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Carbohydrates Contribute to the Interactions between Cockroach Allergen Bla g 2 and a Monoclonal Antibody

Mi Li,*,† Alla Gustchina,* Jill Glesner,‡ Sabina Wünschmann,‡ Lisa D. Vailes,‡ Martin D. Chapman,‡ Anna Pomés,‡ and Alexander Wlodawer*

The crystal structure of a murine mAb, 4C3, that binds to the C-terminal lobe of the cockroach allergen Bla g 2 has been solved at 1.8 Å resolution. Binding of 4C3 involves different types of molecular interactions with its epitope compared with those with the mAb 7C11, which binds to the N-terminal lobe of Bla g 2. We found that the 4C3 surface epitope on Bla g 2 includes a carbohydrate moiety attached to Asn[268] and that a large number of Ag–Ab contacts are mediated by water molecules and ions, most likely zinc. Ab binding experiments conducted with an enzymatically deglycosylated Bla g 2 and a N268Q mutant showed that the carbohydrate contributes, without being essential, to the Bla g 2–4C3 mAb interaction. Inhibition of IgE Ab binding by the mAb 4C3 shows a correlation of the structurally defined epitope with reactivity with human IgE. Site-directed mutagenesis of the 4C3 mAb epitope confirmed that the amino acids Lys[251], Glu[233], and Ile[199] are important for the recognition of Bla g 2 by the 4C3 mAb. The results show the relevance of x-ray crystallographic studies of allergen–Ab complexes to identify conformational epitopes that define the antigenic surface of Bla g 2. ***The Journal of Immunology*****, 2011, 186: 000–000.**

Cockroach allergy is associated with the development of asthma, especially in inner cities where it affects ≤80% of asthmatic children that are sensitized and exposed to allergens produced by *Blattella germanica* (1, 2). Bla g 2, an inactive aspartic protease with a bilobal structure (3), is the most potent allergen in terms of prevalence of sensitization among German cockroach allergic patients (50–70%) (4, 5). Unlike viral aspartic proteases that have two identical subunits forming a similar bilobal structure, Bla g 2 consists of two fused lobes, with a structure comparable to those of typical active aspartic proteases exemplified by pepsin, renin, or chymosin (6). However, amino acid substitutions in the active site render Bla g 2 inactive (7). Although both lobes are structurally similar and evolved from the fusion of the single-domain subunits of an ancestral protein, presumably similar to the viral aspartic proteases (8), the amino acid sequence of the lobes differs and thus would be expected to cause antigenic differences on their molecular surfaces.

Our goal has been to map the antigenic surface of Bla g 2 by solving the x-ray crystallographic structures of complexes of the allergen with specific murine mAbs. Most of the reported epitope mapping techniques for allergens omit the identification of conformational epitopes. Such methods are based on the use of libraries of overlapping synthetic peptides, fragments from digested allergens, or parts of allergens expressed as recombinant proteins. The use of synthetic peptides has been proven useful to identify linear B cell epitopes, especially in foods (9, 10). However, epitope mapping of globular proteins such as Bla g 2 with allergen peptides or fragments has an important limitation of missing the identification of conformational epitopes. Crystallographic techniques have been proven to be an effective strategy to perform epitope mapping of globular proteins (11). This approach aims to map the antigenic surface of an allergen by solving complexes of the allergen with specific nonoverlapping Abs.

We reported previously the x-ray crystal structure of Bla g 2 alone (12) and in complex with mAb 7C11 that binds to the N terminus of Bla g 2 (13). In this paper, we describe the epitope of a murine mAb 4C3 that binds to the opposite, C-terminal, lobe of Bla g 2. Binding of 4C3 involves different types of molecular interactions than the binding of 7C11 to its epitope. We found that the 4C3 surface epitope on Bla g 2 included a carbohydrate moiety and that a large number of Ag–Ab contacts were mediated by water molecules.

Materials and Methods

Expression and purification of rBla g 2

Recombinant Bla g 2 mutants (partially deglycosylated by a substitution of either N93Q or N268Q in one of the three glycosylation sites and the mutants of the 4C3 mAb epitope) were expressed in *Pichia pastoris* and purified by affinity chromatography using 7C11 mAb, as described previously (3, 12–14). Briefly, the DNA encoding for the mature form of Bla g 2 was inserted into a pGAPZα expression vector (Invitrogen, Carlsbad, CA). The plasmid was mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The linearized plasmid was electroporated into *P. pastoris* for constitutive expression of the allergen,

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The coordinates and structure factors presented in this article have been submitted to the Protein Data Bank under accession code 3LIZ.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: Bla g 2-DG, enzymatically deglycosylated allergen; GlcNAc, *N*-acetylglucosamine; Man, mannose; NAG, *N*-acetylglucosamine; S_c, shape correlation statistic.

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followed by purification from culture media. The purity of rBla g 2 was >95% as judged by SDS-PAGE.

Production of Fab from the 4C3 mAb and formation of the rBla g 2–Fab complex

Monoclonal anti-Bla g 2 Ab 4C3 (clone 4C3 H5 F6 F7) was raised against affinity-purified natural Bla g 2 and purified from ascites by affinity chromatography through a protein A column. Purified Ab was fragmented using papain. Fab fragments were purified (>95%) by protein A chromatography and eluted in 20 mM sodium phosphate and 150 mM NaCl (pH 7.2). Fab was mixed with rBla g 2-N93Q at a 1.2:1 molar ratio. The mixture was purified further over a HiPrep 16/60 Sephacryl S-100 HPLC column equilibrated with the buffer 0.2 M NaCl and 20 mM Tris (pH 7.2) and concentrated to ~5.2 mg/ml for crystallization.

Sequencing of the mAb 4C3

The cell line producing the anti-Bla g 2 mAb 4C3 H5 F6 F7 was grown at the Lymphocyte Culture Center (University of Virginia, Charlottesville, VA). Total RNA (20 μg per 3×10^6 cells) was isolated from the 4C3 mAb cell line using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs encoding for the L and H chains of the mAb were obtained by reverse transcription from RNA (SuperScript III; Invitrogen), and the DNA was PCR-amplified using specific primers, sequenced, and analyzed.

Two and four pairs of primers were tested initially for amplification of the L and H chains of the Ab, respectively. A degenerate primer for the N terminus [VHb, 5′-GAG GTG CAG CTG GTG GA(AG) TC-3′, encoding for EVQLVE] and the C-terminal primer (CH2, 5′-TT AGG AGT CAG AGT AAT GGT GAG CAC ATC C-3′, encoding for DVLTITLTP) amplified the H chain. Forward (5′-GCC AAA ACG ACA CCC CAT C-3′, encoding for AKTTPH) and reverse (5′-A GGT CAC TGT CAC TGG CTC AGG-3′, encoding for PEPVTVT) middle primers were used to complete H chain sequencing. The L chain was amplified with primers for the N terminus (Vκ4, 5′-CAA ATT GTT CTC ACC CAG TCT CCA-3′, encoding for QIVLTQSP) and the C terminus (Cκ, 5′-GAT GGA TAC AGT TGG TGC-3′, encoding for APTVSI). The mAb was isotyped G1 for the H chain and κ for the L chain.

Structure determination

Crystals of the complex of Bla g 2 with Fab 4C3 were obtained at room temperature using the hanging-drop, vapor diffusion method. Each drop contained 2 μl of ~5 mg/ml allergen–Ab complex in 20 mM Tris buffer, 0.2 M NaCl, and 2 mM DTT at pH 7.2 as well as 2 μl of reservoir solution consisting of 20% polyethylene glycol 8000, 8% ethylene glycol, 5 mM DTT, and 0.2 mM $CdCl_2$ in 0.1 M Tris buffer at pH 7. The crystals grew to the dimensions of ~$0.15 \times 0.2 \times 0.1$ mm in a week. They belong to the monoclinic space group *C*2, and each unit cell contains one molecule of Bla g 2 as well as one molecule of Fab.

Diffraction data extending to 1.8 Å resolution were collected from one crystal at the SER-CAT beamline 22-ID, located at the Advanced Photon Source synchrotron (Argonne, IL). Data were measured with a Mar300CCD detector and were integrated and scaled with the HKL2000 package (15). Prior to data collection, the crystal was cooled rapidly to 100 K in a nitrogen stream, after being transferred to the cryo-solution containing 20% polyethylene glycol 8,000, 10% ethylene glycol, and 0.2 mM $CdCl_2$ in Tris buffer at pH 7 (Table I).

The structure of Bla g 2–4C3 complex was solved by molecular replacement using the program Phaser (16). The search model for Bla g 2 was the structure of Bla g 2 in complex with Fab of 7C11 mAb. Bla g 2 and Fab were searched separately, and solution of the molecular replacement was unambiguous, with the final *Z* score 35.4 and log likelihood gain 2745. The structure was refined using Phenix (17) at an early stage and then finalized with the program Refmac5 (18). The program Coot (19) was used for model building. The final model contains one Bla g 2 molecule, one Fab molecule, 3 Cd^{2+} ions, 6 Zn^{2+} ions, and 871 water molecules and is characterized by R_{free} of 20.2%. Identification of metal ions is tentative and largely based on the previous observation that Zn^{2+} is strongly bound to Bla g 2 and on proper behavior of the ions during refinement.

ELISA to test 4C3 mAb binding to Bla g 2-N268Q mutant

Plates were coated with 1 μg/ml of either 7C11 or 4C3 mAb, followed by a blocking step by incubation with 1% BSA, PBS, and 0.05% Tween 20 at pH 7.4 for 1 h. All of the steps were performed at room temperature, and plates were washed three times with PBS and 0.05% Tween 20 at pH 7.4 between incubation steps. The Bla g 2-N268Q mutant was quantified by Advanced Protein Assay (Cytoskeleton, Denver, CO) and used at 1:2 dilutions across the plate starting at a concentration of 250 ng/ml. A rabbit polyclonal anti-Bla g 2 Ab was used as a detection Ab followed by peroxidase-labeled goat anti-rabbit polyclonal Ab (Jackson Immuno Research Laboratories, West Grove, PA). Plates were developed and read at OD of 405 nm as described previously (20).

Deglycosylation of rBla g 2

Bla g 2 was deglycosylated by adding *N*-glycosidase F (14 μl of the enzyme at a concentration of 500,000 U/ml; New England Biolabs, Ipswich, MA) to ~1 ml rBla g 2-N93Q (700 μg in PBS at pH 7.4) and incubating at 37°C for 72 h. Deglycosylation was confirmed by SDS-PAGE and silver staining. Deglycosylated Bla g 2 was purified further by affinity chromatography as described above. The allergen was eluted with 0.1 M glycine and 0.15 M NaCl (pH 2.5), concentrated, and dialyzed against PBS (pH 7.4). The allergens were quantified by measuring absorbance at 280 nm.

Comparison of rBla g 2 versus the deglycosylated allergen by ELISA

Dose–response curve experiments were performed using rBla g 2 (N93Q, glycosylated at positions N268 and N317) versus the enzymatically deglycosylated allergen (Bla g 2-DG). Microplates (96-well) were coated with 1 μg/ml of either 7C11 or 4C3 mAb in 50 mM carbonate–bicarbonate buffer (pH 9.6). Plates were blocked with PBS containing 0.05% Tween 20 and 1% BSA (pH 7.4). Subsequent steps and washes between steps with PBS containing 0.05% Tween 20 were performed at room temperature. Known concentrations of allergen were added in the first well, and 1:2 serial dilutions were performed along the plate. Detection Abs were biotinylated 4C3 mAb for plates coated with 7C11 mAb and vice versa (at 1:1000 dilution). Peroxidase-labeled streptavidin (0.25 μg/ml) was added in the last step (Sigma-Aldrich, St. Louis, MO). Plates were developed and read at OD 405 nm as described previously 20).

Inhibition of IgE Ab binding by 4C3 mAb

Microplates were coated with 5 μg/ml rBla g 2-N93Q in 50 mM carbonate–bicarbonate buffer (pH 9.6). The blocking step and washes were performed as described above. The Abs tested for inhibition of IgE Ab binding were the anti-Bla g 2 mAb 4C3 and positive and negative controls. The anti-Bla g 2 mAb 4C3 was used at 0, 0.1, 1, 10, and 100 μg/ml concentrations. The rabbit polyclonal anti-Bla g 2 Ab was used as a positive control (at 1:4 and 1:10 dilutions), and the anti-mite Der p 1 allergen 4C1 mAb was used as a negative control (at 10 and 100 μg/ml). These Abs were added after the blocking step, followed by addition of sera (1:5 final dilution). Sera of cockroach allergic patients with high anti-Bla g 2 IgE Ab titer were used (see next section). Incubation was at room temperature for 2.5 h. Affinity-purified peroxidase-labeled goat anti-human Ab IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used for detection at 1:1000. Plates were developed and read at OD 405 nm as above.

Sera from cockroach allergic patients

Sera from cockroach allergic patients were obtained from stored samples that had been collected from patients enrolled in 1988–1989 in Wilmington (Delaware) and Charlottesville (Virginia) for epidemiological studies performed at the University of Virginia (21, 22). The studies performed at the University of Virginia were approved by the Human Investigation Committee, and blood was drawn after informed consent was obtained from the patient. IgE Ab levels in sera were 64 and 27 ng of total IgE, measured by radioallergosorbent test (22), and 65 and 29 ng of IgE against Bla g 2 per milliliter, measured by mAb-based RIA (4).

Multiplex array for the measurement of Ab binding to Bla g 2

mAb 7C11 (20 μg) was coupled to a Luminex carboxylated fluorescent microsphere bead set (Luminex, Austin, TX), as described previously (23). The multiplex array assay was performed in 96-well filter plates in the dark at room temperature with incubation steps of 1 h, followed by filter-washing twice. After the filter plate was wet with assay buffer (1% BSA, PBS, and 0.02% Tween 20 at pH 7.4) and vacuum filtering, mAb-coupled beads were added along with rBla g 2 at a concentration of 400 ng/ml. The 4C3 mAb mutants were assayed in a single-plex manner. Beads and allergen were mixed and incubated. In the following step, biotinylated 4C3 mAb was added to the wells, mixed, and incubated. Finally, streptavidin–PE was added to all of the wells and mixed. The plate was incubated for 30 min and filter-washed twice. Assay buffer (100 μl) was added to wells and mixed. Plate was read in a Bio-Plex fluorescent suspension array reader (Bio-Rad, Hercules, CA), consisting of Luminex xMAP instrumentation (Luminex) supplied with Bio-Rad proprietary software. Data were analyzed by two- or one-tailed, paired Student *t* test. The *p* values <0.05 were considered significant.

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Results

Description of the Bla g 2–4C3 mAb–Fab complex

The crystal structure of the complex between Bla g 2 and mAb 4C3 was solved at the resolution of 1.8 Å (Table I). The structure of the complex demonstrated that the Ab 4C3 binds tightly to Bla g 2 in 1:1 ratio, revealing a novel epitope located in the C-terminal domain of the allergen molecule (Fig. 1, Supplemental Fig. 1). This epitope is composed of the residues from three loops of the allergen (199–202, 248–259, and 268–272) as well as of the residues from the C-terminal end of the helix 225–235 (Fig. 1, *inset*). The loop 248–259 represents a continuous part of the epitope. This fragment of the structure of the allergen is composed of a helical segment that includes residues 248–254, which adopt the conformation of a 3_{10} helix, with the remainder of this fragment assuming an extended conformation. The latter segment interacts with the most extended part of the CDR H1 in an antiparallel fashion, maintaining the main chain–main chain contact between residues 255–257 of the allergen and Ser^{H31}–Ala^{H33} of CDR H1 of the Ab. In the text that follows, residue numbers of the H and L chains of the Ab are preceded with letters H and L, respectively. The side chain of Asn^{268} of Bla g 2 is glycosylated, and a carbohydrate molecule attached to this residue is involved in extensive interactions with the Ab, predominantly water mediated.

The allergen molecule interacts with all three CDRs of the H chain (H1–H3) of the 4C3 Ab, either directly or via solvent. The interactions of the allergen with the variable loops L1 and L3 of the L chain of 4C3 are, with one exception, mediated by solvent. The only direct contacts involve the side chain of Lys^{251}, which forms electrostatic interactions with the residues from both L1 and L3 CDRs. The remaining L2 CDR is not involved in any interactions with Bla g 2, either directly or via solvent.

The combined solvent-accessible surface buried in the Bla g 2–4C3 complex is 1831 $Å^2$ (calculated with the program CNS, including both the allergen and Ab surfaces), which is within the range of 1400–2300 $Å^2$ observed for the known Ag–Ab complexes (24, 25). To quantify the shape complementarity in the Bla g 2–4C3 complex, we calculated a shape correlation statistic (S_c) that estimates the geometric match for the interface (26). We found that the value of S_c is 0.62 for the Bla g 2–4C3 complex, compared with S_c = 1.0 for the interfaces with a geometrically perfect fit.

The epitope on the surface of a Bla g 2 molecule recognized by the 4C3 Ab is of the conformational type and is composed of residues originating from several secondary structure elements of the C-terminal domain of the allergen (Fig. 1, *inset*). On the basis of the type of the allergen–Ab interactions, the overall interface involves four kinds of contacts: direct contacts between residues from the counterparts, solvent-mediated interactions, interactions of mixed character, and carbohydrate-mediated interactions.

Specific interactions between Bla g 2 and 4C3 mAb

The overall views of the areas with numerous Ag–Ab interactions are shown in Supplemental Figs. 2 and 3. The most specific interactions between Bla g 2 and 4C3 are charge–charge interactions between the guanidinium group of Arg^{H103} from CDR H3 and a negative charge of the C terminus of the helix composed of residues 225–235, enhanced by the presence of the properly oriented, negatively charged side chain of Glu^{233} (Fig. 2). In a similar manner, the positive charge of Arg^{103} is enhanced by the close proximity of Arg^{L50} and Arg^{L53}—two positively charged residues from CDR L2. Although the latter two residues do not interact directly with the allergen, they contribute to allergen–Ab interactions by forming a cluster of positively charged residues on the surface of the Ab that is able to specifically recognize its negatively charged counterpart on the surface of the allergen. These long-distance, charge-driven interactions bring the side chain of Trp^{H105} of the variable loop H3 to the exactly right position to dock it into the hydrophobic pocket on the surface of the allergen molecule composed of Ala^{234}, Ile^{199}, and Pro^{256} (Fig. 2). A main chain–main chain hydrogen bond is formed between the carbonyl oxygen of Ala^{234} and the amide nitrogen of Arg^{H103} (Fig. 3).

An area adjacent to the one described above involves the interactions between CDRs L1 and L3 of the L chain of 4C3 and the residues from two fragments of the allergen epitope. The first of these fragments comprises residues 248–252 from the loop 248–259 (the continuous part of the epitope), and the second involves Ala^{234} from the helix 225–235. There are no direct contacts between Bla g 2 and the Ab in this area, with the exception of Lys^{251}. The side chain of this residue is involved in two types of electrostatic interactions with the Ab. On one hand, it forms an ion pair with Asp^{L92} of the variable loop L3, whereas, on the other hand, it is involved in cation–π type interactions with the side chain of Tyr^{L32} of CDR L1 (Fig. 3, *inset*). The orientation of the aromatic ring of Tyr^{L32}, favorable for that interaction, is supported by another strong cation–π interaction formed with Arg^{L50} of L2 CDR on the opposite side of the ring (Fig. 3, *inset*). All of the other interactions in this region are mediated by water molecules. Two metal ions (presumably Zn^{2+}) participate in this extensive hydrogen-bonded network (Fig. 3). In addition to the involvement

Table I. *Data collection and refinement statistics*

Parameter	Value
Wavelength (Å)	1.000
Space group	*C*2
Unit cell parameters (Å)	a = 155.2, b = 105.3, c = 109.1, β = 132.6°
Resolution (Å)	50–1.8
No. of reflections (unique/total)	118,390 (441,093)
Completeness (last shell)	99.3% (94.5%)
R_{merge} (last shell)	6.8% (49.1%)
No. of complexes in a.u.	1
No. of protein atoms	5897
No. of solvent molecules	871
No. of heteroatoms	98
R_{cryst}	17.8%
R_{free} (3% of data)	20.2%
r.m.s. deviations from ideality	
Bond lengths	0.011 Å
Angles	1.4°

r.m.s., root-mean-square.

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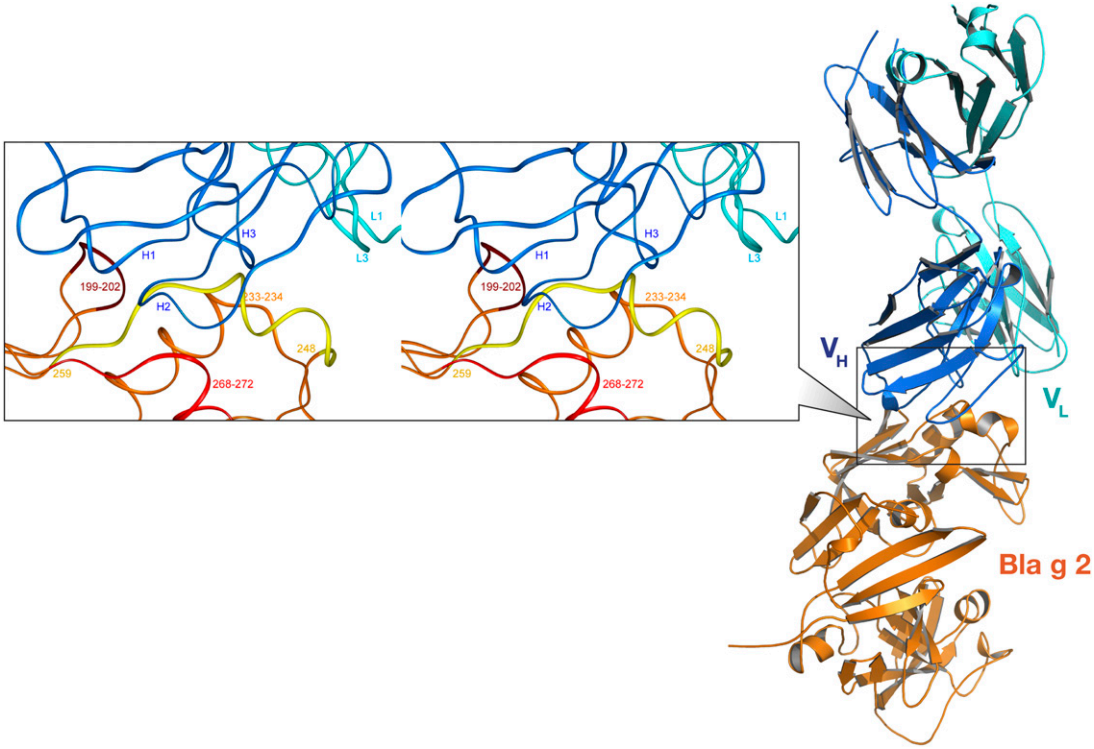

FIGURE 1. Crystal structure of the complex between Bla g 2 and mAb 4C3. A cartoon representation of the complex with the Bla g 2 molecule colored orange and 4C3 in two shades of blue. *Inset*, Stereoview of the interface between Bla g 2 and mAb 4C3 shown in ribbon representation. The chain of the Ag is colored orange, with the epitope loops brown, red, and yellow. The H chain of 4C3 is dark blue, and the L chain is cyan, with the CDRs identified and numbered.

in the cation–π interactions described above, Tyr^{L32} of CDR L1 is water bridged (W1 in Fig. 3) to the carbonyl oxygen of Ile^{235} of Bla g 2, whereas the side chain of Asn^{L30} contributes to the network of hydrogen bonds between CDR L3 and the fragment 248–252 of Bla g 2 by being hydrogen-bonded to one of the two water molecules (W2) in the coordination sphere of Zn2 (Fig. 3). The metal ion is also directly coordinated by Asp^{L92} of L3 as well by Glu^{L93} via a water molecule (W3). The other metal ion (Zn1) is coordinated on the side of the allergen by the side chains of Ser^{250} and Asp^{248}, whereas its contact with the Ab is mediated by the water molecule (W4). The latter water molecule is polarized by a strong hydrogen bond with Lys^{251} of Bla g 2 and thus able to maintain a short hydrogen bond with Glu^{L93} of CDR L3 of the Ab. The side chain of Asp^{248} was modeled in two conformations in the

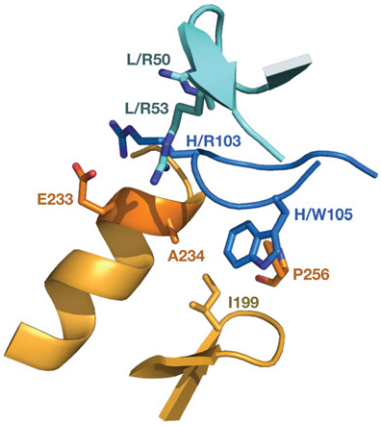

FIGURE 2. The region of specific interactions between Bla g 2 and mAb 4C3. Ionic interactions are formed between the C terminus of helix 225–235 in Bla g 2 and an arginine cluster originating from the H3 and L2 CDRs. A salt bridge is formed by the guanidinium group of Arg^{H103} from the CDR H3 and the side chain of Glu^{233}, with an additional negative charge provided by the C terminus of the helix. Residues from the H chain of the Ab are dark blue, residues from the L chain are cyan, and the Ag is gold.

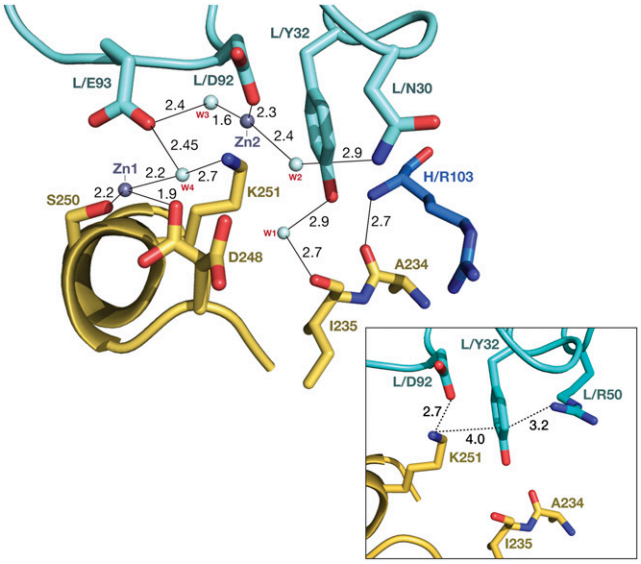

FIGURE 3. Interactions involving CDRs L1 and L3. The contacts are predominantly solvent-mediated and involve residues from two fragments of the allergen epitope, comprising residues 248–252 from the loop 248–259 (the continuous part of the epitope), and Ala^{234}–Ile^{235} from the helix 225–235. Residues from the H chain of the Ab are dark blue, residues from the L chain are cyan, and the Ag is gold. Hydrogen bonds and metal–ligand interactions are shown as thin lines, with marked distances. *Inset*, Ionic and cation–π interactions involving Tyr^{L32} and Asp^{L92} with Lys^{251}.

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structure of the complex, with only one of them participating in the interactions described above (Fig. 3).

Three CDRs of the H chain of the Ab interact with all four loops involved in the formation of the epitope recognized by 4C3 (Fig. 1, *inset*). Loop H1 of 4C3 is involved in direct, predominantly hydrophobic interactions with the fragments 200–201 from the loop 199–202 of Bla g 2, and 256–257 from the loop 248–259 (Fig. 4*A*). In addition, two residues of CDR H1, Thr^{H28} and Ser^{H31}, make polar contacts with the residues from the same two loops of the epitope. A short hydrogen bond is formed between the side chain of Asp^{201} from the first loop and the hydroxyl of Thr^{H28} of H1, whereas the carbonyl oxygen of H1 Ser^{H31} maintains a direct hydrogen bond with the amide nitrogen of Asp^{257} from the second loop of Bla g 2 (Fig. 4*A*). In turn, the side chains of the latter two residues also are hydrogen-bonded via a water molecule (W5 in Fig. 4*B*) (i.e., the hydroxyl of the Ser^{H31} side chain interacts with the OD1 atom of Asp^{257} of the Bla g 2 epitope) (Fig. 4*B*).

Residues from the variable loop H2 form predominantly polar interactions with two loops of the allergen that carry residues Asp^{257} and Ser^{270}. A short hydrogen bond is formed between the OD2 atom of Asp^{257} and the hydroxyl of Ser^{H53} of H2. Ser^{270} is found in two conformations, maintaining different types of contacts with the Ab. One direct hydrogen bond is formed between the serine hydroxyl in the first orientation and the amino group of the side chain of Asn^{H54} of CDR H2, but when Ser^{270} adopts the second conformation, it interacts with the Ab via solvent molecules (W6–W8 in Fig. 4*B*). A single hydrophobic contact in this area is found between Pro^{253} of Bla g 2 and the Cβ atom of the side chain of Asn^{H52} of H2 (Fig. 4*A*).

The contacts with H3 are rather limited in this area, flanking the continuous hydrophobic contacts of CDRs H1 and H2. The Cβ atom of Ser^{254} makes an important hydrophobic contact with Ala^{H101} of H3 (Fig. 4*A*), which, when combined with the interactions formed by Trp^{H105} (Fig. 2), completes the hydrophobic pillow in this area of the allergen–Ab interface.

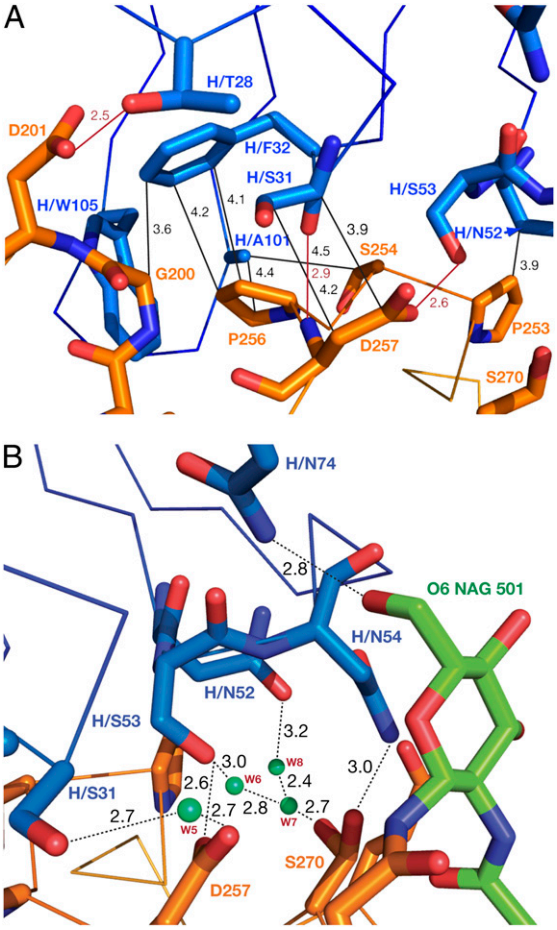

FIGURE 4. Interactions involving CDRs H1 and H2 of 4C3. *A*, CDR H1 makes contacts with fragments 200–201 from the loop 199–202 of Bla g 2 as well as with residues 256–257 from the loop 248–259. Residues from the H chain of the Ab are dark blue, and the Ag is gold. Hydrogen bonds are shown as red dotted lines, and hydrophobic interactions are shown as black dotted lines, with the distances marked in respective colors. *B*, Solvent-mediated interactions between CDR H2 of 4C3 mAb and a part of the epitope on Bla g 2 that includes a glycosylation site. The side chain of Ser^{H31} of CDR H1 also is shown. Hydrogen bonds are marked with dotted lines and their lengths are given.

Carbohydrate-mediated interactions

The epitope on the surface of Bla g 2 involved in binding mAb 4C3 includes a glycosylation site on the side chain of Asn^{268} of the allergen. The well-defined carbohydrate molecule attached to this residue interacts with the Ab either directly or via solvent (Fig. 5*A*). The interactions with the Ab significantly stabilize the conformation of the oligosaccharide. Two mannose (Man) residues that follow the first two *N*-acetylglucosamine moieties (GlcNAc, also abbreviated here as NAG1 and NAG2) can be traced in the electron density map of the complex (Supplemental Fig. 4), whereas these mannoses are much more mobile and thus not visible in the electron density map of the uncomplexed allergen.

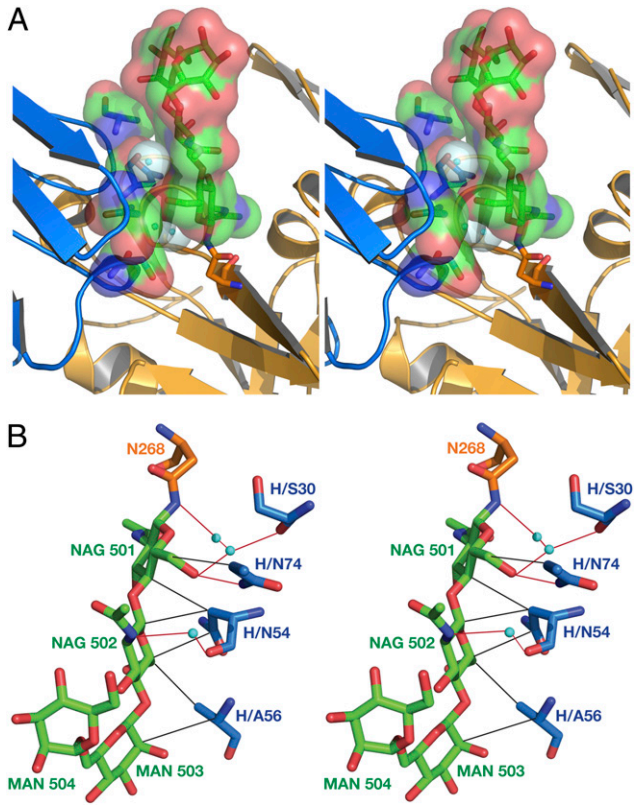

FIGURE 5. Location of the carbohydrate moieties on Bla g 2. *A*, Stereoview of the carbohydrate molecule bound to Asn^{268} of Bla g 2 (orange) shown as sticks covered by the van der Waals surface. The fragments of mAb 4C3 interacting with the carbohydrate are shown as a blue cartoon. *B*, Interactions between the oligosaccharide (carbon atoms green) and Ab (carbon atoms blue). The residue glycosylated in Bla g 2, Asn^{268}, is shown in orange. Hydrogen bonds are shown by red lines, and the hydrophobic contacts are shown in black.

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An additional intramolecular contact between the side chain of Arg^{324} of the allergen molecule and O7 of NAG2, absent in unbound Bla g 2, supports the conformation of the sugar in the complex with the Ab as well as its interactions with the Fab residues. Involvement of a sugar moiety in the interactions with Ab increases the total buried surface (for allergen and Fab) from 1585 to 1831 $Å^2$. An extensive network of the interactions between the sugar and the Ab includes both hydrophobic and polar interactions (Fig. 5*B*). The polar interactions are maintained mostly via water molecules, with the exception of a single strong hydrogen bond between Asn^{74} from the third loop (which does not contain a CDR and is located between the second and third CDRs) of the H chain and the O6 atom of NAG1. Several hydrophobic contacts are maintained between the residues from the Ab and three out of four sugar rings that could be traced in the electron density maps. Very good surface complementarity for this region is observed. To the best of our knowledge, this is the first structure that places a carbohydrate as part of the Ag epitope that is recognized by an Ab and reveals direct contacts between them.

Structural comparison of the two Ab complexes

Crystal structures of the complexes of Bla g 2 with two mAbs, 7C11 (13) and 4C3 (this work), mark two epitopes on the surface of allergen. These epitopes are located in two different domains of the molecule. Although 7C11 recognized an epitope in the N-terminal domain of Bla g 2, the second epitope, recognized by 4C3 mAb, is located in the C-terminal domain (Supplemental Fig. 5). Both epitopes have similar structural organization, which includes their continuous part as well as three short loop areas. Combined, these elements form the epitopes of the conformational type for both Abs. The residues from the continuous parts of both epitopes are involved in cation–π interactions. The epitopes are also of comparable sizes (1739 and 1831 $Å^2$), although there are significant differences between them in the nature of the Ag–Ab interactions. The interface between Bla g 2 and 4C3 includes more solvent molecules compared with those in the complex with 7C11 (21 water-mediated interactions as compared with only 4 such interactions).

The other distinctive feature of the 4C3 epitope is the involvement of the glycosylation site at Asn^{268}. The carbohydrate molecule interacts with the Ab directly and via solvent. When included in the calculations, sugar increases the contact area in the complex from 1586 to 1831 $Å^2$.

The most profound differences in the conformations of the variable loops in two Abs are detected for the loops H3 (with maximum deviation over 6 Å between the corresponding Cα atoms). The residues from H3 CDR in 4C3 complex are involved in most specific direct contacts with the allergen (see above); therefore, the differences in the conformations of H3 CDRs reflect the specific properties of two different epitopes on the surface of the allergen molecule.

Relevance of carbohydrates that mediate the Bla g 2–4C3 mAb interaction

Binding of 4C3 mAb to Bla g 2-N268Q mutant by ELISA. To assess the relevance of the carbohydrate involved in 4C3 mAb binding, a recombinant Bla g 2-N268Q mutant, expressed in *P. pastoris*, was tested for Ab binding by ELISA. The mutant N268Q that retained two of the three N-glycosylation sites present in wild-type Bla g 2 (Asn^{93} and Asn^{317}) was able to bind the Abs (either 7C11 or 4C3 mAb used for capture and the rabbit polyclonal used for detection), and the dose–response curves were parallel to those obtained with rBla g 2-N93Q and natural Bla g 2 (data not shown). These results prove that the N268Q mutant folds similarly to wild-type Bla g 2 and that the presence of carbohydrate at position 268 is not essential for the allergen–Ab interaction.

Effect of enzymatic deglycosylation on Ab binding. Effective deglycosylation of rBla g 2 was confirmed by a reduction of the allergen m.w. on SDS-PAGE. Bla g 2-DG was compared with nondeglycosylated rBla g 2-N93Q (Bla g 2) by similar ELISAs that used either 7C11 mAb as capture and biotinylated 4C3 mAb (Fig. 6*A*) or vice versa (Fig. 6*B*). ELISA experiments showed parallel dose–response curves for Bla g 2 and the enzymatically deglycosylated allergen Bla g 2-DG. In both ELISAs, the dose–response curves were parallel, but the one for Bla g 2-DG displayed a ~40-fold displacement to the right compared with that for the nondeglycosylated allergen.

Inhibition of IgE Ab binding by 4C3 mAb

At increasing concentrations (from 0.1 to 100 μg/ml), the mAb 4C3 showed increased inhibition of IgE Ab binding of ≤45%. The positive control (an anti-Bla g 2 rabbit polyclonal Ab) showed inhibition of IgE Ab binding of ≤83%, whereas the negative control (the anti-mite allergen Der p 1 mAb 4C1) showed inhibition below 7% at the highest concentration of 100 μg/ml (Fig. 7*A*).

Site-directed mutagenesis analysis of the 4C3 mAb epitope

Site-directed mutagenesis of the 4C3 mAb epitope was performed based on structural analysis of the allergen–Ab interactions. Mutants K251A-N93Q, E233A-N93Q, E233R-N93Q, and E233R-I199W resulted in a significant decrease of 4C3 mAb binding

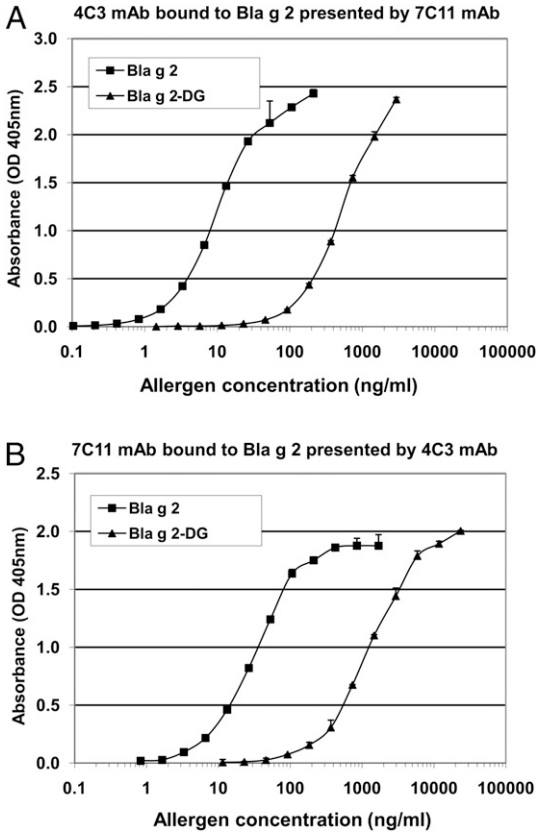

FIGURE 6. Dose–response curves of Bla g 2 deglycosylated versus nondeglycosylated allergen using sandwich mAb ELISAs. *A*, Binding of biotinylated 4C3 mAb to Bla g 2 presented by 7C11 mAb. *B*, Binding of biotinylated 7C11 mAb to Bla g 2 presented by 4C3 mAb. Values represent average of duplicates and SD from one representative experiment of the two performed for each of the ELISAs.

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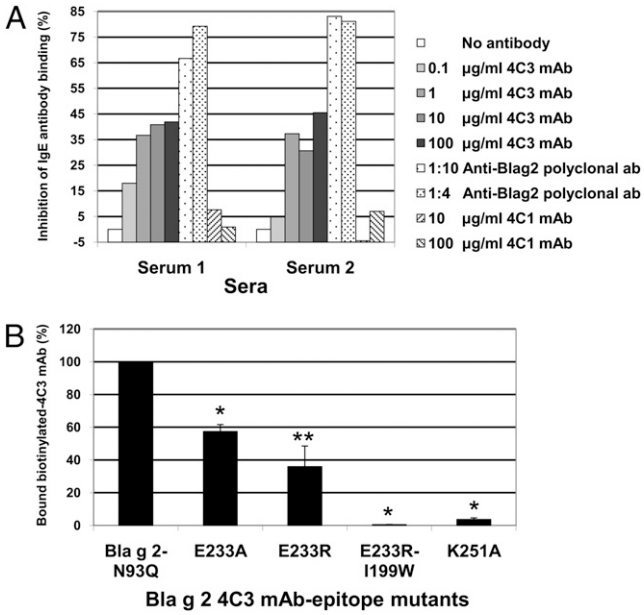

FIGURE 7. *A*, Overlap of 4C3 mAb and IgE Ab epitopes. Inhibition of IgE Ab binding to Bla g 2 by 4C3 mAb (0.1, 1, 10, and 100 μg/ml), an anti-Bla g 2 rabbit polyclonal Ab (as a positive control), and an anti-Der p 1 mAb 4C1 (as a negative control) at concentrations of 10 and 100 μg/ml. *B*, Binding of biotinylated 4C3 mAb to Bla g 2-N93Q and four epitope mutants (E233A, E233R, E233R-I199W, and K251A). An average of two out of three experiments performed is given ($p < 0.05$ paired t test compared with the wild type, two- (*) and one-tailed (**) distribution).

versus the wild type in a validated multiplex array assay (27) (Fig. 7*B*). Bla g 2-specific polyclonal Ab bound Bla g 2-N93Q and the epitope mutants (presented by 7C11 mAb) in a similar manner, showing that the overall folding of the mutants was similar to that of the wild type (data not shown). A decrease of 4C3 mAb binding to the mutants confirmed the importance of the amino acids Lys^{251}, Glu^{233}, and Ile^{199} in the interaction and highlighted the importance of a rational design for site-directed mutagenesis for the identification of key amino acids for the allergen–Ab interaction. Interestingly, K132A and K251A, two single mutants from Bla g 2 epitopes for 7C11 and 4C3, respectively, that significantly reduce Ab binding have very similar interaction patterns in both complexes.

Discussion

The conformational epitope on the surface of the cockroach allergen Bla g 2 for the mAb 4C3 has been defined by x-ray crystallography, and the biological significance of the individual contacts within the interface was evaluated by site-directed mutagenesis. The key residues responsible for specific recognition of the epitope by the 4C3 Ab were identified. A carbohydrate moiety (*N*-acetylglucosamine) attached to the allergen appears to play a role in Ab recognition. Finding a positive contribution of carbohydrates to allergen–Ab interactions contrasts with many previous studies that report the "masking" role of carbohydrates covering the parts of the epitope and thus interfering with the Ab recognition and impairing Ab binding (28, 29). The masking of epitopes by carbohydrates could be compared with the blockage of IgE Ab binding observed by the "pro" part of the mite cysteine protease allergen Der p 1 (30). The structure of Bla g 2 in complex with 4C3 mAb suggests a possibility of another role of such glycosylation.

Whereas a number of structures of Abs in complex with their carbohydrate Ags have been published [exemplified by the complexes between a trisaccharide and scFv based on Se155-4 (31), between human Ab 2G12 and an oligosaccharide, $Man_9GlcNAc_2$ (32), or between hu3S193 and Lewis Y tumor Ag (33, 34)], few if any structures show Abs interacting simultaneously with both the protein and the carbohydrate. However, a recent structure of the broadly neutralizing human Ab CR6261 Fab in complex with the major surface Ag from the H5N1 avian influenza virus (35) also describes an epitope on the Ag surface with a sugar molecule located at its border. Because some atoms in the sugar molecule attached to Asn^{154} in HA2 are in close proximity to the Ab, (i.e., ~4 Å), we can hypothesize that, similarly to our structure, a solvent-mediated Ag–Ab interaction that could include sugar moieties might be present in that case, although few water molecules were actually seen in this comparatively low-resolution structure. The involvement of carbohydrates in the interactions, as seen in the structure of Bla g 2 in complex with 4C3 mAb, suggests the possibility of a dual role for such glycosylation sites, which can either mask the surface of the epitope preventing direct interactions with the Ab or facilitate the contacts for the parts of the Ab that cannot otherwise interact with the Ag.

The role of the carbohydrates will depend on the particular orientation of the Ab toward the epitope, which is dictated by the majority of specific interactions within the Ag–Ab interface. When carbohydrates cover the essential part of the epitope on the surface of the Ag that carries a majority of the residues specifically recognized by the Ab, then their role can be considered to mask Ab interactions. In a case like ours, when the presence of the sugars at the periphery of the epitope does not obstruct the specific Ag–Ab interactions within the major part of the epitope, involvement of carbohydrates in the interactions (either direct or via solvent) with the Ab may contribute to the stability of the complex. Although taking into account the nonspecific nature of such interactions (predominantly via solvent molecules), combined with the generally high *B* factors of the sugar moieties, we would not like to overemphasize their significance, but our data on deglycosylated allergen indicate that such interactions might be helpful for increasing Ab binding.

N-glycans involved in IgE Ab recognition have been reported for plant and insect allergens and are named cross-reactive carbohydrate determinants (36). N-glycans play an important role in IgE Ab binding for some allergens such as latex Heb v 4 and yellow jacket Ves m 2. For both allergens, a total reduction of IgE Ab recognition of nonglycosylated recombinant allergen versus native or allergen expressed in *P. pastoris* has been reported (37, 38). However, the clinical relevance of cross-reactive carbohydrate determinants is controversial (39-41). In this study, we compared two rBla g 2 allergens (enzymatically deglycosylated versus nondeglycosylated Bla g 2 expressed in *P. pastoris*) that retain a similar fold, as proven by Ab binding. The selection of *N*-glycosidase F as a deglycosylating enzyme was based on its capacity to remove the trimannosyl core that is involved in the 4C3 mAb interaction with Bla g 2 and is the minimal identical structure common to all *N*-glycans, regardless of the species in which they have been synthesized. We showed a displacement of the dose–response curves to the right for the deglycosylated versus the nondeglycosylated allergen using two similar ELISAs that involved 4C3 mAb as either detection or capture Ab. Because carbohydrates are not involved in the 7C11 Bla g 2–mAb interaction (13), the displacement to the right indicates that the 4C3 mAb binds less to the deglycosylated allergen. These results show that the carbohydrates at position Asn^{268} contribute to the allergen–4C3 mAb interaction. However, the carbohydrate at this position is not essential for the interaction because 4C3 mAb binds to the mutant N268Q. An overlap between the 4C3 mAb epitope and the

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IgE Ab binding site was proven by high levels of inhibition (≤45%) of IgE Ab binding to Bla g 2 by 4C3 mAb compared with those of a negative control (anti-mite Der p 1 mAb 4C1). We also have reported an overlap between 4C3 mAb and IgE Ab epitopes by mutagenesis analysis as well as a contribution of carbohydrates and certain amino acids in the 4C3 mAb epitope to the allergen–IgE interaction (27). These results show the clinical relevance of the 4C3 mAb epitope described in this study. The present study highlights the importance of X-ray crystallographic studies on allergen–Ab complexes to map the conformational epitopes on the surface of inhaled allergens such as Bla g 2 and the molecular mechanisms that contribute to allergen–Ab interactions.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure Legends

Figure S1 A stereoview of the crystal structure of the complex between Bla g 2 and mAb 4C3. Bla g 2 molecule is colored orange, 4C3 two shades of blue, and the carbohydrate bound to Asn268 of Bla g 2 is shown as sticks (green for carbon, red for oxygen, and blue for nitrogen).

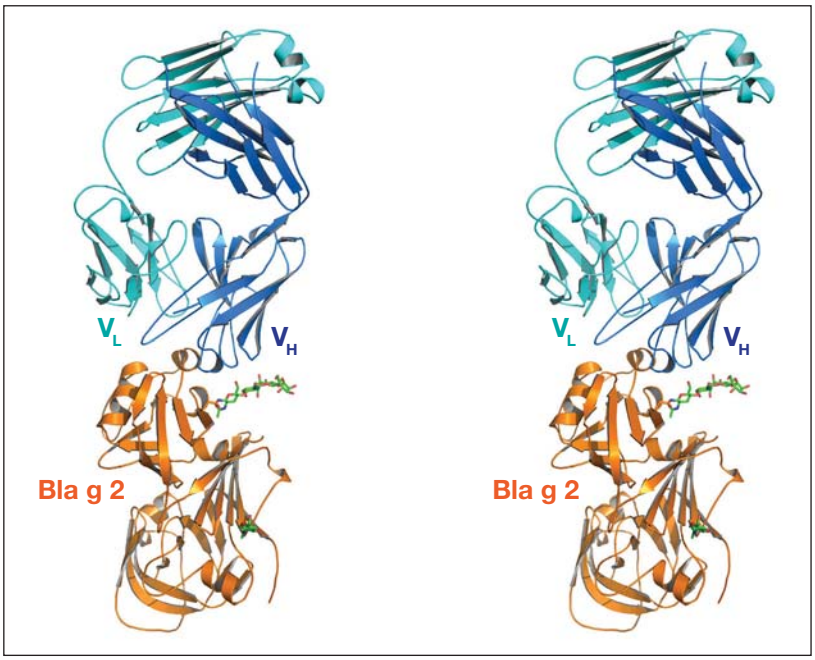
Figure S2 Interactions involving CDRs L1, L3, and H3 of mAb 4C3. Parts of the antibody chain involved in the interactions are colored pink for V_L and red for V_H, and the interacting part of the antigen is yellow. For details, see Fig. 3.

Figure S3 Interactions involving CDRs of chain V_H of mAb 4C3. Parts of the antibody chain involved in the interactions are colored red, and the interacting part of the antigen yellow. A part of the carbohydrate molecule involved in the interactions is green. For details, see Fig. 4.

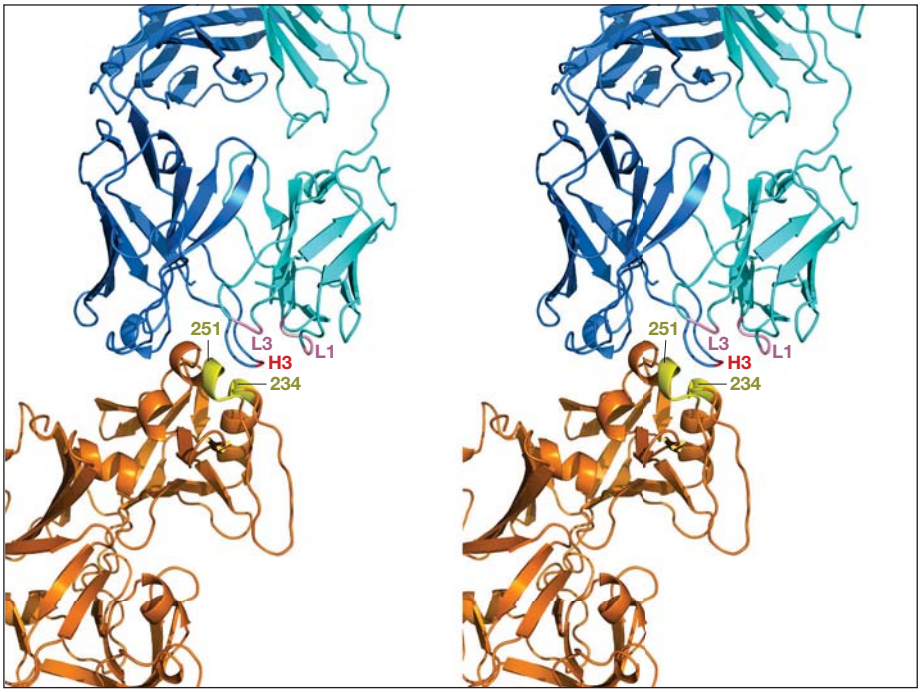
Figure S4 An electron density map covering the carbohydrate bound to Asn268 and the water molecules that participate in the interactions with the mAb. The map is calculated as 2Fo-Fc, contoured at 1σ level.

Figure S5 Superposition of the complexes of Bla g 2 with 4C3 and 7C11 mAb. The Cα coordinates of Bla g 2 were used to calculate the superposition. The complex with 7C11 is pink, and with 4C3 is cyan.

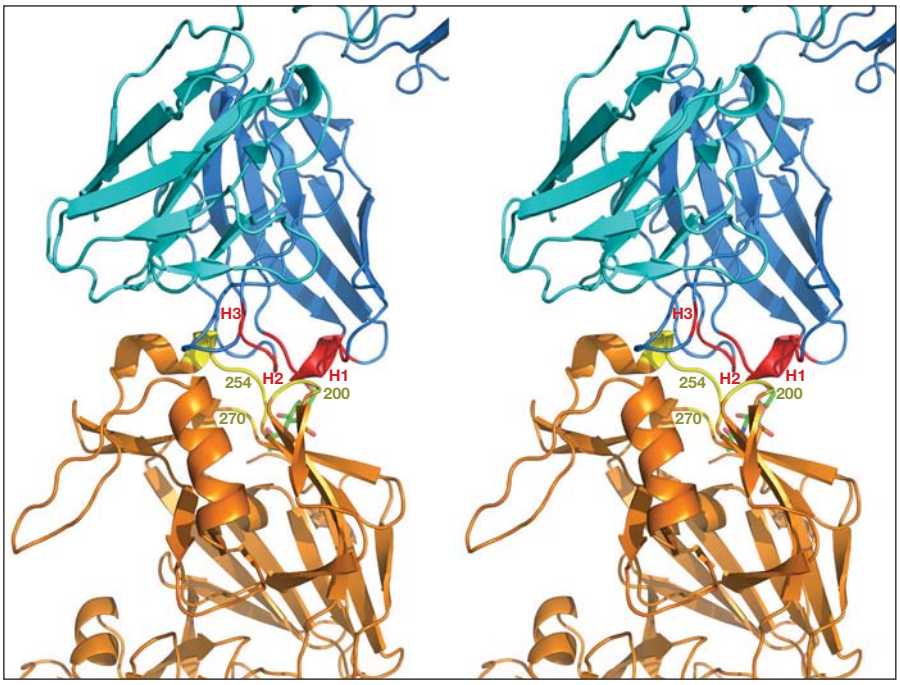
Supplemental Figure 1



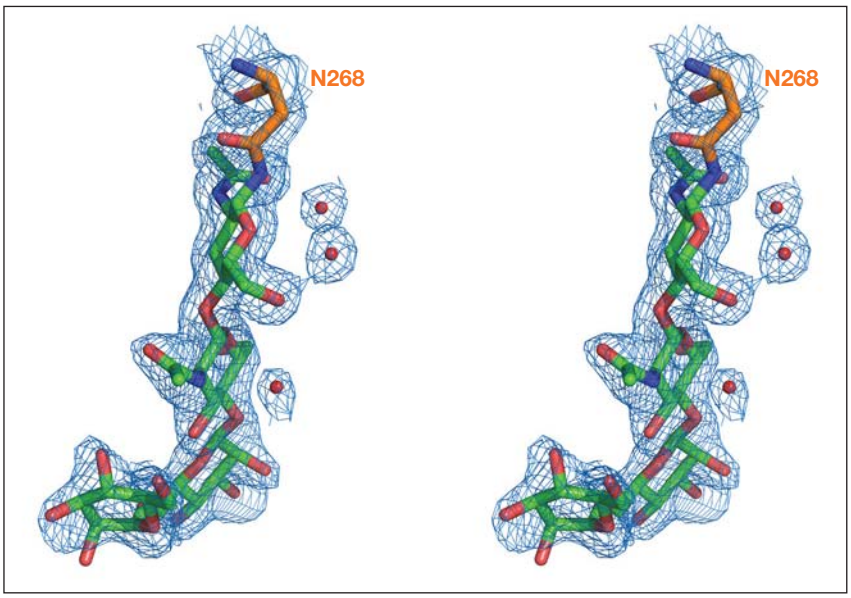
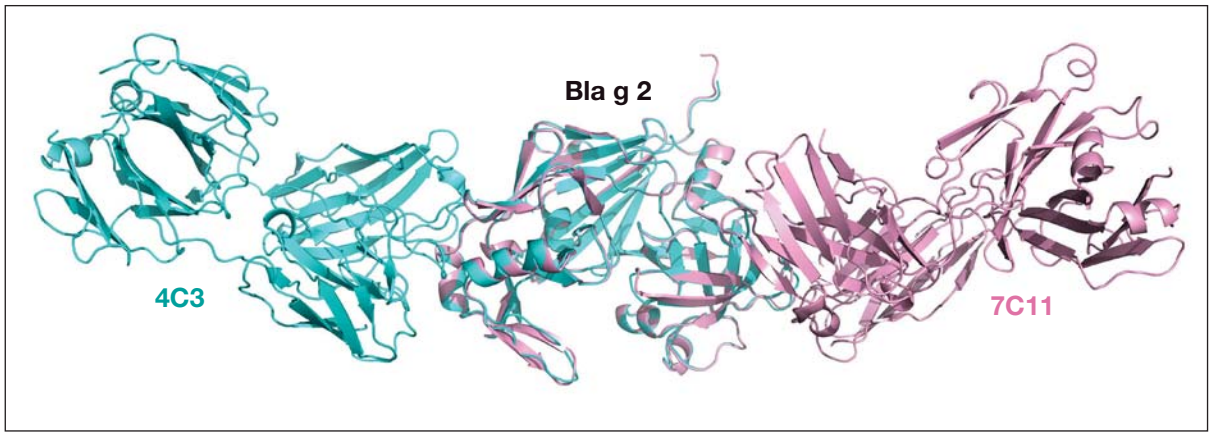
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5