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

Received for publication, August 2, 2012, and in revised form, August 29, 2012 Published, JBC Papers in Press, September 4, 2012, DOI 10.1074/jbc.M112.407502

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IgE antibodies interact with the high affinity IgE Fc receptor, FceRI, and activate inflammatory pathways associated with the allergic response. The IgE-Fc region, comprising the C-terminal domains of the IgE heavy chain, binds FceRI 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, FceRIα. 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 (FceRI) expressed on mast cells and basophils (1). Cross-linking of FceRI 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 FceRI 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 IgFc 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 (KD = ~10−9–10−10 m) to FceRI. 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 FceRI α-chain extracellular domains (FceRIα) have provided significant insights into their interactions. We have previously determined the crystal structures of the FceRIα (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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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-Fc3–4-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–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 IgE 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).

Gen Filteration 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 e280nm of 1.32 cm−1 (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% (v/w) PEG 4000, 2% (v/w) 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, 15% (w/v) ethylene glycol) and flash cooled in liquid nitrogen.

X-ray Data Collection, Molecular Replacement, and Refinement—Data were collected at −160 °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 P21, with unit cell dimensions a = 106.8 Å, b = 104.8 Å, c = 45.9 Å, and β = 96.2°. 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 Rmerge 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 Å using |Fo| > 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

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VOLUME 287 • NUMBER 43 • OCTOBER 19, 2012
building yielded a structure with an $R_{\text{work}}$ of 22.8% and an $R_{\text{free}}$ 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 $R_{\text{work}}$ of 19.9% and an $R_{\text{free}}$ 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 FceRIα—100 µl of purified FceRIα 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 62.5 nM) were added in duplicate to wells coated with Fc with 5% dry milk. WT or Cys-335 IgE-Fc samples (ranging from 0.1% (v/v) Tween 20 and blocked with the Tris/NaCl buffer containing 5% dry milk) were added to wells coated with FceRIα. The binding of IgE-Fc proteins to FceRIα 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 FceRIα 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% 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 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 Ce2-Ce3 Linker—Because previous x-ray crystal structures of the IgE-Fc$_{3–4}$ suggested that the IgE-Fc can adopt multiple conformational states in solution, populating intermediate states between closed and open arrangements of the Ce3 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-Fc$_{2–4}$, two interchain disulfides are formed between the Ce2 domain residue Cys-241 of one chain and the Ce2-Ce3 linker residue Cys-328 of the other chain. However, in the shorter IgE-Fc$_{3–4}$ protein that lacks residue Cys-241, a single interchain disulfide is instead formed between the linker region Cys-328 residues. To trap the Ce3 domains in the closed conformation, we systematically introduced cysteines into the Ce2-Ce3 linker region (residues 328–335) all the way up to and including the first Ce3 domain residue, 336 (Fig. 1, A–C). In previous free IgE-Fc$_{3–4}$ structures (9, 13, 16), the linker region was flexible and not observed, but in the complex structure with the high affinity recep-
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 Ce3 domain (linker residues 334 and 335 and Ce3 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 Ce3-Ce4 hinge or Ce3 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 Ce3 domains, locks the Fc in the closed state (Fig. 1D). We tested the binding of WT and Cys-335 IgE-Fc3–4 to purified soluble FcRI using gel filtration chromatography (Fig. 2, A–C). Free WT IgE-Fc3–4 and Cys-335 mutants behaved similarly, eluting with an apparent molecular mass of ~8.8 kDa (Fig. 2A). The soluble receptor α chain (FcRIα) 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).
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 \( P_2_1_1 \) and were observed (Fig. 2C, red trace). When the Cys-335 IgE-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 Ce3 domains to open. Although the native residue Gly-335 is in the Fc binding interface, the cysteine is compatible with receptor binding. Comparison of Cys-335 and WT IgE binding to Fc receptor, the complex peak was observed (Fig. 2D). In free IgE-Fc structures, 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 Ce3 domain movements involve a combination of an open-closed motion relative to the Ce4 domain and a swinging motion relative to the dimer axis and the other Ce3 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 Ce3 and Ce4 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.

### TABLE 1

| Crystallographic data and refinement statistics | Crystal | IgE-Fc Cys-335 |
|-----------------------------------------------|---------|---------------|
| **Space group**                               | \( P_2_1_1 \) |               |
| **Unit cell dimensions**                      |         |               |
| \( a \) (Å)                                   | 106.8   |               |
| \( b \) (Å)                                   | 104.8   |               |
| \( c \) (Å)                                   | 45.9    |               |
| \( \alpha, \beta, \gamma \) (°)               | 90, 96.2, 90 |               |
| **Molecules/asymmetric unit**                 | 2       |               |
| Resolution (Å)\( ^{a} \)                     | 30-2.61 | 2.72-2.61     |
| Completeness\( ^{b} \)                       | 97.1%   | 93.7%         |
| Reflections, unique (total)                   | 29,836  | (114,148)     |
| Average redundancy                            | 3.82    |               |
| \( <1/\sigma_I> \)                            | 14.04   |               |
| \( R_{cryst} \)\( ^{c} \)                    | 6.9%    | (35.4)        |
| Number of reflections (free)                  | 29,836  | (1,395)       |
| \( R_{cryst}/R_{free} \)\( ^{c} \)           | 19.9/25.8 |               |
| **Atoms, total**                              | 7,082   |               |
| Protein                                       | 6,723   |               |
| Water                                         | 126     |               |
| Carbohydrate                                  | 233     |               |
| **Average B factor (Å\(^{2}\))**              | 60.6    |               |
| Protein                                       | 40.3    |               |
| Water                                         | 88.2    |               |
| Carbohydrate                                  |         |               |
| **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.

\( ^{b} \) \( R_{cryst} \) = \( \sum \{[F(h) - |\langle F(h) \rangle|]/\sum |F(h)| \} \), where \( |F(h)| \) = the observed intensity, and \( |\langle F(h) \rangle| \) = the mean intensity for multiple measurements.

\( ^{c} \) \( R_{cryst}/R_{free} \) = \( \sum [\langle F(h) \rangle_{calc} - |\langle F(h) \rangle_{obs}]/\sum |\langle F(h) \rangle_{calc} | \) 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 Ce3 domains to open. Although the native residue Gly-335 is in the Fc binding interface, the cysteine is compatible with receptor binding. Comparison of Cys-335 and WT IgE binding to Fc receptor, the complex peak was observed (Fig. 2D). In free IgE-Fc structures, 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.

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The elbow region of the Cys-335 Fc corresponding to the linker between the Ce3 and Ce4 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.

**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-Fc 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 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 Ce3 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 Ce3 domains that allows FcεRIα binding.
Each chain retains a small hydrophobic pocket at the elbow region, found in other closed form IgE-Fc structures (16).

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**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 FcɛRI binding interface (33). Despite the conformational restriction imposed by the Cys-335 disulfide, the omalizumab epitope remains accessible to binding.

DISCUSSION

Structural studies have previously documented a range of IgE-Fc Cε3 domain and FcɛRI-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 FcɛRI 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-

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**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 FcɛRI binding interface (33). Despite the conformational restriction imposed by the Cys-335 disulfide, the omalizumab epitope remains accessible to binding.
Structure of a Covalently Closed IgE-Fc

Demonstrating the feasibility of controlling receptor binding through a conformational mechanism. Using disulfide bond scanning mutagenesis, we demonstrated that the flexibility of the IgE-Fc allows disulfide bond formation to occur through every residue of the Ce2-Ce3 linker region (residues 328–335) but not at the first residue of the Ce3 domain (residue 336). The disulfide bond formed closest to the Ce3 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 E2_79, 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.

Acknowledgments—We thank Hadar Feinberg for helpful discussions and past and present members of the Jardetzky Laboratory.

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