

Dominating IgE-Binding Epitope of Bet v 1, the Major Allergen of Birch Pollen, Characterized by X-ray Crystallography and Site-Directed Mutagenesis

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Specific allergy vaccination is an efficient treatment for allergic disease; however, the development of safer vaccines would enable a more general use of the treatment. Determination of molecular structures of allergens and allergen-Ab complexes facilitates epitope mapping and enables a rational approach to the engineering of allergen molecules with reduced IgE binding. In this study, we describe the identification and modification of a human IgE-binding epitope based on the crystal structure of Bet v 1 in complex with the BV16 Fab' fragment. The epitope occupies ~10% of the molecular surface area of Bet v 1 and is clearly conformational. A synthetic peptide representing a sequential motif in the epitope (11 of 16 residues) did not inhibit the binding of mAb BV16 to Bet v 1, illustrating limitations in the use of peptides for B cell epitope characterization. The single amino acid substitution, Glu⁴⁵-Ser, was introduced in the epitope and completely abolished the binding of mAb BV16 to the Bet v 1 mutant within a concentration range 1000-fold higher than wild type. The mutant also showed up to 50% reduction in the binding of human polyclonal IgE, demonstrating that glutamic acid 45 is a critical amino acid also in a major human IgE-binding epitope. By solving the three-dimensional crystal structure of the Bet v 1 Glu⁴⁵-Ser mutant, it was shown that the change in immunochemical activity is directly related to the Glu⁴⁵-Ser substitution and not to long-range structural alterations or collapse of the Bet v 1 mutant tertiary structure. *The Journal of Immunology*, 2003, 171: 3084–3090.

Determination of three-dimensional structures of allergens constitutes the basis for rational mapping of specific serum IgE-binding B cell epitopes, and for addressing molecular mechanisms of Ab specificity and allergic cross-reactivity. The three-dimensional structure reveals which amino acids are surface exposed and accessible for interactions with Abs. In combination with primary sequence alignment analysis, identification of conserved surface structures between homologous allergens from different species is enabled. Conserved surface areas large enough to encompass Ab-binding epitopes cause allergic cross-reactivity, and they are proposed to constitute dominating IgE-binding epitopes (1, 2). A strategy based on determination of molecular structures and site-directed mutagenesis enables rational manipulation of the molecular surface, and thus the Ab-binding characteristics of the molecule.

Birch pollen-allergic individuals often, if not always, show clinical reaction to other pollens of the *Fagales*, including the related trees alder and hazel (3, 4). This cross-reaction has been shown to be caused by the presence of major allergens homologous to Bet v 1 in these pollens (5, 6). The amino acid sequences of Bet v 1 from birch (*Betula verrucosa*) (7), Aln g 1 from alder (*Alnus glutinosa*) (8), Cor a 1 from hazel (*Corylus avellana*) (9), and Car b 1 from

hornbeam (*Carpinus betulus*) (10) share ~75% identity. Sequence alignment combined with analysis of the molecular surface of Bet v 1 identified surface areas common to the *Fagales* tree pollen major allergens, providing a molecular basis of cross-reactivity (1). Thus, IgE directed toward conserved surface areas bind to any *Fagales* major allergen with similar affinity, and through cross-linking of receptor-bound IgE on the surface of mast cells and basophils give rise to allergic symptoms.

A more direct approach to the identification of B cell epitopes is structure determination of allergen-Ab complexes. The study of human IgE-binding B cell epitopes is hampered, however, by the technical difficulties in obtaining relevant amounts of IgE mAbs from allergic patients. Murine monoclonal IgG, however, may serve as a useful model system, particularly when inhibiting the binding of human IgE to the allergen. The murine IgG1 mAb BV16, which was raised by immunizing mice with purified Bet v 1 from pollen, inhibits the binding of human serum IgE to Bet v 1 by ~40% (2). Moreover, the BV16 mAb shows cross-reactivity with the homologous major allergens from alder and hornbeam (2). The successful crystallization (11) and structural determination of Bet v 1 in complex with the Fab' fragment of the murine mAb BV16 (2) for the first time revealed the three-dimensional architecture of an Ab-binding epitope located on the molecular surface of a major allergen involved in IgE-mediated allergic responses.

Inhalation allergy is a disease with two major components, i.e., a chronic state of inflammation in the airway mucosa and transient bursts of histamine and other mediators produced by mast cells and basophils. Symptoms are triggered by a combination of the two, regardless of the disease being hay fever in the nose or asthma in the lungs (12). According to current models, both processes are regulated by cytokines produced by allergen-specific T cells, i.e., IL-5 mobilizes eosinophils and IL-4/IL-13 stimulates IgE production. Soluble IgE in turn binds to specific high affinity receptors

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(FcεRI) anchored in the membrane of mast cells and basophils. Exposure to allergen leads to cross-linking of receptor-bound IgE, which is the initial event in a signal cascade leading to release of mediators directly responsible for triggering of symptoms, and thus, the interaction between IgE and allergen is a crucial step in the triggering of allergic symptoms.

Specific allergy vaccination, i.e., specific immunotherapy, is an effective and well-tolerated treatment of allergic disease in selected patients (13). Although recent years have brought substantial insight into the immunological mechanisms underlying successful vaccination, some aspects are still debated. Early serological studies demonstrated an increase in allergen-specific IgG as a resulting effect (14, 15), whereas allergen-specific IgE is largely unaffected (16). The reason for this difference in response to the allergen, i.e., stimulation of IgE when encountered through natural exposure vs stimulation of IgG when injected, is not known. It may theoretically rely on differences in administration route, presence of adjuvant, and dose, or it may reflect differences in compartmentalization in the human body of the cells of the acquired immune response.

A number of observations indicate that allergen-specific IgG may play an important role in the mechanism of successful allergy vaccination: first, IgG compete with IgE for binding to the allergen reducing histamine release from mast cells and basophils in vitro by interfering with the cross-linking of receptor-bound IgE (17). Second, the allergen-specific IgG disturb allergen-IgE complexes, which bind to CD23, i.e., the low affinity IgE receptor, on APCs facilitating Ag presentation to T cells (18); and third, IgG may mediate inhibition of downstream FcεRI signaling in human basophils (19). Furthermore, Abs are likely to play an important role in Ag presentation, which may affect T cell cytokine profiles, because T cells cannot respond directly to allergens (20).

Several strategies for the modification of allergen molecules for specific allergy vaccination have been proposed in recent years (21). Most strategies aim at a disruption of the allergen structure to avoid IgE binding, enabling a safer way of addressing allergen-specific T cells. As opposed to these strategies, the current concept aims at inactivating dominating IgE-binding epitopes by point mutation, carefully avoiding a collapse of the tertiary structure for the purpose of conserving surface structures that will induce protective IgG Abs reactive with the natural allergen.

Materials and Methods

Antibodies

Serum samples were derived from birch pollen-allergic patients with clinical reactions to birch pollen during season and with Radio allergosorbent test class 2 or more. The local ethics commission approved the study.

Hybridoma mAb BV16 was derived from BALB/c mice immunized with Bet v 1 purified from *B. verrucosa* pollen. The BV16 mAb was of the

IgG1 isotype, and supernatants from cells grown in the absence of serum were used.

Synthetic peptide

The synthetic peptide representing Bet v 1 residues 39–53 was obtained from Schafer-N (Copenhagen, Denmark). The peptide was synthesized on a MK-III fully automatic synthesizer using Fmoc chemistry. More than 80% purity of the peptide was verified by high-pressure liquid chromatography.

Cloning of the gene encoding Bet v 1

cDNA cloning of the gene encoding Bet v 1 was performed by PCR using mRNA purified from birch pollen, as previously described (22). Subcloning of the gene encoding Bet v 1 into the pMAL-c vector (New England Biolabs, Beverly, MA) was performed by PCR, as described (23). The particular isoallergenic variant used in this study is Bet v 1.2801, as defined in the official allergen nomenclature (24) administered by the International Union of Immunological Societies Allergen Nomenclature Subcommittee (<http://www.allergen.org>). The sequence has European Molecular Biology Laboratory (EMBL) accession number Z80104 and Swiss-PROT accession number P15494, and deviates from Bet v 1.0101 (7) only in position 62, which is occupied by Leu in Bet v 1.2801 as opposed to Phe in Bet v 1.0101. The three-dimensional structure of rBet v 1.2801 has been solved by x-ray crystallography (Protein Data Bank (PDB): 1BV1) as well as by nuclear magnetic resonance spectroscopy (PDB: 1BTv) (1).

In vitro mutagenesis

In vitro mutagenesis was performed by PCR using Bet v 1.2801 inserted in pMAL-c as template. Each mutant Bet v 1 gene was generated by three PCR using four primers. Mutant-specific primers were synthesized for both DNA strands and used in combination with generally applicable primers yielding ~1-kb PCR products in a standard PCR, with the exception that only 20 temperature cycles were performed to reduce the error frequency. The two PCR products containing the mutation were purified by gel electrophoresis and ethanol precipitation, combined in a third PCR reaction, and amplified using the generally applicable primers. The resulting PCR products were purified and directionally cloned into pMAL-c using restriction endonucleases BsiWI and EcoRI.

Nucleotide sequencing

All mutants were sequenced full length on both DNA strands. For sequencing, plasmid DNAs were purified using Qiagen (Valencia, CA) columns and sequenced using the Sequenase version 2.0 DNA sequencing kit (USB) following the recommendations of the suppliers.

Expression and purification of recombinant Bet v 1 and Bet v 1 mutants

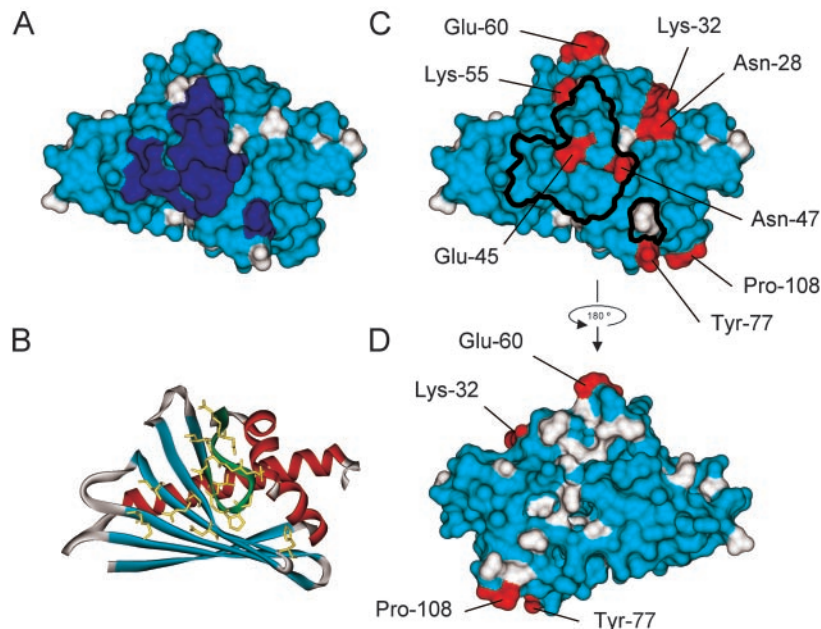
Recombinant Bet v 1 and mutants were expressed in *Escherichia coli* K12, strain DH5α (25), in fusion with maltose-binding protein and purified, as previously described (23). The purified rBet v 1 mutant preparations appeared as single bands with apparent molecular mass of 17.5 kDa after SDS-PAGE and silver staining. N-terminal sequencing demonstrated the expected amino-terminal sequences, and quantitative amino acid analysis showed the expected amino acid compositions (data not shown).

Table I. Summary of the crystallographic data

Data Collection Statistics		Final Refinement Statistics		RMS Deviations	
Space group (mutant)	C222 ₁	R factor (%) ^a	27.4	Bond lengths (Å)	0.003
abcαβγ (mutant)	31.87 74.94 120.00 90 90 90	R _{free} (%)	30.1	Bond angles (degrees)	0.8
Outer shell	3.12–3.06			Dihedral angles (degrees)	23.9
No. of observations	11,258 (2,668)			Improper angles (degrees)	0.54
(unique observations)					
Completeness (%)					
All data (outer shell)	89.5 (86.1)				
R _{sym}					
All data (outer shell)	0.173 (0.377)				

^a Calculated without the test-set reflections.

FIGURE 1. Bet v 1 structure and mutated amino acids. **A**, Surface representation of Bet v 1.2801 (PDB: 1BV1) showing contact residues in Bet v 1-BV16 Fab' complex (dark blue). **B**, Secondary structure elements of the Bet v 1 structure. Side chains of contact residues are shown in yellow. Green color highlights the backbone of aa 39–53 represented in the synthetic peptide used for immunoinhibition assays. **C** and **D**, Molecular surface representation of Bet v 1 showing location of mutated amino acids highlighted in red. Blue color indicates amino acids >95% identical in 57 *Fagales* major allergen sequences. Contact residues in Bet v 1-BV16 Fab' complex are encircled by a solid line. The two panels represent the model rotated 180° around a vertical axis.



Mass spectrometry and quantitative amino acid analysis

The purity and molecular mass of purified recombinant Bet v 1.2801 and Bet v 1 Glu⁴⁵-Ser mutant were determined by mass spectrometry using a Compact Maldi IV instrument (Kratos Analytical, Manchester, U.K.). Proteins were dissolved in the matrix mixture (saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in a 2:3 mixture of acetonitrile/0.1% aqueous trifluoroacetic acid) at a concentration of 1 mg/ml. A total of 0.5 μ l of the protein solution was applied to the sample target and dried in an air stream. Masses were determined in the linear flight mode with time-delayed extraction. Trypsin from bovine pancreas (Sigma-Aldrich, St. Louis, MO) was used as internal standard. The molecular mass of Bet v 1.2801 was determined as 17,405.6 Da (theoretical value 17,405.6 Da), and the mass of Bet v 1 Glu⁴⁵-Ser mutant was 17,323.5 Da (theoretical value 17,363.5 Da).

The molar extinction coefficients of purified Bet v 1.2801 and Bet v 1 Glu⁴⁵-Ser mutant were determined by quantitative amino acid analysis performed using the Waters Pico-Tag system. Samples were hydrolyzed at 110°C in 6 N gaseous HCl for 14 h and derivatized with phenylisothiocyanate (26). Derivatized amino acids were separated on a Hewlett-Packard (Palo Alto, CA) 1100 HPLC system with a Waters Nova-Pak (Milford, MA) C₁₈ column (3.9 $\times$ 300 mm) using the elution conditions recommended in the Waters Pico-Tag manual.

Crystallization and structural determination of Bet v 1 Glu⁴⁵-Ser mutant

Crystals of rBet v 1 Glu⁴⁵-Ser mutant were grown by vapor diffusion at 25°C by applying the microseeding technique using crystals of rBet v 1.2801 (27) as a source of seeds. After ~2 mo, crystals were harvested and analyzed. Data were collected in house using a Rigaku R-axis IIC image plate system with a Rigaku RU200 rotating anode (Rigaku Instruments, Tokyo, Japan). The system was equipped with a graphite monochromator and a 0.5-mm collimator. The data consist of a total of 30 frames of 2° of oscillation and 60-min exposure per frame. The crystal to detector distance was 120 mm. Data collection statistics are given in Table I. Data integration, reduction, and merging were performed using DENZO and SCALEPACK (28).

Due to a slight, but significant change in the cell c-axis (2 Å), it was necessary to solve the structure by molecular replacement, using the Bet v 1.2801 structure (1) as search model and the AmoRe (29) program package. Refinement of the structure was performed using the programs X-PLOR (30) and CNS (31) with the Engh-Huber (32) parameters. Both maximum likelihood and residual-based target functions were used. Molecular modeling using 2Fo-Fc and Fo-Fc maps was done in the program O (33). Throughout the refinement, 10% of the data was set aside for cross-validation. Before refinement, model bias was removed with 3000 K simulated annealing run. Due to the low data to parameter ratio, torsion angle dynamics and restrained cartesian dynamics were applied. An overall

B factor of 25 Å (2) was used. The structure has been deposited at the PDB (Rutgers, The State University of New Jersey, New Brunswick, NJ), with the accession code 1LLT.

Peptide and mutant ELISA inhibition

Peptide and mutant immunoinhibition experiments were performed in triplicate in ELISA trays coated with murine mAb BV16 using standard procedures. Biotinylated rBet v 1.2801 was mixed with 2-fold dilution series of nonbiotinylated rBet v 1.2801, Bet v 1 synthetic peptide 39–52, or rBet v 1 mutants, respectively, and added to the mAb BV16-coated ELISA wells. After incubation and washing, the wells were incubated with streptavidin-labeled alkaline phosphatase (DAKO, Glostrup, Denmark) and washed, substrate was added, and the initial velocities of alkaline phosphatase-catalyzed hydrolysis of 4-nitro-phenyl-phosphate were determined on an ELISA reader. The degree of binding (DoB) was calculated according to equation 1:

$$\text{DoB} = \frac{V_{\text{inhibitor}} - V_{\text{blank}}}{V_{\text{buffer}} - V_{\text{blank}}}$$

Inhibition percentage was calculated as $(1 - \text{DoB}) 100$. V_{buffer} is the mean velocity determined without inhibitor, $V_{\text{inhibitor}}$ is the mean velocity determined at a given inhibitor dilution, and V_{blank} represents the average autohydrolysis of the substrate.

Specific serum IgE inhibition assay

For specific serum IgE inhibition by rBet v 1.2801 and Bet v 1 mutants, sera from individual patients or a pool of seven birch-allergic patients was used. Purified rBet v 1.2801 was biotinylated at a molar ratio of 1:5 (Bet v 1:biotin). The inhibition assay was performed in triplicate using ADVIA Centaur System (Bayer, Lyngby, Denmark), as follows: serum samples (25 μ l) were incubated with solid-phase anti-IgE (ALK-Abelló), washed, re-suspended, and further incubated with a mixture of biotinylated Bet v 1 and nonbiotinylated Bet v 1 or Bet v 1 mutant. The amount of biotinylated Bet v 1 bound to the solid-phase serum IgE was estimated from the measured relative light units (RLU)³ after incubation with acridiniumester-labeled streptavidin. The degree of binding (DoB) was calculated according to equation 2:

$$\text{DoB} = \frac{\text{RLU}_1 - \text{RLU}_{100\%}}{\text{RLU}_{0\%} - \text{RLU}_{100\%}}$$

Inhibition percentage was calculated as $(1 - \text{DoB})100$. $\text{RLU}_{100\%}$ and $\text{RLU}_{0\%}$ are mean RLU values obtained using rBet v 1.2801 and buffer as inhibitor, respectively.

³ Abbreviations used in this paper: RLU, relative light unit; RMS, root mean square.

Table II. Amino acid sequence of Bet v 1.2801^a

10	20	30	40	50	60	70	80
GVFN ⁹⁰ YETET	SVIPAARLFK	AFILDGDNL ¹¹⁰ F	PKVAPQAIS ¹²⁰ S	<u>V</u> ENIEGNGG ¹³⁰ P	<u>G</u> TIKKISFPE ¹⁴⁰ S	GLPFKYVKDR ¹⁵⁰	VDEVDHTNFK ¹⁵⁹
YNYSVIEGGP	IGDTLEKISN	EIKIVATPDG	GSILKISNKY	HTKGDHEVKA	EQVKASKEMG	ETLLRAVESY	LLAHSDAYN

^a Contact residues in Bet v 1-BV16 Fab' complex are underlined. Mutants described in this study are indicated with substituting amino acids shown below the Bet v 1.2801 sequence.

Statistical analysis

For quantitative immunoassays, means of triplicate measurements were converted into percentage of inhibition, as outlined above. Five or six data points symmetrically distributed around 50% inhibition were selected for statistical analyses relating the percentage of inhibition to log dose. Statistical analyses comprised tests for linearity and parallelism. All curves produced a coefficient of regression above 0.9 and were considered linear. Equality of slopes was evaluated (double-sided Student's *t* test, $p < 0.05$), and the potency was expressed relative to nonmutated rBet v 1 (mutant ELISA inhibition) or a pool of tree pollen-allergic individuals' sera (specific serum IgE inhibition). Graphical display and curve fitting were performed using GraphPad Prism v. 4.0 (GraphPad Software, San Diego, CA).

Results

Complex and epitope structure

The crystal structure of Bet v 1 in complex with the Fab' fragment generated from murine IgG1 mAb BV16 as determined at 2.9 Å resolution was previously reported (2). Fig. 1A shows a molecular surface representation of Bet v 1 with the epitope defined by BV16 in dark blue. The epitope is clearly conformational because six segments of the polypeptide chain are represented in the epitope (Fig. 1B); however, 11 of the 16 amino acids form a strong sequential motif (Table II).

Peptide inhibition

To test the relative binding strengths of the linear motif and the entire epitope with BV16, an immunoinhibition experiment using rBet v 1.2801 and a 15-aa-long synthetic peptide representing residues 39–53 was performed. mAb BV16 was immobilized in the ELISA tray, and the binding of biotinylated rBet v 1 was inhibited by addition of a dilution series of the peptide. In the concentration range tested, Bet v 1 (mole):peptide (mole) 1:1000, the peptide was not able to inhibit the binding of Bet v 1 to mAb BV16 (Fig. 2).

Surface modulation

In the interface of the Bet v 1-BV16 Fab' complex, the Glu⁴⁵ residue stretches its side chain directly outward from the molecular

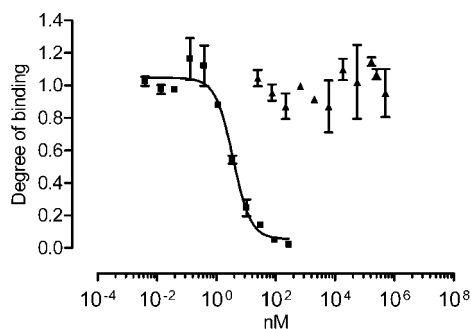


FIGURE 2. Bet v 1-BV16 interaction is not inhibited by synthetic peptide. Binding of biotinylated rBet v 1.2801 to Ab BV16 inhibited by dilution series of rBet v 1.2801 (■) and synthetic peptide 39–53 (▲), respectively. Solid line represents a four-parameter logistic curve fit.

surface of Bet v 1 into a pocket formed by themAb (2). A series of mutations in surface-exposed amino acid residues expected to be important for mAb binding and including Glu⁴⁵-Ser were generated by site-directed mutagenesis, and the effect on mAb BV16 binding was analyzed by immunoinhibition assays. Bet v 1 mutants carrying the following substitutions, Asn²⁸-Thr + Lys³²-Gln (a double mutant), Glu⁴⁵-Ser, Lys⁵⁵-Asn, Glu⁶⁰-Ser, Thr⁷⁷-Ala, and Pro¹⁰⁸-Gly, respectively (Fig. 1, C and D), were expressed in *E. coli*, followed by purification to electrophoretic homogeneity. The ability of the different Bet v 1 mutants to inhibit the binding of Bet v 1 to mAb BV16 is shown in Fig. 3. In agreement with the structure of the complex, substitution of Bet v 1 residues in positions 28, 32, 60, 77, and 108 did not significantly affect the binding of mAb BV16. Substitution of glutamic acid in position 45 to serine, however, had a dramatic effect, completely abolishing the binding of the mutant to mAb BV16 in the concentration range tested. Substitution of Lys⁵⁵, which is located at the edge of the epitope, had marginal effect on BV16 binding.

Structure of Glu⁴⁵-Ser mutant

The structural effect of the glutamic acid 45 substitution was evaluated by x-ray crystallography to a resolution of 3 Å, using protein crystals of the Bet v 1 Glu⁴⁵-Ser mutant and x-ray diffraction analysis (PDB: 1LLT) (see Table I).

Overall, good electron density was found at the site of the mutation and throughout the structure, which was refined to a final $R_{\text{crystallography}}$ of 30.1% and R_{free} of 27.4%. Both R values are quite reasonable for this resolution. All, except Asp⁹³, fall in most favored or additionally allowed Ramachandran regions. The conformation of Asp⁹³ is clearly defined by the 2Fo-Fc map of the mutant, and has previously been found in the same conformation in the native structure. When calculated, the Fo-Fc difference map

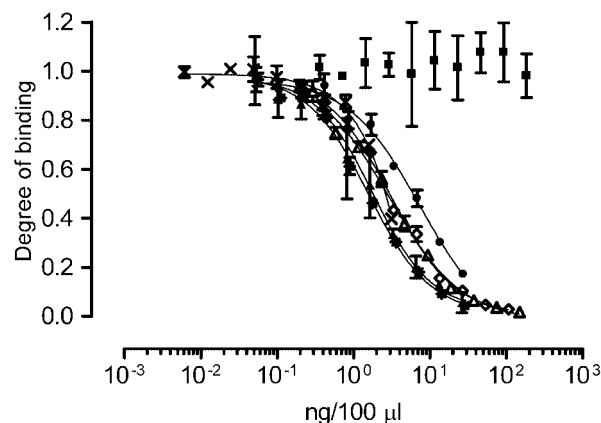


FIGURE 3. Bet v 1-BV16 interaction inhibited by mutants. Binding of biotinylated rBet v 1.2801 to BV16 inhibited by dilution series of rBet v 1.2801 and individual mutants: Glu⁴⁵-Ser (■), Lys⁵⁵-Asn (●), Glu⁶⁰-Ser (◇), Thr⁷⁷-Ala (▲), Asn²⁸-Thr + Lys³²-Gln (△), Pro¹⁰⁸-Gly (×), rBet v 1.2801 (*). Solid lines represent four-parameter logistic curve fits.

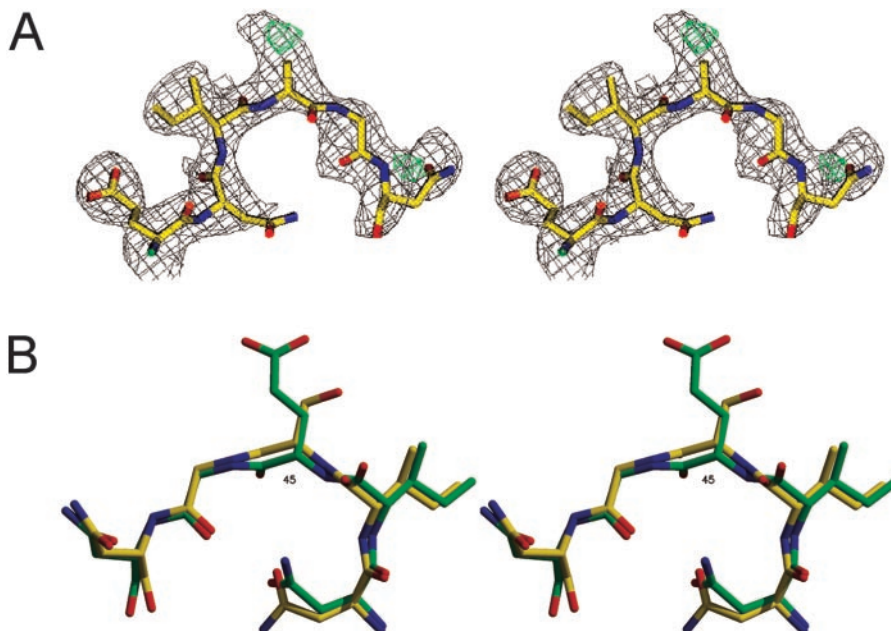


FIGURE 4. Structure of Glu⁴⁵-Ser mutant. *A*, Stereo representation of the 1 σ 2Fo-Fc electron density (gray) along with 2.5 σ Fo-Fc electron density (green). Both maps were calculated with an alanine as residue 45. *B*, The section of the structure surrounding the mutation (residues 43–47) in stereo representation showing Bet v 1.2801 (PDB: 1BV1) in green, overlaid with the structure of the Glu⁴⁵-Ser mutant (PDB: 1LLT) in yellow.

clearly showed a positive peak, corresponding to the O γ of a serine residue, demonstrating that this is indeed the Glu⁴⁵-Ser mutant (Fig. 4A). The overall root mean square (RMS) deviation between the C α atoms of the native and mutant structures is 0.46 Å, and the cross-validated Luzzati coordinate error is estimated to 0.5 Å. The RMS deviation between the C α atoms of region 43–55 is 0.24 Å. For residues 43–47 (shown in Fig. 4B), this deviation is slightly higher (0.3 Å). No change in the folding pattern of the polypeptide chain has occurred as a consequence of the mutation, because all RMS deviations fall within the cross-validated estimation of the coordinate error. Therefore, the change in immunochemical activity of the Glu⁴⁵-Ser mutant can be directly ascribed to the single amino acid substitution.

Effect on specific serum IgE binding

The effect of the Glu⁴⁵-Ser mutation on specific serum IgE binding was addressed in an IgE-inhibition assay using sera from individual birch pollen-allergic individuals and a serum pool derived from seven birch-allergic patients. Serum IgE was captured by anti-IgE

immobilized on paramagnetic beads. After washing, the binding of biotinylated rBet v 1 to IgE was inhibited by addition of dilution series of rBet v 1 and the Glu⁴⁵-Ser mutant, respectively. IgE binding to the Glu⁴⁵-Ser mutant was significantly reduced for all individual sera as well as for the serum pool (Fig. 5). The degree of reduction in IgE binding to the mutant differed from patient to patient, which was reflected in the relative binding potencies varying between 0.51 (0.40–0.64; 95% confidence limits) and 0.75 (0.58–0.95) relative to rBet v 1. Fig. 6 shows the effect on serum IgE binding of mutations in various surface-exposed amino acid residues. Mutations located in coherent surface areas conserved throughout the *Fagales* group 1 allergens, i.e., Asn²⁸-Thr + Lys³²-Gln, Glu⁴⁵-Ser, and Pro¹⁰⁸-Gly, reduced the binding of serum pool IgE to a relative potency of 0.52 (0.39–0.70; 95% confidence limits), 0.53 (0.32–0.86), and 0.52 (0.38–0.70), respectively. The mutation Glu⁶⁰-Ser, although conserved throughout the *Fagales* is

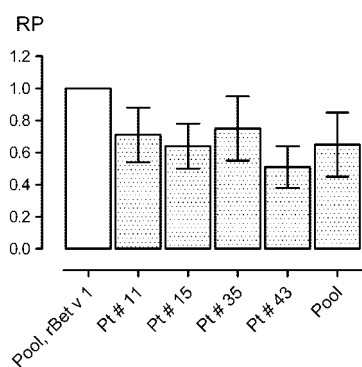


FIGURE 5. Glu⁴⁵-Ser inhibition of individual patients' IgE. Individual patients' serum IgE binding to Glu⁴⁵-Ser mutant. Serum IgE from individual patients was bound to solid phase-coupled anti-IgE. The binding of biotinylated rBet v 1 was inhibited by a dilution series of Glu⁴⁵-Ser mutant, and the inhibitory potency was expressed relative to the inhibitory potency of rBet v 1 (open bar) toward a pool of allergic patients' serum IgE. Ninety-five percent confidence limits are indicated. RP denotes relative potency.

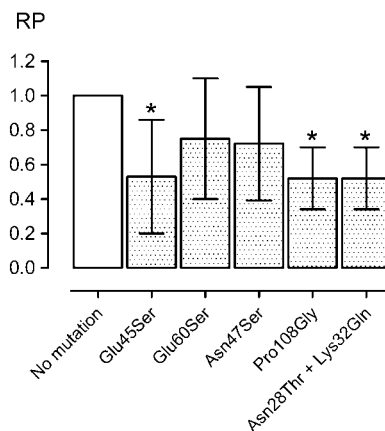


FIGURE 6. IgE inhibition by different mutants. Individual mutants binding to a pool of allergic patients' serum IgE. Serum IgE was bound to solid phase-coupled anti-IgE. The binding of biotinylated rBet v 1 was inhibited by dilution series of individual mutants, and the inhibitory potency was expressed relative to the inhibitory potency of rBet v 1 (open bar). Ninety-five percent confidence limits are indicated. RP denotes relative potency. Asterisks indicate statistical significant differences in relative potency ($p < 0.05$).

located outside coherent conserved patches, did not significantly reduce the binding of serum pool IgE, yielding a relative potency of 0.75 (0.51–1.10) (Fig. 6).

Discussion

We have previously reported the first three-dimensional structure of an important inhalation allergen (1). The structural studies, which aim at the study of allergen–Ab interactions, led to the first report on the structure on an allergen–Ab Fab' fragment complex (2). In this study, we report further studies on this Bet v 1 epitope using x-ray crystallography, site-directed mutagenesis, and IgE-binding analyses. The purpose of these studies is to explore the potential of structure-based rational design of safer vaccines for specific allergy vaccination.

The epitope defined by the murine mAb BV16 (2) is conformational, because it is constituted by six segments of the polypeptide chain; however, it contains a dominating sequential element. Despite the fact that the sequential motif makes up more than half of the number of amino acids in the epitope, even at a 1000-fold molar excess, no inhibition of the binding of BV16 to Bet v 1 could be exerted by a synthetic peptide representing the sequential element. This observation clearly illustrates the limitations in using peptides for B cell epitope characterization.

The mAb BV16 was raised by immunization using purified natural Bet v 1 from pollen and has been shown to inhibit the binding of human-specific serum IgE to Bet v 1 by ~40% (2). This observation suggested a complete or partial overlap between the epitope defined by the murine IgG1 mAb BV16 and human IgE-binding epitopes, a result that is confirmed in this work by the relatively large decrease in Bet v 1-specific IgE reactivity following site-directed mutagenesis in the epitope. Glutamic acid in Bet v 1 position 45 is centrally located in the epitope defined by BV16 (Fig. 1), and shows a high degree of solvent exposure (65%) (1). A serine residue was found to occupy the corresponding position in some Bet v 1 homologous plant pathogenesis-related (PR-10) proteins (34), for example, from parsley (35) and asparagus (36), suggesting that glutamic acid can be replaced by serine without distortion of the three-dimensional structure. In addition, because none of the known tree pollen allergen sequences have serine in position 45, the substitution of glutamic acid with serine gives rise to a nonnaturally occurring Bet v 1 molecule.

The electron density map of the mutant structure, which also showed preservation of the overall folding pattern of the molecule, verified the substitution of glutamic acid to serine in position 45 (Fig. 4). This is a crucial result because the decrease in specific serum IgE binding to the mutant can be directly ascribed to the single amino acid substitution and is not caused by long-range structural alterations or a collapse of the structure of the molecule. In the complex structure (2), Glu⁴⁵ fits into a groove in the Fab' surface, and its carboxyl oxygen atoms form two hydrogen bonds (~2.9 Å) with Fab' backbone nitrogen atoms. Substituting this acidic residue with serine is likely to disturb the pattern of hydrogen bonds forming upon complex formation, and furthermore result in alteration of the local molecular surface area and its electrostatic potential, which might lead to reduced fit in the Bet v 1–Ab interaction, explaining a reduction in binding affinity. A perfect fit in the topographies of the complementary surfaces of the epitope/paratope interaction has been described as a major factor in determining binding affinity (37).

The total Ab-accessible molecular surface of Bet v 1 is 7203 Å² (2), of which Glu⁴⁵ accounts for 71.6 Å² (2) corresponding to 1.0%. The relatively large effect on the binding of specific serum IgE due to this minor change in molecular surface topography suggests that

glutamic acid in position 45 is an important residue with respect to the binding of specific serum IgE.

Whereas the Glu⁴⁵–Ser mutation reduced the binding of a pool of tree pollen-allergic patients' IgE up to 50%, the substitution of Glu⁶⁰, which is located outside surface areas conserved among the *Fagales*, had no significant effect on IgE binding (Fig. 6). Although this result does not demonstrate a statistical significant difference between the two mutants due to a relatively large uncertainty on the quantitative IgE-binding results, neither does it invalidate the concept of dominating IgE-binding epitopes, and the notion that dominating IgE-binding epitopes may be preferentially located in conserved surface areas. Other point mutations in conserved surface areas, i.e., Pro¹⁰⁸–Gly and Asn²⁸–Thr + Lys³²–Gln (a double mutant), also reduced IgE binding ~50%, whereas the mutant Asn⁴⁷–Ser did not significantly reduce IgE binding.

Results from immunoassays comparing the effect of the Glu⁴⁵–Ser mutation on individual patients' IgE indicate that IgE from a single patient react with a restricted area of the allergen surface, and that these restricted areas differ from patient to patient. Thus, for example, in the serum of patient 43, 49% of the IgE seems to be directed toward the surface area at and immediately surrounding Glu⁴⁵, whereas this is only true for 25% of the IgE in the serum of patient 35 (Fig. 5). The results emphasize that although the concept of dominating IgE-binding epitopes is supported, certainly there is more than one dominating epitope, even when considering IgE specificities in individual patients. It is therefore necessary to introduce more than one surface-exposed amino acid substitution to produce a molecule with reduced IgE binding in a larger patient population. All four serum samples applied in this study did, however, show reduced IgE binding to the artificial Glu⁴⁵–Ser mutant as compared with Bet v 1.2801.

Similar results with respect to patient-to-patient variations were observed in another study of the effect on IgE binding of single-point mutations, however, designed to reflect naturally occurring amino acid substitutions (38). In this study, some, but not all, patients showed reduced IgE binding to each of six single-point mutants investigated. Combining all six mutations into one molecule resulted in reduction in IgE binding for all patients tested, again indicating that several mutations are needed to achieve a molecule with reduced anaphylactic potential in a larger patient population.

In this study, we have demonstrated that structural determination of allergen–Ab complexes can be used as a valuable tool for detailed mapping of epitopes and amino acid residues crucial for interaction with specific serum IgE. By the approach described, modified recombinant allergens can rationally be designed with substantially reduced IgE reactivity by introduction of a limited number of amino acid substitutions in identified IgE-binding epitopes. Verification of the folding pattern of the mutated allergen is essential to ensure preservation of surface structures capable of inducing protective allergen-specific IgG Abs during the course of specific allergy vaccination. Furthermore, a limited number of substitutions may theoretically be designed without preventing the modified allergen from the ability to stimulate polyclonal Th cell responses in allergic patients.

The structure-based design of recombinant mutated allergens is a promising approach for the development of safer and true pharmaceutical vaccines for future specific allergy vaccination.

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