

Crystal structure of IgE bound to its B-cell receptor CD23 reveals a mechanism of reciprocal allosteric inhibition with high affinity receptor FcεRI

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The role of IgE in allergic disease mechanisms is performed principally through its interactions with two receptors, FcεRI on mast cells and basophils, and CD23 (FcεRII) on B cells. The former mediates allergic hypersensitivity, the latter regulates IgE levels, and both receptors, also expressed on antigen-presenting cells, contribute to allergen uptake and presentation to the immune system. We have solved the crystal structure of the soluble lectin-like “head” domain of CD23 (derCD23) bound to a subfragment of IgE-Fc consisting of the dimer of Cε3 and Cε4 domains (Fcε3-4). One CD23 head binds to each heavy chain at the interface between the two domains, explaining the known 2:1 stoichiometry and suggesting mechanisms for cross-linking membrane-bound trimeric CD23 by IgE, or membrane IgE by soluble trimeric forms of CD23, both of which may contribute to the regulation of IgE synthesis by B cells. The two symmetrically located binding sites are distant from the single FcεRI binding site, which lies at the opposite ends of the Cε3 domains. Structural comparisons with both free IgE-Fc and its FcεRI complex reveal not only that the conformational changes in IgE-Fc required for CD23 binding are incompatible with FcεRI binding, but also that the converse is true. The two binding sites are allosterically linked. We demonstrate experimentally the reciprocal inhibition of CD23 and FcεRI binding in solution and suggest that the mutual exclusion of receptor binding allows IgE to function independently through its two receptors.

antibody–receptor interactions | X-ray crystallography

IgE antibodies play a key role in the mechanisms of allergic disease, not only recognizing allergens through their Fab regions but also interacting via their Fc regions with two very different cell surface receptors (1). FcεRI, the receptor found on mast cells and basophils, binds IgE with high affinity ($K_A = 10^{10}$ – 10^{11} M^{−1}) and is responsible for allergic sensitization and the immediate (type I) hypersensitivity reaction in which minute amounts of allergen cross-link receptor-bound IgE and trigger cell degranulation. The IgE-binding α-chain of FcεRI consists of two extracellular Ig-like domains [sFcεRIα (1, 2)]. In contrast, CD23 (FcεRII), expressed on B cells, consists of three C-type lectin “head” domains connected to the membrane by a trimeric α-helical coiled-coil “stalk” (3). A single head domain binds to IgE-Fc with lower affinity ($K_A = 10^5$ – 10^6 M^{−1}) than FcεRI (4–8), although avidity of the trimer can substantially enhance this interaction (7, 9–11). Membrane CD23 (mCD23) is cleaved from the cell surface by endogenous proteases such as ADAM10 (12, 13) to yield soluble trimeric and monomeric forms (sCD23), which have been implicated in both positive and negative feedback mechanisms for the regulation of IgE synthesis by B cells that have switched to IgE production (1, 7, 8, 14–16). Both FcεRI and CD23 are also expressed on a range of antigen-presenting cells (APCs), where they play similar roles in trapping IgE–allergen complexes and promoting the allergic response (1, 14, 15), but the functional interplay—cooperation or competition—between these two receptors in the context of APCs is not well understood. CD23 expressed on B cells also has the potential to contribute to the clinically serious phenomenon of the spreading

of allergic reactivity to unrelated allergens, through its ability to internalize IgE–allergen complexes irrespective of the allergen, in contrast to mIgE-mediated allergen-specific presentation through the B-cell receptor (1). CD23 expressed on gastrointestinal epithelial cells also contributes to IgE–allergen transport across the gut epithelial barrier to trigger food allergenic reactions (17) and similarly on respiratory tract epithelial cells to contribute to airway allergic inflammation (18). Understanding the IgE–CD23 interaction thus has implications for many aspects of allergic disease.

Both receptors bind to the Cε3 domains of IgE-Fc (1, 6, 19–21). Cε2 was also implicated in FcεRI binding (5), but crystal structures of the sFcεRIα–Fcε3-4 complex (20) (Fcε3-4 is a subfragment of IgE-Fc consisting of a dimer of the Cε3 and Cε4 domains) and most recently the sFcεRIα–IgE-Fc complex, including the Cε2 domains, show that the Cε2 domains exert their influence only indirectly upon the formation of this 1:1 complex (21). The location of the CD23 binding site has also been mapped to Cε3 by mutagenesis (22), and monomeric sCD23 has been shown to bind to Fcε3-4 and IgE-Fc with 2:1 stoichiometry (4, 7). The fact that sCD23 can compete with FcεRI binding, albeit at high concentrations (23), was thought to be due to overlap of the two receptor binding sites. This competition was observed at considerably lower concentrations of a trimeric sCD23 molecule, presumably owing to the avidity effect (10, 11), and subsequently an intriguing effect of temperature upon the relative affinities of IgE for FcεRI and CD23 was discovered (24). We now report the crystal structure of the complex between the head domain of CD23 [termed derCD23 because it corresponds to the sCD23 fragment generated by the house dust mite allergenic protease *Der p* 1 (25)] and Fcε3-4. This 2:1 complex unexpectedly reveals a direct involvement of the Cε4 domain and also shows that the binding sites for FcεRI and CD23 are remote from each other, at opposite ends of the Cε3 domains. Furthermore, the binding of IgE to either receptor precludes interaction with the other, although an allosteric mechanism, which also provides an explanation for the temperature effect. This mutual exclusion of CD23 and FcεRI binding is important for the functioning of IgE.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4EZM).

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Results

Overall Topology of the Complex. The structure of the complex was determined at 3.1-Å resolution and reveals one derCD23 molecule bound to each heavy chain at the interface between Ce3 and Ce4, making contact with residues from both domains (Fig. 1 and Table S1; Fig. S1 shows the electron density map at the interface). The crystal form contained three independent copies of the complex in the asymmetric unit, and the six heavy chains (labeled A to F) bound to six derCD23 heads (labeled G to L) showed essentially identical modes of interaction, exemplified by the structure depicted (Fig. 1; chains A, B, G, and H). The angle between the Ce3 and Ce4 domains varies only slightly between the six heavy chains (Fig. S24), and there are virtually no differences between the six derCD23 head domains (Fig. S2B). All three complexes thus display approximately twofold symmetry, although one (consisting of chains C, D, I, and J) includes the two “extremes” in terms of the angle between the Ce3 and Ce4 domains, but these differ by only 7°. On all six heavy chains, electron density was present for five *N*-linked sugar units at Asn394 ([*N*-acetylglucosamine]₂-Mannose)₃; Fig. 1), and an additional two mannose residues were visible on chain C.

Flexibility in the Ce3–Ce4 interdomain angle has been well-documented through studies of unliganded Fce3-4 structures in different crystal forms (26, 27), as well as the unliganded IgE-Fc structure (28) and the sFceRIα complexes with both Fce3-4 and IgE-Fc (20, 21). Among all of these structures the interdomain angle varies over a range of ~25° between the most “open” (in the sFceRIα complexes) and the most “closed” (chain D in the derCD23 complex reported here) (Fig. S3 A–D). Previously, the most closed conformation had been seen in one of the unliganded Fce3-4 structures [chain D of PDB 3HA0 (27)], and the conformations seen in the derCD23 complex all range within 3° or 4° of this closed conformation (Fig. S3D).

Nature of the IgE/CD23 Interface. The 2:1 stoichiometry agrees with earlier measurements in solution by analytical ultracentrifugation, recorded for both Fce3-4 (4) and IgE-Fc (7), but direct contact with Ce4 was not anticipated by previous mutagenesis or peptide inhibition studies. Three salt bridges between Fce3-4 and derCD23 (Asp409-Arg188, Glu412-Arg188, and Glu412-Arg224) and a potential fourth (Glu414-His186 if protonated), together with four additional hydrogen bonds (Arg376-Tyr189, Asp409-Tyr189, and Arg440-Ser254, both side-chain and main-chain) dominate the interaction (Fig. 2 and Fig. S1). In the four salt bridges, all negative charges reside on IgE, all positive charges on CD23. Arg440, although H-bonding to Ser254 of derCD23, retains its intrachain salt bridge interaction with Glu529 of Ce4 that is seen in other,

unliganded Fce3-4 structures (27). These salt-bridging and hydrogen-bonding residues of IgE are not conserved in the other human antibody classes (Fig. S4), consistent with the specificity of CD23 for IgE.

Remarkably few additional residues make well-defined van der Waals contacts in all six interfaces (Ile411, Gly413, Pro439, Glu529, and Gln535 in Fce3-4; Trp184, Val185, and Asp227 in derCD23), and the surface complementarity is poor [calculated *Sc* values for the six interfaces range from 0.63 to 0.71 (29, 30)]; indeed there is a substantial cavity at the interface between Glu412 (in Fce3-4), Arg224, and the Cys259-Cys273 disulphide bridge (in derCD23). The buried surface area for each interaction ranges from 860 to 890 Å² for all except one (chains F and L) at 920 Å²; in this latter case the derCD23 loop at residues Ser256 and Glu257 is not as disordered as it is in the other five independent views of the interaction. The Ce3 domain dominates the interface with 63% of the contact area, a further 25% involves the linker region (residues 437–440), and Ce4 contributes 12%. All of the salt-bridge and hydrogen-bond interactions involve the Ce3 domain with the exception of Arg440 in the linker region, whereas Ce4 contributes only van der Waals interactions. Earlier studies had implicated the AB loop of Ce3, and residue Lys352 in particular, in CD23 binding (22), but this loop is not directly involved at all, and Lys352 makes no contact with CD23; any effects of mutagenesis in this loop must therefore be indirect. It is the EF loop/helix of the Ce3 domain that is centrally placed at the interface (Fig. 2). Surprisingly, no Ca²⁺ ions were found bound to derCD23 in the complex; the role of Ca²⁺ in the CD23–IgE interaction will be discussed later.

Conformational Change upon CD23 Binding to IgE-Fc. In the free state, IgE-Fc adopts an asymmetrically bent structure with the (Ce2)₂ domain pair folded back against one of the Ce3 domains (28), but although the two Ce3–Ce4 pairs have different interdomain angles, both are more open than that seen in the derCD23–Fce3-4 complex (Fig. S34). To assess whether the presence of the Ce2 domains might be expected to affect derCD23 binding, the (Ce2)₂ domain pair was modeled onto the derCD23–Fce3-4 complex. This was achieved by superimposing the Ce3 domain of free IgE-Fc that contacts the (Ce2)₂ domain pair onto the Ce3 domain (chain A) of the derCD23–Fce3-4 complex. One of the derCD23 molecules (chain G) lay very close to the Ce2 domains, with a minor rearrangement of the N-terminal residues of Ce2 required to prevent a steric clash. Contact between side chains of this derCD23 molecule and Ce2 in a complex with IgE-Fc (or indeed IgE) certainly cannot be ruled out; the other bound derCD23 molecule (chain H), however, lay far from the Ce2 domains. Modeling of the Ce2 domains onto the Fce3-4 complex also revealed that the AB loop of Ce2 (chain A) would have to adopt a slightly different conformation to avoid clashing with Ce3 (chain B) in the very closed conformation that this Ce3 domain adopts in the complex.

Mutual Exclusion of FceRI and CD23 Binding to IgE-Fc. The closed conformation for the Ce3 domains in the derCD23 complex is clearly incompatible with FceRI binding. This may be seen by superimposing the derCD23–Fce3-4 and the sFceRIα–Fce3-4 (20) complexes on their (Ce4)₂ domain pair and noting the very different orientations of the Ce3 domains (Fig. 34). The steric clashing of sFceRIα with the Ce3 domains in their CD23-bound conformation, and of both derCD23 molecules with the Ce3 and Ce4 domains in their sFceRIα-bound conformations, is clear from the superposition of the complexes upon one or other of the Ce3 domains (Fig. 3 B and C). Thus, IgE cannot bind both FceRI and CD23 simultaneously; binding of sFceRIα causes conformational changes that are incompatible with binding of *either* derCD23 molecule, and the binding of *either* derCD23 molecule ensures that the two subsites required for high-affinity binding of FceRI are not both accessible.

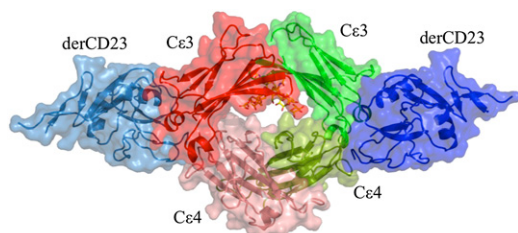


Fig. 1. Structure of the derCD23–Fce3-4 complex. The two molecules of derCD23 (light and dark blue α traces with surfaces) bind one to each heavy chain between the Ce3 (dark red and green) and Ce4 domains (light red and green). The carbohydrate is shown in all-atom representation (red and yellow, without surfaces) and can be seen behind the (red) Ce3 domain. The adjacent N and C termini of each derCD23 molecule, the former being the connection to the “stalk,” the latter to the “tail” region, can be seen at the extreme left and right of the figure. [The complex shown here comprises chains A (red), B (green), G (light blue), and H (dark blue)].

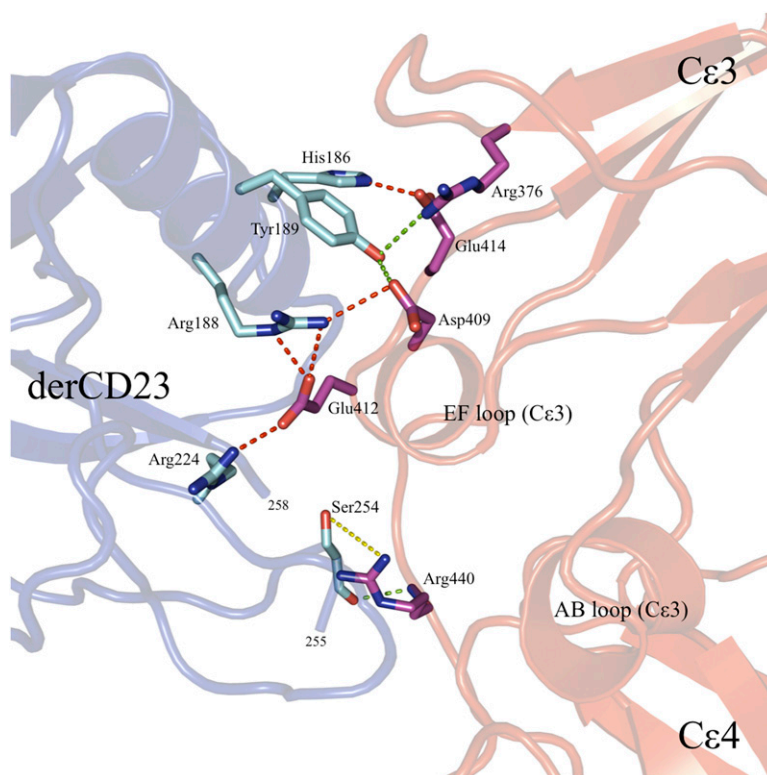


Fig. 2. Salt bridges and hydrogen bonds at the derCD23–Fcε3-4 interface. The H-bonds associated with the four salt bridges are shown in red, additional H-bonds present in all six independent interactions are shown in green, and a further H-bond present in five of six molecules is shown in yellow.

This conclusion was verified experimentally in solution using acceptor fluorophore-labeled IgE-Fc or Fcε3-4 and competitively displacing either donor fluorophore-labeled derCD23 by unlabeled sFcεRIα, or labeled sFcεRIα by unlabeled derCD23

(Fig. 4). It can be seen that sFcεRIα readily and completely displaces derCD23 from the complex with either IgE-Fc or Fcε3-4. Similarly, derCD23 can displace sFcεRIα almost completely from either IgE-Fc or Fcε3-4, but at very much higher concentrations as

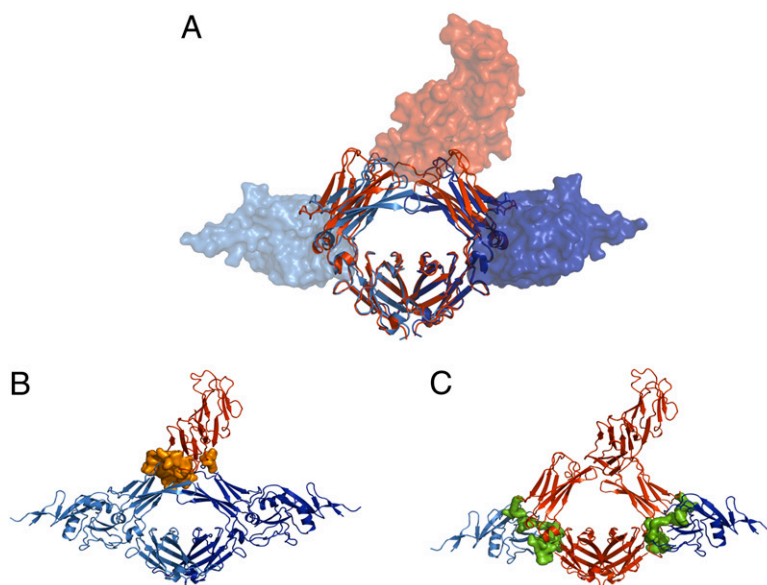


Fig. 3. Composite images of the derCD23 and sFcεRIα complexes with Fcε3-4 to show the mutual incompatibility of their binding modes. (A) derCD23–Fcε3-4 and sFcεRIα–Fcε3-4 (Protein Data Bank ID code 1F6A) complexes superposed on their (Cε4)₂ domain pairs. The receptors are shown as surfaces (derCD23, light and dark blue; sFcεRIα, red), and the Fcε3-4 structures are shown as Cα traces (in corresponding colors). The closed (derCD23-binding) and open (sFcεRIα-binding) conformations of the Cε3 domains may be seen. (B) Steric clashes between the sFcεRIα structure (red Cα trace) and both chains of the derCD23–Fcε3-4 complex (blue) are indicated (orange surfaces). (C) Steric clashes of both derCD23 molecules (blue Cα traces) with the sFcεRIα–Fcε3-4 complex (red) are indicated (green surfaces).

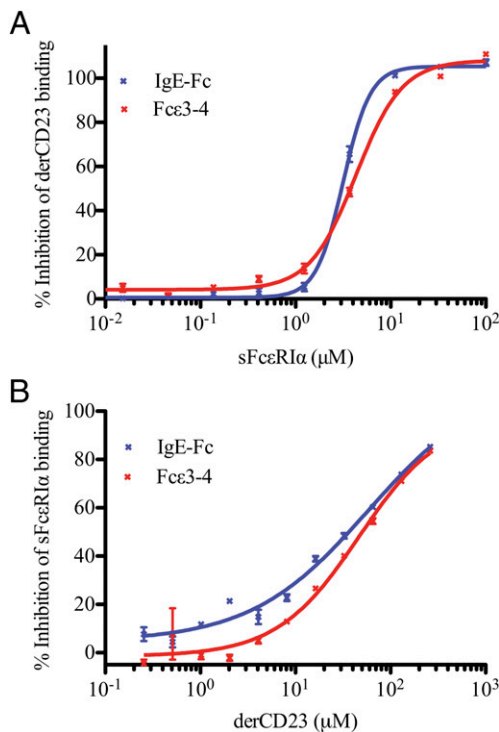


Fig. 4. sFcεRIα displaces derCD23 and vice versa in a FRET inhibition assay. (A) Competition of derCD23 binding to IgE-Fc (blue) and Fcε3-4 (red) by unlabeled sFcεRIα. (B) Competition of sFcεRIα binding to IgE-Fc (blue) and Fcε3-4 (red) by unlabeled derCD23. All data: $n = 3$; error bars $\pm$ SEM.

expected from the considerably lower binding affinity of derCD23. Positive controls for these FRET measurements were provided by displacement of labeled sFcεRIα by unlabeled sFcεRIα, and labeled derCD23 by unlabeled derCD23, which are shown together with the raw FRET data (Fig. S5 A–D).

Discussion

The interaction between IgE and CD23 is critically involved in the allergic response at several stages, including allergen presentation, the regulation of IgE synthesis, and transport of IgE and immune complexes across epithelial barriers in the gut and airways (1, 14, 15, 17, 18). At the cell surface, mCD23 is trimeric (9, 31, 32), and sCD23 fragments shed from the membrane that contain sufficient stalk region are also trimeric (31), although the structure of the trimer has only been modeled either on the basis of the structures of other C-type lectins (25) or guided by NMR chemical shift data (6). In the crystal structure of the complex reported here, two derCD23 “heads” bind to IgE, one to each heavy chain at a location between the Cε3 and Cε4 domains, and remote from the FcεRI binding site. The interaction is predominantly hydrophilic and dominated by salt bridges between positively charged CD23 residues and negatively charged IgE residues, despite the overall net positive charge (+9) of the Cε3 domain. The site on CD23, in agreement with that identified by NMR chemical shift mapping by titration of ¹⁵N-labeled derCD23 with monomeric Cε3 (6) (titration with IgE-Fc led to the formation of high molecular weight oligomers), is diametrically opposed to the connection to the α-helical coiled-coil stalk region (Fig. 1). This topology is such that an IgE molecule could not engage two heads from the same (modeled) CD23 trimer [as depicted in earlier cartoons (10, 33)] but could readily cross-link two mCD23 molecules. (The distance of 136 Å between the Cα atoms of the N-terminal Phe158 residues of the two derCD23 molecules, which are immediately adjacent to the top of the predicted α-helical coiled-coil stalk region (3), is

such that it would require unraveling of approximately 30 residues, or almost one third of the stalk, to allow two heads from the same CD23 trimer to bind to the same IgE-Fc.) Furthermore, the exposed location of the IgE-binding site on derCD23 strongly suggests that trimeric sCD23 could cross-link IgE molecules either in solution or as mIgE on the cell surface, but a definitive statement must await determination of the structure of the CD23 trimer.

The binding of derCD23 in the cleft between the Cε3 and Cε4 domains fixes the angle between these two domains. Comparisons between Fcε3-4 and IgE-Fc structures (the latter including Cε2 domains), both free and bound to sFcεRIα (20, 21, 26–28), show that whereas the (Cε4)₂ domain pair display no conformational variation and provide a fixed point of reference, the Cε3 domains can adopt a range of “open” and “closed” conformations. The most open of these have been found in the complexes of Fcε3-4 and IgE-Fc with sFcεRIα (20, 21) (Fig. S3 A–D) and occur in both ε-chains because this receptor engages with both Cε3 domains (Fig. 3A). In contrast, CD23 binding causes the Cε3 domains to adopt the most closed conformation that has yet been observed in any Fcε3-4 or IgE-Fc structure. This opening and closing of the Cε3 domains in fact results from a flexing *within* the Cε3 domain, an effect first noted in earlier structural comparisons (26, 27). The AB loop/helix of Cε3 interacts closely with Cε4 (Fig. 2), and a change in the relative orientation of the EF loop/helix, which is contacted by derCD23 (Fig. 2), can clearly be seen when the FcεRI and CD23 complexes are compared (Fig. 3A). This intradomain flexibility may be a result of the unique packing/stability profile of Cε3 compared with all other C_H domains (34), and in fact the isolated Cε3 domain displays molten globule-like characteristics (35, 36).

The consequence of these extreme open (FcεRI-bound) and closed (CD23-bound) conformations for the Cε3 domains of IgE is that the binding of the two receptors are incompatible with each other (Fig. 3A–C). Clearly this is not, as previously thought, due to overlapping binding sites, but to an allosteric linkage between the two distant sites. The idea that the open and closed conformations might interact differently with FcεRI and CD23 was in fact anticipated by Conrad et al. (24) after their surprising discovery that CD23 bound more strongly to IgE at 4 °C than 37 °C, whereas the opposite was true for FcεRI. They hypothesized that a change in the relative proportion of these open and closed conformational states might account for this temperature effect, and it will certainly be revealing to explore further the temperature dependence of the kinetics and thermodynamics of these receptor interactions as well as the conformational dynamics of the IgE molecule, particularly with respect to the Cε3 domains.

IgE-Fc and IgE of course additionally contain the Cε2 domains, and it is important to consider their effect upon the crystallographic results presented here for Fcε3-4. In IgE-Fc the (Cε2)₂ domain pair packs asymmetrically against one of the Cε3 domains in the bent IgE-Fc structure (28) and moves together with that Cε3 domain when it opens up to accommodate sFcεRIα binding (21). Modeling the (Cε2)₂ domains onto the derCD23–Fcε3-4 complex shows that although there is no steric conflict that would prevent binding of either derCD23 molecule, the Cε2 domains lie immediately adjacent to one of the derCD23 molecules and suggest that an interaction (stabilizing or destabilizing) might occur. This is consistent with the observed 2:1 stoichiometry for derCD23 binding to both Fcε3-4 and IgE-Fc (4, 7). Although there is no evidence of any significant difference between the binding affinity of derCD23 for Fcε3-4, IgE-Fc, or IgE (4–7), or between the binding of the two derCD23 molecules to IgE-Fc, a slightly faster on-rate for derCD23 binding to Fcε3-4 compared with both IgE-Fc and IgE has been reported (5). The biphasicity or dual affinity of CD23 binding to IgE that has been reported (9, 37) is a function of its oligomeric state and ability to engage IgE through more than one head, although as pointed out above, two heads of one CD23 almost certainly cannot engage the same IgE molecule, and the

avidity effect must result from trimeric CD23 binding to more than one IgE molecule.

The structural comparison of the derCD23 and sFcεRIα complexes (Fig. 3 *B* and *C*) also shows that sFcεRIα binding prevents binding of *both* derCD23 molecules, and similarly that binding of *either* derCD23 molecule will prevent sFcεRIα binding. The experimental data presented here (Fig. 4) confirm this observation. sFcεRIα readily displaces derCD23 and, as expected, at approximately equimolar (5-μM) concentrations of sFcεRIα and IgE-Fc (or Fcε3-4) the displacement of derCD23 is virtually complete because this concentration of sFcεRIα is very much greater than the $K_D \sim 1$ nM for sFcεRIα binding. [The difference between the two curves in Fig. 4A may reflect the slightly lower affinity reported for sFcεRIα binding to Fcε3-4 compared with IgE-Fc (5, 21, 38)]. derCD23 can similarly cause almost complete displacement of sFcεRIα, although much higher concentrations are required, greater than 100 μM, to overcome its lower affinity ($K_D \sim 10^{-5}$ – 10^{-6} M) (4–8).

CD23 belongs to the C-type (calcium-dependent) lectin superfamily, and the presence of Ca^{2+} is known to enhance the affinity for IgE approximately sevenfold (6, 39), although it is not essential for binding. A crystal structure of the head domain with a single bound Ca^{2+} ion has been solved, together with the Ca^{2+} -free form (40), and an NMR structure also reports Ca^{2+} binding, but at an alternative site (6). However, no Ca^{2+} ions were observed in any of the six derCD23 molecules in the Fcε3-4 complex, despite the presence of 2 mM Ca^{2+} in the crystallization medium. In this regard, the existence of a disordered loop in derCD23 at the edge of the interface [residues 256–257 of loop 4, following earlier terminology (40)] is intriguing. The crystal structure of the lectin head domain showed Ca^{2+} bound at a site involving residues Glu249 and Thr251 of loop 4 (40). In that structure, residues 253–257 of loop 4 were also disordered, paradoxically becoming ordered in the *absence* of Ca^{2+} owing to a rearrangement of the side chain of Arg253, which occupied the Ca^{2+} site. The conformation of loop 4 seen in the complex differs from either the apo- or Ca^{2+} -bound structures; together with the adjacent loop 1, they represent the only main-chain conformational changes in derCD23 upon Fcε3-4 binding (Fig. S6). The role of Ca^{2+} in IgE binding to CD23 thus remains unresolved at present, but it is tempting to speculate that it may involve reorganization of residues of the flexible loop 4 in the context of the complex, providing additional protein–protein interactions and thus enhancing affinity. There are precedents for the stabilizing effects of Ca^{2+} upon this and other loops in C-type lectin domains, such as mannose-binding protein, in which carbohydrate binding is affected (41), and others where the binding of a protein partner is affected (42).

What is the functional significance of IgE's inability to engage both FcεRI and CD23 simultaneously? Although the affinity of derCD23 for IgE is considerably lower than that of FcεRI, avidity effects for trimeric CD23 can considerably enhance its ability to compete with FcεRI binding (7, 9–11). Indeed, a recombinant sCD23 species trimerized via a leucine-zipper motif, termed *lz*CD23, more effectively inhibited IgE binding to mast cells expressing FcεRI (10, 11). The mutual exclusion of FcεRI and CD23 binding to IgE is essential to prevent mast cell and basophil activation by trimeric sCD23, which could otherwise cross-link FcεRI-bound IgE on these cells. Similarly, it ensures that FcεRI cannot be cross-linked either by soluble IgE–CD23 complexes, or IgE bound to mCD23 (on B cells or APCs) in the absence of allergen. The mutual exclusion of FcεRI and CD23 binding is thus an important aspect of IgE biology, allowing it to function independently through its two receptors.

Furthermore, the location of the CD23 binding sites at points where the two heavy chains are most widely separated (in contrast to the FcεRI binding site, where the chains approach each other most closely) and the connections to the stalk region (Fig. 1),

maximize the propensity for cross-linking of mIgE on B cells committed to IgE synthesis by trimeric sCD23, and also mCD23 on B cells by IgE or IgE–allergen complexes. We have hypothesized that through such interactions, the former leading to up-regulation of IgE synthesis and the latter to down-regulation, CD23 contributes to the mechanism of IgE homeostasis (43), and this notion has received experimental support from studies with monomeric and oligomeric sCD23 species (7, 8, 16, 44). The structure of the complex is also consistent with the cocross-linking of mCD21 and mIgE by trimeric sCD23 (proposed to enhance IgE up-regulation), because CD21 binds to the “tail” sequence that is, although only partially present in derCD23, located adjacent to the connection to the stalk (Fig. 1) (6). However, the precise spatial arrangement of the IgE and CD21 binding sites on each derCD23 head and the relative positions of the heads in the CD23 trimer (as yet only modeled), together with the fact that two heads from one trimer almost certainly cannot engage a single IgE molecule, undoubtedly places topological constraints upon the formation of signaling complexes of these three molecules at the B-cell surface (1)—a process that clearly requires further investigation.

The crystal structure of the derCD23–Fcε3-4 complex reported here provides a structural basis for designing inhibitors with the potential to interfere with IgE regulation and other CD23-mediated processes. Of wider significance to IgE and allergic disease, the structure also reveals the mechanism for an allosteric connection between the distantly located binding sites for IgE's two principal receptors and demonstrates that allosteric inhibition is a viable strategy to target either of these receptor interactions for therapeutic purposes.

Materials and Methods

Protein Expression and Purification. Recombinant human derCD23 (Ser156–Glu298) was expressed, refolded, and dialyzed into 25 mM Tris-HCl (pH 7.5) (“purification buffer”) as previously described (6). It was purified on a heparin-Sepharose column (GE Healthcare) preequilibrated with purification buffer, eluting with 25 mM Tris-HCl (pH 7.5), 200 mM NaCl. Fractions were pooled, concentrated to 1 mL, and loaded onto a HiLoad 16/60 Superdex G75 column (GE Healthcare), preequilibrated, and subsequently washed with purification buffer. Folding was assessed by $^1\text{D}^1\text{H}$ NMR at 500 MHz (large dispersion and strong signals of methyl groups between 1.0 and -1.0 ppm). Human IgE-Fc (N265Q, N371Q) was expressed in NS0 cells and purified by affinity chromatography with sFcεRIα-IgG₄-Fc fusion protein as previously described (4, 45). The genes for recombinant human Fcε3-4 (Cys328–Lys547, with N-terminal ADP) and sFcεRIα-Cys-His (Val1–Lys176, with C-terminal cysteine and His₆ tag) were synthesized by DNA2.0 and cloned as HindIII/EcoRI fragments into proprietary mammalian expression vectors. The DNAs were transiently transfected into HEK293 cells using 293-fectin (Life Technologies) according to the manufacturer's instructions, and the supernatants were harvested 6 d after transfection. Human Fcε3-4 was purified by cation exchange chromatography on a SPHP matrix (GE Healthcare) in 50 mM NaOAc buffer (pH 6.0), followed by gel filtration on a Superdex S200 matrix (GE Healthcare) in PBS (pH 7.4). Human sFcεRIα-Cys-His was purified on a Ni-NTA column (Qiagen), followed by gel filtration on a Superdex S200 matrix (GE Healthcare) in PBS (pH 7.4), and stored under nitrogen to prevent reactivity of the free cysteine residue.

Crystallization and Data Collection. Fcε3-4 was concentrated to 20 mg/mL, and derCD23 to 18 mg/mL, in 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 0.05% sodium azide (“crystallization buffer”). The complex was formed with 0.4 mM derCD23 (6.2 mg/mL), 0.2 mM Fcε3-4 (10 mg/mL), and 4 mM CaCl_2 , diluted with an equal volume of 3% (wt/vol) PEG 8,000, 0.1 M Tris-HCl (pH) 7.5 as the precipitant. Crystals grew to ~ 400 μm within 8 d at 295 K, were flash-cooled to 100 K [using 14% (wt/vol) PEG 8,000, 0.1 M Tris-HCl (pH) 7.5, and 30% (vol/vol) PEG 200] and data collected at beamlines I02 and I04, Diamond Light Source. Data processing and structure determination details are provided in *SI Materials and Methods*.

FRET Assay. Labeling of the proteins is described in *SI Materials and Methods*. Inhibition assays were performed by competing 5% of 1 μM terbium-labeled (46) derCD23 and 5% of 5 μM Alexa Fluor 647-labeled IgE-Fc or Fcε3-4 with a dilution series of unlabeled sFcεRIα-Cys-His. Assays were conducted in 384-well hi-base, white plates (Greiner BioOne) using Lanthascreen buffer

(Invitrogen) as a diluent. The plate was left to incubate for 1 h at room temperature with shaking and read by the Artemis plate reader (Berthold Technologies). Time resolved-FRET ratios were then calculated for each well as the emission of acceptor at 665 nm divided by the emission of donor at 620 nm multiplied by 10^4 . Data were analyzed using GraphPad Prism 5. Similarly, 2 nM terbium-labeled sFc ϵ R1 α and 10 nM Alexa Fluor 647-labeled IgE-Fc or Fc ϵ 3-4 were competed with a dilution series of unlabeled derCD23. Assays were conducted as described above, with the exception of an overnight incubation. Positive controls for the two experiments were provided by displacement

of labeled sFc ϵ R1 α by unlabeled sFc ϵ R1 α , and labeled derCD23 by unlabeled derCD23 (Fig. S5 B and D); these defined the start- and end-point values for each titration, which were treated as zero and 100% inhibition for calculation of the “% inhibition” values for displacement of one receptor by the other (Fig. 4 A and B).

ACKNOWLEDGMENTS. This study was supported by the Wellcome Trust, Asthma UK, the Medical Research Council (United Kingdom), and by Diamond Light Source.

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Supporting Information

Dhaliwal et al. 10.1073/pnas.1207278109

Materials and Methods

Crystallization. Crystals with a different morphology were also found after 6 d with 12% (wt/vol) PEG 4,000, 0.1 M Tris-HCl (pH 8.75), and 0.2 M sodium acetate trihydrate as precipitant after streak seeding. Diffraction data were collected to 3.6 Å, and the crystal form was found to be the same as that reported in Table S1.

Crystallographic Data Collection and Structure Determination. Indexing, integration, and merging of data to 3.1 Å resolution were carried out with the *HKL2000* suite of programs (1). Nine previously determined Fcε3-4 dimers (2–4) were used as search models in molecular replacement. However, because of the high degree of conformational flexibility of the two Fcε3-4 chains (4), 14 additional dimers were manually generated from crystallographically independent chains and were also used as search models. Solutions were only found using the “closed” conformations of the Fcε3-4 dimer. For example, using a dimer generated from chain A of Protein Data Bank (PDB) 3H9Z, two copies of the Fcε3-4 dimer were identified in the asymmetric unit using *PHASER* (5). Subsequently, four derCD23 (PDB 2H2R) molecules were located. A third derCD23–Fcε3-4 complex could only be found by searching with one of the previously located complexes.

Because of the high degree of non-crystallographic symmetry (NCS), reflections were selected for the R_{free} set in thin resolution shells (6) using *SFTOOLS* (7). Iterative cycles of refinement using *PHENIX* (8), *REFMAC5* (9), and *BUSTER-TNT* (10) alternated with manual model building with *COOT* (11). To minimize bias, the model was built into $2F_o - F_c$ composite

omit, $2F_o - F_c$ and $F_o - F_c$ electron density maps. Initially during refinement, tight NCS restraints were used. These were gradually relaxed, and finally local structure similarity restraints (or “local NCS”) (9, 12) were applied. TLS groups (13) were generated using the *TLSMD* Web server (14). Carbohydrate atoms and a single water molecule were subsequently incorporated into the structure. No electron density was observed for Fcε3-4 residues 363–364 of chain B; 367–371 and 421–428 of chain C; 367–370, 419–422, 429–430, 479–480, and 517–519 of chain E; and derCD23 residues 256–257 of chains G, H, I, J, and K. Therefore, these residues were not built into the model. Refinement statistics are shown in Table S1.

Labeling of Proteins for FRET Assay. IgE-Fc and Fcε3-4 were labeled with acceptor fluorophore by reacting 4 mg/mL protein in 100 mM sodium bicarbonate, 50 mM NaCl (pH 9.3), with a fivefold molar excess of Alexa Fluor 647 succinimidyl ester (Invitrogen). After 1 h incubation at room temperature with agitation, excess unreacted fluorophore was removed by dialyzing into HBS (10 mM Hepes, 150 mM NaCl, and 4 mM CaCl₂, pH 7.4). derCD23 and the sFcεRIα-Cys-His mutant were labeled with donor fluorophore. derCD23 at 3.5 mg/mL in 100 mM Hepes and 125 mM NaCl (pH 8.3) was reacted with a fivefold molar excess of terbium chelate isothiocyanate (Invitrogen) for a 3-h incubation at room temperature with agitation. Excess fluorophore was removed by dialyzing into HBS. sFcεRIα-Cys-His at 5 mg/mL in PBS [20 mM PBS (pH 7.4)] was reacted with a 10-fold molar excess of terbium chelate maleimide (Invitrogen) overnight at 4 °C via thiol coupling.

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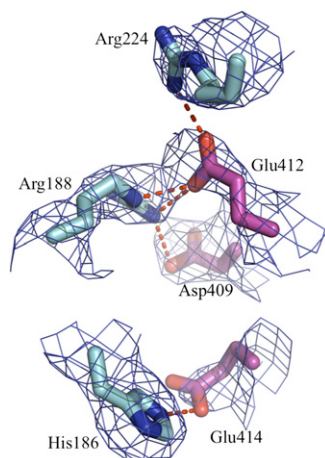


Fig. S1. Electron density in the interface region. Electron density $2F_o - F_c$ map contoured at 1σ for key side chains involved in salt bridges at the derCD23/Fc ϵ 3-4 interface. The side chains shown in blue are from derCD23, those in purple from Fc ϵ 3-4.

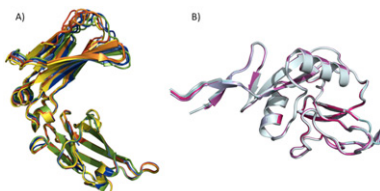


Fig. S2. (A) The six independent Fc ϵ 3-4 heavy chains superposed on their C ϵ 4 domains, showing the variation in the relative positions of the C ϵ 3 domains. (B) The six independent derCD23 molecules superposed, showing virtually no overall structural variation.

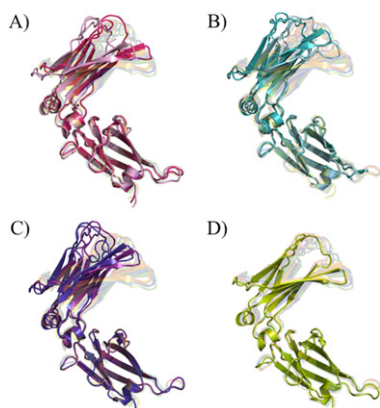


Fig. S3. Comparison of Fc ϵ 3-4 heavy chains taken from various Fc ϵ 3-4 and IgE-Fc structures superposed by their C ϵ 4 domains upon the set of six independent heavy chains of the derCD23-Fc ϵ 3-4 complex (shown lightly colored). (A) Free IgE-Fc (light and dark red) [PDB 2WQR (1)]; (B) sFc ϵ RI α -IgE-Fc complex (light and dark cyan) [PDB 2Y7Q (2)]; (C) sFc ϵ RI α -Fc ϵ 3-4 complex (light and dark purple) [PDB 1F6A (3)]; (D) single chain of free Fc ϵ 3-4 (green), the most closed structure observed to date [PDB 3HA0 (4)].

1. Wan T, et al. (2002) The crystal structure of IgE Fc reveals an asymmetrically bent conformation. *Nat Immunol* 3:681–686.
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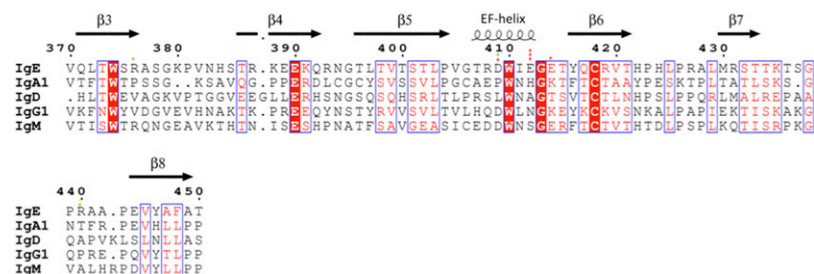


Fig. S4. Alignment of the region of the heavy chain of human IgE that includes the salt-bridge and hydrogen-bonding residues involved in CD23 binding, with human IgA1, IgD, IgG1, and IgM sequences (SwissProt IDs P01854, P01876, P01880, P01857, and P01871, respectively), drawn with *ESPrnt* (1). Residues are numbered with reference to IgE and boxed according to an alignment produced with *ClustalW2* (2); those strictly conserved have a red background and those well-conserved are indicated by red lettering. Secondary structural elements of IgE and residues involved in a salt bridge (red asterisk) or hydrogen bond interaction (green asterisk) with CD23 are indicated; a residue involved in a further hydrogen bond in five of the six molecules is denoted (yellow asterisk).

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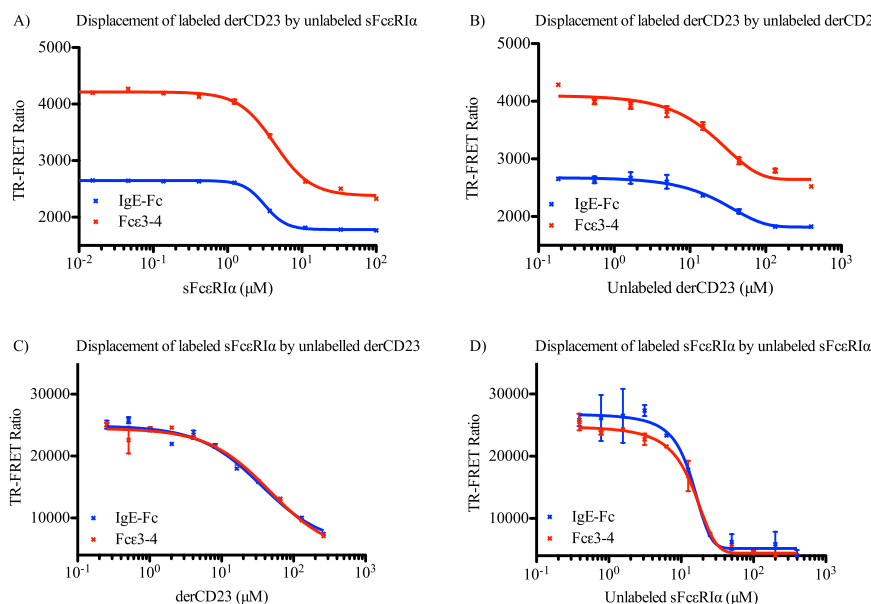


Fig. S5. All four panels show the raw data for the time resolved-FRET ratio (emission of acceptor at 665 nm divided by emission of donor at 620 nm multiplied by 10^4) as a function of unlabeled sFcεRIα or derCD23 added to either IgE-Fc (blue) or Fcε3-4 (red) in complex with labeled sFcεRIα or derCD23. (A) Displacement of labeled derCD23 by unlabeled sFcεRIα. (B) Displacement of labeled derCD23 by unlabeled derCD23, showing that the end points of the titrations are very similar to those in panel A, i.e., unlabeled derCD23 is a positive control for displacement of labeled derCD23 by sFcεRIα. (C) Displacement of labeled sFcεRIα by unlabeled derCD23. (D) Displacement of labeled sFcεRIα by unlabeled sFcεRIα, showing that the end points of the titrations are very similar to those in C (i.e., unlabeled sFcεRIα is a positive control for displacement of labeled sFcεRIα by derCD23). In C, the end point is not quite reached because derCD23 has a much lower affinity for IgE-Fc/Fcε3-4 than sFcεRIα (refer to text). All data: $n = 3$; error bars $\pm$ SEM.

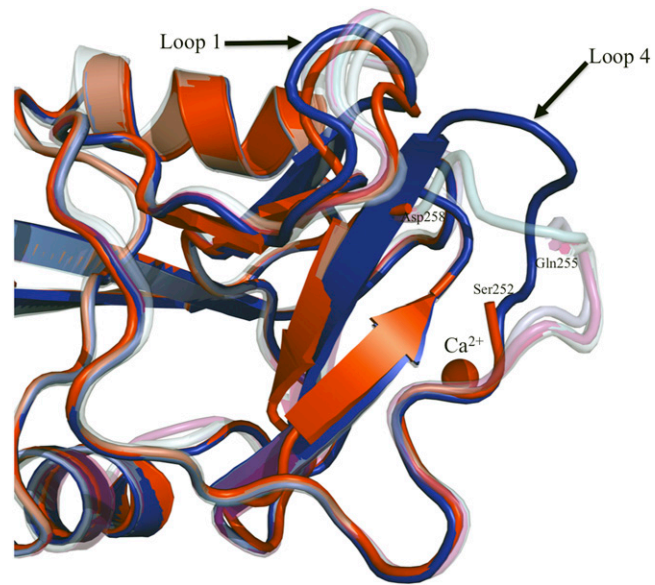


Fig. S6. Superposition of apo- and calcium-bound derCD23 structures. The apo-CD23 (dark blue) and calcium-bound CD23 (dark red) structures [PDB 2H2R, 2H2T (1)] superposed upon the six derCD23 molecules from the Fcε3-4 complex (lightly colored), showing differences in loops 1 and 4. The Ca²⁺ ion is shown (red sphere); note the disordered loop 4 between residues Ser252 and Asp258 in the calcium-bound structure (1).

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Table S1. Crystallographic data collection and refinement statistics

Data processing statistics	
Beamline	I04, Diamond Light Source
Wavelength (Å)	0.9763
Space group	$P2_12_12_1$
Unit cell parameters (Å)	$a = 62.89, b = 110.75, c = 376.56$
Resolution range (Å)*	50.0–3.10 (3.21–3.10)
Observations	162,066
Unique reflections	48,290
Average redundancy	3.6 (4.0)
Completeness (%)	92.8 (94.7)
Wilson B factor (Å ²)	117
$I/\sigma(I)$	14.5 (1.68)
$R_{\text{merge}}^{\dagger}$	0.086 (0.588)
Refinement statistics	
Resolution range (Å)	29.9–3.1
Total no. of reflections	48,257
No. of working reflections	45,820
No. of test reflections	2,437
R factor [‡]	0.2649
R_{working}	0.2640
R_{free}	0.2831
No. of atoms	
Protein	16,107
Others [§]	389
rms bond-length deviation (Å)	0.041
rms bond-angle deviation (°)	1.29
Mean B factor (Å ²)	111
Main chain	107
Side chain	115
Others [§]	131
rms backbone B factor deviation [¶]	2.6
Ramachandran statistics (%)	
Favored	92.9
Allowed	100
Outliers	0

*Values in parentheses are for the outer resolution shell.

[†] $R_{\text{merge}} = \sum |I_{\text{obs}} - \langle I \rangle| / \sum \langle I \rangle$.

[‡] R factor = $\sum_{hkl} ||F_o(hkl)| - |F_c(hkl)|| / \sum_{hkl} |F_o(hkl)|$.

[§]Carbohydrate and one water molecule.

[¶]rmsd between B factors for bonded main-chain atoms.

^{||}As defined by MolProbity (1).

1. Chen VB, et al. (2010) MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* 66:12–21.