Three-Dimensional Structure of the Cross-Reactive Pollen Allergen Che a 3: Visualizing Cross-Reactivity on the Molecular Surfaces of Weed, Grass, and Tree Pollen Allergens

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Three-Dimensional Structure of the Cross-Reactive Pollen Allergen Che a 3: Visualizing Cross-Reactivity on the Molecular Surfaces of Weed, Grass, and Tree Pollen Allergens

Petra Verdino,* Rodrigo Barderas,† Mayte Villalba,† Kerstin Westritschnig,§ Rudolf Valenta, §§ Rosalia Rodriguez, † and Walter Keller3*

Two EF-hand calcium-binding allergens (polcalcins) occur in the pollen of a wide variety of unrelated plants as highly cross-reactive allergenic molecules. We report the expression, purification, immunological characterization, and the 1.75 Å crystal structure of recombinant Che a 3 (rChe a 3), the polcalcin from the weed Chenopodium album. The three-dimensional structure of rChe a 3 resembles an α-helical fold that is essentially identical with that of the two EF-hand allergens from birch pollen, Bet v 4, and timothy grass pollen, Phl p 7. The extensive cross-reactivity between Che a 3 and Phl p 7 is demonstrated by competition experiments with IgE Abs from allergic patients as well as specific Ab probes. Amino acid residues that are conserved for the two EF-hand allergen family were identified in multiple sequence alignments of polcalcins from 15 different plants. Next, the three-dimensional structures of rChe a 3, rPhl p 7, and rBet v 4 were used to identify conserved amino acids with high surface exposition to visualize surface patches as potential targets for the polyclonal IgE Ab response of allergic patients. The essentially identical three-dimensional structures of rChe a 3, rPhl p 7, and rBet v 4 explain the extensive cross-reactivity of allergic patients IgE Abs with two EF-hand allergens from unrelated plants. In addition, analyzing the three-dimensional structures of cross-reactive Ags for conserved and surface exposed amino acids may be a first approach to mapping the conformational epitopes on disease-related Ags that are recognized by polyclonal patient Abs. The Journal of Immunology, 2008, 180: 2313–2321.

A
terallergies represent a health problem of increasing importance in the industrialized world (1–3). The immediate symptoms of type I allergy (rhinitis, conjunctivitis, asthma, and anaphylactic shock) are caused by IgE recognition of environmental allergens and the subsequent activation of inflammatory cells. Pollen from trees, grasses, and weeds belong to the most potent and abundant allergen sources.

To date, three families of cross-reactive pollen allergens have been described. The first family, represented by the major birch pollen allergen Bet v 1, shows high sequence homology to proteins that are activated when plants are wounded, infected, or subjected to other stressful conditions (4, 5). Allergens belonging to this pathogenesis-related protein family can be found as cross-reactive allergens in the pollen of certain trees (i.e., order Fagales) and in plant-derived food (fruits, vegetables, nuts, and spices) (6, 7).

The second family of cross-reactive plant allergens are profilins (8, 9). These ubiquitous actin-binding proteins are involved in signal transduction and, accordingly, can be found as cross-reactive pan-allergens in almost all plant species and plant tissues.

Furthermore, a third family of highly cross-reactive allergens was discovered (10). Proteins belonging to this allergen family constitute a novel class of calcium-binding proteins that contain two EF-hand calcium-binding motifs. Two EF-hand allergens, also termed polcalcins, have been characterized in pollens of various plants (trees, bushes, grasses, weeds, and flowering plants) as extremely potent allergens and, due to their pollen-specific expression, are cross-reactive allergens for patients with pollen polysensitization (11, 12). To date, cDNAs coding for polcalcins that exhibit the canonical EF-hand signature motifs have been identified in at least 15 different plant pollens. Interestingly, no relevant cross-reactivity between two EF-hand pollen allergens and other calcium-binding proteins could be observed (10, 13). Characteristic features specific for polcalcins are a short linker connecting the two EF-hand domains and a rather hydrophobic helix C-terminal of the second calcium-binding domain. Recently, the three-dimensional structures of the polcalcins from timothy grass, Phl p 7, and birch, Bet v 4, were solved (14, 15).

To analyze the IgE-cross-reactivity between pollens of grasses, trees, and weeds, we expressed, purified, and conducted immunological competition experiments with recombinant Che a 3 (rChe a 3), the polcalcin from the weed common lambsquarters. To determine the structural basis for the extensive immunological cross-reactivity among polcalcins, we determined the crystal structure of rChe a 3 and compared its molecular surface with that of rPhl p 7.

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and rBet v 4 as a novel approach for the localization of putative cross-reactive IgE epitopes.

### Materials and Methods

**Cloning, expression, and purification of recombinant Che a 3**

The complete cDNA of Che a 3 (previously cloned into the pCR2.1 plasmid (Invitrogen Life Technologies); Ref. 16) was subcloned into the NdeI/EcoRI restriction sites of the pET11b expression plasmid essentially as described earlier (17). Overnight cultures of *Escherichia coli* BL21(DE3) transformed with the pET11b/Che a 3 construct were diluted 10-fold with Luria-Bertani medium containing 0.1 mg/ml ampicillin and grown to an OD~600~ of 1.0. After induction with 0.4 mM isopropyl-β-D-thiogalactoside cultures were maintained for 4 h at 37°C and harvested by centrifugation. The pellet was resuspended in 20 mM ammonium bicarbonate (pH 8.0) and 1 mM PMSF, and lysed by three cycles of freezing and thawing (4°C). After centrifugation at 10,000 x g at 4°C, the soluble fraction was lypophilized, dissolved in 0.2 M ammonium bicarbonate (pH 8.0), and chromatographed on a Sephadex G-50 gel filtration column equilibrated with the same buffer. Fractions containing recombinant Che a 3 (rChe a 3) were detected with a specific-polyclonal antiserum obtained against the polyclin from olive pollen, Ole e 3. The rChe a 3 fractions were lypophilized, dissolved in 20 mM ammonium bicarbonate (pH 8.0), and bound to a DEAE-cellulose column equilibrated with the same buffer. rChe a 3 was eluted in 10-nl fractions by a discontinuous ionic strength gradient (0.1, 0.2, 0.3, 0.4, and 0.5 M of ammonium bicarbonate (pH 8.0)), and the protein-containing fractions were pooled and concentrated.

**Amino acid composition and sequence analysis**

Protein samples (1 nmol) were hydrolyzed with 5.7 M HCl at 105°C for 24 h. Hydrolyzed samples were analyzed on a Beckman System 6300 amino acid analyzer. NH2-terminal Edman degradation of the rChe a 3 was performed on an Applied Biosystems model 477A sequencer. Protein sequences for the amino acid sequence alignments were retrieved from the SwissProt database and aligned using ClustalW (18): Che a 3 from common lambsquarters (*Chenopodium album*), SwissProt accession no. Q84V36; Phl p 7 from common timothy grass (*Phleum pratense*), SwissProt accession no. O82040; Bet v 4 from white birch (*Betula verrucosa*), SwissProt accession no. Q39419; Amb a 9 from short ragweed (*Ambrosia artemisiifolia*), SwissProt accession no. Q5SRP7; *Q9LF54*; Art v 5 from mugwort (*Artemisia vulgaris*), SwissProt accession no. AOPJ17; Bra v 1/2 from rape (*Brassica napus*), SwissProt accession no. P69196/P69198B; Bra v 1/2 from turnip (*Brassica rapa*), SwissProt accession no. P69196/P69198B; Cyn d 7 from bermuda grass (21:21 DE3) transformed with the pET11b/Che a 3 construct were diluted 10-fold with Luria-Bertani medium containing 0.1 mg/ml ampicillin and grown to an OD~600~ of 1.0. After induction with 0.4 mM isopropyl-β-D-thiogalactoside cultures were maintained for 4 h at 37°C and harvested by centrifugation. The pellet was resuspended in 20 mM ammonium bicarbonate (pH 8.0) and 1 mM PMSF, and lysed by three cycles of freezing and thawing (4°C). After centrifugation at 10,000 x g at 4°C, the soluble fraction was lyophilized, dissolved in 0.2 M ammonium bicarbonate (pH 8.0), and protein-containing fractions were pooled and concentrated.

**Crystallography, data collection, and processing**

rChe a 3 crystals were grown at 20°C by sitting drop vapor diffusion. Drops consisted of 3.0 μl of 6.0 mg/ml rChe a 3 in MilliQ water mixed with 0.7 μl of the reservoir (0.2 M ammonium sulfate, 22% polyethylene glycol 4000, 0,1 M alanine/HCl, adjusted to pH 3.4). rChe a 3 was crystallized in space group P2_12_2 with unit cell dimensions of a = 72.2 Å, b = 73.9 Å, and c = 68.9 Å and an estimated solvent content of 49.8% (V_M = 2.45 Å³/Da for four monomers in the asymmetric unit). Crystals were cryoprotected by short soaks in a solution consisting of 10% polyethylene glycol 200 and 90% reservoir and flash frozen in liquid nitrogen. A data set to 1.75 Å was collected at beamline ID14-4 (European Synchrotron Radiation Facility, Grenoble, France) and reduced and scaled with HKL2000 (21) (Table I).

**Phasing and refinement**

The structure of rChe a 3 was solved by molecular replacement using the coordinates of its homologue Phl p 7 (Protein Data Bank accession no. 1K9U) (14) as a template. Clear molecular replacement solutions were found for two dimers with BEAST (22) (as implemented into the CCP4i suite; Ref. 23). The molecular replacement model was subjected to rigid body refinement and restrained all atom refinement using REFMAC5 (24). The σ_A-weighted electron density maps as visualized with O (25) clearly indicated the amino acids exchanged between Che a 3 and Phl p 7: A11D, S19N, S23A, I27V, R49Q, S64D, D66N, T69I, D70S, and V79M. The Che a 3 amino acid sequence was implemented and the model was refined first with simulated annealing and restrained all atom refinement using REFMAC5 (24).

### Table I. Data collection and refinement statistics

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<th>Data Collection/Refinement</th>
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<td>Wilson B</td>
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</table>

**a** Highest resolution shell is shown in parenthesis.

**b** R_cryst = Σ(|F| - |<F>|)/Σ|F|, where |<F>| is the intensity of an individual measurement and |F| is the corresponding mean value.

**c** R_free = Σ|F_o| - |<F_o>|)/|Σ|F_o|, where |F_o| is the observed and |<F_o>| the calculated structure factor amplitude.

**d** R_total is the same as R_cryst calculated with a randomly selected test set of 5.0% of all reflections that was never used in the refinement calculations.
dimers (monomer A,B: residues 6–86; monomer C: residues 2–86; mono-
mer D: residues 5–86), eight calcium ions, four sulfate molecules, and 292
water molecules (eight of those providing calcium-coordination) (Table I).
The quality of the structure was ascertained with PROCHECK (28),
WHATCHECK (29), and MOLPROBITY (30). Of the residues, 92.3% are
in the core regions, 7.7% in the additional allowed regions, and no residues
are in the generously allowed or disallowed regions. The atomic coordi-
nates and structure factors were deposited in the Protein Data Bank with
accession no. 2OPO.

Surface analysis and comparison of the three-dimensional
structures of rChe a 3, rPhl p 7, and rBet v 4

The relative surface accessibility of each residue (with the exception of the residues that were disordered in the crystal structure, namely 2–5 of rChe a 3 monomer A,B,D and residues 2 of rPhl p 7 monomer A,B) in the three-dimensional structures of the rChe a 3 dimer (Protein Data Bank accession no. 2OP0), the rPhl p 7 dimer (Protein Data Bank accession no. 1KUJ) dimer, and the rBet v 4 monomer (Protein Data Bank accession no. 1H4B) was calculated with GETAREA 1.1 (31). Residues with a relative surface accessibility >50% were considered to be solvent exposed and are indicated for each allergen monomer as spheres in the alignment (Fig. 4).

For the surface accessibility histograms (Fig. 5), the GETAREA 1.1 analysis data were transferred into MS EXCEL. For each allergen, the percent-
ages of surface exposure of the individual monomers were averaged on a residue basis and graphically displayed. For rChe a 3 (with two
in the asymmetric unit) this means that each bar displays the av-
eraged surface exposure of the individual monomers. In case of rPhl p 7, each bar is the averaged surface exposure of a particular residue in the two monomers, whereas for the monomeric rBet v 4 no averaging was applied. A residue was considered to be part of a putative cross-reactive epitope based on two criteria: 1) its averaged surface exposure has to be >50%; and 2) the residue must be at least 90% conserved
or 100% homologous (Glu/Asp, Gln/Asn, Arg/Lys, Ser/Thr, Ile/Leu/Val,
and Tyr/Phe) within 19 presently known polcalcin primary sequences, be-
cause until the structural information of Ab complexes with different mem-
bers of the same cross-reactive allergen family becomes available, there is
no evidence that cross-reactivity between homologous allergens must be
restricted to the same side chain interactions and thus require complete
conservation. Conservation/homology was highlighted in different col-
ors in the alignment, and putative cross-reactive residues were colored
accordingly in the three-dimensional surface representations (Fig. 6).

Graphical representations of the molecular structures were produced with PyMOL (32).

Abs, sera, and immunological assays

Sera were obtained from Austrian and Spanish patients with polysensi-
tization to pollens from various unrelated plants (e.g., trees, grasses, and
weeds) and tested for the presence of IgE Abs specific for two EF-hand
calcium-binding allergens (16, 33). Rabbit Abs and the mouse mAb raised
against the two EF-hand allergen from olive pollen, Ole e 3, have been
described elsewhere (17). rChe a 3 was analyzed by SDS-PAGE according
to Laemmli (34) in 15% polyacrylamide gels. Proteins were visualized by
Coomassie brilliant blue staining or electrophoretically transferred onto
nitrocellulose membranes (Amersham Biosciences) for immunodetection
as described previously (35). A pool of five sera from Spanish Che a 3-all-
ergic patients (diluted 1/10) or a rabbit polyclonal antiserum raised against
rOle e 3 (diluted 1/5000) were used as primary Abs. A mouse monoclonal
anti-human IgE Ab (1/5000 diluted) and an HRP-labeled goat anti-mouse
IgG (1/2500 diluted) or an HRP-labeled goat anti-rabbit IgG (1/3000 di-
luted) were used as secondary Abs. The blots were developed using the
ECL Western blotting reagent (Amersham Biosciences).

ELISA inhibition assays were performed as previously described (11, 36). Sera from patients sensitized to calcium-binding pollen allergens were
diluted 1/10 in PBS, 0.05% (v/v) Tween 20, and 0.5% (w/v) BSA and
incubated with either rPhl p 7, rChe a 3, or BSA (1 μg of protein per
milliliter of serum dilution) overnight at 4°C. ELISA plates (Nunc Maxi-
sorp) were coated with 5 μg/ml rPhl p 7 or 5 μg/ml rChe a 3 overnight at

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Expression, purification, and molecular characterization of rChe a 3. A, Coomassie blue-stained SDS/PAGE. Protein extracts from E. coli were obtained after 0, 30, 60, 120, and 240 min of induction; lanes S and I represent the soluble and insoluble fraction, respectively. M, Molecular mass marker; the arrow indicates the position of rChe a 3. B, Mass spectrometry analysis of purified rChe a 3. The ratio of mass/charge (m/z) is shown on the x-axis and the signal intensity on the y-axis. C, Immunoreactivity of purified rChe a 3. The lanes show purified rChe a 3 (Coomassie blue), and rChe a 3 exposed to various probes (pAb, rabbit polyclonal anti-Ole e 3 Ab serum; mAb, monoclonal anti-Ole e 3 Ab; Sera, sera pool from chenopod pollen allergic patients).
4°C. After blocking the plates with PBS, 0.05% (v/v) Tween 20, and 1% (w/v) BSA, plates were incubated with serum dilutions preadsorbed overnight at 4°C. Plates were washed and bound IgE Abs were detected with an alkaline phosphatase-labeled anti-human IgE Ab (BD Pharmingen) diluted 1/1000 in PBS, 0.05% (v/v) Tween 20, and 0.5% (w/v) BSA. OD values (means of duplicates with a SD of 10%) correspond to the amount of bound IgE Abs. The percentage inhibition of IgE binding was calculated as follows: percentage inhibition = 100 × ([A - B]/A); A represents the OD values obtained after incubation of serum with BSA, and B represents the OD values after incubation of serum with rPhlp7 or rChe a 3, respectively.

Results

Expression, purification, and characterization of recombinant Che a 3

Recombinant Che a 3 (rChe a 3) was expressed as soluble protein in E. coli (Fig. 1A) and purified by gel filtration and ion-exchange chromatography yielding ~50 mg of homogenous protein per liter of cell culture. In MALDI-TOF mass spectrometry, rChe a 3 displays two peaks, a major one at 9398 Da (Fig. 1B) corresponding to the monomer and a minor one at 18830 Da corresponding to the dimer (data not shown). Edman degradation of rChe a 3 rendered NH₂-AEDT, indicating processing of the N-terminal methionine. Immunoblotting of purified rChe a 3 with Ole e 3-specific rabbit IgG antiserum and Ole e 3-specific mAb, as well as with sera from allergic patients, was performed to study the immunological properties of the recombinant protein. rChe a 3 was recognized by both IgG and IgE Abs after SDS-PAGE and transfer to membranes (Fig. 1C), demonstrating the integrity of the recombinant protein. Circular dichroism investigations of rChe a 3 yielded spectra of a well-folded α-helical protein illustrated by minima at 222 nm and 208 nm and a maximum at 192 nm (data not shown). Fitting of the circular dichroism spectra resulted in a secondary structure estimation of 57% α-helix, 5% β-strand, and 39% undefined secondary structure.

Extensive cross-reactivity of recombinant Che a 3 with other two EF-hand pollen allergens

The cross-reactivity between Che a 3 and Phl p 7 was studied by cross-wise IgE competition experiments using sera from five Phl p 7-allergic patients (Table II). Preincubation of sera with rChe a 3 inhibited IgE binding to rPhlp7 between 27 and 73% (mean inhibition 50%), and IgE reactivity to rChe a 3 was inhibited by rPhlp7 between 69 and 87% (mean inhibition 76%). Similar results were obtained with a serum pool from Spanish Che a 3-allergic patients. Cross-reactivity was also found when a mAb and

FIGURE 2. Inhibition studies of IgG and IgE binding to rChe a 3. ELISA inhibition experiments were conducted with rPhlp7 as the inhibitor for the binding to rChe a 3-coated wells using a pool of sera from patients allergic to Chenopod pollen (A), specific polyclonal rabbit antiserum raised against Ole e 3 (B), or a specific mAb obtained against rOle e 3 (C). The data were obtained in comparison with the inhibition by rChe a 3 as positive control. rOle e 1 allergen from olive pollen and BSA were used as nonreactive proteins.

FIGURE 3. Three-dimensional structures of rChe a 3, rPhlp7, and rBet v 4. A, Schematic representation of the crystal structure of rChe a 3 (dimer AB). Shown are EF-hand I (blue), EF-hand II (green), and the C-terminal α5 helix (red), with dark colors for monomer A and lighter colors for monomer B. N and C termini as well as the helices and short β-strands are labeled. The calcium ions are represented as spheres. B, Wall-eyed stereo representation of the superimposed structures of the rChe a 3 dimer (red, monomer A; light red, monomer B), the rPhlp7 dimer (blue, monomer A; light blue, monomer B), and the rBet v 4 monomer (green). The calcium ions are shown in the same colors as the corresponding molecules, and the N and C termini of the monomers are indicated.
polyclonal Abs specific for the two EF-hand allergen from olive, Ole e 3, were tested.

Quantitative inhibition experiments were performed with the serum pool, polyclonal anti-Ole e 3 Abs, and the monoclonal anti-Ole e 3 Ab with increasing concentrations of rChe a 3, rPhl p 7, and unrelated control proteins (rOle e 1, BSA). A strong inhibition (\(>60\%\)) of IgE reactivity to rChe a 3 was obtained already at low concentrations (\(<10^{-2} \mu g/ml\)) of rPhl p 7 and rChe a 3, but not with the control proteins (Fig. 2A). rChe a 3 and, to a lower degree, rPhl p 7 inhibited the reactivity of both the polyclonal Ole e 3 Abs and the monoclonal anti-Ole e 3 Ab to rChe a 3 (Fig. 2, B and C, and Table II).

Similarity of the crystal structure of rChe a 3 with the structures of rPhl p 7 and rBet v 4

The three-dimensional structure of rChe a 3 was solved by x-ray crystallography at 1.75-Å resolution from crystals obtained under similar conditions as those described for rPhl p 7 (14). Diffraction data were collected from native crystals at a synchrotron radiation source. Che a 3 and Phl p 7 exhibit 66.3\% sequence identity; thus, the structure of rChe a 3 was readily solved by molecular replacement using dimeric rPhl p 7 (Protein Data Bank accession no. 1K9U) as a template. Two rChe a 3 dimers (AB and CD) were located in the asymmetric unit. Apart from different degrees of disorder in the N termini, the structures of the two rChe a 3 dimers are essentially identical and the polypeptide backbones superimpose with a root mean square deviation of 0.75 Å. Therefore, until otherwise noted, only the structure of the AB dimer will be discussed further on.

The overall structure of rChe a 3 resembles a barrel-like shape with approximate dimensions of 45 \(\times\) 43 \(\times\) 44 Å and a total molecular surface area of 8,186 Å\(^2\) (AB dimer) and 8,507 Å\(^2\) (CD dimer) (37). Each rChe a 3 monomer consists of five \(\alpha\)-helices \((1-5)\), two short \(\beta\)-strands \((1, 2)\), and two calcium-coordinating loops (Figs 3A and 4). Helices \(\alpha 1\) and \(\alpha 2\) compose the N-terminal EF-hand I, and \(\alpha 3\) and \(\alpha 4\) form the C-terminal EF-hand II (Fig. 4). Two rChe a 3 monomers form a highly symmetric interdigitating dimer (Fig. 3A) and bury an extensive molecular surface area of some 4,400 Å\(^2\) (4,349 Å\(^2\) in the AB dimer, 4,441 Å\(^2\) in the CD dimer) (37). The EF-hand I of one monomer pairs with the EF-hand II of the other monomer and vice versa. A pair of EF-hand motifs constitutes the upper and the lower part of the barrel-like assembly. The calcium-binding loops are located at the very top and bottom, and the C-terminal \(\alpha 5\) helix is at the very bottom.
helices compose the middle part. The N-terminal α1 helices protrude from the molecular surface into the solvent and are disordered (except for monomer C, where the N terminus was ordered due to crystal packing interactions). The N and C termini as well as the hinge regions connecting the EF-hand motifs display increased flexibility as indicated by higher than average atomic B-factors as well as by minor structural differences between the four rChe a 3 monomers in the asymmetric unit. Similar observations were also made for the nuclear magnetic resonance structure of rBet v 4 (15) and the X-ray structure of rPhl p 7 (14).

Despite their different oligomerization states, dimeric rChe a 3 and rPhl p 7 and monomeric rBet v 4 have highly similar three-dimensional structures: the root mean square deviation for the superposition of the polypeptide backbones of the three molecules is some 1.3–1.4 Å (1.3 Å for rChe a 3 and rPhl p 7, 1.4 Å for rChe a 3 and rBet v 4, and 1.4 Å for rPhl p 7 and rBet v 4) (Fig. 3B).

**FIGURE 5.** Surface exposure analysis of rChe a 3, rPhl p 7, and rBet v 4 residues. The relative surface accessibility of individual amino acids was calculated for each monomer of each allergen using GETAREA 1.1 (31). Residue-by-residue, for each protein the percentages of surface exposure were averaged from the monomers and plotted against the residue number for Che a 3 (red), Phl p 7 (blue), and Bet v 4 (green). The black bar indicates the 50% cut-off line, above which residues were considered solvent exposed. The numbering on the x-axis corresponds to Che a 3 residue numbers as shown in the alignment in Fig. 4.

**FIGURE 6.** Location of putative cross-reactive residues on the molecular surface of rChe a 3. The rChe a 3 monomers are distinguished by lighter (monomer A) and darker colors (monomer B). Putative cross-reactive residues are indicated (orange, 90% conservation; green, homologous exchanges) analogously to Fig. 4 and labeled in white for monomer A and in black for monomer B. A, Hinge (linker) region. B, 90° rotation along the vertical axis. C, The kink region (180° vertical rotation). D, 70° vertical rotation. E, The upper calcium-binding site. F, The lower calcium-binding site.
Che a 3 and Bet v 4 have N termini that are, respectively, eight and seven residues longer than that of Phl p 7. In the rChe a 3 structure, the first five residues are either partly disordered or adopt undefined secondary structures. Because the very N-terminal residues have the highest sequence variability among polcalcins (Fig. 4), they are a likely location for protein-specific epitopes. The C-terminal α5 helices adopt slightly different conformations in rChe a 3, rPhl p 7, and rBet v 4 (Fig. 3B).

Like rPhl p 7, the rChe a 3 dimer also exhibits a hydrophobic cavity in its interior. The accessible volume (1.4-Å probe radius) of the rChe a 3 cavity is 183 Å³ and 205 Å³ for dimer AB and dimer CD, respectively. These values compare well with the 178 Å³ cavity volume of rPhl p 7. Even though the dimensions and the hydrophobic nature of the rChe a 3 cavity are comparable to that of rPhl p 7, no electron density hinting at the presence of a bound ligand as seen for rPhl p 7 (14) could be observed for rChe a 3. In contrast to the enclosed cavity of rPhl p 7, the rChe a 3 cavity is solvent-accessible via two tunnels with diameters of ~3 Å. Instead of a cavity, monomeric rBet v 4 has a hydrophobic groove that is only partly covered by the packing of the hydrophobic face of the α5-helix, thus leaving a major part of the groove solvent exposed (15). These findings demonstrate that polcalcins exhibit significant structural plasticity that might be essential for binding of a hydrophobic ligand.

Sequence and surface analysis to identify putative cross-reactive amino acid residues

The molecular surfaces of rChe a 3, rPhl p 7, and rBet v 4 were analyzed for highly conserved, solvent-accessible residues that can compose the cross-reactive epitopes. Sequence conservation was evaluated for the 19 known two EF-hand pollen allergen sequences (Fig. 4). Solvent exposure was calculated for each residue in the structures of rChe a 3, rPhl p 7, and rBet v 4, which represent polcalcins from unrelated plant species (weeds, grasses, and trees, respectively), and is displayed in surface accessibility histograms (Fig. 5). To identify putative cross-reactive amino acids, the following criteria were applied: 1) the residues must either be 90% conserved or entirely homologous for all 19 of the polcalcin sequences known to date; and 2) 50% of the total surface of the residue must be solvent accessible. In this way, twelve putative cross-reactive amino acids were identified and mapped onto the surface of rChe a 3: Arg15, Lys18, Asn23, Gly24, Asp35, Ser42, Thr44, Asp58, Asp59, Gly60, Ala74, and Lys80 (Fig. 6). Arg15/Lys18, Asn23/Gly24, Asp35/Ser42, Thr44, Asp58, Gly59, Ala74, and Lys80 (Fig. 6). Arg15/Lys18/Asn23/Gly24, Ser42/Thr44/Asp46, and Asp58/Gly59 form three clusters, which, due to the dimeric nature of rChe a 3, are present twice on the molecular surface. Asp58 may contribute to either of the former two clusters due to its close proximity to each of them. Another cluster is formed by Lys80 and Ala74 (located at the kink region between the α4 and α5 helix) from both monomers (Fig. 6C).

Discussion

Polcalcins, a family of two EF-hand calcium binding proteins, constitute a group of highly cross-reactive allergens in the pollen of various plants. To date, 15 members of this allergen family have been identified in grasses, trees, bushes, weeds, and other flowering plants. Two EF-hand allergens share a high degree of sequence homology, which may explain the extensive cross-reactivity of allergic patients’ IgE Abs with the various members of the family.

It has been demonstrated that the IgE recognition of polcalcins critically depends on the presence of protein-bound calcium; the use of calcium-chelating agents or the mutation of the calcium-coordinating residues affects the structural integrity of EF-hand allergens, and it has been assumed that polcalcin IgE epitopes belong to the conformational type. The latter hypothesis is supported by data showing that two polypeptides comprising residues 2–37 and 37–78 of the timothy grass polcalcin, Phl p 7, lose their structural fold and IgE reactivity (39). This finding is in agreement with data obtained for many other important respiratory allergens that contain primarily conformational IgE epitopes (40). Disruption of the structure of these allergens strongly reduces their IgE reactivity and is currently used as strategy for the construction of hypoallergenic allergen derivatives useful for allergy vaccination (41).

In contrast to respiratory allergens that apparently sensitize allergic patients as intact, structurally folded proteins via the respiratory mucosa, many food allergens such as milk and egg allergens become digested in the gastrointestinal tract and may sensitize in the form of unfolded polypeptides (42, 43). IgE Abs of food allergic patients are therefore frequently directed against continuous and sequential epitopes also. Continuous epitopes can be mapped with overlapping peptides synthesized according to the primary Ag sequence or with recombinant Ag fragments. In contrast, the determination of conformational epitopes represents a difficult if not impossible task, especially for polyclonal Abs that occur in immunologically mediated diseases (e.g., allergy, autoimmunity, infectious diseases, cancer etc.) and when sufficient homogenous amounts of Abs are not available.

We have solved the three-dimensional structure of the cross-reactive two EF-hand weed allergen rChe a 3 and compared it to the structures of its timothy grass and birch pollen homologues, Phl p 7 and Bet v 4, respectively. All three allergens assume essentially identical structural folds despite the observation that rPhl p 7 and rChe a 3 form dimers while rBet v 4 occurs as monomer (which may be explained by different purification protocols and structure determination techniques). It is likely that the extensive cross-reactivity of polyclonal IgE Abs from allergic patients with several polcalcins might thus be due to the significant structural similarity of two EF-hand allergens. This hypothesis is also supported by the fact that, while their structures are highly similar, the sequence identity between Che a 3 and Phl p 7 is only 66.3% (with an additional 5.8% homology), a level at which the IgE cross-reactivity of other homologous allergens is usually found to diminish considerably.

To obtain more information about the amino acids that may be involved in IgE cross-reactivity of polcalcins, we used multiple sequence alignments to identify conserved amino acids in 19 two EF-hand allergen sequences. These amino acids were then analyzed regarding their surface exposure in the three-dimensional structures of rChe a 3, rPhl p 7, and rBet v 4. This approach allowed us to pinpoint 12 amino acids that are arranged into four clusters of putative cross-reactive epitopes on the surface of the allergens. All but one residue in the clusters on the surface of the monomeric polcalcins are hydrophilic and six of them carry a carboxylate corresponding to Asn23 and mutation of the two residues immediately following Gly24 as well as Gly59 reduced the IgE reactivity of Bra r 1 (45).

Two structures of allergens in complex with mAbs have been determined to date: a Bet v 1-IgG Fab complex (46) and a hyaluronidase-IgG Fab complex (47). In both cases the Abs are binding to surface exposed loops of the allergens. The Bet v 1 epitope for the BV16 Fab consists of mostly hydrophilic and charged residues (46). The hyaluronidase-Fab complex interface (47) is characterized by mainly hydrophilic interactions (four salt bridges and nine...
References

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Disclosures

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References


