Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry (*Prunus avium*) allergen

Regina WICHE*, Michaela GUBESCH*, Herbert KÖNIG†, Kay FÖTISCH*, Andreas HOFFMANN*, Andrea WANGORSCH*, Stephan SCHEURER* and Stefan VIETHS*¹

*Division of Allergology, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51–59, 63225 Langen, Germany, and †Division of Haematology and Transfusion Medicine, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51–59, 63225 Langen, Germany

Birch (*Betula verrucosa*) pollen-associated food allergy is a well-characterized syndrome, which is due to the cross-reactivity of IgE antibodies to homologous allergens in various foods. One cross-reacting area on the major birch pollen allergen Bet v 1 and its homologue in cherry (*Prunus avium*) Pru av 1 has already been identified. This is the so-called ‘P-loop’ region, which encompasses amino acid residues around position 45 and is found on the two virtually identical tertiary protein structures. We tried to determine an additional IgE cross-reacting patch on Pru av 1 and Bet v 1. The putative IgE-binding region on Pru av 1 was localized with a mAb (monoclonal antibody) that was generated against Bet v 1, and cross-reacts with several Bet v 1 homologues in food and inhibits the binding of patients’ IgE to Pru av 1. mAb reactivity pattern was analysed and amino acid positions 28 and 108 of Pru

av 1 were selected and mutated by site-directed mutagenesis. The Pru av 1 mutants were produced as recombinant proteins and characterized for their folding, mAb- and IgE-binding capacity and allergenic potency with a cellular assay using the humanized rat basophilic leukaemia cell line RBL-25/30. Amino acid position 28 is involved in a second major IgE-binding region on Pru av 1 and probably on Bet v 1. The identification of this second major IgE-binding region is an essential prerequisite to understand the phenomenon of cross-reactivity and its clinical consequences, and to produce hypoallergenic proteins for an improved immunotherapy of type I allergy.

Key words: allergy, antibody, cross-reactivity, hypoallergenic mutant, IgE epitope analysis, *Prunus avium* (cherry).

INTRODUCTION

Allergies to birch (*Betula verrucosa*) and grass pollen are present in 15–20 % of the population in central and northern Europe [1]. Between 50 % and 93 % of birch pollen-allergic patients develop a pollen-related food allergy [2–4], resulting in an estimated prevalence of adverse reactions to foods, such as fruits, nuts and vegetables, of 2–4 % within the population. Food allergies of birch pollen-allergic patients are caused by common IgE epitopes on allergenic birch pollen proteins and food homologues [5,6]. Primary sensitization to birch pollen allergens gives rise to specific IgE antibodies, which are cross-reactive with homologous proteins in various fruits and vegetables. Bet v 1, the major allergen from birch pollen, is also the main cause of this pollen-associated food allergy [7]. Homologues of Bet v 1 have been identified in various fruits, such as cherry (*Prunus avium*), apple (*Malus domestica*), hazelnut (*Corylus avellana*), peach (*Prunus persica*), carrot (*Daucus carota*), celery (*Apium graveolens*) and soya bean (reviewed in [4]). In contrast to ‘classical’ food allergens, such as Ara h 1 from peanut (*Arachis hypogaea*) or cow’s milk caseins, which have been shown to contain sequential epitopes [8,9], the IgE reactivity of Bet v 1 and its homologues in foods appears to be strictly dependent on the intact tertiary fold of the protein [10–12].

The tertiary structure of Bet v 1 was solved as an important prerequisite for studying the molecular structure of its B-cell epitopes [13]. We have selected Pru av 1, the Bet v 1 homologue from cherry, as a model to study the molecular properties of pollen-related food allergens. The prevalence of cherry allergy is 58 % among birch pollen-allergic individuals [14]. Pru av 1 was identified as the only major allergen in cherry, and was produced as pure

recombinant protein, and initial epitope data were obtained by site-directed mutagenesis [6,15]. Recombinant Pru av 1 proved to be a useful tool for *in vivo* diagnosis of cherry allergy [16]. Pru av 1 is the first pollen-related food allergen for which a highly resolved solution structure became available [17]. Thus Pru av 1 is probably the best-characterized pollen-related food allergen.

Three conserved surface patches have been suggested as potentially cross-reactive IgE epitopes on Bet v 1 and homologous tree pollen allergens [13,18]. The amino acid sequences of these surface patches are also conserved on Bet v 1 homologous food proteins [17]. One putative IgE-binding epitope region on Bet v 1 has been identified [10]. This area is located around the ‘P-loop’ region between the β -strands 2 and 3, which indeed represents a conserved area in this protein family. By investigating a Pru av 1 Glu⁴⁵ → Trp (amino acid in P-loop region) mutant, we have recently shown that this region is also a cross-reactive IgE-binding epitope on Pru av 1 [11]. Because at least two IgE-binding epitopes are required to activate effector cells, such as mast cells and basophils, via cross-linking of IgE bound to Fc ϵ -receptors, further studies on the IgE epitope structure of this important allergen family are required. The knowledge of such major IgE epitopes creates the possibility for a specific vaccination or immunotherapy in allergic patients [19–21]. Altered surfaces of allergens obtained by amino acid exchange may lead to a lower IgE-binding capacity and maintain the T-cell response to these molecules [22–24]. Therefore we tried to identify a second IgE-binding region by using a mAb (monoclonal antibody) with similar binding properties as IgE from patients’ sera. Site-directed mutagenesis of Pru av 1 wt (wild-type) was used to identify the location of the IgE-reactive region.

Abbreviations used: mAb, monoclonal antibody; wt, wild-type; EAST, enzyme allergosorbent test; r, recombinant; RBL, rat basophilic leukaemia.

¹ To whom correspondence should be addressed (email viest@pei.de).

EXPERIMENTAL

Antibodies and allergens

Serum samples were collected from 28 birch pollen-allergic patients with a clear history of cherry allergy. Ninety percent of these patients reported oral allergy syndrome, and 10 % urticaria and/or gastrointestinal symptoms after ingestion of fresh cherries. Sera with specific IgE to recombinant Pru av 1 [class 1 to 4 by EAST (enzyme allergosorbent test)] were selected for the study. The study was approved by the ethical committee of the Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany. Written informed consent was obtained from all participants. All patients, with the exception of three, were monosensitized to Pru av 1, as indicated by IgE ELISA obtained with Pru av 1, 3 and 4.

The mAbs mP10 and mP16 were obtained after immunization of Balb/c mice with birch pollen extract and selection for reactivity to Bet v 1. mAbs G4a, C10b, F11e and G4d1 were generated against Pru av 1. The mP16 hybridoma supernatant was produced in an Integra cell line CL 350 (Integra Biosciences AG, Chur, Switzerland) using Hybridoma-SFM medium (Invitrogen GmbH, Karlsruhe, Germany) without fetal calf serum in the cell compartment. For inhibition assays mAb mP16 was purified by Protein G-affinity chromatography (Amersham Biosciences Europe, Freiburg, Germany) and dialysed against PBS. All other antibodies were applied as cell culture supernatants.

The allergens rBet v 1a (birch, UniProt number P15494), Bet v 1l (P43185) and Cor a 1.0101 (hazel pollen, *Corylus avellana*, S30053) were purchased from Biomay (Vienna, Austria). Pru av 1 (cherry, O24248), Pru av 1 Ser¹¹² → Pro and Glu⁴⁵ → Trp mutant, Cor a 1.0401 (hazelnut, Q9SWR4), Mal d 1 (apple, AF124823), Api g 1.0101 (celery, P49372), Api g 1.0201 (P92918) and Dau c 1.0104 (carrot, Z81362) were produced as recombinant proteins in our laboratory [11,15,25–28].

ELISA

IgE Inhibition ELISA

Maxisorb plates (96-well, Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with 50 ng/100 µl of Pru av 1 dissolved in PBS and blocked for 1 h at 25 °C with PBS containing 1 % BSA (Sigma–Aldrich, Deisenhofen, Germany). The mAb mP16 (0.5–0.01 µg/100 µl) and patient's serum (3 single sera, 1:50) were incubated simultaneously overnight. An anti-(mitogen-activated protein kinase)-specific mAb (Sigma–Aldrich) was used as negative control for the specific inhibition with mAb mP16. For the detection of bound human IgE antibodies, it was incubated successively with rabbit anti-human IgE (DAKO, Hamburg, Germany; 1:4000), goat anti-rabbit IgG–biotin (DAKO; 1:6000) and streptavidin–horseradish peroxidase (Calbiochem, Bad Soden, Germany; 1:10 000). The substrate for horseradish peroxidase was 3,3',5,5'-tetramethylbenzidine (Merck, Darmstadt, Germany), the reaction was stopped with 3 M H₂SO₄ and the absorbance was measured at 450 nm.

IgG ELISA

Maxisorb plates were coated with 50 ng of protein/50 µl of PBS per well (coating and blocking procedure described above) and incubated with mAb (cell culture supernatant, 1:10 diluted, overnight at room temperature). Mouse IgG was determined with a goat anti-mouse IgG conjugated with peroxidase (Sigma–Aldrich). The substrate and stopping reagent were the same as described above.

Site-directed mutagenesis of Pru av 1

Three Pru av 1 variants were generated carrying single mutations at positions 28 (Lys²⁸), 108 (Ala¹⁰⁸) and mutations at both positions (Lys²⁸ and Ala¹⁰⁸). The multi-site-directed mutagenesis kit from Stratagene (Amsterdam, The Netherlands) and PCR conditions were applied according to manufacturer's instructions. Pru av 1 cDNA (accession number, O24248) in the vector pET-16b was utilized as template [15]. 5' primers (Sigma–Aldrich) were used: CCTTTGTCCTCGATGCTGACAAGCTTGTCCCTAAGATTGG (Lys²⁸, underlined), CCAAGTTGGTGGCATCCGCCAGC-GGAGGATCCATCATCAAGAGC (Ala¹⁰⁸).

Plasmid DNA was purified with a plasmid purification kit (Qiagen, Hilden, Germany) and sequenced full length on both strands with T7 promoter and T7 terminator primers (MWG-Biotech, Ebersberg, Germany). After sequencing plasmids were transformed in *Escherichia coli* BL21(DE3) competent cells for protein expression.

Expression and purification of Pru av 1 mutants

Protein synthesis was induced by adding IPTG (isopropyl β-D-thiogalactoside) (Carl Roth, Karlsruhe, Germany) to a final concentration of 1 mM at *D*₆₀₀ of 0.6–0.7. Cultures were incubated at 37 °C for 4 h. Cells were harvested by centrifugation, resuspended in buffer [50 mM Na₂HPO₄, 500 mM NaCl, 20 mM imidazole, pH 7.5, and 5 units/ml Benzonase (Merck)] and disrupted by repeated freeze–thawing. Cell debris was removed by centrifugation for 30 min at 25 000 g and 4 °C. The allergens were purified from the soluble fraction by Ni²⁺-chelate affinity chromatography as described previously [15], and dialysed against 10 mM potassium phosphate buffer (pH 7.2).

CD spectroscopy of natural and recombinant Pru av 1

Protein spectra were recorded on a Jasco J-810 spectropolarimeter (Groß-Umstadt, Germany), step width 0.2 nm, band width 1 nm, spectral range 255–185 nm, scanning speed 50 nm/min. Ten scans were accumulated at a temperature of 21 °C. The mean residue ellipticity [θ]_{m.r.w.} was calculated [11].

Binding analyses of mAb mP16

Surface plasmon resonance measurements were carried out on a BIAcore 1000 system (BIAcore AB, Uppsala, Sweden). mAb mP16 (2 ng) was immobilized in 10 mM sodium acetate (pH 4.5) on a CM5 sensor chip using standard amine-coupling chemistry. Excess reactive groups were blocked with ethanolamine. Binding analyses were performed in buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P20, pH 7.4) at a flow rate of 50 µl/min at 25 °C. Association (30 s) and dissociation (60 s) times were analysed with several concentrations of Pru av 1 wt, Pru av 1 Asn²⁸ → Lys and Bet v 1a in running buffer. The surface was regenerated with 10 mM HCl. The kinetic rate constants (*k*_a and *k*_d), as well as the equilibrium dissociation constant (*K*_D), were determined using BIAevaluation version 3.0 software supplied by the manufacturer. The Langmuir 1:1 interaction model was chosen for calculation.

EAST and EAST inhibition

Specific IgE was semi-quantified (IgE ≤ 0.35 units/ml, class 0; 0.35 ≤ IgE ≤ 0.7 units/ml, class 1; 0.7 ≤ IgE ≤ 3.5 units/ml, class 2; 3.5 ≤ IgE ≤ 17.5 units/ml, class 3; IgE > 17.5 units/ml, class 4) by EAST according to the manufacturer's instructions (Allergopharma Spez. IgE ELISA, Allergopharma, Reinbek,

Germany). Recombinant Pru av 1 wt and its mutants Asn²⁸ → Lys, Pro¹⁰⁸ → Ala and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala were coupled to CNBr-activated paper disks (Hycor, Kassel, Germany) at a protein concentration of 250 ng/disk. Sera from 25 cherry-allergic patients were analysed for their IgE-binding reactivity to Pru av 1 wt and mutants. The data were analysed for statistically significant differences of IgE reactivities to Pru av 1 wt and mutants. Dose-related EAST inhibition experiments with Pru av 1 as solid-phase antigen were performed as described previously [15]. Pooled serum samples were diluted 1:2 and incubated with Pru av 1 wt, Asn²⁸ → Lys, Pro¹⁰⁸ → Ala and the Asn²⁸ → Lys/Pro¹⁰⁸ → Ala mutants, inhibitor concentrations ranging from 0.01 to 20 µg of protein/ml. Absorbance (A) was measured at 405/650 nm and inhibitions calculated as follows (s, serum; s + i, serum plus inhibitor; nsb, non-specific binding):

$$\text{Inhibition (\%)} = [(A_s - A_{s+i}) / (A_s - A_{nsb})] \times 100$$

Statistical analysis

The Friedman test (two-sided hypothesis, with size of test $\alpha = 0.05$) was performed to test if specific IgE antibody reactivities to Pru av 1 mutants differed significantly from those to Pru av 1 wt (global hypothesis). Within-group comparisons were performed with the Wilcoxon signed-ranks test (two-sided hypothesis to multiple size of test $\alpha = 0.05$ with Bonferroni–Holm adjustment for multiple comparisons).

Mediator release assay

The assay was carried out with the humanized rat basophilic leukaemia cell line (RBL-30/25) established in our laboratory [29]. The cell line had been transfected with the α -chain of the human Fc_ε receptor. Cells (1.5×10^5 /well) were plated in 96-well flat bottomed plates. After adherence (4 h) the cells were sensitized with patients' sera (optimal dilution serum A, C and D, 1:20; serum B, 1:15; see Figure 9) and with a serum of a non-allergic subject (control) overnight. Sera with a Pru av 1-specific IgE level of at least 15 unit/ml, as determined by CAP[©], were selected for the experiments. Cells were stimulated with Pru av 1 and mutants in 10-fold dilutions (highest concentration, 10 µg/ml in Tyrode's buffer). Allergens recombinant (r)Pru av 1 wt or serum alone, as well as recombinant shrimp tropomyosin (Pen a 1, 10 µg/ml; donated by Gerald Reese, Paul-Ehrlich-Institut), served as additional antigen negative controls. Released β -hexosaminidase was measured in supernatants using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma–Aldrich) dissolved in 0.1 M phosphate buffer (pH 4.5) as substrate [29]. The specific release was calculated as the percentage of total β -hexosaminidase content from RBL cells treated with 1 % Triton X-100 in PBS. The spontaneous release was subtracted from this total value. For the mediator release inhibition, cell culture supernatants of mAb mP16 and G4a were used and their concentrations were adjusted to the molar concentration of rPru av 1 at 1 µg/ml. Pru av 1 and mAbs were pre-incubated for 20 min before stimulation of cells. The colour intensity of the Pru av 1 reference solution was adjusted to the mAb-containing culture supernatants.

RESULTS

Screening of mAbs for recognition of putative IgE binding regions

Monoclonal antibodies mP10, mP16, C10b, F11e, G4a, G4d1 were selected for their specificity to Bet v 1 and its homologues Pru av 1, Api g 1 (celery) and Mal d 1 (apple) and Pru av 1 mutants

Figure 1 Patients' IgE inhibition assay

Inhibition of IgE binding of three different sera from cherry allergic patients (sera no. 2, 5 and 6) to rPru av 1 by purified mAb mP16, as measured by ELISA.

Glu⁴⁵ → Trp and Ser¹¹² → Pro by immunoblots and ELISA (results not shown). Results of these experiments identified mAb mP16 as a candidate for epitope analysis, because it was reactive with Bet v 1 and cross-reacted with Pru av 1, Mal d 1 and Cor a 1.0401 (hazelnut homologue), a pattern that is typical for patients with pollen-related food allergy [5–7,14]. Moreover mP16 did not react with the Pru av 1 Ser¹¹² → Pro mutant. This mutant displayed a CD spectrum typical for a mostly unfolded protein, and the IgE reactivity of 95 % of patients' sera was reduced to a large extent [11]. This reaction pattern prompted us to use this antibody in IgE inhibition assays. Immunoblot inhibition was performed with a serum pool of five human sera (all from birch and cherry allergic patients with an EAST class 3 or 4 to Pru av 1). Patients' sera and mAb mP16 competed for binding to immobilized Pru av 1. The mAb showed a strong binding inhibition of patients' IgE to Pru av 1 (results not shown). This observation was confirmed by results of a competitive ELISA using sera from individual patients. In these experiments, the mAb mP16 showed up to 50 % inhibition of IgE binding to Pru av 1 (Figure 1).

Selection of candidate positions for mutational epitope analysis of Pru av 1

The mAb mP16 reacted with rBet v 1a and its homologous allergens rPru av 1 (cherry), rMal d 1 (apple) and rCor a 1.0401 (hazelnut). It did not react with the hypoallergenic Bet v 1 mutant carrying the mutations Asn²⁸ → Thr, Lys³² → Glu, Glu⁴⁵ → Ser and Pro¹⁰⁸ → Gly (given by Dr M. Spangfort, ALK-Abello, Horsholm, Denmark) [20], the Bet v 1 isoform Bet v 1l, and the major allergen Cor a 1.0101 from hazel pollen. mAb mP16 had also no reactivity to Dau c 1.0104, Api g 1.0101 and Api g 1.0201, the Bet v 1 homologues in carrot and celery tuber (results not shown). Sequence alignment revealed that two amino acids (Asn²⁸ and Pro¹⁰⁸) seemed to be critical for the binding of mAb mP16 to Pru av 1. These two amino acids are surface exposed on Pru av 1 and Bet v 1 and belong to the proposed IgE-binding sites on Bet v 1 [13]. In Figure 2 the putative critical amino acid positions are highlighted on the tertiary surface structure of Pru av 1. The position Asn²⁸ was selected for mutational analysis, because the corresponding amino acids of Bet v 1l and Cor a 1.0101, which both did not react with mP16, have a lysine residue at position

Figure 2 Structure of Pru av 1 (Protein Databank no. 1E09)

Amino acids involved in known and hypothetical IgE-binding regions are highlighted: Glu⁴⁵ (E45), Asn²⁸ (N28), Pro¹⁰⁸ (P108). The structure was modelled with Swiss PDB Viewer 3.7 [33].

28 (Figure 3A). Accordingly an Asn²⁸ → Lys mutant of Pru av 1 was produced. Because Dau c 1.0104 and Api g 1.0101 both have alanine at position 108 and were not recognized by mP16, the mutation Pro¹⁰⁸ → Ala was introduced as the second mutation by site-directed mutagenesis (Figure 3B). In addition a Pru av 1 mutant carrying both substitutions (Asn²⁸ → Lys/Pro¹⁰⁸ → Ala) was produced.

Purity and secondary structure analysis of Pru av 1 mutants

Mutated Pru av 1 sequences were expressed as recombinant proteins with N-terminal histidine-tags and purified. All mutated proteins showed a high (> 95 %) purity by SDS/PAGE analysis and subsequent staining with Coomassie Blue (results not shown). The CD spectra of all Pru av 1 mutants revealed a nearly identical secondary structure compared with the CD spectrum of Pru av 1 wt protein (Figure 4).

Binding of mAb mP16 to rPru av 1 is sensitive to the mutation at position 28

The effect of the Asn²⁸ → Lys and Pro¹⁰⁸ → Ala mutations on the specific binding of mAb to Pru av 1 was analysed by ELISA. The binding of mAb mP16 to the mutant Asn²⁸ → Lys and the double mutant Asn²⁸ → Lys/Pro¹⁰⁸ → Ala was reduced drastically (90 %) in comparison to the wt protein (Figure 5). Binding of mAb mP16 to the Pru av 1 Pro¹⁰⁸ Ala mutant was not reduced. An immunoblot inhibition experiment with Pru av 1 wt and mutants as inhibitors confirmed the reduction of mP16 binding to Pru av 1 Asn²⁸ → Lys and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala mutants (results not shown).

Figure 3 Critical amino acids for human IgE binding

Sequence alignment of Pru av 1 with Bet v 1 isoforms Bet v 1a and Bet v 1l, and the homologues Cor a 1.0401, Cor a 1.0101, Api g 1.0101, Api g 1.0201 and Dau c 1.0104. Reactivity pattern of mAb mP16 was used for the alignment to determine sequence differences between Bet v 1 homologues that reacted with mP16 (Pru av 1, Bet v 1a and Cor a 1.0401) and that did not react with mP16 (Bet v 1l, Cor a 1.0101, Api g 1.0101, Api g 1.0201 and Dau c 1.0104). Alignments are divided into two groups for selection of amino acids critical for putative IgE-binding region (group 1, sequence identity 58–65%; group 2, sequence identity 39–42% to Pru av 1). Group 1 (A, amino acid position Asn²⁸): Pru av 1, Cor a 1.0401 and Bet v 1a have an asparagine residue in position 28, whereas Bet v 1l and Cor a 1.0101 have a lysine residue. Group 2 (B, amino acid position Pro¹⁰⁸): Dau c 1.0104 and Api g 1.0101 have an alanine residue in position 108, instead of proline as in Pru av 1. The output of MultAlin 5.4.1 [34] was edited with ESPript 3.02. White residues on black background, strict identity; bold black residues, similarity within a group; boxed residues, similarity across groups; arrow, mutation sites.

Figure 4 CD spectra of Pru av 1 wt and its mutants

The spectra overlay showed that the mutants possess a nearly identical distribution of secondary structure elements in comparison with Pru av 1 wt.

Figure 5 Direct binding of mAb mP16 to Pru av 1 wt and to the mutants Asn²⁸ → Lys (N28K), Pro¹⁰⁸ → Ala (P108A) and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala (N28K, P108A) measured by ELISA

Affinity constants of mAb mP16 were determined by real-time interaction analysis on BIAcore 1000. Ligand was mAb mP16. Pru av 1 wt, Pru av 1 Asn²⁸ → Lys and Bet v 1a were applied as analytes in different concentrations. Using the BIAevaluation software, the mathematical curve-fit analysis showed that the experimental and theoretical curves could be superimposed (Figure 6). There were no significant differences between the k_a rates of all three analytes (Table 1). The k_d rates of Pru av 1 wt and Bet v 1a were very similar: $3.62 \times 10^{-3} \text{ s}^{-1}$ and $2 \times 10^{-3} \text{ s}^{-1}$ respectively. The k_d rate of the Pru av 1 Asn²⁸ → Lys mutant protein was only 0.08 s^{-1} , which is a significant reduction compared with the two wt allergens. The Pru av 1 Asn²⁸ → Lys mutant had a 22.3-fold lower affinity (K_D) than Pru av 1 wt, mainly due to a faster dissociation. As expected Bet v 1a had a higher affinity (K_D 2.2-fold higher) than Pru av 1, because mAb mP16 was obtained after immunization with birch pollen. This binding analysis confirmed that mAb mP16 binds to Pru av 1 wt and Bet v 1a. In addition, the results support the idea that the antigenic binding site of mP16 is near the amino acid Asn²⁸ on Pru av 1 and probably also on Bet v 1a.

Figure 6 Surface plasmon measurement of the interactions between immobilized mAb mP16 with Pru av 1 wt (A), Bet v 1a (B) and Pru av 1 Asn²⁸ → Lys mutant (C)

Analyte concentrations: 111 nM, 37 nM, 12.3 nM. The broken lines are theoretically calculated.

Table 1 Results of real-time interaction analysis of mAb mP16 binding to Pru av 1 wt, Pru av 1 Asn²⁸ → Lys and Bet v 1a

Allergen	$k_a \text{ (M}^{-1} \cdot \text{s}^{-1})^*$	$k_d \text{ (s}^{-1})^*$	$K_D \text{ (M)}$
rPru av 1 wt	1.67×10^5	3.62×10^{-3}	2.17×10^{-8}
rPru av 1 Asn ²⁸ → Lys	1.74×10^5	0.08	4.83×10^{-7}
rBet v 1a	2.09×10^5	2×10^{-3}	9.55×10^{-9}

* Values represent the mean of data from one experiment in which the binding of triplicate samples of analytes at different concentrations were measured.

Pru av 1 mutants Asn²⁸ → Lys and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala display reduced IgE-binding capacity

Specific IgE to Pru av 1 wt and the generated mutants were measured by an EAST (Figure 7). Reduced binding of patients' IgE was observed for the Pru av 1 Asn²⁸ → Lys mutant and the double mutant Asn²⁸ → Lys/Pro¹⁰⁸ → Ala (Figures 7A and 7C). Up to 80 % of the sera showed weaker IgE binding to these two mutants, whereas only approx. 12 % had decreased reactivity to the Pru av 1 Pro¹⁰⁸ → Ala mutant. The statistical analysis (Friedman test) showed that the groups (IgE-binding capacity of the different proteins) differed significantly from each other ($P < 0.0001$). Specific IgE concentrations measured with the different Pru av 1 antigens were compared by the Wilcoxon-signed rank test. The differences between Pru av 1 wt and the Asn²⁸ → Lys mutant ($P = 0.0003$) and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala mutant ($P < 0.0001$) were significant when pairs of Pru av 1 wt and each mutant were compared. The difference between Pru av 1 wt and the Pro¹⁰⁸ → Ala mutant was not significant ($P = 0.1538$).

The reduced IgE reactivity of the two Pru av 1 mutants was further confirmed by EAST inhibition experiments. A serum pool for four human sera with reduced IgE binding to mutant Asn²⁸ → Lys and the double mutant was selected for a dose-related inhibition experiment (Figure 8). Maximum inhibition with wt protein was 88 %. The ID₅₀ was calculated to be 1 µg/ml.

Figure 7 Comparison of IgE-binding capacity of rPru av 1 wt and mutants

Patients' sera ($n = 25$) were tested for their binding to Pru av 1 wt and its mutants by EAST. A statistically significant reduction in IgE reactivity was found for the mutants Asn²⁸ → Lys (N28K) and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala (N28K, P108A). U/ml, units/ml.

Pre-incubation with the Pru av 1 Asn²⁸ → Lys and the double mutant Asn²⁸ → Lys/Pro¹⁰⁸ → Ala resulted in an inhibition of 73 %. The ID₅₀ value was calculated between 4 to 5 µg/ml.

A functional assay confirms binding of mP16 to a putative IgE epitope region

Mediator release assays were performed with a humanized RBL cell line passively sensitized with sera from cherry allergic patients. To compare the allergenic activity of the different Pru av 1 proteins in a functional assay, sensitized cells were stimulated with Pru av 1 wt and the generated mutants. Two representative sera were selected which showed reduced IgE binding to the Pru av 1 mutant Asn²⁸ → Lys and the double mutant, but not to the Pru av 1 mutant Pro¹⁰⁸ → Ala (EAST results). The results of me-

Figure 8 Inhibition of IgE binding to Pru av 1 wt measured by EAST

The pooled serum of 4 patients was pre-incubated with rPru av 1 and mutants as inhibitors. Pru av 1 Asn²⁸ → Lys (N28K) and Asn²⁸ → Lys/Pro¹⁰⁸ → Ala (N28K, P108A) mutants showed a clearly reduced inhibition capacity compared with Pru av 1 wt.

diator release assays indicated that reduced IgE-binding capacity correlated with lower allergenic activity in this functional test system (Figures 9A and 9B): the Pru av 1 mutant Asn²⁸ → Lys and the double mutant caused a significantly lower mediator release compared with the wt protein and the mutant Pro¹⁰⁸ → Ala. All controls (stimulation of sensitized cells with shrimp tropomyosin as an unrelated allergen, sensitization with non-allergic control sera and subsequent stimulation with Pru av 1 wt or mutants) were negative.

To further confirm that the position Asn²⁸ is involved in an IgE-binding surface area, a mediator release inhibition experiment was performed. Recombinant Pru av 1 wt was incubated with mP16 cell culture supernatant (Figures 9C and 9D). Because the size of the antibody molecule and antigen differ drastically (the molecular mass of Pru av 1 is approx. 9-fold lower), it had to be excluded that an observed reduced mediator release might only be caused by sterical hindrance by the antibody molecule or lower mobility of the immune complex. Therefore a second mAb (G4a) generated against Pru av 1 was included as control. This antibody reacted with the unfolded Pru av 1 Ser¹¹² → Pro mutant in contrast with mAb mP16. At a concentration of 1 µg/ml rPru av 1 mAbs (mP16/G4a) were present in equimolar amounts. Constant concentrations of the mAbs were incubated with a dilution series of rPru av 1, as indicated in Figures 9(C) and 9(D). The addition of mP16 almost abolished mediator release elicited by Pru av 1, whereas allergenic activity was clearly detectable when the allergen was pre-incubated with mAb G4a.

DISCUSSION

Birch pollen-related food allergy is caused by cross-reactive B-cell epitopes on homologous allergens found in pollens and foods [5–7]. Identification and structural characterization of such cross-reacting IgE epitopes enables the production of recombinant allergens with reduced IgE-binding capacity [19–21]. Such allergen derivatives have been suggested as candidates for improved specific immunotherapy, because B-cell epitopes are destroyed and T-cell epitopes are conserved [22–24]. Until now only one cross-reacting IgE binding region has been described

Figure 9 Mediator release of Pru av 1 wt, mutants and mAbs as inhibitors with humanized RBL 30/25 cell line

Cells were sensitized with sera from individual cherry allergic patients and stimulated (i) with Pru av 1 wt and the three Pru av 1 mutants (**A, B**), and (ii) with Pru av 1 wt pre-incubated with mAb mP16 or G4a cell culture supernatant (**C, D**). The mediator release was clearly reduced for the Pru av 1 Asn²⁸ → Lys (N28K) and the Asn²⁸ → Lys/Pro¹⁰⁸ → Ala (N28K, P108A) mutants in comparison with the wt protein (**A, B**). Pre-incubation of mAb mP16 with Pru av 1 wt led to almost total reduction of release, whereas mAb G4a did not greatly reduce the mediator release (**C, D**). Release of Pru av 1 (%) is reduced in (**C**) and (**D**) compared with (**A**) and (**B**), because colour intensity of Pru av 1 reference solution was adjusted to mAb cell culture supernatant colour. 1e-5, 1 × 10⁻⁵ etc.

for tree pollen allergens: the P-loop (amino acids 41–52) of Bet v 1 was identified as putative major IgE-reactive region [10], and site-directed mutagenesis confirmed that position Glu⁴⁵ was important for IgE antibody binding [12]. Our previous work has shown that position 45 is also critical for the epitope structure of pollen-related food allergens; by a combination of site-directed mutagenesis and structural analysis, Glu⁴⁵ on Pru av 1 was identified as part of a major IgE-binding region [6,11].

Our present results strongly suggest that the amino acid Asn²⁸ is involved in a second IgE-reactive epitope of Pru av 1. First, the mAb mP16 inhibits IgE binding to Pru av 1 wt up to 50%, and shows a marked reduced affinity to the Pru av 1 mutant Asn²⁸ → Lys. mAb affinity was found to be even higher to Bet v 1 than to Pru av 1, showing that Bet v 1 was used for specificity selection of mP16 and mimics the human sensitization pattern. As indicated by CD spectroscopy, the Pru av 1 Asn²⁸ → Lys mutant shares a similar distribution of secondary structure elements with the native allergen, supporting the fact that the mutants are correctly folded. Second, EAST data indicate a statistically significant reduction of the IgE-binding capacity of the Pru av 1 mutant Asn²⁸ → Lys and the double mutant Asn²⁸ → Lys/Pro¹⁰⁸ → Ala, but not of the mutant Pro¹⁰⁸ → Ala. Third, EAST inhibition and mediator release experiments consistently showed a marked re-

duction of allergenicity of the mutant Asn²⁸ → Lys and the double mutant Asn²⁸ → Lys/Pro¹⁰⁸ → Ala in comparison with Pru av 1 wt. Fourth, pre-incubation of allergen with mP16 blocked IgE-caused mediator release, whereas this effect was not observed with a control immune complex.

The amino acid Asn²⁸ on Pru av 1 is part of the α -helix 2 which is surface exposed (Figure 2) and belongs to one of the areas that were proposed to be major antigenic sites on the Pru av 1 homologue, Bet v 1. Both the surface area dimension and the observation that this surface patch is highly conserved among allergens of the Bet v 1 family support this assumption [13].

The substitution at position 28 of asparagine to lysine resulted in a change of physico-chemical properties (enlarged side chain without the amidic character). Because the recombinant Pru av 1 Asn²⁸ → Lys mutant showed a CD spectrum superimposable on that of the wt protein, it can be assumed that the single amino acid substitution caused minimal perturbations on the overall folding and structural characteristics of the protein. Therefore, it is likely that the structural impact of this mutation is restricted to the neighbourhood of amino acid 28 on the surface of Pru av 1. Spangfort et al. [12] used Bet v 1 as a model and found evidence for reduced binding of human IgE to the Bet v 1 mutant Asn²⁸ → Thr/Asn³² → Glu. Ferreira et al. [24] described a similar phenomenon for a Bet v 1 Phe³⁰ → Val mutant in sera of a

subpopulation of birch pollen-allergic patients. Although only IgE-binding assays and neither mAb competition nor functional tests were performed by these authors [12,24], the results suggest this surface area as an IgE-binding epitope area of Bet v 1. Our results confirm and substantiate their findings, and show that helix 2 is involved not only in a birch pollen allergen epitope region, but is also responsible for the clinically important pollen–fruit cross-reactivity.

Because mAb mP16 showed binding to the homologous allergens Mal d 1 from apple and Cor a 1.0401 from hazelnut, we hypothesize that this region is also an IgE-binding area on these Bet v 1 homologues. In contrast, mAb mP16 did not react with the other homologous food allergens Api g 1.0101 (celery) and Dau c 1.0104 (carrot). (ELISA and immunoblot, results not shown). This is in agreement with a lower IgE reactivity of these vegetable allergens in general, a lower overall amino acid sequence identity to Bet v 1 (< 40 % versus 59 % in the case of Pru av 1), and the observation of a lack of IgE cross-reactivity between Pru av 1 and Api g 1 [4,6,17]. Therefore, it may be speculated that, although fruit allergens as well as vegetable allergens from this family clearly cross-react with IgE raised against Bet v 1, different subsets of cross-reacting IgE antibodies directed against the surface area around amino acid 28 exist, or that a different surface patch is responsible for cross-reactive binding to the vegetable allergens.

Three discontinuous antigenic sites with a surface area of at least 600 Å² have been suggested as IgE epitopes of Bet v 1: (i) the surface patches comprising the β -strands 6 and 7 (Pro¹⁰⁸), including the turn in between them and part of the long α -helix; (ii) a part of the α -helix 2 (Asn²⁸) and the C-terminus of the long helix; and (iii) the P-loop region (Glu⁴⁵) [13]. The conformational nature of the third putative IgE epitope was verified by Spangfort et al. [12]. They showed that a synthetic peptide representing the sequence of the interaction site did not inhibit the formation of the immune complex between Bet v 1 and the Fab BV16. Asn²⁸ belongs to the second proposed conformational IgE-binding region. The observation that mAb mP16 did not react to the unfolded Pru av 1 Ser¹¹² → Pro mutant (results not shown) [11] indicates that amino acid Asn²⁸ is also part of a conformational IgE-binding region on Pru av 1.

One might be tempted to hypothesize that every mAb that reacts with a small allergen, such as the 17 kDa Pru av 1, might inhibit the binding of IgE due to steric hindrance. The results of mediator release inhibition did not support this notion. One mAb, mP16, produced an almost total reduction of mediator release, and in contrast the effect of the second mAb, G4a, was not as impressive, although both mAbs showed a good reactivity with Pru av 1 in immunoblots and ELISA (results not shown). Laffer et al. [31] observed a similar effect. They described a mAb (BIP1) with anaphylactogenic characteristics and no capability to inhibit the binding of human IgE to Bet v 1. Using a filamentous phage-library strategy they proposed that the mAb antigenic site was between amino acids Phe⁵⁸ and Tyr⁶⁶ [32]. This sequence between β -strands 3 and 4 does not belong to the postulated three major IgE-epitope areas [13]. Therefore the immuno-inhibition assays gave a good clue how to narrow down the location of putative IgE-binding epitopes.

Reactivity of mAb mP16 to the Pru av 1 mutant Pro¹⁰⁸ → Ala compared with the wt protein was not reduced, nor did this mutant display reduced IgE binding for the majority of sera from allergic patients. The position of this amino acid is located in the first hypothetical IgE epitope area of Bet v 1 [13]. In the case of Bet v 1, evidence for IgE binding to this region originated from a reduced IgE reactivity of a Bet v 1 Pro¹⁰⁸ → Gly mutant [12]. We cannot exclude the possibility that this amino acid exchange

on Pru av 1 would also show reduced human IgE binding to this allergen. The observation that the double mutant Pru av 1 Asn²⁸ → Lys/Pro¹⁰⁸ → Ala displayed an even larger reduction of IgE-binding capacity than the single mutant Asn²⁸ → Lys may be taken as evidence for involvement of this area in the epitope structure of Pru av 1.

In conclusion, we identified a second putative IgE-binding area on Pru av 1, which is relevant for the clinical phenomenon of pollen-related allergy to fruits. Further work is in progress to characterize this epitope by structural analysis of an immune complex, and to identify structurally different IgE epitopes on homologous vegetable allergens.

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