REPORT

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Mapping of the CD23 Binding Site on Immunoglobulin E (IgE) and Allosteric Control of the IgE-FcεRI Interaction*[S]

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Background: Immunoglobulin E (IgE) has two cellular receptors, FcεRI and CD23, that mediate distinct functional effects.
Results: We have identified the CD23 binding site on IgE and show that FcεRI and CD23 allosterically compete for binding.
Conclusion: A mechanism of communication exists within the IgE molecule to prevent simultaneous engagement with the two receptors.
Significance: Competition between IgE receptors explains ligand cross-regulation.

IgE, the antibody that mediates allergic responses, acts as part of a self-regulating protein network. Its unique effector functions are controlled through interactions of its Fc region with two cellular receptors, FcεRI on mast cells and basophils and CD23 on B cells. IgE cross-linked by allergen triggers mast cell activation via FcεRI, whereas IgE-CD23 interactions control IgE expression levels. We have determined the CD23 binding site on IgE, using a combination of NMR chemical shift mapping and site-directed mutagenesis. We show that the CD23 and FcεRI interaction sites are at opposite ends of the Cε3 domain of IgE, but that receptor binding is mutually inhibitory, mediated by an allosteric mechanism. This prevents CD23-mediated cross-linking of IgE bound to FcεRI on mast cells and resulting antigen-independent anaphylaxis. The mutually inhibitory nature of receptor binding provides a degree of autonomy for the individual activities mediated by IgE-FcεRI and IgE-CD23 interactions.

Immunoglobulin E (IgE) is the antibody isotype responsible for mediating allergic reactions. It functions through interactions with its two receptors, FcεRI[4] and CD23 (also known as FcεRII). The binding of IgE to FcεRI is essential for type I hypersensitivity, whereas the interaction between CD23 and IgE is crucial for IgE-mediated facilitated allergen binding, processing, and presentation (1). Through interactions with membrane IgE, soluble CD23 fragments can up- or down-regulate synthesis of IgE, depending on the oligomerization state of CD23 (2). IgE expression can also be controlled by a negative feedback mechanism through an interaction of IgE·allergen complexes with membrane-bound CD23 on IgE^+ B cells (3). Because CD23 both positively and negatively regulates IgE expression, a critical role for CD23 in IgE homeostasis has been proposed.

High-resolution structures have been determined for Fc fragments of IgE (4, 5), the extracellular region of FcεRIα (6), the C-type lectin domain of CD23 (7, 8), and complexes of IgE-Fc·FcεRIα (4, 9). The structures of the complex explain the 1:1 stoichiometry observed for the IgE-Fc-FcεRIα interaction; one FcεRIα molecule engages two IgE Cε3 domains simultaneously near the Cε2-Cε3 domain interface. In contrast, the stoichiometry of binding CD23 to IgE is 2:1 (10), with a biphasic affinity, trimeric CD23 apparently binding with an affinity an order of magnitude higher than monomeric CD23 (11).

The structure of IgE is noteworthy for a marked bend between the second and third constant domains of the Fc region. It has been suggested that this bend imparts conformational constraints on the Fab arms, which might favor cross-linking of mast cell-bound IgE by allergens with specific disposition of epitopes (12). The IgE Fc region shows an intriguing mixture of structural rigidity and conformational flexibility, with the aforementioned rigid bend between the Cε2 and Cε3 domains (5) and an unusual degree of intrinsic structural lability within the Cε3 domain (13). Conformational flexibility around the Cε3-Cε4 interface has been noted previously (14); motions around an axis at this interface control whether both Cε3 domains are in a correct orientation to bind simultaneously to the FcεRIα receptor. If only one Cε3 domain binds to FcεRIα, then the affinity is about 10,000-fold weaker than when both Cε3 domains are engaged (15).

In this study, we define the CD23 binding site on the Cε3 domain of IgE using NMR spectroscopy and site-directed mutagenesis. We show that the CD23 and FcεRI binding sites occur on opposite ends of the Cε3 domain of IgE. We demonstrate that allosteric inhibition prohibits simultaneous binding of these two receptors and that this mechanism prevents engagement and cross-linking of IgE bound to mast cells by soluble CD23.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant human IgE-Fc (composed of domains Cε2-Cε4) (5), the αγ-fusion pro-

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[4] The abbreviations used are: FcεRI, Fcε receptor I; sFcεRI, soluble Fcε receptor I; SPR, surface plasmon resonance; TR-FRET, time-resolved FRET.

tein (the FcεRIα extracellular region fused to an IgG4 Fc) (10), soluble FcεRIα (13), derCD23 (7), and the Cε3 domain (13) were each produced and purified as described previously. mAb 7.12 was produced from a B cell hybridoma (16).

NMR Spectroscopy—NMR spectroscopy was performed on protein samples in a buffer containing 25 mM Tris, 125 mM NaCl, 4 mM $CaCl_2$, pH 6.8, at protein concentrations between 120 and 900 μM. Data were collected at 25 °C on Bruker spectrometers equipped with CryoProbes operating at 500 and 700 MHz. For chemical shift perturbation experiments, unlabeled derCD23 ligands were concentrated to 2 mM and then titrated in small aliquots to samples of 200 μM ^{15}N-labeled Cε3 until saturation was seen. The NMR chemical shifts of the urea denatured and native state Cε3 domain are available from the BioMagResBank database under accession numbers 18482 and 18483.

Surface Plasmon Resonance—All experiments were performed on a Biacore T100 instrument (GE Healthcare), essentially as described previously (7, 9). All measurements were done independently at least twice, using standard double reference subtraction methods for data analysis (17). Specific binding surfaces were prepared using standard amine coupling methods for derCD23 and the αγ-fusion protein, whereas IgE-Fc was biotinylated and captured on a streptavidin surface. Ligands in HEPES-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl, 4 mM $CaCl_2$, 0.005% surfactant P20) were injected at 25 μl/min with a 1-min association phase followed by a 15-min dissociation phase. For the sandwich binding experiments, ~90 resonance units of IgE-Fc was captured on an αγ-fusion protein surface during a 1-min injection of a 10 nM IgE-Fc sample; after a 3-min stabilization period, 0–100 μM derCD23 was injected for 1 min followed by a 15-min dissociation phase.

FRET Assay—Inhibition assays were performed by competing 1 μM terbium chelate-labeled derCD23 and 0–20 μM Alexa Fluor 647-labeled IgE-Fc with a range of concentrations of unlabeled αγ-fusion protein. Protein mixtures were prepared in LanthaScreen buffer (Invitrogen) in triplicate, in 384-well plates (Greiner Bio-One), and equilibrated overnight at room temperature. FRET measurements were made on an Artemis plate reader (Berthold Technologies). TR-FRET ratios were calculated for each well as the emission of acceptor at 665 nm divided by the emission of donor at 620 nm and then multiplied by 10,000.

Mast Cell Degranulation Assay—The human mast cell line LAD-2 (National Institutes of Health) was primed by the addition of 2.5 nM IgE (National Institute for Biological Standards and Control) or a buffer-only control for 1 h before the addition of cross-linking reagents. Polyclonal rabbit anti-human IgE (Dako) was added at 20 nM, and then soluble CD23 constructs were added at 0.1, 1 and 10 μM and incubated for 1 h at 37 °C. Supernatants were harvested and tested for β-hexosaminidase release, as described previously (18). The level of degranulation measured for Triton X-treated cells was defined as 100% release, and all samples were compared with that.

B Cell Activation Assays—Human tonsillar B cells were activated with IL-4 (200 IU/ml) (R&D Systems), anti-CD40 antibody (1 μg/ml) (G28.5; ATCC), and either 1 μM derCD23 or 1 μM triCD23, as described previously (19).

RESULTS

In an earlier study, we identified the IgE binding site on CD23 using NMR chemical shift perturbation studies (7). Here we performed the reciprocal NMR binding experiment, mapping the interaction site of CD23 onto the Cε3 domain from IgE. Using an approach described by Schulman *et al.* (20), we assigned the backbone resonances of the molten globule Cε3 domain by first performing resonance assignments of Cε3 denatured in 6 M urea and then, through gradual titration of buffer conditions, tracking those resonances to the native state Cε3 domain. Next, we titrated unlabeled monomeric CD23 protein (derCD23) against an ^{15}N-labeled Cε3 sample and used the assigned NMR spectra to identify residues that were affected by the addition of ligand. Similar to what was observed on derCD23 in the reciprocal titration (7), a small number of Cε3 residues showed peak shifting and line broadening during the derCD23 titration (Fig. 1*A*), consistent with an interaction showing intermediate and fast/intermediate exchange kinetics. When mapped onto the surface of the protein, the identified residues from three discontinuous sequences (amino acids 405–407, 409–411, and 413 from the E-F helix, amino acids 377–380 from the C-D loop, and residue 436 from the C-terminal region) form a contiguous surface representing the binding site on Cε3 for CD23 (Fig. 1*B*). A plot of change in peak intensity *versus* residue number can be seen in supplemental Fig. S1.

This region is at the end of the Cε3 domain, near to the interface with Cε4, in contrast to the interaction site for FcεRI, which is at the other end of Cε3 near the interface with Cε2 (4, 9) (Fig. 1*B*). Among other immunoglobulin-receptor interactions, sites analogous to the Cε3-Cε4 interface are utilized in the interactions of FcαRI with IgA (21), CHIR-AB1 with IgY (22), and FcRn, protein A, and protein G with IgG (23–25). A comparison of the CD23 binding surface on IgE with the analogous IgA and IgG binding surfaces shows areas of overlap but a nonconserved interaction motif, in contrast to the striking conservation of interaction surfaces for IgE·FcεRIα and IgG·FcγR complexes (4).

The identification of this CD23 interaction site on IgE provides a structural explanation for the experimentally observed 2:1 (CD23:IgE) stoichiometry (10) as the dimeric IgE-Fc can bind to two separate CD23 lectin head domains. The two CD23 interactions were shown to have slightly different binding affinities and thermodynamic characteristics (10), as was also observed for the FcαRI-IgA interaction (21). The two binding affinities imply an asymmetry of the two CD23 binding sites, which may possibly be allosterically induced. The capacity of CD23 to induce a conformational change in IgE is discussed further below.

Following the NMR mapping of interaction epitopes for both proteins, we used site-directed mutagenesis to validate the interaction site in the context of the full IgE-Fc construct and to define the energetic contributions of individual residues. Ten mutants from derCD23 and 11 mutants from IgE-Fc (domains Cε2–4) were produced, purified, and characterized; their binding affinities were measured using an SPR assay (7). Table 1 summarizes the results of the site-directed mutagenesis studies. Mutations on both proteins that affect binding are entirely

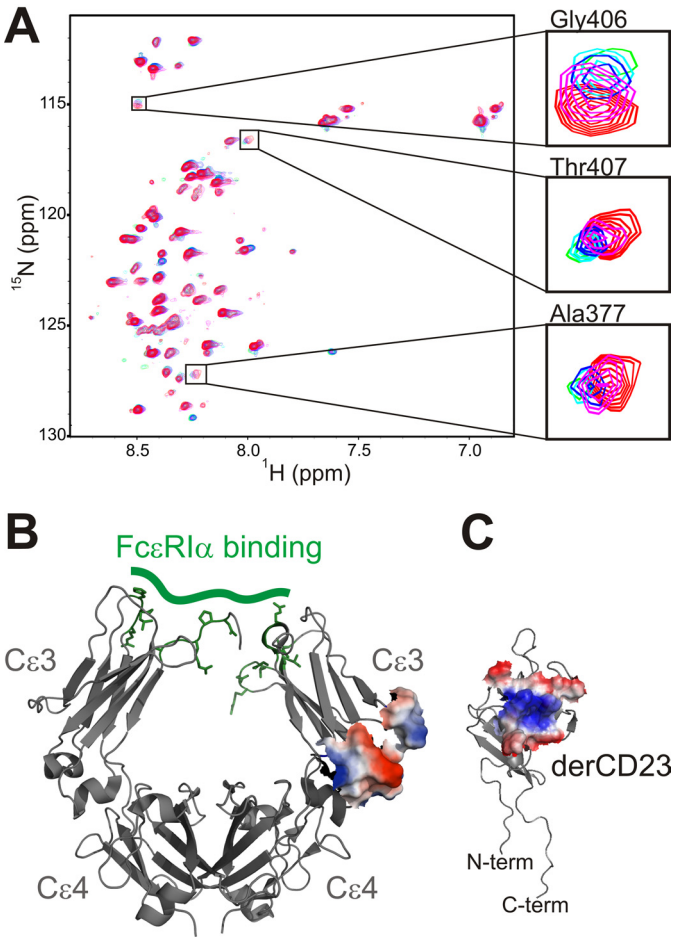

FIGURE 1. **NMR mapping of the CD23 and IgE interaction surfaces.** *A*, increasing amounts of unlabeled derCD23 were added to a 200 μM sample of ^{15}N-labeled Cε3; five spectra of the titration are overlaid (*red*, zero derCD23; *magenta*, 50 μM; *blue*, 100 μM; *cyan*, 150 μM; *green*, 200 μM). *Insets* show magnified views of the indicated regions. *B*, the NMR-derived derCD23 interaction site on Cε3 was mapped onto the structure of IgE-Fc (1F6A (6)) and shown as surface representation. For comparison, the residues of IgE that interact with FcεRI are indicated in *green*. *C*, the IgE interaction surface on CD23 was defined previously (Ref. 7 and shown here as a surface representation). The interacting surfaces of IgE and CD23 are colored according to electrostatic potential and coded such that regions with a potential <-4 k_BT are red, whereas those >4 k_BT are blue (k_B, Boltzmann constant; T, absolute temperature).

consistent with the NMR-defined interaction sites. Charged residues have the largest energetic effect on binding. CD23 mutations D227A, E257A, R224A, and R188A all show a change in binding free energy ($\Delta\Delta G$) of about +6 kJ mol^{-1} (Table 1). Uncharged residues also contributed to the binding energy; a prominently exposed tyrosine residue (Tyr-189) in the center of the IgE binding site of CD23 made a substantial contribution to binding energy. The CD23 binding surface on IgE was also predominantly electrostatic, with residues Asp-409, Glu-412, Arg-376, and Lys-380 showing the largest effects on CD23 binding energetics. Because the NMR data indicated a site on Cε3 very near to the Cε4 interface (Fig. 1*B*) and because binding sites from several other immunoglobulin-receptor interactions involve sites analogous to the Cε3-Cε4 interface (21–23), we also made a pair of mutations in the F-G loop of the Cε4 domain, close to the CD23 binding site in Cε3. However, neither Q535A nor Q538A appeared to affect CD23 binding, leading us to believe that the CD23 binding surface on IgE is largely restricted to residues from Cε3.

TABLE 1
Effects of mutations on the IgE-CD23 interaction

IgE-Fc mutation	K_D	$\Delta\Delta G$	derCD23 mutation	K_D	$\Delta\Delta G$
	μM	kJ mol^{-1}		μM	kJ mol^{-1}
Wild type	2.3		Wild type	2.3	
D409A	26.3	+6.0	D227A	30.9	+6.4
E412A	24.2	+5.8	E257A	26.7	+6.1
R376A	19.7	+5.3	R224A	26.2	+6.0
K380A	13.3	+4.3	R188A	25.0	+5.9
K435A	5.0	+1.9	Y189A	15.6	+4.7
K352A	3.8	+1.2	K276A	10.9	+3.9
R351A	2.6	+0.3	L226A	6.6	+2.6
D347A	2.5	+0.2	D236A	5.8	+2.3
P439A	2.5	+0.2	D192A	4.3	+1.6
Q535A	2.5	+0.2	E265A	2.5	+0.2
Q538A	2.4	+0.1			

Earlier studies indicated that soluble CD23 can compete with FcεRI binding, and this was attributed to steric competition for an overlapping binding site within the Cε3 domain (26, 27). However, our data show that the CD23 and FcεRI binding sites are spatially distinct and suggest that the mechanism of mutual inhibition must be allosteric in nature. We performed a set of competitive binding assays to confirm this experimentally. Firstly, using an SPR assay, we showed that derCD23 can bind to IgE-Fc immobilized to an SPR chip but cannot bind to IgE-Fc captured by immobilized FcεRIα (Fig. 2, *A* and *B*); a positive control, a Fab fragment of the anti-IgE antibody 7.12 (16), directed against the Cε2 domain, did bind to FcεRIα-captured IgE-Fc (data not shown). Secondly, we showed that IgE-Fc can bind to immobilized derCD23, but an IgE-Fc·sFcεRIα complex cannot bind to derCD23 (Fig. 2, *C* and *D*). These data indicated that CD23 and FcεRI interactions with IgE are mutually inhibitory. Finally, we also tested the ability of the receptors to compete for binding to IgE in a solution TR-FRET experiment (28). This assay can be performed under equilibrium binding conditions, allowing a different set of mechanistic properties to be tested than in the kinetic SPR experiments. Under equilibrium conditions, different inhibition patterns are observed for competitive and allosteric inhibitors. A competitive inhibitor affects the apparent binding affinity, with inhibitor I reducing the apparent affinity by a ratio of $(1+[I]/K_I)$, whereas an allosteric inhibitor affects the apparent B_{max} of the interaction without changing the apparent K_D (29). Soluble FcεRIα inhibited the IgE-Fc-derCD23 interaction (Fig. 2*E*) and derCD23 inhibited the IgE-Fc-FcεRIα interaction (Fig. 2*F*), and both inhibitors resulted in a decrease of apparent interaction B_{max} values without affecting the apparent K_D of the interactions. These experiments confirmed mutual inhibition by the two IgE receptors and offer experimental evidence that an allosteric mechanism is involved.

Given the location of the CD23 binding site, the most obvious mechanism for allostery is a conformational change around the interface between the Cε3 and Cε4 domains. Crystal structures of IgE-Fc and IgE-Fc·FcεRIα complexes indicate that the Cε3 domains can exist in "open" and "closed" states, with only an open state being capable of binding FcεRI (4, 9, 14). A detailed study of the open and closed states concluded that it is the motions around the Cε3 A-B helix, sitting at the Cε3-Cε4

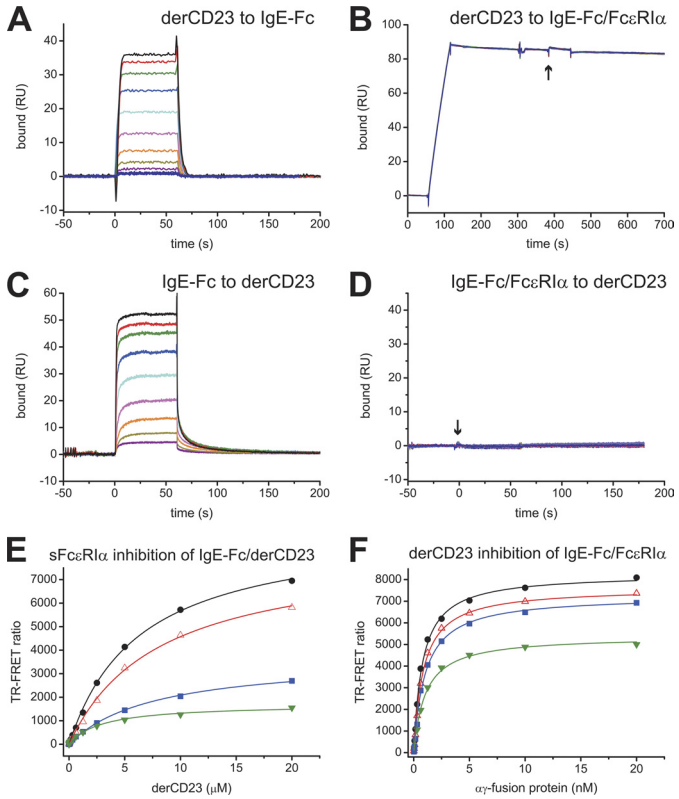

FIGURE 2. **Competition binding experiments between derCD23 and sFcεRIα for IgE-Fc.** *A* and *B*, the binding of derCD23 was tested against IgE-Fc immobilized on a sensor surface (*A*) and IgE-Fc captured on an FcεRIα-immobilized surface (*B*); the start of the derCD23 injection is indicated with an *arrow*. DerCD23 bound to immobilized IgE-Fc with a K_D of 2.3 μM; no measureable binding was observed for derCD23 to IgE-Fc complexed to FcεRIα. *RU*, resonance units. *C* and *D*, the binding of IgE-Fc to immobilized derCD23 (*C*) was compared with the binding of a complex of IgE-Fc·sFcεRIα (*D*) to the same surface; the start of the injection of the complex is indicated with an *arrow*. IgE-Fc bound to derCD23 with a K_D of 2.4 μM, but the IgE-Fc·sFcεRIα complex did not bind to derCD23. All SPR binding experiments were performed using identical 2-fold serial dilutions of ligands, from 40 μM to 78 nM. *E*, binding between terbium-labeled derCD23 and Alexa Fluor 647-labeled IgE-Fc was measured in a solution TR-FRET assay in the presence of increasing concentrations of unlabeled αγ-fusion protein as inhibitor: 0 nM (*black*), 0.5 nM (*red*), 2.5 nM (*blue*), and 5 nM (*green*). *F*, binding between terbium-labeled αγ-fusion protein and Alexa Fluor 647-labeled IgE-Fc was measured with increasing concentrations of unlabeled derCD23 as inhibitor: 0 μM (*black*), 25 μM (*red*), 50 μM (*blue*), and 185 μM (*green*).

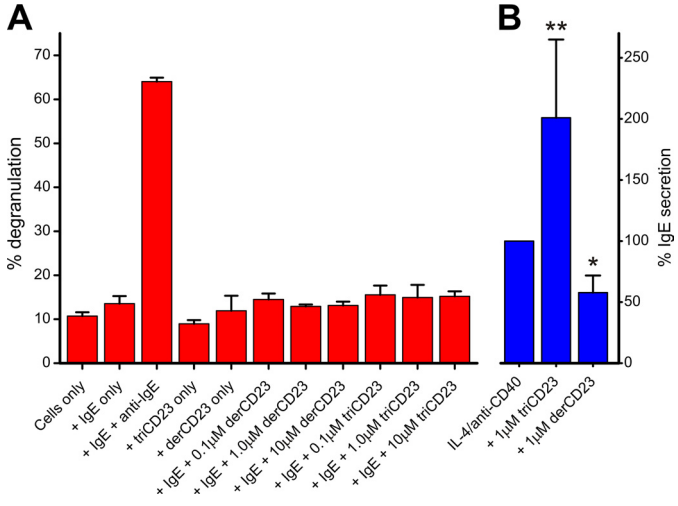

FIGURE 3. **Soluble CD23 does not cross-link IgE bound to FcεRI on mast cells.** The ability of soluble CD23 to engage IgE on B cells and mast cells was tested. *A*, after preincubation of IgE, the addition of anti-IgE antibody resulted in activation of the FcεRIα$^+$ LAD-2 mast cell line, as measured by release of β-hexosaminidase. Neither monomeric derCD23 nor trimeric triCD23 was able to cross-link IgE and activate mast cells in this assay. *B*, in contrast, triCD23 effectively cross-links membrane IgE on the surface of IgE$^+$ human tonsillar B cells, resulting in activation of these cells and increased secretion of IgE. B cell cultures were activated with IL-4 and anti-CD40, and soluble CD23 was added at 1 μM; supernatants were harvested 12 days after activation, tested for IgE levels, and compared with levels for cells treated with IL-4/anti-CD40 alone (* = $p < 0.05$; ** = $p < 0.01$). The regulatory activities of soluble CD23 on IgE$^+$ B cells are described in detail in Ref. 19.

interface, that control the orientation of the two Cε3 domains (14). Indeed, Wurzburg *et al.* (14) suggested that the Cε3-Cε4 domain interface might serve as a drug target for allosteric inhibitors of FcεRI binding. It appears that nature has already utilized this approach to modulate FcεRI binding of IgE by CD23.

Soluble trimeric CD23 has been shown to bind to and cross-link membrane IgE on B cells, resulting in B cell activation (19). However, it is essential that trimeric CD23 not cross-link IgE bound to FcεRI on the surface of mast cells. If this were to occur, then high levels of CD23 would result in mast cell activation in the absence of allergens. Our data from binding experiments (Fig. 2*B*) predicted that soluble CD23 cannot directly cross-link IgE bound to FcεRI on mast cells. We tested this prediction in a mast cell degranulation assay using the FcεRI$^+$ LAD-2 human mast cell line. In this assay, cells were first primed by adding IgE followed by the addition of potential cross-linking reagents and measurement of release of the mast cell granule-associated enzyme β-hexosaminidase. An anti-IgE antibody resulted in FcεRI-mediated activation of the mast cell and robust β-hexosaminidase release, but the addition of either the monomeric derCD23 or a trimeric CD23 construct (triCD23) failed to induce mast cell degranulation (Fig. 3*A*). In contrast, trimeric CD23 effectively cross-linked IgE on B cells, resulting in activation of these cells and increased production of soluble IgE (19) (Fig. 3*B*).

DISCUSSION

Immunoglobulins have evolved two separate sites for binding to receptors. One site is near the hinge region in IgG and at the Cε2-Cε3 interface in IgE, whereas the other is at the interface of the C-terminal domain and the penultimate domain: the Cε3-Cε4 interface in IgE. A mechanism of communication has evolved within the IgE molecule between these two distant sites to prevent simultaneous engagement of CD23 and FcεRI. This may be a unique property of IgE; it is known, for example, that IgG binding of either FcRn or protein A at the Cγ2-Cγ3 interface does not affect binding of FcγRIIa at the hinge region (30). Because IgE and CD23 both exist in membrane-bound and soluble forms, and soluble FcεRIα has also recently been shown to exist at functionally relevant concentrations (31), there is considerable potential for receptor cross-regulation. Mutually exclusive receptor binding ensures independent functions for IgE-FcεRI and IgE-CD23 interactions.

IgE is a clinically important drug target. An anti-IgE antibody (omalizumab) is an effective therapy, currently used in the treatment of moderate to severe asthma that is not controlled by corticosteroids. Omalizumab binds to the Cε3 domain of IgE

and competitively inhibits FcεRI binding, although its *in vivo* activity relies on more than just inhibition of this interaction (32). Results presented here demonstrate that IgE is amenable to allosteric inhibition, an approach that may have significant advantages over competitive inhibition (33) and lay the foundation for the development of allosteric modulators of IgE-receptor interactions.

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