

Crystal structure of a dimerized cockroach allergen Bla g 2 complexed with a monoclonal antibody

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Abstract:

The crystal structure of a 1:1 complex between the German cockroach allergen Bla g 2 and the Fab' fragment of a monoclonal antibody 7C11 was solved at 2.8-Å resolution. Bla g 2 binds to the antibody through four loops that include residues 60-70, 83-86, 98-100, and 129-132. Cation- π interactions exist between Lys-65, Arg-83, and Lys-132 in Bla g 2 and several tyrosines in 7C11. In the complex with Fab', Bla g 2 forms a dimer, which is stabilized by a quasi-four-helix bundle comprised of an α -helix and a helical turn from each allergen monomer, exhibiting a novel dimerization mode for an aspartic protease. A disulfide bridge between C51a and C113, unique to the aspartic protease family, connects the two helical elements within each Bla g 2 monomer, thus facilitating formation of the bundle. Mutation of these cysteines, as well as the residues Asn-52, Gln-110, and Ile-114, involved in hydrophobic interactions within the bundle, resulted in a protein that did not dimerize. The mutant proteins induced less β -hexosaminidase release from mast cells than the wild-type Bla g 2, suggesting a functional role of dimerization in allergenicity. Because 7C11 shares a binding epitope with IgE, the information gained by analysis of the crystal structure of its complex provided guidance for site-directed mutagenesis of

The atomic coordinates and structure factors (code 2NR6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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the allergen epitope. We have now identified key residues involved in IgE antibody binding; this information will be useful for the design of vaccines for immunotherapy.

Keywords: cockroach allergy | IgE | mast cells | basophils | immunotherapy

Article:

Cockroach allergy is associated with the development of asthma and is a risk factor for emergency room admission of asthmatic patients, especially among inner city children living in low-income houses infested with cockroaches (1, 2). Cockroaches release allergens to the environment, which are carried by particles (5-40 μm of diameter) that reach the lung by inhalation. Bla g 2 is one of the most important cockroach allergens, eliciting production of specific IgE in ~70% of cockroach-allergic patients at exposure levels that are 10-100-fold lower than those from other common indoor allergens from dust mite and cat (3-5). Exposure to Bla g 2 results in cross-linking of IgE bound to the surface of mast cells or basophils from sensitized patients (*i.e.* in immunological terms, cross-linking refers to the non-covalent linkages between the allergen and two IgE molecules at the surface of mast cells or basophils), and induces release of potent mediators (histamine, leukotrienes, prostaglandins, etc.) of allergic reactions.

We recently solved the crystal structure of Bla g 2, confirming that the overall fold of this allergen corresponds to that of pepsin-like aspartic proteases, and revealing structural elements that explain why it is enzymatically inactive (6, 7). Bla g 2 contains important amino acid substitutions in the area corresponding to the catalytic site. These modifications impair enzymatic function, and the allergen did not show proteolytic activity in standard *in vitro* assays using casein and hemoglobin as substrates (7). Bla g 2 belongs to a group of inactive aspartic proteases that also includes the family of pregnancy-associated glycoproteins. The latter proteins have similar active site substitutions to Bla g 2 and are produced in the chorion of pregnant females from ungulate species such as pig, sheep, cow, and horse (8). Unlike other members of the pepsin family, Bla g 2 is a zinc-binding protein and has five instead of the usual two or three disulfide bonds that are present in typical aspartic proteases. These two features provide stability to the protein, thus increasing the persistence of this allergen in the environment (6).

The three-dimensional structures of eight indoor allergens have been solved to date, but there are few studies of epitopes or allergen-antibody interactions. Only three allergen-antibody complex structures have been resolved to date: birch pollen allergen, Bet v 1 (9) and bee venom allergen, Api m 2 (hyaluronidase) (10), both in the complexes with recombinant Fab fragments of IgG antibodies, and β -lactoglobulin in complex with Fab fragment of IgE (11). A majority of reports describing epitope mapping have not been based on the tertiary structure, but rather have used different approaches, such as libraries of overlapping synthetic peptides, *e.g.* for food allergens (12), the expression of allergen peptides as recombinant proteins, and the creation of epitope expression cDNA libraries (13, 14). A serious limitation of these approaches is that they exclude conformational epitopes. For inhaled allergens, such as Bla g 2, that are globular and stable, approaches to map antigenic determinants involving the tertiary structure of the protein are essential. Determination of the structure of allergen-antibody complexes facilitates epitope mapping and reveals the precise architecture of a conformational epitope. The mapping strategy utilized in this study involved co-crystallization of the allergen with Fab' fragments of a

monoclonal antibody that partially overlaps with IgE antibodies. Site-directed mutagenesis of the epitope identified by this procedure will allow rational design of hypoallergens for immunotherapy.

MATERIALS AND METHODS

Expression and Purification of rBla g 2—A partially deglycosylated recombinant Bla g 2 mutant protein (rBla g 2 N93Q) was expressed in *Pichia pastoris* and purified by affinity chromatography using 7C11 mAb, as previously described (6). Briefly, the DNA encoding for the mature form of Bla g 2 with a mutation (N93Q) in one of the three *N*-glycosylation motifs was inserted into the *P. pastoris* expression vector pGAPZ α C (Invitrogen) for constitutive expression of the allergen. Recombinant Bla g 2 was expressed and purified from culture media by affinity chromatography over a 7C11 monoclonal antibody (mAb)³ column as described (15, 16). The purity of rBla g 2 was >95% by SDS-PAGE and mass spectroscopy.

Production of Fab' from the 7C11 Monoclonal Antibody and Formation of the rBla g 2-Fab' Complex—Monoclonal anti-Bla g 2 antibody 7C11 (clone 7C11 C2 A6) was purified from ammonium sulfate-precipitated ascites by affinity chromatography through a protein G column. Purified antibody was fragmented using pepsin and F(ab')₂ fragments were purified by protein A chromatography and size exclusion chromatography. In brief, F(ab')₂ fragments were applied to a HiTrap 16/60 Sephacryl S-100 gel filtration column (GE Healthcare) in 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.2. Eluted fractions containing F(ab')₂ were further purified by protein A chromatography (Protein A-Sepharose Fast Flow, GE Healthcare). Purity of the (Fab')₂ was analyzed by non-reducing SDS-PAGE followed by silver staining.

F(ab')₂ were reduced with 3 mM dithiothreitol in 20 mM phosphate buffer containing 0.15 M NaCl at pH 7.2 for 30 min at 4 °C. The free disulfide bonds were blocked by adding 300 mM iodoacetamide for 3 h at 4 °C. The solution was dialyzed against 20 mM Tris, 0.2 M NaCl at pH 7.2 overnight, concentrated to 15 mg/ml, and loaded onto a Superdex 75 (10/30) gel filtration column. The peak containing Fab' was collected, concentrated, and mixed with Bla g 2 at a 1:1 molar ratio. The mixture was further purified over a Superdex 200, HPLC column and concentrated to ~5 mg/ml for crystallization.

Sequencing of the Monoclonal Antibody 7C11—The cell line producing the anti-Bla g 2 mAb 7C11 C2 A6 was grown at the Lymphocyte Culture Center (University of Virginia). Total RNA (44 μ g/ 3×10^6 cells) was isolated from the 7C11 mAb cell line using an RNeasy Mini kit (Qiagen, Valencia, CA). cDNAs encoding for the light and heavy chains of the mAb were obtained by reverse transcription from RNA (SuperScriptTM III, Invitrogen), and the DNA was PCR-amplified using specific primers, sequenced, and analyzed.

To amplify the light and heavy chains of the antibody, two and four pairs of primers were initially tested, respectively. A degenerate primer for the N terminus (VHa: 5'-gag gtt cag ctg cag cag(ct)c-3', encoding for EVQLQQ), and the C-terminal primer (CH2: 5'-tt agg agt cag agt aat ggt gag cac atc c-3', encoding for DVLITLTTP) amplified the heavy chain. Forward (5'-c aac aca gcc tac ctg c-3', encoding for NTAYL) and reverse (5'-a ggt cac tgt cac tgg etc agg-3', encoding for PEPVTVT) middle primers were used to complete heavy chain sequencing. To

amplify the light chain, a primer was designed based on the N-terminal sequence of the antibody and the sequence of homologous light chains from a BLAST search (primer L2F, 5'-cac aaa ttc atg tcc aca tca-3', encoding for HKFMSTS). The mAb was isotyped G1 for the heavy chain and kappa (κ) for the light chain using the IsoStrip (Roche Applied Science).

Crystallization of the rBla g 2-Fab' Complex—Crystals of complex of Bla g 2 with Fab' were obtained using the hanging-drop vapor diffusion method at room temperature. Each drop contained 2 μ l of \sim 5 mg/ml allergen/antibody complex in 20 mM Tris buffer, 0.2 M NaCl, and 2 mM dithiothreitol at pH 7.2, as well as 2 μ l of reservoir solution consisting of 16% PEG 10,000 in 0.1 M sodium citrate buffer at pH 5.6. The crystals are shaped as thin plates, with the dimensions of \sim 0.15 \times 0.2 \times 0.01 mm. They belong to the monoclinic space group P2₁, and each asymmetric unit contains two molecules of Bla g 2, as well as two molecules of Fab'.

Data Collection, Structure Solution, and Refinement—Diffraction data extending to nominal 2.8 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 Mar225CCD detector and were integrated and scaled with the HKL2000 package (17). Prior to data collection, the crystal was rapidly cooled to 100 K in a nitrogen stream, after being transferred to the cryo-solution containing 16% PEG 10K and 10% glycerol, pH 5.6, in sodium citrate buffer. The highest resolution shell, which is over 75% complete, is 2.93-3.0 Å, thus the actual resolution of the structure might be slightly lower than the nominal one of 2.8 Å (Table 1).

TABLE 1. Data collection and refinement statistics

Wavelength (Å)	1.000
Space group	P2 ₁
Unit cell parameters (Å)	$a = 77.0, b = 103.2, c = 146.3,$ $\beta = 94.8^\circ$
Resolution (Å)	50-2.8
Number of reflections (unique/total)	51,667 (170,997)
Completeness % (total/last shell)	93.3 (65.2)
R_{merge} % (total/last shell)	9.8 (30.1)
No. of molecules in a.u.	2
No. of protein atoms	11600
No. of solvent molecules	111
No. of heteroatoms	86
R_{cryst}	23.2%
R_{free} (5%)	28.1%
R.m.s. deviations from ideality	
Bond lengths	0.006 Å
Angle distances	1.05°
Ramachandran plot (most favored/allowed/ generously allowed/disallowed)	88.9%/10.0%/0.8%/0.2%

The structure of Bla g 2/Fab' complex was solved by molecular replacement using the program PHASER (18). The search model for Bla g 2 was based on the previously solved 1.3-Å structure (PDB accession code 1YG9 and Ref. 6). A number of different models of Fab were used in the search for this component, with the best result provided by Fab D44.1 (PDB accession code 1MLB). Solution of the molecular replacement was unambiguous, with the final Z-score 49.3 and LLG 4915. The structure was refined using the program REFMAC5 (19) and rebuilt using the program COOT (20). The refinement procedure was very conservative (21), with the overall B-factor fixed at 15 Å². The final model contains two Bla g 2 molecules, two Fab' molecules,

and 111 water molecules, and is characterized by R_{free} of 28.1% and R of 23.2%. The coordinates and structure factors have been deposited in the PDB with accession code 2NR6.

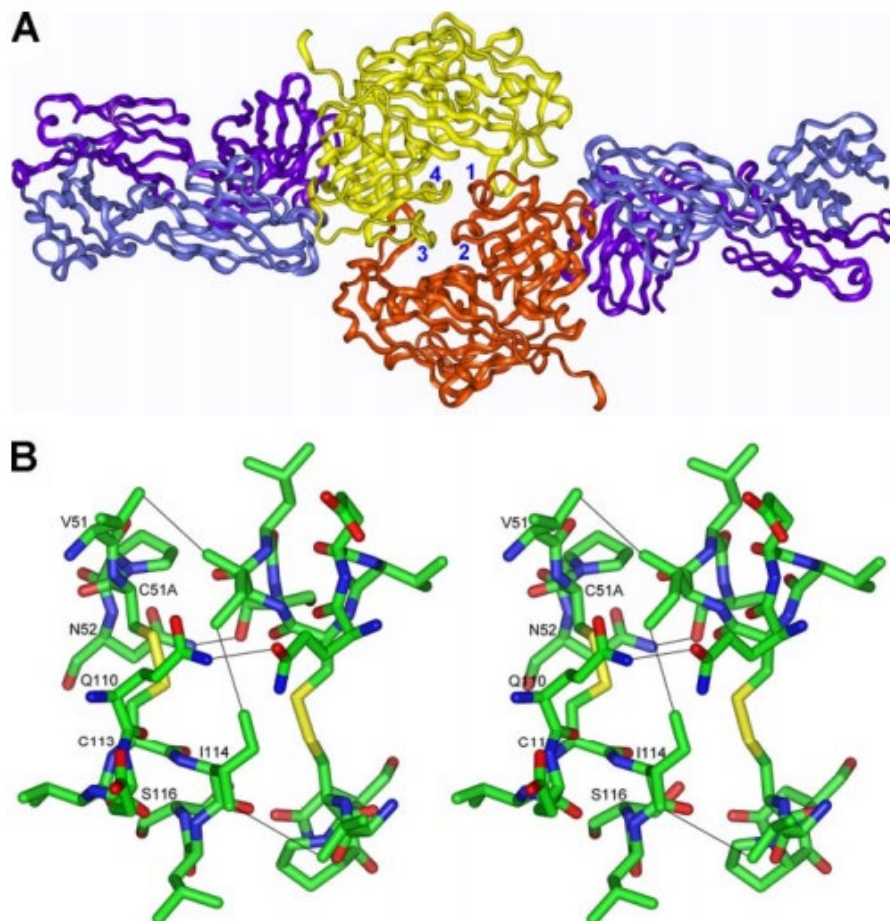


FIGURE 1. The structure of the Fab' fragments of mAb 7C11 and a dimer of Bla g 2. *A*, ribbon diagram of the complex. Bla g 2 monomers are shown in yellow and orange, and heavy and light chains of Fab' are shown in dark and light shades of purple, respectively. The quasi-helical bundle 1-4 contains cysteines 51a and 113 from both monomers. *B*, stereoview of the area of interaction between Bla g 2 monomers in the bundle, showing the contacts between amino acids involved in dimerization. Disulfide bonds between cysteines 51a and 113 are indicated in yellow. Potential hydrogen bonds and non-bonded interactions are marked with black lines.

Expression of Bla g 2 Mutant Proteins in P. pastoris—Two mutant proteins of either the two cysteines alone (C51aS and C113A), or both cysteines plus additional residues that may be involved in dimerization (C51aA, N52A, Q110N, C113A, and I114A), were obtained by site-directed mutagenesis. The DNA template consisted of Bla g 2 cDNA inserted into the *P. pastoris* expression vector pGAPZaC that allows constitutive expression of the allergen. Site-directed mutagenesis was performed sequentially with two sets of primers per mutant using QuikChange™ (Stratagene, La Jolla, CA). Mutated DNA was used to transform the XL1-Blue supercompetent cells and bacterial clones were sequenced to confirm the desired amino acid substitutions. DNA was linearized and electroporated into *P. pastoris* for expression. Recombinant allergens were purified by 7C11 mAb affinity chromatography. The presence of dimers in the expressed allergens was analyzed by size exclusion chromatography. Bla g 2 proteins in PBS or protein standards (low molecular weight gel filtration kit, GE Healthcare)

were applied to a HiTrap 16/60 Sephacryl S-100 gel filtration column (GE Healthcare) and elution profiles were compared.

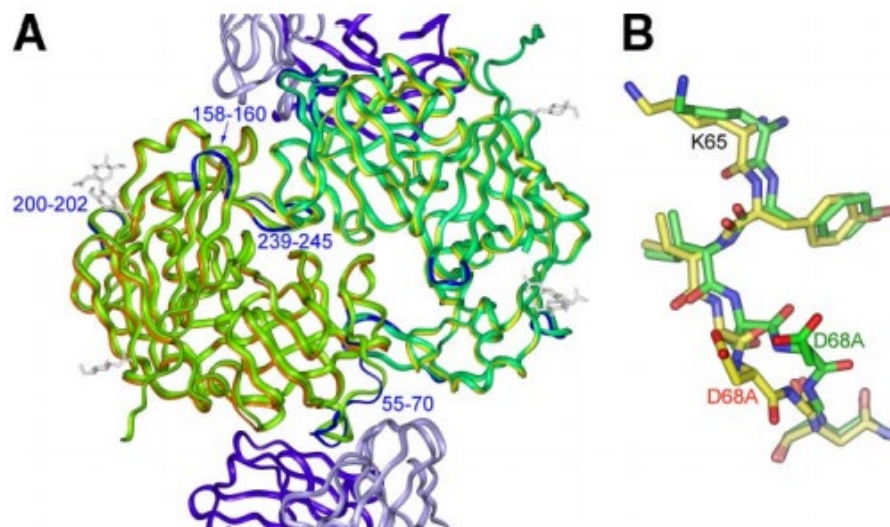


FIGURE 2. Superposition of the structures of the unbound and antibody-bound Bla g 2. *A*, two monomers of allergen in the unbound state (*green*) superimposed on the antibody-bound Bla g 2 (*yellow and orange*). The areas with significant conformational changes are *colored blue* and labeled. *B*, area of the largest differences in the vicinity of Asp68a.

CD Spectra of Wild Type and Mutant Proteins—Circular dichroism (CD) spectroscopy experiments were performed using an AVIV model 202 CD spectropolarimeter with a 1-mm cuvette. The scanning was set using 0.5-nm steps, 7.5 s averaging time, from 260 to 194 nm. Bla g 2 proteins, wild type and mutants, were dissolved in phosphate-buffered saline at 0.34–0.66 mg/ml. Data were analyzed with the program CDNN v2.1 (22). Bla g 2 analysis required CDNN reference set 33 for complex CD spectra proteins.

Effect of Bla g 2 Mutant Proteins on IgE-mediated Degranulation from Sensitized Skin Mast Cells—The β -hexosaminidase assay was used to test the effect of the Bla g 2 mutant proteins on IgE-mediated degranulation from sensitized skin mast cells (23). This assay is based on the measurement of β -hexosaminidase released from mast cells from three non-allergic donors, which were sensitized with sera from a cockroach allergic patient (64 ng/ml of anti-cockroach IgE; CAP class 5). Skin mast cells were sensitized overnight with a 1:25 dilution of serum from a cockroach-sensitive patient. The next day, cells were washed and challenged without the allergen (testing for spontaneous release) or with different concentrations of allergen (0.01–10 μ g/ml). β -Hexosaminidase activity was measured as a percent of total within the cell, and statistical differences were assessed by Student's *t* test. Skin mast cells were obtained from human surgical specimens. All studies were approved by the Human Studies Committee at Virginia Commonwealth University Health Systems.

RESULTS

Description of the Complex and Dimerization Mode—The crystal structure of the complex formed between Bla g 2 and the Fab' fragment of monoclonal antibody 7C11 was solved at 2.8-Å resolution. In the crystals, a dimer of Bla g 2 interacts with two Fab' molecules, forming two

1:1 complexes, related by a non-crystallographic 2-fold symmetry operation (Fig. 1A). Each antibody only interacts with one out of two monomers. Superposition of the two crystallographically independent complexes yielded an r.m.s. deviation of 0.42 Å for 739 C α atoms, with even lower deviations for the individual components (0.16 Å for 317 C α atoms of Bla g 2, 0.26 Å for 206 atoms in the antibody light chain, and 0.33 Å for the 206 atoms in antibody heavy chain). The differences in the r.m.s. values for the individual molecules are most likely related to their involvement in the crystal contacts.

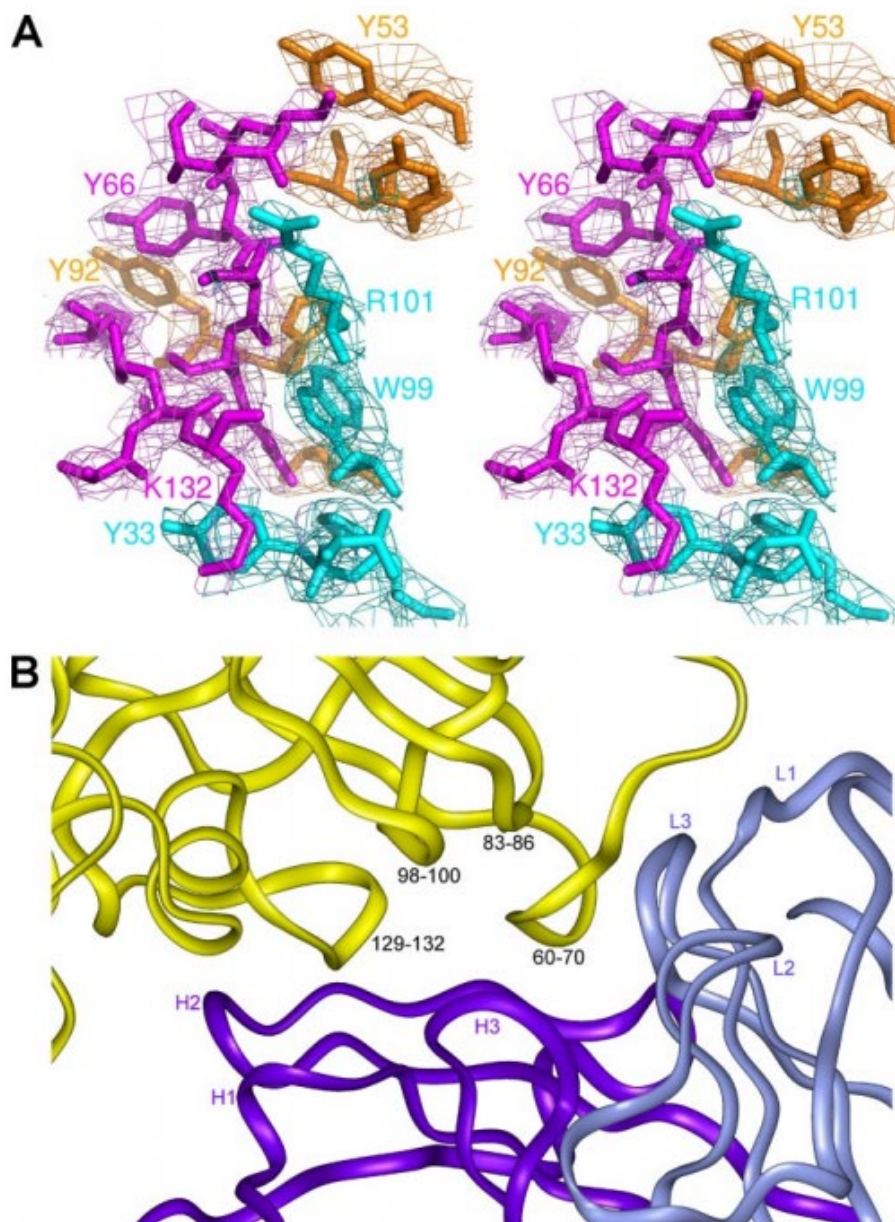


FIGURE 3. The allergen-antibody interface. *A*, electron density superimposed on the final model in the area of the interface. The final 2F_o-F_c map was contoured at 1.5 σ level. The model and map of Bla g 2 are colored *purple*, the Fab' light chain *orange*, and the Fab' heavy chain *teal*. Selected residues are labeled. *B*, *ribbon representation* of the interacting loops in the allergen (*yellow*), and the light (*light blue*) and heavy (*dark blue*) chains of the Fab'. Residues in four loops of Bla g 2 (marked with numbers in *black*) compose the epitope, and the interacting loops in the light (*L*) and heavy (*H*) chain are labeled.

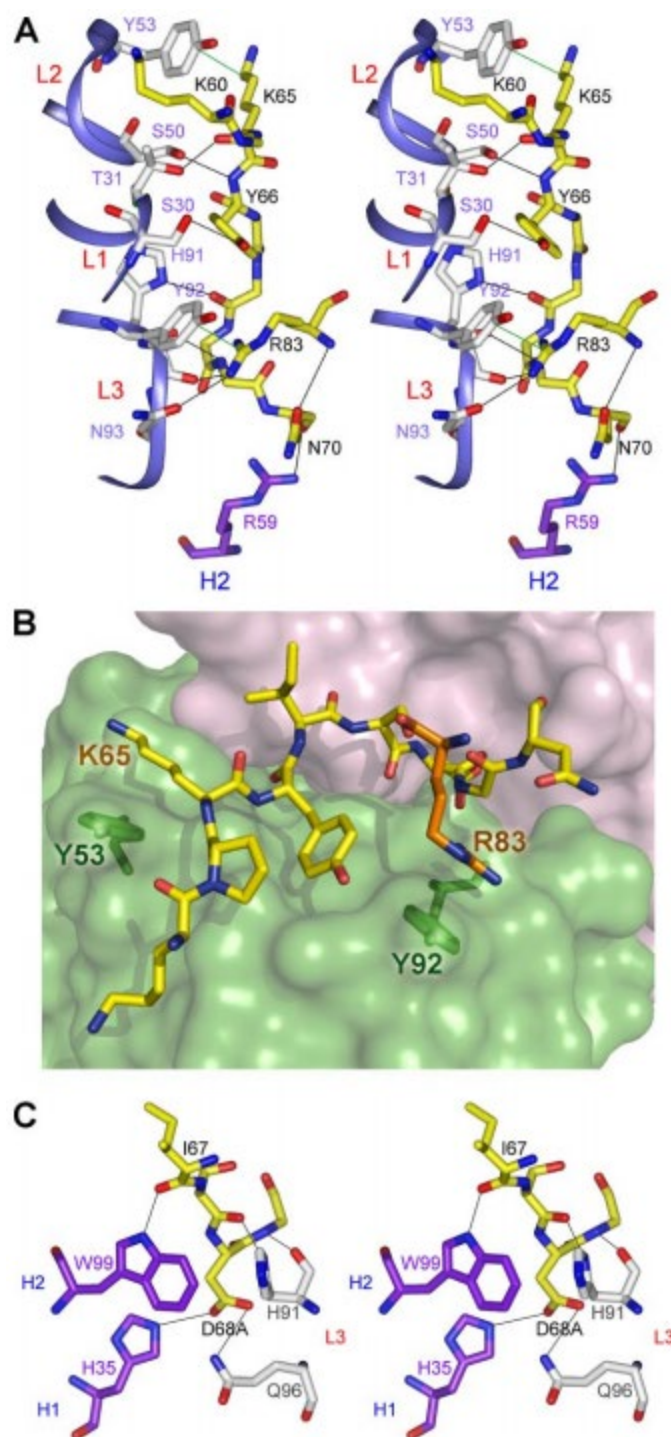


FIGURE 4. Continuous epitope in the allergen. *A*, interactions of the longest consecutive fragment in Bla g 2, containing the residues 60-70 of loop 1 plus Arg-83 of loop 2, with three variable loops of the light chain of the Fab'. Allergen residues are represented by *yellow sticks* and the residues of the antibody light chain in *gray*. Arg-59 of H2 is shown in *purple*. In *panels A* and *C*, potential hydrogen bonds are marked with *black lines*. *B*, cation- π interactions between Lys-65 and Arg-83 in the allergen and Tyr-53 and Tyr-92 in the antibody. *C*, interactions between the Bla g 2 fragment containing residues 67-69 and the Fab'. The side chain of D68a docks in between the heavy and light chains of Fab', forming potential hydrogen bonds with the residues His-35 (H1 loop) and Gln-96 (L3 loop). Flanking residues Ile-67 and Gly-69 interact with Trp-99 (H3 loop) and His-91 (L3 loop), respectively.

The Bla g 2 dimer observed in the structure of the allergen-antibody complex is stabilized by the formation of a quasi-four-helical bundle comprised of one short α -helix and one helical turn from each monomer. The α -helix includes residues 48-51, and the helical turn contains residues 110-114, with these elements related by a non-crystallographic 2-fold symmetry axis in a dimer (Fig. 1, *A* and *B*). These two fragments are present as two short helices in almost all other pepsin-like aspartic proteases (24). The insertion of two residues (Cys51a and Pro51b; see Fig. 4 in Ref. 6) within the first fragment distorts its conformation, transforming a helix into a helical turn. The interactions within the bundle include hydrogen bonds and hydrophobic contacts, and are formed between the residues from both monomers (Fig. 1*B*). A unique disulfide bridge (Cys51a-Cys113) connects the helix and the helical turn within each Bla g 2 monomer. The overall area of the dimer interface calculated with CNS was 1139 Å² per molecule (25).

A comparison between free and bound state of the allergen did not reveal any major conformational changes (Fig. 2*A*). The overall r.m.s. deviations between free Bla g 2 and the two allergen molecules in the dimer within the complex were 0.28 Å and 0.30 Å for 309 and 307 Ca atoms, respectively. The largest conformational changes occurred in the area interacting with the antibody in the vicinity of Asp68a (2.8 Å) (Fig. 2*B*). Other differences (r.m.s. deviations 0.8-1.6 Å) include three surface loops, composed of the residues 158-160, 200-202, and 239-245 (Fig. 2*A*). The latter loop also contributed to the interactions between the monomers in the dimer.

The Allergen/Antibody Interface: Continuous versus Discontinuous Epitope—Monoclonal antibody 7C11 recognizes a large epitope on the surface of the N-terminal domain of a Bla g 2 monomer, which includes four loops (loop 1, residues 60-70; loop 2, residues 83-86; loop 3, residues 98-100; and loop 4, residues 129-132), interacting with all six complementarity determining regions (CDRs) of the Fab' and forming tight 1:1 complex (Fig. 3, *A* and *B*). In the crystals, two Fab' molecules (chains C, E and D, F for the light and heavy chains, respectively, in the PDB coordinate file) interact with a dimer of Bla g 2 (chains A and B in the PDB file) in a symmetrical manner, forming two 1:1 complexes (ACD and BEF). The contact areas between the allergen monomer and the Fab' are calculated to be 862 Å² for the former and 877 Å² for the latter (program CNS, Ref. 25).

The light chain of the antibody recognizes predominantly one continuous epitope on the surface of Bla g 2 molecule, which consists of nine consecutive residues Lys-60-Asn-70 from loop 1 plus the side chain of Arg-83 of loop 2, protruding through loop 1 in the direction of L3 of the light chain (Fig. 4*A*). All three CDRs of the light chain (L1, L2, and L3) are involved in the extensive and complex network of the interactions with the residues 60-70 and Arg-83 of Bla g 2. Although the exact nature of the interactions is not always accurately defined at the moderate resolution of 2.8 Å, the identity and orientation of the potentially interacting residues should suffice to postulate the type of contacts. These interactions are predominantly polar, involving a number of potential hydrogen bonds and ion pairs between the antibody and both the main chain and the side chains of the allergen, thus defining this continuous, almost linear epitope (Fig. 4*A*). Two pairs of residues, Lys-65 (Bla g 2) and Tyr-53 from L2 on one end, and Arg-83 (Bla g 2) and Tyr-92 from L3 on another, are involved in cation- π type interactions (Fig. 4*B*). The only interactions with the heavy chain within this epitope are found in the vicinity of Asp68A (Fig. 4*C*). This is a unique residue in the allergen that interacts with both the light and heavy chains of

the antibody, involving both carboxyl oxygens of the side chain of Asp68A. One of them forms an ionic pair with His-35 of H1, and the other is hydrogen-bonded to Gln-96 of L3. In addition, several hydrogen bonds are formed between the main chain atoms of residues 67-70 in the allergen with Arg-59 (H2) (Fig. 4A), as well as with Trp-99 (H3) and His-91 (L3) (Fig. 4C). As pointed out before, this is also the area with the largest conformational changes in the allergen bound to the antibody (Fig. 2B).

Glu-86 is another residue of loop 2 that interacts with the antibody. This residue, together with Gln-98 and Asp-100 of loop 3, as well as Lys-132 of loop 4, maintain a discontinuous part of the allergen-antibody epitope. All three residues of loops 2 and 3 are involved in charge and polar interactions with Arg-101 of H3 (Fig. 5A). The orientation of the side chain of Arg-101 is stabilized by the hydrophobic contacts with Ile-67 of loop 1.

Lys-132 is a major contributor to the interactions between the residues of small loop 4 and two heavy chain CDRs, H1 and H2 (Fig. 5, B and C). It forms an ion pair with Asp-52 (H2) in both monomers. In addition, Lys-132 is hydrogen-bonded to the carbonyl oxygen of Lys-30 (H1) in one monomer (Fig. 5B), whereas in the other monomer it is involved in cation- π interactions with Tyr-33 (H1) in the heavy chain (Fig. 5C). The orientation of the ring of Tyr-33 (H1) seems to be maintained by the hydrophobic and possible cation- π interactions with Arg-50 (H2) (Fig. 5C). No significant conformational changes were found in the antibody-bound allergen around Lys-132 compared with its unbound state. At the same time, the mobility of the residues in the antibody that interact with Lys-132 is restricted by the intrinsic interactions within H1 and H2 CDRs. The surfaces of both the allergen and the antibody within this epitope appear to possess a high degree of complementarity prior to binding. Therefore, Lys-132 is a good candidate for site-directed mutagenesis aimed at reducing the antibody binding properties of Bla g 2.

Relevance of Dimerization to IgE Cross-linking—Mutant proteins that were expected to be unable to dimerize were designed, expressed in *P. pastoris*, and purified by affinity chromatography. Cysteines 51a and 113 alone (Mut 1), or both cysteines as well as the residues Asn-52, Gln-110, and Ile-114 (Mut 2) were substituted by either alanines or serines by site-directed mutagenesis. Analysis of CD spectra confirmed a similar folding of Bla g 2 wild type and mutants. In agreement with these results, ELISA showed parallel dose-response curves for wild type and mutant proteins (Fig. 6, A and B). No decrease of IgE antibody binding was observed by the mutations (data not shown). Size exclusion chromatography showed that only Mut 2 behaved as a monomer in phosphate buffer, whereas Bla g 2 wild type and Mut 1 behaved as dimers (Fig. 6C). Therefore, the additional disruption of hydrophobic interactions involving residues Asn-52, Gln-110, and Ile-114 was necessary to prevent dimerization.

IgE cross-linking by wild type Bla g 2 and the monomeric Mut 2 was assessed by a cell release assay based on the measurement of β -hexosaminidase released from mast cells sensitized with sera from a cockroach allergic patient. The dimeric wild-type Bla g 2 induced significantly more β -hexosaminidase release than the monomeric Mut 2 (Fig. 7).

DISCUSSION

Crystallographic studies of allergen-antibody complexes are the most direct approach to determine the precise molecular structure of a conformational epitope. Given the polyclonal nature and limited amount of IgE antibodies in sera ($<1\text{ }\mu\text{g/ml}$ compared with $\sim 10\text{ mg/ml}$ for IgG) to study the allergen-antibody interaction we selected the Bla g 2-specific monoclonal antibody 7C11, which shows evidence of binding to an overlapping epitope with IgE.

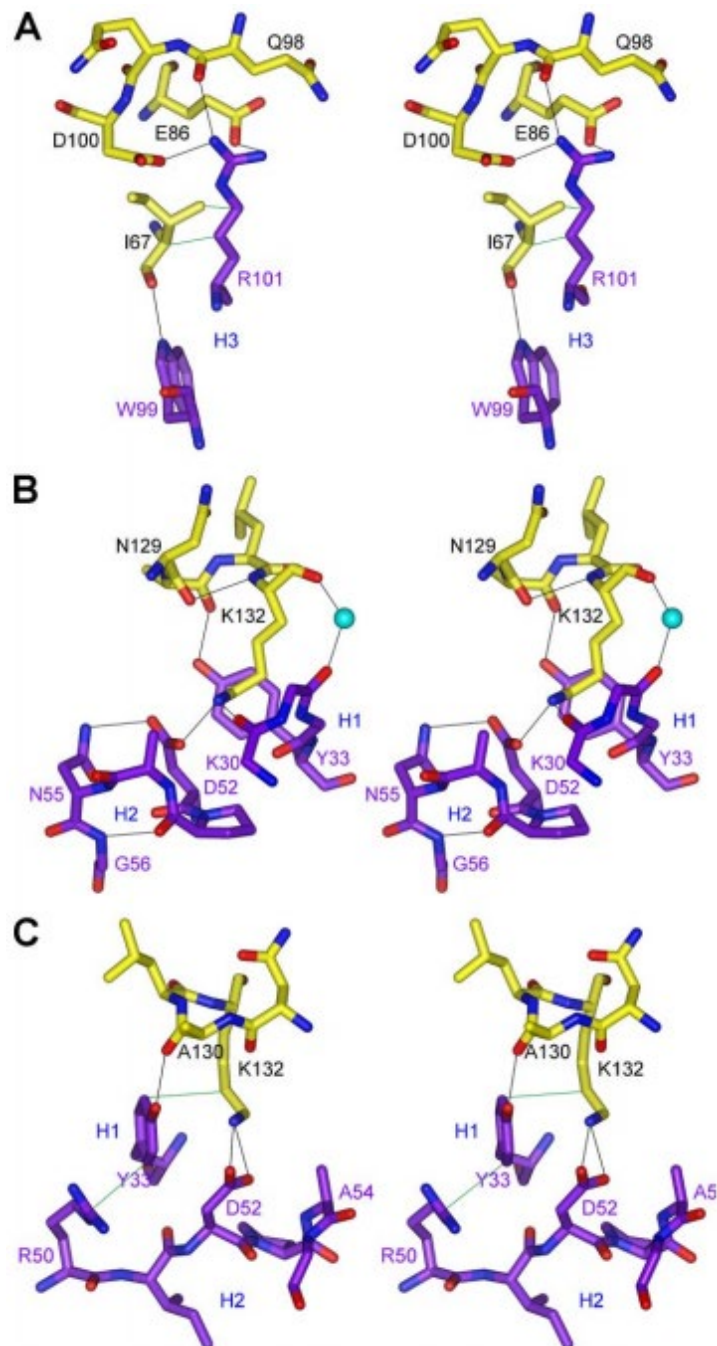


FIGURE 5. Discontinuous epitope in the allergen. *A*, interactions of the residues belonging to loops 2 and 3 in the allergen with the H3 loop of the antibody. In all panels, potential hydrogen bonds are marked with *black lines*, and non-bonded interactions with *green lines*. *B* and *C*, the side chain of Lys-132 links two variable loops H1 and H2 in the heavy chain of Fab' by forming an ion pair with Asp-52 and a hydrogen bond with the carbonyl oxygen of Lys-30 in the first monomer (*B*), and a cation- π interaction with Tyr-33 in the second monomer (*C*).

The crystal structure of the complex reveals that two antibody molecules bind to a dimer of Bla g 2 in a manner resembling the structure of the Fab complex with β -lactoglobulin (11). Interestingly, in the complex, Bla g 2 forms a dimer stabilized by an unusual quasi-four-helical bundle comprised of one α -helix and a helical turn from each allergen monomer. This form of dimerization and the presence of a disulfide bridge connecting the short helix and the helical turn within each monomer have not been seen in any other previously investigated aspartic proteases. Distinguishing functional dimer interfaces from contacts that are artifacts of crystallization is a difficult task, because it is known that features such as hydrophobicity, hydrogen bonding, and amino acid composition vary widely in subunit interfaces (26). The x-ray crystal structure of a homodimer of mature recombinant Derp 1 has been reported, and the study suggested that dimerization under natural conditions contributed to allergenicity (27). However, we recently found that natural Der f 1 did not dimerize in crystals.⁴ Whereas it was not possible to conclude unambiguously that the dimeric state of mature Der p 1 observed in crystals is physiologically relevant, Bla g 2 is predicted to be a dimer under physiological conditions. The dimer interface between Bla g 2 monomers is extensive (1139 Å² per monomer), above the consensus cut-off value (~850 Å²) that allows distinction between contacts caused by crystal packing and real physiological contacts, but it is only 620 Å² for Der p 1 (27).

The functional relevance of dimerization on IgE cross-linking on mast cells was investigated using two Bla g 2 mutant proteins expressed in yeast with amino acid substitutions that were designed to affect the dimerization interface, without significantly changing the protein folding. The substitution of the cysteines alone was expected to create distortions at the level of the α -helices involved in dimerization, and the additional amino acid substitutions were supposed to eliminate hydrophobic interactions. The overall similarity of the folding of the wild-type Bla g 2 and both mutant proteins was confirmed by CD spectra analysis, and ELISA data were in agreement with the CD spectra results. Only Mut 2 behaved as a monomer, indicating that the hydrophobic interactions are important for dimerization of Bla g 2. Dimerization increases IgE cross-linking in mast cells, because the dimeric wild type Bla g 2 induced more β -hexosaminidase release from mast cells than the monomeric Mut 2. Mediator release is induced when two IgE antibodies are cross-linked by simultaneously binding two epitopes on the allergen surface. If the allergen dimerizes naturally, each IgE antibody epitope is more likely to be involved in cross-linking with another epitope of the dimer (including the same epitope on the other molecule of the pair if location is favorable for cross-linking). In fact, because of dimerization, one epitope in each allergen would be enough to cross-link IgE antibodies. Recently, the crystal structure of a complex of β -lactoglobulin and Fab from IgE antibodies of a combinatorial scFv phage display library showed that the allergen is a dimer, and that the Fab fragments are located in positions such that the dimeric β -lactoglobulin would be able to cross-link two IgE antibodies (11). Our results suggest that allergen dimerization plays a role in allergenicity to Bla g 2, by increasing IgE antibody cross-linking, in agreement with a study with engineered dimers of carrot allergen Dau c 1 (28). Oligomerization of allergens in their natural form is very common (*e.g.* Feld1, β -lactoglobulin, Equ c 1 and Arah1 among others), and could contribute to allergenicity (11, 29-31).

Analysis of the interactions in the allergen-antibody structure allows us to define the Bla g 2 epitope for the 7C11 mAb. The size of the allergen-antibody interface (~870 Å²) is similar to the

interfaces in a variety of other antibody-antigen complexes (32). In particular, the size of the interface is comparable to those found in three other allergen-antibody complexes (10, 11, 33).

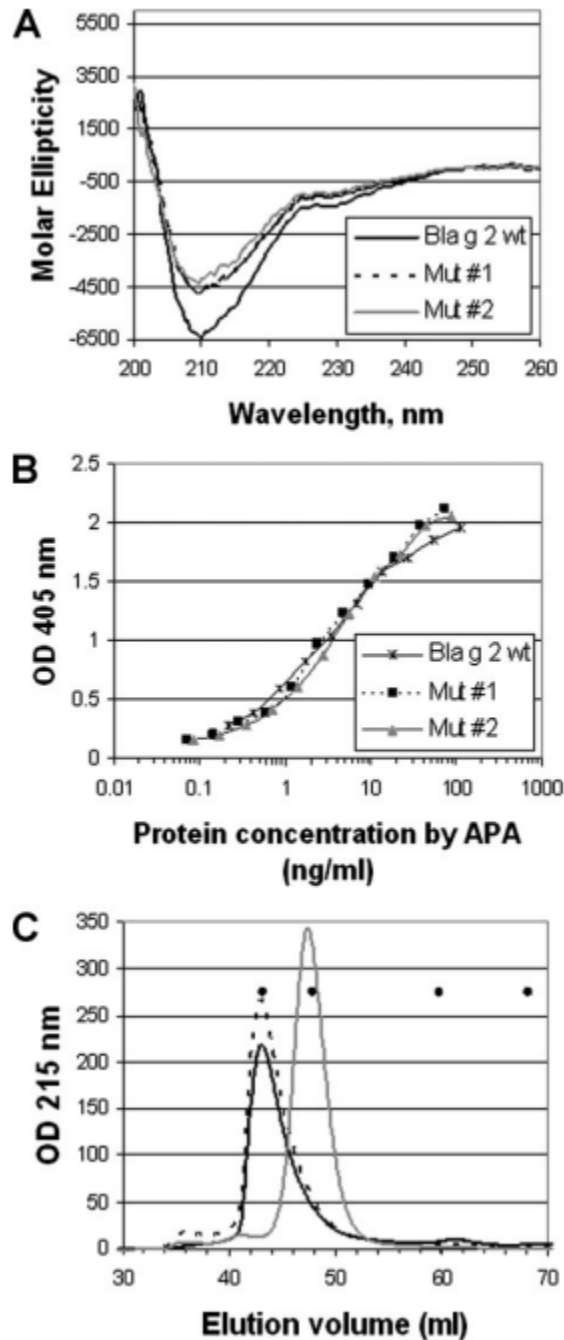


FIGURE 6. Characterization of Bla g 2 and its mutants. *A*, CD spectra of rBla g 2 and mutant proteins Mut 1 and Mut 2. *B*, comparison of the rBla g 2 mutant proteins and rBla g 2 wild-type (N1G2) by ELISA. *C*, size-exclusion chromatography of Bla g 2 wild type, Mut 1 and Mut 2.

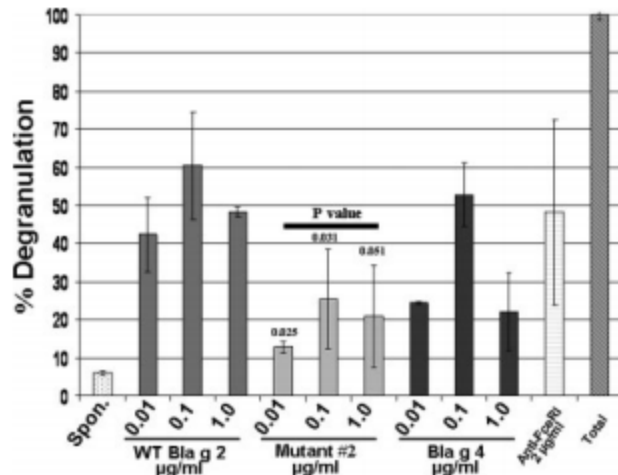


FIGURE 7. Effect of Bla g 2 wild type and Mut 2 on IgE-mediated degranulation from sensitized skin mast cells (MC). Skin mast cells were sensitized overnight with a 1:25 dilution of serum from a cockroach-sensitive patient. The next day, cells were washed and challenged without (spontaneous release; *spon.*) or with the indicated concentrations of antigen. β -Hexosaminidase was measured as a percent of total within the cell. The result is representative of three experiments from separate MC preparations. The *p* values are from a Student's *t* test performed comparing release using each mutant concentration with the same cross-linker concentration of wild-type Bla g 2.

The overall epitope on the Bla g 2 surface that is utilized for binding of 7C11 mAb ought to be defined as discontinuous since it is comprised of four loops, interacting with six CDRs of the antibody. However, two-thirds of the residues in Bla g 2 interacting with the antibody belong to a continuous part of the epitope that includes nine consecutive residues (60-70) of loop 1, as well as Arg-83 of loop 2 (Fig. 4, *A* and *B*), which predominantly interact with the light chain of the antibody. This type of recognition pattern involving a continuous section of the allergen as the largest part of the contact surface is very similar to the patterns found in the birch pollen and bee venom allergen/antibody complexes. In Bla g 2, this fragment is also characterized by the largest conformational changes in the allergen structure upon antibody binding, which implies “induced fit” mechanism of antibody-antigen interactions (32). Two cation- π interactions were observed between the positively charged side chains in the allergen (Arg, Lys) and the aromatic side chains (Tyr) of the antibody. Such interactions have been reported to be important for protein recognition in antibody-protein complexes (34), as well as in other protein-protein interactions (35); they were also observed in the complexes of antibodies with Bet v 1, Api m 2, and β -lactoglobulin mentioned above. We propose that cation- π interactions are important for the long range recognition of the epitopes that interact utilizing the “induced fit” mechanism, which requires some conformational changes in the interacting counterparts.

Three other loops in the allergen epitope interacting with the antibody (*loops 2, 3, and 4*, Fig. 5) are composed of very short stretches of residues (4, 3, and 4, respectively). Conformation of loops 2 and 3 is fairly extended and is supported by main chain-main chain hydrogen bonds between residues 85 and 87 of loop 2 and residues 99 and 101 of loop 3, respectively. Therefore, these two fragments from both loops could be described as a short β -sheet, which is recognized by 7C11 mAb in a manner similar to the one described in β -lactoglobulin-Fab structure. Four residues in loop 4 adopt a helical turn conformation supported by a hydrogen bond between the carbonyl oxygen of Asn-129 and the main chain amide of Lys-132, which restricts the flexibility

of this fragment. On the other hand, residues in the fragments of the antibody interacting with loops 2-4 are also involved in various intramolecular contacts. Consequently, the three loops seem to recognize the antibody by a “lock-and-key” mechanism (32), thus suggesting that they might be good candidates for mutation studies.

Site-directed mutagenesis of the conformational epitope allows the identification of key amino acids for IgE binding. Several mutants of the epitope residues have been produced and analyzed by antibody binding assays.⁵ A specific mutation, K132A, predicted on the basis of the structure of the complex described here strongly affected mAb 7C11 and IgE antibody binding, without producing significant structural changes.⁵ The knowledge of antigenic determinants involved in B cell response is important for understanding the contribution of the allergen structure to the disease and will enable a rational design of vaccines with reduced IgE antibody binding for immunotherapy.

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Footnotes

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2. Supported by a grant from the Food Allergy and Anaphylaxis Network.
3. The abbreviations used are: mAb, monoclonal antibody; r.m.s., root mean-squared; PEG, polyethylene glycol; ELISA, enzyme-linked immunosorbent assay; CDR, complementarity determining regions.
4. M. Chruszcz, M. D. Chapman, L. D. Vailes, W. Minor, and A. Pomés, unpublished data.
5. S. Wünschmann *et al.*, in preparation.

References

1. Arruda, L. K., Vailes, L. D., Ferriani, V. P., Santos, A. B., Pomés, A., and Chapman, M. D. (2001) *J. Allergy Clin. Immunol.* **107**, 419-428

2. Rosenstreich, D. L., Eggleston, P., Kattan, M., Baker, D., Slavin, R. G., Gergen, P., Mitchell, H., McNiff-Mortimer, K., Lynn, H., Ownby, D., and Malveaux, F. (1997) *N. Engl. J. Med.* **336**, 1356-1363
3. Arruda, L. K., Vailes, L. D., Mann, B. J., Shannon, J., Fox, J. W., Vedvick, T. S., Hayden, M. L., and Chapman, M. D. (1995) *J. Biol. Chem.* **270**, 19563-19568
4. Satinover, S. M., Reefer, A. J., Pomés, A., Chapman, M. D., Platts-Mills, T. A., and Woodfolk, J. A. (2005) *J. Allergy Clin. Immunol.* **115**, 803-809
5. Sporik, R., Squillace, S. P., Ingram, J. M., Rakes, G., Honsinger, R. W., and Platts-Mills, T. A. (1999) *Thorax* **54**, 675-680
6. Gustchina, A., Li, M., Wunschmann, S., Chapman, M. D., Pomés, A., and Wlodawer, A. (2005) *J. Mol. Biol.* **348**, 433-444
7. Wünschmann, S., Gustchina, A., Chapman, M. D., and Pomés, A. (2005) *J. Allergy Clin. Immunol.* **116**, 140-145
8. Guruprasad, K., Blundell, T. L., Xie, S., Green, J., Szafranska, B., Nagel, R. J., McDowell, K., Baker, C. B., and Roberts, R. M. (1996) *Protein Eng.* **9**, 849-856
9. Spangfort, M. D., Mirza, O., Ipsen, H., Van Neerven, R. J., Gajhede, M., and Larsen, J. N. (2003) *J. Immunol.* **171**, 3084-3090
10. Padavattan, S., Schirmer, T., Schmidt, M., Akdis, C., Valenta, R., Mittermann, I., Soldatova, L., Slater, J., Mueller, U., and Markovic-Housley, Z. (2007) *J. Mol. Biol.* **368**, 742-752
11. Niemi, M., Jylha, S., Laukkanen, M. L., Soderlund, H., Makinen-Kiljunen, S., Kallio, J. M., Hakulinen, N., Haahtela, T., Takkinen, K., and Rouvinen, J. (2007) *Structure* **15**, 1413-1421
12. Burks, A. W., Shin, D., Cockrell, G., Stanley, J. S., Helm, R. M., and Bannon, G. A. (1997) *Eur. J. Biochem.* **245**, 334-339
13. Ball, T., Fuchs, T., Sperr, W. R., Valent, P., Vangelista, L., Kraft, D., and Valenta, R. (1999) *FASEB J.* **13**, 1277-1290
14. Flicker, S., Vrtala, S., Steinberger, P., Vangelista, L., Bufe, A., Petersen, A., Ghannadan, M., Sperr, W. R., Valent, P., Norderhaug, L., Bohle, B., Stockinger, H., Suphioglu, C., Ong, E. K., Kraft, D., and Valenta, R. (2000) *J. Immunol.* **165**, 3849-3859
15. Pomés, A., Chapman, M. D., Vailes, L. D., Blundell, T. L., and Dhanaraj, V. (2002) *Am. J. Respir. Crit. Care Med.* **165**, 391-397
16. Pomés, A., Vailes, L. D., Helm, R. M., and Chapman, M. D. (2002) *Eur. J. Biochem.* **269**, 3086-3092
17. Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* **276**, 307-326
18. Storoni, L. C., McCoy, A. J., and Read, R. J. (2004) *Acta Crystallogr. Sect. D* **60**, 432-438
19. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr. Sect. D* **53**, 240-255

20. Emsley, P., and Cowtan, K. (2004) *Acta Crystallogr. Sect. D* **60**, 2126-2132
21. Jaskolski, M., Gilski, M., Dauter, Z., and Wlodawer, A. (2007) *Acta Crystallogr. Sect. D* **63**, 611-620
22. Bohm, G., Muhr, R., and Jaenicke, R. (1992) *Protein Eng.* **5**, 191-195
23. Kepley, C. L., Taghavi, S., Mackay, G., Zhu, D., Morel, P. A., Zhang, K., Ryan, J. J., Satin, L. S., Zhang, M., Pandolfi, P. P., and Saxon, A. (2004) *J. Biol. Chem.* **279**, 35139-35149
24. Sielecki, A. R., Fedorov, A. A., Boodhoo, A., Andreeva, N. S., and James, M. N. (1990) *J. Mol. Biol.* **214**, 143-170
25. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D* **54**, 905-921
26. Ponstingl, H., Henrick, K., and Thornton, J. M. (2000) *Proteins* **41**, 47-57
27. de Halleux, S., Stura, E., VanderElst, L., Carlier, V., Jacquemin, M., and Saint-Remy, J. M. (2006) *J. Allergy Clin. Immunol.* **117**, 571-576
28. Reese, G., Ballmer-Weber, B. K., Wangorsch, A., Randow, S., and Vieths, S. (2007) *J. Allergy Clin. Immunol.* **119**, 944-951
29. Kaiser, L., Velickovic, T. C., Badia-Martinez, D., Adedoyin, J., Thunberg, S., Hallen, D., Berndt, K., Gronlund, H., Gafvelin, G., van Hage, M., and Achour, A. (2007) *J. Mol. Biol.* **370**, 714-727
30. Lascombe, M. B., Gregoire, C., Poncet, P., Tavares, G. A., Rosinski-Chupin, I., Rabillon, J., Goubran-Botros, H., Mazie, J. C., David, B., and Alzari, P. M. (2000) *J. Biol. Chem.* **275**, 21572-21577
31. Maleki, S. J., Kopper, R. A., Shin, D. S., Park, C. W., Compadre, C. M., Sampson, H., Burks, A. W., and Bannon, G. A. (2000) *J. Immunol.* **164**, 5844-5849
32. Davies, D. R., Sheriff, S., and Padlan, E. A. (1988) *J. Biol. Chem.* **263**, 10541-10544
33. Mirza, O., Henriksen, A., Ipsen, H., Larsen, J. N., Wissenbach, M., Spangfort, M. D., and Gajhede, M. (2000) *J. Immunol.* **165**, 331-338
34. Livesay, D. R., and Subramaniam, S. (2004) *Protein Eng. Des. Sel.* **17**, 463-472
35. Gallivan, J. P., and Dougherty, D. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9459-9464