a closed conformational state. The omalizumab epitope on IgE has not been fully determined but has been mapped to Cε3 domain residues (residues 421–432) that overlap (33), but are

not identical to, the FcεRI-binding site (Fig. 4, *C* and *D*). These residues lie to one side of the Cε3 domain. The binding of omalizumab to the exterior of the Cε3 domain, engaging only one of the Cε3 loops involved in FcεRI binding (accessible in the Cys-335 structure), is consistent with the binding of omalizumab to the closed Cys-335 IgE-Fc. We have recently mapped the binding of E2_79 to the edge of the Cε3 domain by x-ray crystallography, which is also in accord with the ability of E2_79 to bind both WT and Cys-335 IgE-Fc proteins. The selective effect of the Cys-335 mutation on inhibiting FcεRI but not omalizumab or E2_79 binding further indicates that there are no gross conformational distortions of the Cys-335 IgE-Fc.

DISCUSSION

Structural studies have previously documented a range of IgE-Fc Cε3 domain and FcεRI-binding loop conformations, indicating that IgE antibodies populate an ensemble of diverse conformational states (16). The recent observation that the low affinity IgE receptor, CD23, binds to the IgE-Fc at the Cε3–Cε4 domain junction and competes with FcεRI through a potential allosteric mechanism (18) provides further evidence for the physiological importance of these conformational changes. Here, we engineered a disulfide bond into the IgE-Fc that stably but reversibly traps a closed, non-receptor binding state, dem-

onstrating the feasibility of controlling receptor binding through a conformational mechanism. Using disulfide bond scanning mutagenesis, we demonstrated that the flexibility of the IgE-Fc₃₋₄ allows disulfide bond formation to occur through every residue of the Cε2-Cε3 linker region (residues 328–335) but not at the first residue of the Cε3 domain (residue 336). The disulfide bond formed closest to the Cε3 domain (Cys-335) quantitatively forms an interchain disulfide bond. The structure of this covalently closed Fc is consistent with the observed conformational range documented in other IgE-Fc structures (16). The Cys-335 disulfide bond locks the IgE-Fc into a closed state incapable of receptor binding but does not induce any apparent distortions in the Fc structure. By contrast, a hinge deleted IgG-Fc exhibiting an altered Cγ2 domain arrangement shows significant distortions at the Cγ2-Cγ3 hinge region (7, 13). Interestingly, the Cys-335 IgE-Fc retains high affinity binding to the anti-IgE inhibitors omalizumab and DARPin E2₇₉, demonstrating the possibility of identifying selective, conformational state-dependent IgE ligands. This stabilized, closed conformation of the Cys-335 IgE-Fc may prove useful for identifying new monoclonal antibodies or synthetic proteins, such as the DARPins, that specifically recognize the closed conformational state and could act as allosteric inhibitors of the allergic response. In addition, the Cys-335 protein may prove useful in the search for novel, small molecule inhibitors that could selectively bind to the closed state, providing a novel route to inhibiting IgE-mediated allergic reactions.

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