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The Importance of Lys-352 of Human Immunoglobulin E in FcεRII/CD23 Recognition*

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The interaction of immunoglobulin E (IgE) with its low affinity receptor (FcεRII/CD23) plays a central role in the initiation and regulation of type I hypersensitivity responses. We have previously identified the importance of amino acid residues in the A-B loop of the Cε3 domain of human IgE and implicated a region close to the glycosylation site at asparagine 371 as contributing to IgE-CD23 interaction. These residues were now targeted by site-directed mutagenesis. The IgE-CD23 interaction was assessed by semiquantitative flow cytometry. Replacement of the entire Cε3 A-B loop (residues 341–356) with the homologous rat IgE sequence resulted in complete loss of human CD23 recognition, as did replacement of residues 346–353, indicating that class-specific effector residue(s) are contained within these eight amino acids. Lysine 352 within the A-B loop was identified as contributing directly to human CD23 interaction. Mutation to the rodent homologue glycine or glutamate resulted in a significant reduction in binding compared with native IgE, whereas conservative substitution with arginine effected a small, but statistically significant, enhancement of CD23 binding. Mutation of the Cε3 glycosylation site at asparagine 371 to threonine or glutamine did not significantly affect CD23 recognition. Our results yield new insights into the structural basis of the hIgE-CD23 interaction and hold promise for the rational design of drugs that can manipulate IgE-mediated regulation of the allergic response.

Antibodies of the immunoglobulin E (IgE)[1] isotype have a central role in the initiation and regulation of allergic disorders (1). IgE mediates these functions via the interaction of the Fc region of the molecule with its Fc receptors. The crystal structure of the high affinity receptor (FcεRI, K_A ~10^{10} M^{-1}) complexed to IgE-Fc has been determined, identifying critical residues involved in the interaction (2). FcεRII/CD23 has been identified as a low affinity receptor for human (h) IgE (K_A ~10^7 M^{-1}) (3). Structurally, CD23 is not a member of the Ig superfamily but a type II transmembrane receptor expressed on a variety of cells of the immune system (4). In the absence of a high resolution structure, a composite molecular model has been proposed based on homology of the lectin head with the rat mannose-binding protein (5, 6). The model predicts that five heptadic repeats of hydrophobic Leu/Ile residues form a trimeric α-helical stalk linking the N-terminal extracellular domain to the transmembrane and C-terminal cytoplasmic domain of the receptor (7).

Previous studies based on the interaction of hIgE-Fc with a soluble 16-kDa form of the lectin homology domain expressed in NS-O cells identified this region as containing the IgE binding site(s) (8). More recent studies indicate the necessity of the stalk region for IgE interaction (9). Using site-specific mutagenesis, the IgE binding region of CD23 has been mapped to two discontinuous segments between residues 165–190 and 224–256 (10). Although several other ligands for CD23 have been identified (11), the role of the receptor and receptor-derived fragments in the regulation of the IgE response has attracted particular interest (12, 13). A greater understanding of the IgE-CD23 interaction, based on the identification of complementary binding regions on each molecule, could assist the development of strategies for the manipulation of the allergic response via this regulatory mechanism.

The interaction of IgE with CD23 is species-specific. Human IgE does not bind to the rodent receptor, and rat and mouse IgE will not engage the human homologue, although rat IgE recognizes the mouse receptor (14). Previous studies have identified the hCε3 domain as the site of interaction with CD23 (15, 16), although monoclonal antibodies that map to epitopes in the Cε2 and Cε4 domains have been described and are thought to inhibit receptor interaction by steric hindrance (15, 17). Our own studies (18) and those by Nissim *et al*. (16) highlighted the importance of residues in the A-B loop region of hCε3 for binding to CD23.

The present investigation aimed to identify class-specific effector residue(s) in the A-B loop influencing the hIgE-CD23 interaction. The A-B loop was targeted by site-directed mutagenesis. Substitutions involved the introduction of highly conservative changes, *e.g.* Leu to Ile or Asp to Glu in order to minimize potential structural perturbations caused by the mutagenesis. Residues targeted were also exchanged for rodent homologues in order to investigate the species specificity of the CD23 interaction (19).

The use of a eukaryotic expression system (20) facilitated an investigation of the role of hIgE glycosylation. IgE is heavily glycosylated (12–15%), but only *N*-linked glycosylation has been detected to date. Glycosylation of IgE is not essential for CD23 recognition, in contrast with the interaction of this receptor with CD21 or CD11b/c, which involves carbohydrate residues (21, 22). The role of *N*-linked glycosylation in IgE is still unresolved, although it has been demonstrated that enzymatically deglycosylated IgE (PS) and hIgE-Fc fragments ex-

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[1] The abbreviations used are: IgE, immunoglobulin E; hIgE, human IgE; CHO, Chinese hamster ovary; RBL, rat basophilic leukemia; WT, wild type.

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TABLE I
Summary of IgE-CD23 interaction data

IgE variants generated along with the rationale for construction are listed together with an evaluation of the effect of receptor interaction. IgE binding was compared to WT using median channel numbers at 2.5 μg/assay and expressed as a percentage of the WT binding (designated 100%). The median channel numbers from three independent experiments were compared by *t* test. FcϵRI interactions for some variants were not determined by flow cytometry (ND) but have previously been shown to be as wild type.

hIgE variant	Rationale for mutation	Receptor interaction compared to WT		Significance (*t* test, *p* value)	
		FcϵRI	CD23	FcϵRI	CD23
		% ± S.E.			
Cϵ3 A-B Loop					
R16	Homologous rat residues (amino acids 341–356) grafted into the human Fc region to replace loop A-B. Is loop A-B a critical determinant for the CD23 interaction?	88.7 ± 4.6	3.0 ± 1.8	0.13	0.02[a]
R8	Homologous rat residues (amino acids 346–353) grafted into the human Fc region to replace loop A-B. Limited substitutions based upon crystal structure.	91.2 ± 1.6	3.2 ± 1.2	0.03[a]	0.02[a]
P345A	Pro-345 is conserved in human, mouse, and rat IgE. Mutation to Ala is computed to remove fixed bend while maintaining the hydrophobicity and size of the residue.	ND	118 ± 2.4		0.09
D347N	Asp-347 is conserved in human, mouse, and rat IgE. Mutation to Asn maintains the size of the amino acid side chain but alters the charge.	ND	101.3 ± 2.9		0.79
D347E	Mutation to Glu maintains the charge of the residue but increases the length of the side chain.	ND	111.5 ± 2.9		0.14
L348I	Leu-348 is conserved in human, mouse, and rat IgE. Mutation to Ile is highly conservative, changing only the position of a methyl group on the side chain.	ND	92.0 ± 4.6		0.27
R351K	Arg-351 is not conserved in mouse and rat IgE. The rodent homologue is Asn. Mutation to Lys maintains the positive charge of the residue while decreasing the length of the side chain.	ND	105.7 ± 2.4		0.28
K352G	Lys-352 is not conserved; the rodent homologue is Gly.	96.5 ± 2.7	50.0 ± 4.53	0.32	0.0007[a]
K352R	Conservative substitution to maintain charge.	95.1 ± 1.2	114.6 ± 0.5	0.06	0.0006[a]
K352E	Non-conservative substitution alters charge and size of residue.	94.7 ± 5.9	2.4 ± 6.0	0.55	0.0019[a]
R16/K352	Human residue restored within the R16 variant.	105 ± 1.6	38.6 ± 8.1	0.09	0.043[a]
Asn-371 glycosylation site					
N371T	Asn-371 is conserved in human and mouse IgE; the rat homologue is Thr. Mutation to Thr may change the glycosylation from type A to O or inhibit glycosylation	98.7 ± 1.5	84 ± 2.7	0.48	0.024[a]
N371Q	Mutation to Gln will inhibit glycosylation.	96.1 ± 3.8	87.7 ± 5.3	0.37	0.15

[a] p values <0.05 were considered significant.

pressed in *Escherichia coli* engage both Fc receptors, apparently enhancing the affinity of the CD23 interaction by ~10-fold (15). To clarify the role of glycosylation, the solvent-exposed Cϵ3 glycosylation site at Asn-371 was targeted and assessed for a role in the folding, processing, and secretion of IgE variants in mammalian cells.

The functional consequences of site-specific mutagenesis were assessed by examining the binding of hIgE mutants to CD23 on the surface of the RPMI-8866 cell line employing a semiquantitative flow cytometry assay. We show that substitution of Cϵ3 A-B loop residues in hIgE with rodent homologues is associated with loss of human CD23 recognition. Site-directed mutagenesis of specific residues within the A-B loop highlighted Lys-352 as an effector determinant residue in the human IgE-CD23 interaction.

EXPERIMENTAL PROCEDURES

Gene Constructs and Site-directed Mutagenesis—Human epsilon (ϵ) gene variants were constructed by overlap extension mutagenesis PCR and subcloned into the mammalian expression vector pSV-V_{NP}-Hϵ as described (20). The numbering scheme for the hϵ-chain has been maintained as in previous publications (18). IgE variants were generated by transfection of expression vectors into the J558L cell line. Positive clones were selected, and IgE was purified from cell culture supernatant by affinity purification on NIP-CAP-Sepharose columns (19). All pSV-V_{NP}Hϵ variants were sequenced (DyeDeoxy sequencing; Applied Biosystems, Warrington, UK) to ensure the desired mutation had been engineered into the ϵ gene and spurious errors had not been introduced during the PCR reaction. The IgE variants were quantified using the BIAcore biosensor (Amersham Biosciences) as described (19).

Assessment of Cell Surface Receptor Interactions—Binding of IgE to cell surface receptors was assessed using flow cytometry. Three cell lines expressing different IgE receptors were used in this investigation: RPMI-8866 cells expressing human CD23 (23), Chinese hamster ovary (CHO) cells transfected with mouse CD23 (Fc1.7 cells; Ref. 24) (a gift from D. Conrad), and rat basophilic leukemia (RBL) cells transfected with human FcϵRI α-chain (25). Cells were maintained in culture as described previously (23–25). For RPMI-8866 cell analysis, cells were washed twice in wash buffer (phosphate-buffered saline, 1% fetal calf serum, 0.1% sodium azide) and resuspended at 5×10^6 cells/ml. 100 μl of cell suspension was added to 20 μl of recombinant IgE (0.125–5 μg). After incubation on ice for 30 min, cells were washed twice with 1 ml of wash buffer and then incubated with 100 μl of biotinylated anti-IgE (Vector Laboratories, Ltd., Peterborough, UK) diluted 1/400 in wash buffer. Following a 30-min incubation on ice, cells were washed and incubated on ice with 100 μl of streptavidin R-phycoerythrin (PE) (1/20 dilution) (Sigma) for 30 min. Before analysis by flow cytometry, cells were washed twice and resuspended in 200 μl of wash buffer. To determine background levels of fluorescence, phosphate-buffered saline or control antibody (mouse IgE SPE7; Sigma) was used in place of the recombinant IgE. Fc1.7 cells or RBL cells transfected with human FcϵRIα cells were first detached from the culture dish using cell dissociation solution. Substitution of anti-IgE with biotinylated anti-λ antibody (Amersham Biosciences) allowed direct comparison between mouse and recombinant human IgE binding using the Fc1.7 cells. Data were collected using a Coulter Etics Elite flow cytometer and analyzed using Datamate (Dako, Ely, Cambridgeshire, UK).

Data Analysis—Each assay was carried out using chimeric IgE with wild type (WT) human Fcϵ sequence (Serotec, Kidlington, Oxford, UK) as a reference control. This commercial preparation of recombinant IgE was prepared using the pSV-V_{NP}Hϵ expression plasmid and J558L host cell line, identical to that used in the current study. The median log channel number was used for the quantification of WT and variant IgE receptor binding interactions. The mean values from at least three independent experiments were calculated and compared by analysis of variance assay and two sample *t* tests. Statistical analysis was com-

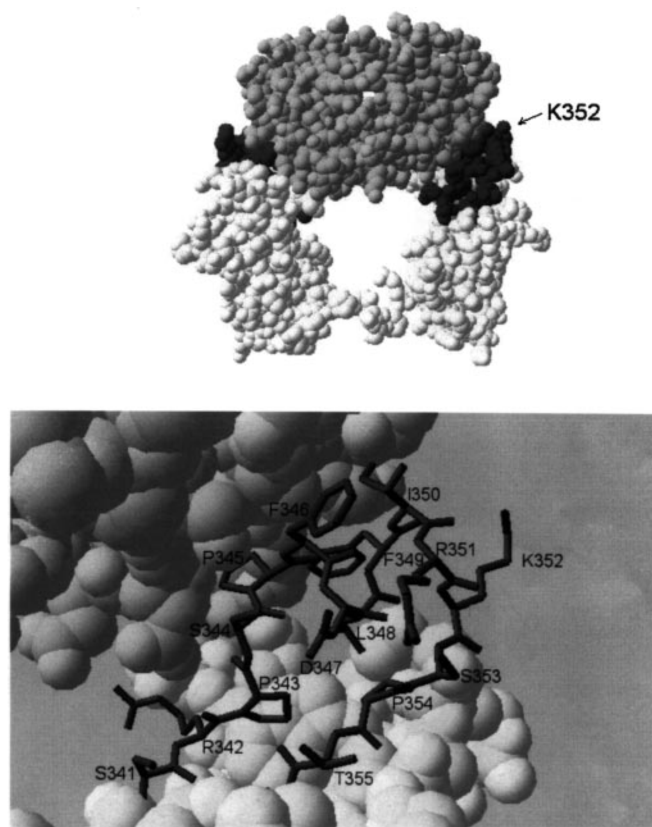

FIG. 1. **The position of Cε3 A-B loop residues within IgE-Fc.** *Upper panel*, solid three-dimensional rendering of IgE Cε3 and -4 in open conformation (2). Cε4 domains are shown a shade *darker* than Cε3 domains. A-B loop residues within Cε3 are *highlighted* close to the Cε4. *Lower panel*, arrangement of A-B loop residues within the closed conformation crystal structure (26). Other residues of the Cε3 and -4 domains are in *solid* rendering with Cε4 in *darker gray*. Residues 341–356 were targeted by mutagenesis. Lys-352, an exposed residue, was identified as a key effector residue of the human IgE-CD23 interaction.

pleted using Minitab (Minitab Inc., State College, PA); a p value <0.05 was considered significant. To facilitate discussion, results were expressed as percentages of WT binding (100%).

RESULTS

IgE variants generated, together with the rationale explaining the mutations, are shown in Table I. The positions of Cε3 A-B loop residues within the crystal structure of IgE-Fc′ are shown (Fig. 1) The interaction of IgE with cell surface high and low affinity Fc receptors was observed employing a semiquantitative flow cytometry assay. Measurement of fluorescence over a range of IgE concentrations allowed the construction of binding curves (Figs. 2 and 3) and comparison of IgE variants with wild type IgE (Table I). WT IgE binding to RPMI-8866 cells reached saturation at 2.5 μg/assay.

Replacement of the entire Cε3 A-B loop (residues 341–356) in the hIgE-Fc for the homologous rat IgE residues (variant R16) resulted in complete loss of hCD23 binding. Binding of the R16 variant was found to be not statistically different from the background level fluorescence (mean value 3% of wild type fluorescence at 2.5 μg of IgE) and significantly reduced compared with wild type binding (p = 0.02, Table I). Throughout this study, FcεRI interactions were assessed in parallel as an indicator of potential structural perturbations that might have occurred as a consequence of mutagenesis. Changes in FcεRI interaction observed for this variant (Fig. 2*A*) were not statistically different from the wild type molecule (88.7%, p = 0.13, Table I).

The R16 variant was constructed prior to crystal structure determination of receptor-bound IgE. When the structural coordinates for IgE/FcεRI became available (2), it could not be ruled out that mutation of residues within the β-strands of the immunoglobulin fold (Ser-341, Arg-342, Thr-355, and Ile-356) could induce significant structural perturbations affecting receptor recognition. To address this, a further IgE variant (R8) was generated with the more limited substitution of residues 346–353 of the Cε3 A-B loop with the homologous rat residues. The binding characteristics of the R8 variant confirmed earlier results obtained with the R16 chimera: the interaction with CD23 was completely inhibited (97% reduction (*versus* WT, p = 0.02), identical to background, p = 0.08). The FcεRI interaction (Fig. 2*A*) was modestly reduced with a marginal statistical significance for this variant (91%, p = 0.03, Table I).

Previous observations suggested Lys-352 within the A-B loop may be a key effector residue, with mutation to Gly, the homologous residue in rodent IgE, resulting in a considerable reduction in receptor interaction (50% decrease (*versus* WT, p = 0.0007), Table I) (11). We interpret the effect of this substitution as identifying Lys-352 as a class-specific effector residue contributing directly to the binding of hIgE to CD23, possibly facilitating docking via an electrostatic interaction. The elimination of this interaction appeared to account, at least in part, for the observed loss of CD23 binding associated with the R16 and R8 variants, which contained this substitution. Of all the A-B loop variants generated within this study, the most prominent change in the IgE-CD23 interaction was observed for the K352G substitution. Point mutations involving D347N, D347E, L348I, or R351K had no significant effect on the IgE-CD23 interaction (Table I).

Mutation of Pro-345 to Ala resulted in an apparent increase in CD23 binding compared with the wild type molecule, although statistical analysis shows this is of marginal significance (12.5% increase, p = 0.09, Table I). The molecular basis of this observation is currently uncertain but could be because of a potential role of Pro-345 in the restriction of structure and maintenance of the rigid conformation of the A-B loop. An increase in loop flexibility may account for the apparent enhancement of the docking process of hIgE and CD23, at the same time suggesting that the Pro-345 residue is not critical for the overall maintenance of the A-B loop CD23 binding conformation.

To substantiate the identification of Lys-352 within the A-B loop as a key effector residue involved in the IgE-CD23 interaction, further point mutations were generated (Fig. 2*B*). Conservative substitution of Lys-352 with Arg resulted in a small, but statistically significant, increase in the CD23 receptor interaction (14% increase, p = 0.0006). Substitution with the negatively charged Glu residue effected the complete loss of CD23 receptor interaction (98% decrease (*versus* WT, p = 0.0019), not significantly different to background, p = 0.61, Table I).

These results substantiate a role for residue Lys-352 in the interaction of hIgE with CD23 and suggest the possibility of an electrostatic interaction between Lys-352 of IgE and the receptor. Mutation of Lys-352 to Arg or Glu maintained the FcεRI interaction, and a binding level similar to the wild type molecule was observed (Table I).

Variant R16/K352 (Lys-352 of hIgE restored within the R16 variant) was generated to evaluate the contribution of residue 352 to the loss of interaction seen with replacement of the entire A-B loop. This variant showed a limited but significant degree of restoration of CD23 recognition (binding level 39% (*versus* WT, p = 0.043), significantly different from background, p = 0.025, Table I). The specificity of the interaction

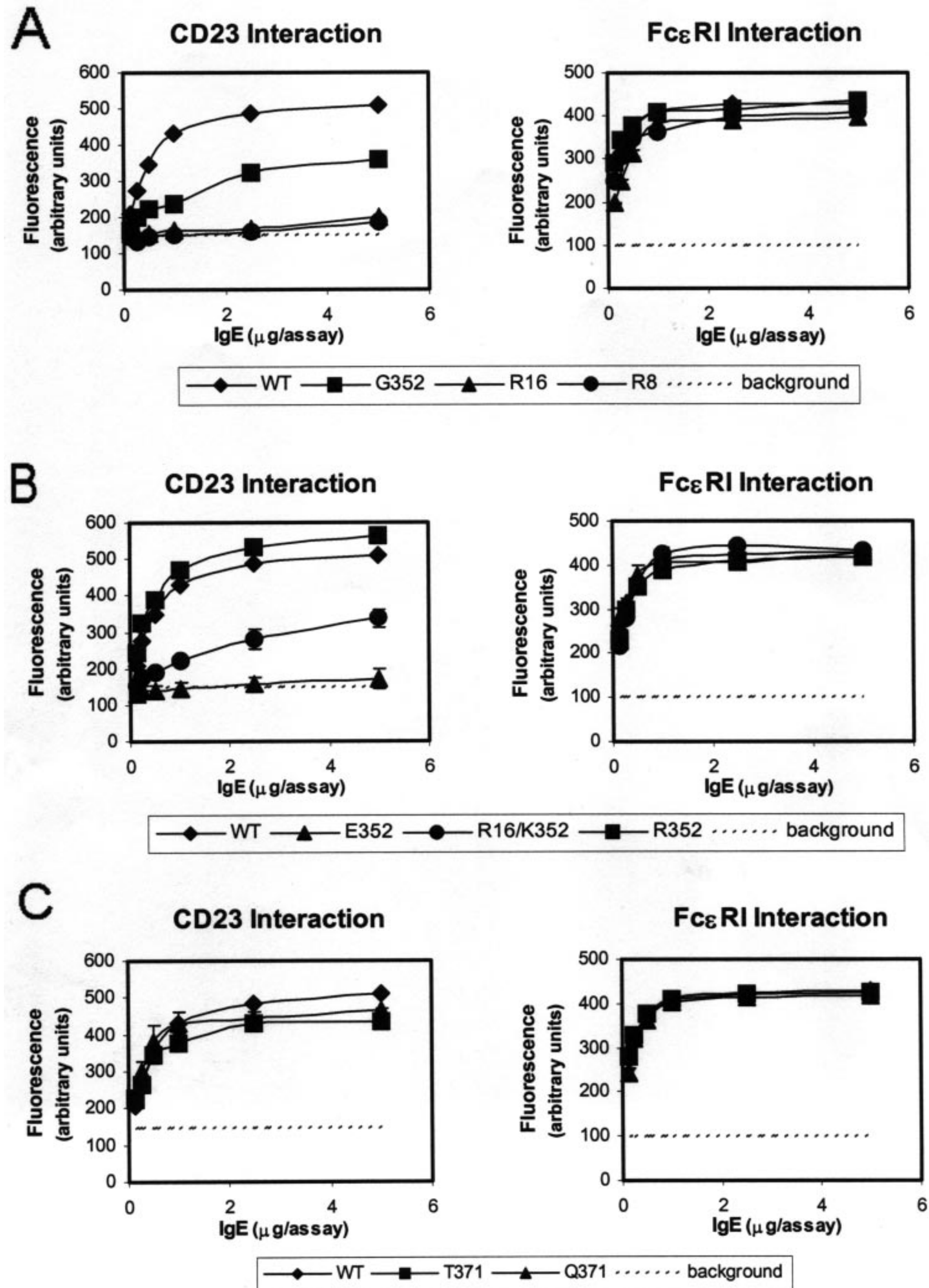

FIG. 2. **Interactions of human IgE variants with human CD23 and FcεRI.** Cells bearing CD23 (RPMI-8866 cells) and cells bearing FcεRIα (transfected RBL) were harvested and washed three times in cold wash buffer (phosphate-buffered saline with 0.1% NaN_3, 1% fetal calf serum) and then resuspended in the same at 5×10^6/ml. 100 μl of cell suspension was added to 20 μl of IgE (0.125–5 μg) incubated on ice for 30 min and then washed (2×1 ml of wash buffer). Cells were incubated with 100 μl of biotinylated anti-IgE antibody (1/400) for a further 30 min, washed as before, incubated for 30 min with 100 μl of streptavidin R-phycoerythrin (1/25 dilution), and washed again. Cells were resuspended in 200 μl of wash buffer and analyzed by flow cytometry. For a positive control, chimeric human IgE (Serotec) was used and the background fluorescence determined in the absence of IgE, using mouse IgE (SPE7) on RPMI-8866 cells or parental RBLs. Median channel numbers show the mean ± S.E. of three separate determinations.

was confirmed by the finding that this interaction could be inhibited using anti-CD23 monoclonal antibody (MHM6) (data not shown), which is known to inhibit hIgE/CD23 binding. As observed for the R16 variant, the FcεRI binding levels for the R16/K352 were similar to WT binding, supporting a localized effect of mutagenesis (Table I, Fig. 2).

To evaluate whether substitution with homologous rat A-B residues conferred the ability to interact with the rodent receptor, IgE variants were screened by flow cytometry analysis using CHO cells transfected with mouse CD23 (Fc1.7 cells). Neither the R16 chimera or hIgE-Gly-352 variants conferred binding to rodent CD23; however, the R8 variant was found to have an increased interaction with Fc1.7 cells (20% compared with WT mouse IgE; Fig. 3). Although these data indicated that the R8 variant was able to interact with rodent CD23, further analysis showed that this interaction was observed with parental CHO cells (Fig. 3), indicating that this interaction was not specific to CD23.

Two additional variants were generated to assess the effect of Asn-371-linked glycosylation on the IgE-CD23 interaction

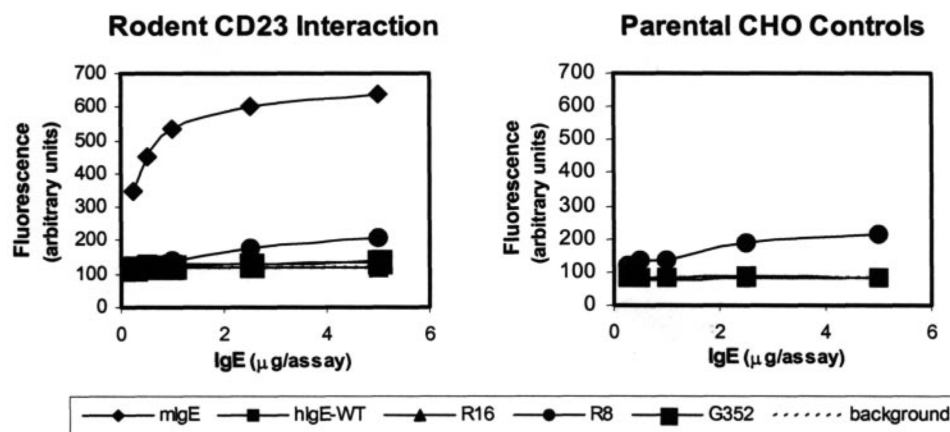

FIG. 3. **Interactions of human IgE variants with rodent CD23.** CHO cells bearing mouse CD23 (Fc1.7 cells) were harvested and washed three times in cold wash buffer (phosphate-buffered saline with 0.1% NaN_3, 1% fetal calf serum) and resuspended in the same at 5×10^6/ml. 100 μl of cell suspension was added to 20 μl of IgE (0.125–5 μg), incubated on ice for 30 min, and then washed (2 × 1 ml of wash buffer). Cells were incubated with 100 μl of biotinylated anti-λ antibody (1/400) for a further 30 min, washed as before, incubated for 30 min with 100 μl of streptavidin R-phycoerythrin (1/25 dilution), and washed again. Cells were resuspended in 200 μl of wash buffer and analyzed by flow cytometry. For a positive control, mouse IgE (SPE7) was used and the background fluorescence determined in the absence of IgE. Control experiments using parental CHO cells are also shown. Median channel numbers show the mean ± S.E. of three separate determinations.

(Fig. 2*C*). Thr-371 and Gln-371 aglycosylated variants both demonstrated a modest decrease in CD23 binding (84%, p = 0.024, and 88%, p = 0.15, respectively, Table I) indicating that enhanced binding reported for deglycosylated IgE cannot be attributed to a lack of glycosylation at this residue.

DISCUSSION

In the present study, we have confirmed the role of the Cε3 A-B loop of human IgE in the IgE-CD23 interaction and identify Lys-352 as an important determinant in CD23 receptor recognition, although the scope of flow cytometry is limited by the fact that it does not yield kinetic data for the interaction studied. These data are in agreement with our previous study (18) and the study of Nissim *et al.*, who replaced residues within the N-terminal of the hIgE Cε3 domain with the homologous mouse residues (16). Replacement of residues 330–346 (variant C3BX) maintained the interaction. However, replacement of residues 330–356 (variant C3HD) dramatically reduced activity, suggesting a role for this region in the CD23 interaction (16). It is unlikely that the substitutions generated within the R16 variant have effected the overall Cε3 domain conformation with concomitant loss of CD23 recognition because FcεRI interaction, which is dependent on residues in hCε3, was fully maintained in this study (Table I, Fig. 2) and in a previous study that used surface plasmon resonance to assess the interaction of IgE variants with soluble truncated FcεRIα (19).

A significant role for the A-B loop in IgE function has previously been proposed by Wurzburg *et al.* (26). Based on crystal structures of IgE-Fc fragments, Wurzburg *et al.* describe how interactions of A-B loop residues facilitate transition between an open conformation when bound to FcεRI and a closed conformation when uncomplexed. Wurzburg proposes that this conformational flexibility may allow IgE to form optimal interactions with both FcεRI and CD23.

It could be argued that substitution with homologous rat residues in the R16 and R8 constructs has effectively constricted domain movement and locked the molecule in a non-binding conformation with respect to CD23. However, maintenance of normal FcεRI interactions would argue in favor of a functionally active molecule. In addition, the absence of noteworthy contacts made by Lys-352 within the available IgE structures and the considerable loss of receptor interaction observed with the Gly-352 variant point to direct involvement in the interaction with CD23. We propose therefore that residues within CD23 bind directly to Lys-352 in hIgE and probably involve electrostatic interaction and concomitant conformational changes to provide optimal docking.

The IgE-CD23 interaction has been proposed to have a 2:1 binding stoichiometry, with two lectin heads of the trimeric receptor interacting with the two ε-chains of an individual IgE molecule (27), and dimerization of the hIgE molecule is required (15). Dual kinetics have been reported for IgE binding to CD23 (24). The most straightforward explanation for this phenomenon in the context of this model is that one lectin head would initially dock with low affinity, providing the basis for a second interaction of higher affinity. This could involve two sets of distinct interactions, possibly involving different points of contact in the IgE molecule and/or within the receptor. The IgE binding site on CD23 has been mapped to two discontinuous segments between residues 165–190 and 224–256 (10). Our observation demonstrating that mutation of a single residue has a profound effect on receptor recognition suggests that Lys-352 makes a definitive contribution, either by establishing an initial point in the docking with CD23 and/or by contributing to the stability of the interaction. Although these data strongly suggest a dominant role for Lys-352 (*e.g.* mutation to Glu resulted in an equivalent loss of CD23 binding as observed for the replacement of the entire A-B loop in the R16 and R8 variants), we cannot exclude the contribution of other residues in the A-B loop, including residues at positions 346, 349, and 353 that were not evaluated directly in the current study.

Abrogation of the higher affinity interaction using an anti-CD23 stalk region antibody has been shown to result in cells that appear negative for IgE binding as determined by flow cytometry, whereas low affinity binding is still detectable using ^{125}I-labeled IgE (28). The use of flow cytometry to assess IgE-CD23 interaction does not allow us to conclude whether loss of binding observed with mutants in the present study results from abrogation of one or both of these interactions.

The localization of Asn-371 on the same face as Lys-352 and our preliminary data regarding the interaction of mutants inhibiting glycosylation led us to propose a role of glycosylation at Asn-371 in the inhibition of hIgE-CD23 interaction. Our present study shows, however, that Asn-371 glycosylation is not involved in this process. Others have suggested that glycosylation of the Cε2 domain accounts for this effect. Deglycosylation

of IgE has been shown to expose epitopes on the Cϵ2 domain (29), and a role for Asn-265 has been postulated (30) to which our results would lend some tentative support.

At present, limited structural information is available concerning the interaction of hIgE and CD23. In the absence of high resolution structural data, we identify key regions of the hIgE molecule involved in CD23 recognition that provide new insights into the structural nature of this interaction. The A-B loop in Cϵ3 was identified as a critical region for the interaction, with Lys-352 as a class-specific residue. Acquiring information concerning the structure of complementary binding sites on IgE and its receptors holds potential for the rational design of drugs that could modulate the allergic response.

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