

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 Allergens¹

Petra Verdino,* Rodrigo Barderas,^{2†} Mayte Villalba,[†] Kerstin Westritschnig,[§] Rudolf Valenta,^{§‡} Rosalia Rodriguez,[†] and Walter Keller^{3*}

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.

Allergies 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

*Institute of Chemistry, Structural Biology, University of Graz, Graz, Austria; [†]Department of Biochemistry and Molecular Biology, Faculty of Chemistry, Complutense University, Madrid, Spain; and [‡]Christian Doppler Laboratory for Allergy Research and [§]Division of Immunopathology, Department of Pathophysiology, Medical University of Vienna, Vienna, Austria

Received for publication September 20, 2007. Accepted for publication December 2, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Grants F1805 and F1815 of the Austrian Science Fund, by the Christian Doppler Research Association, and by Grants SAF02-02711 and SAF05-01847 from the Ministerio de Educación y Ciencia, Spain.

² Current address: Protein Technology Unit, Biotechnology Program, Spanish National Cancer Center, Centro Nacional de Investigaciones Oncológicas, 28029 Madrid, Spain.

³ Address correspondence and reprint requests to Dr. Walter Keller, Institute of Chemistry, Structural Biology, University of Graz, Heinrichstrasse 28, A8010 Graz, Austria. E-mail address: walter.keller@uni-graz.at

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 *Nde*I/*Eco*R1 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 nm} of 1.0. After induction with 0.4 mM isopropyl- β -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 (45°C). After centrifugation at 10,000 $\times$ g at 4°C, the soluble fraction was lyophilized, 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 polcalcin from olive pollen, Ole e 3. The rChe a 3 fractions were lyophilized, 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-ml 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 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. Q2KN27; Aln g 4 from alder (*Alnus glutinosa*), SwissProt accession no. O81701; Ara t 1/2 from mouse-ear cress (*Arabidopsis thaliana*), SwissProt accession no. Q9SRP7/Q9LF54; Art v 5 from mugwort (*Artemisia vulgaris*), SwissProt accession no. A0PJ17; Bra n 1/2 from rape (*Brassica napus*), SwissProt accession no. P69196/P69198; Bra r 1/2 from turnip (*Brassica rapa*), SwissProt accession no. P69197/P69199; Cyn d 7 from bermuda grass (*Cynodon dactylon*), SwissProt accession no. P94092; Nic t 1/2 from common tobacco (*Nicotiana tabacum*), SwissProt accession no. Q8VWY6/Q8VWY7; Ole e 3 from common olive (*Olea europaea*), SwissProt accession no. O81092; Ory s 7 from rice (*Oryza sativa* subsp. *japonica*), SwissProt accession no. Q6YYX3; Syr v 3 from common lilac (*Syringa vulgaris*), SwissProt accession no. P58171; and polcalcin from grape (*Vitis vinifera*), SwissProt accession no. A7PW14.

Mass spectrometry analysis

Protein samples (1 μ g) were mixed with a matrix of saturated α -cyano-4-hydroxycinnamic acid in 30% aqueous acetonitrile and 0.1% trifluoroacetic acid. Measurements were performed using a Bruker Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytik) equipped with an ion source with visualization optics and a nitrogen laser (337 nm). The equipment was externally calibrated using singly, doubly, and triply charged signals from either cytochrome *c* (12,360 Da) or BSA (66,430 Da).

Circular dichroism analysis

Circular dichroism investigations of rChe a 3 were performed at a protein concentration of 1.6×10^{-5} M (determined with the BCA protein assay reagent; Pierce). The measurements were conducted on a Jasco J-715 spectropolarimeter using a 0.1-cm path length cell equilibrated at 20°C. Spectra were recorded for a wavelength range of 180–260 nm with 1-nm resolution at a scan speed of 50 nm/min and resulted from averaging three acquisitions. The final spectra were baseline-corrected by subtracting the corresponding solvent spectra obtained under identical conditions. Results were expressed as the mean residue ellipticity (Θ) at a given wavelength. The data were fitted using the secondary structure estimation program DicroProt (19) and the least-square fitting algorithm according to Fasman (20).

Table I. Data collection and refinement statistics

Data Collection/Refinement	
Data Collection	
Space group	P2 ₁ 2 ₁ 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	72.2, 73.9, 68.9
α , β , γ (°)	90.0, 90.0, 90.0
Wavelength (Å)	0.93930
Resolution (Å)	30.0–1.75 (1.81–1.75) ^a
<i>R</i> _{sym} (%) ^b	5.7 (28.3)
$\langle I/\sigma(I) \rangle$	18.1 (3.2)
Completeness (%)	97.9 (84.5)
Measured reflections	287,800
Unique reflections	37,132
Redundancy	7.8
Wilson B	25
Refinement	
Resolution (Å)	30–1.75
No. reflections	33,503
<i>R</i> _{cryst} / <i>R</i> _{free} (%) ^d	19.8/24.7
No. atoms	
Protein	2752
Calcium	8
Water	292
Sulfate	20
B-values (Å ²)	
Protein	30.8
Calcium	19.7
Water	36.8
Sulfate	54.9
Rmsd bonds (Å)	0.018
Rmsd angles (°)	1.6

^a Highest resolution shell is shown in parenthesis.

^b $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where *I* is the intensity of an individual measurement and $\langle I \rangle$ is the corresponding mean value.

^c $R_{\text{cryst}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where $|F_o|$ is the observed and $|F_c|$ the calculated structure factor amplitude.

^d *R*_{free} 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.

Crystallization, diffraction data collection, and processing

rChe a 3 crystals were grown at 20°C by sitting drop vapor diffusion. Drops consisted of 3.0 μ l of 6.0 mg/ml rChe a 3 in MilliQ water mixed with 0.7 μ l 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 P2₁2₁2 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, S30L, G34T, K38R, V43T, T44S, P45A, R49Q, S64D, D66N, T69I, D70S, A72C, R73N, R76P, V79M, S83A, and I85V. The Che a 3 amino acid sequence was implemented and the model was refined first with simulated annealing using the Crystallography & NMR System (CNS) (26) and further with alternating cycles of model building with COOT (27) and refinement with REFMAC5. Chain tracing in the more flexible regions of rChe a 3, namely the N and C termini as well as in the hinge region that connects the two EF-hand motifs, was assisted by simulated annealing omit maps. The final model (*R*_{cryst} = 19.8%; *R*_{free} = 24.7%) includes two rChe a 3

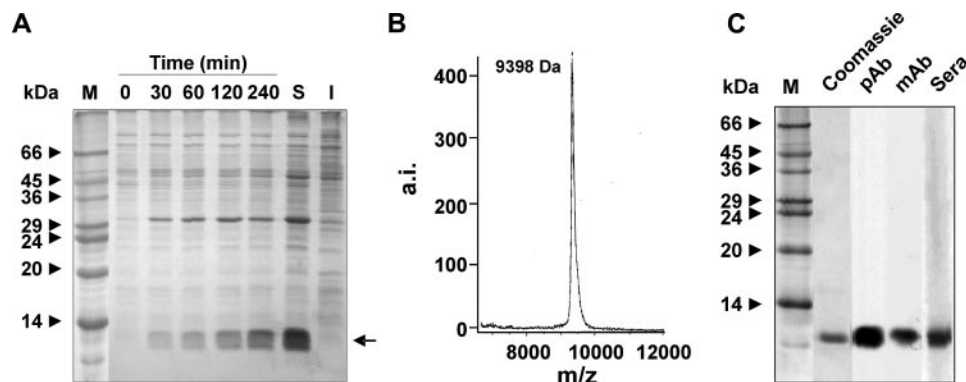


FIGURE 1. Expression, purification, and molecular characterization of rChe a 3. *A*, Coomassie blue-stained SDS/PAGE. Protein extracts from *E. coli* were obtained after 0, 30, 60, 120, and 240 min of induction; lanes *S* and *I* represent the soluble and insoluble fraction, respectively. *M*, Molecular 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).

dimers (monomer A,B: residues 6–86; monomer C: residues 2–86; monomer 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 coordinates 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. 2OPO), the rPhl p 7 dimer (Protein Data Bank accession no. 1K9U) 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 percentages of surface exposure of the individual monomers were averaged on a residue-by-residue basis and graphically displayed. For rChe a 3 (with two dimers in the asymmetric unit) this means that each bar displays the averaged surface exposure of one residue in the four different 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, because until the structural information of Ab complexes with different members 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 colors 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 polysensitization 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-allergic 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 diluted) 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 μ g of protein per milliliter of serum dilution) overnight at 4°C. ELISA plates (Nunc Maxi-sorp) were coated with 5 μ g/ml rPhl p 7 or 5 μ g/ml rChe a 3 overnight at

Table II. ELISA cross-inhibition of calcium-binding pollen allergen-sensitized patients' IgE binding to rPhl p 7 and rChe a 3^a

Patient	OD			Percentage Inhibition of IgE Binding to rPhl p 7 by	
	BSA	rPhl p 7	rChe a 3	rPhl p 7	rChe a 3
1	0.32	0.09	0.13	72	59
2	0.22	0.07	0.12	68	46
3	0.44	0.08	0.12	82	73
4	0.20	0.08	0.11	60	45
5	0.22	0.08	0.16	64	27
Mean				69	50

Patient	OD			Percentage Inhibition of IgE Binding to rChe a 3 by	
	BSA	rPhl p 7	rChe a 3	rPhl p 7	rChe a 3
1	0.47	0.06	0.16	87	66
2	0.24	0.07	0.10	71	58
3	0.41	0.07	0.08	82	80
4	0.26	0.08	0.11	69	58
5	0.27	0.08	0.08	70	70
Mean				76	66

^a Sera from five patients were preadsorbed with BSA, rPhl p 7, and rChe a 3 and probed for IgE binding to ELISA plate-bound rPhl p 7 or rChe a 3. OD values corresponding to IgE antibodies bound to coated rPhl p 7 (*top portion* of Table) or rChe a 3 (*bottom portion* of Table) after preadsorption are shown. Percentages of the inhibition of IgE binding are displayed and the mean inhibitions (in bold type) were calculated.

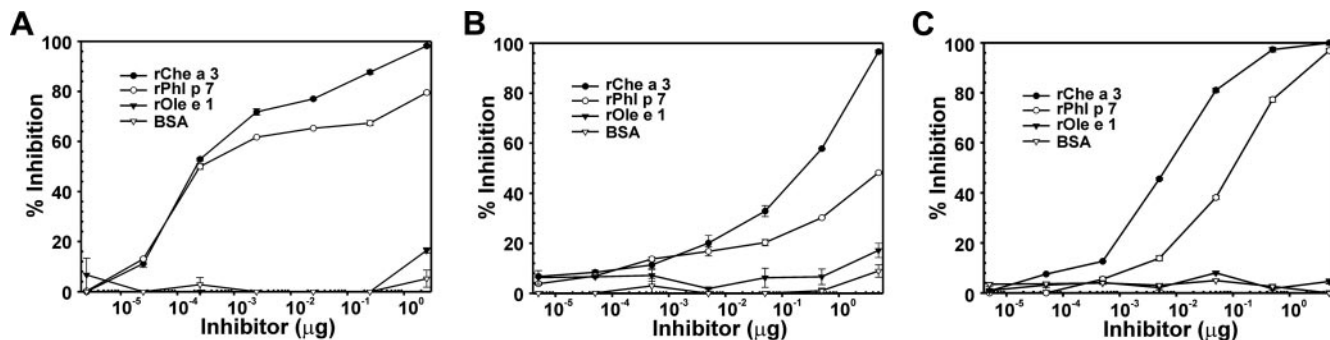


FIGURE 2. Inhibition studies of IgG and IgE binding to rChe a 3. ELISA inhibition experiments were conducted with rPhl p 7 as the inhibitor for the binding to rChe a 3-coated wells using a pool of sera from patients allergic to chenopod pollen (A), specific polyclonal rabbit antiserum raised against rOle 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.

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 = $100 \times [(A - B)/A]$; A represents the OD values obtained after incubation of serum with BSA, and B represents the OD values after incubation of serum with rPhl p 7 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-AAEDT, 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 rPhl p 7 between 27 and 73% (mean inhibition 50%), and IgE reactivity to rChe a 3 was inhibited by rPhl p 7 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

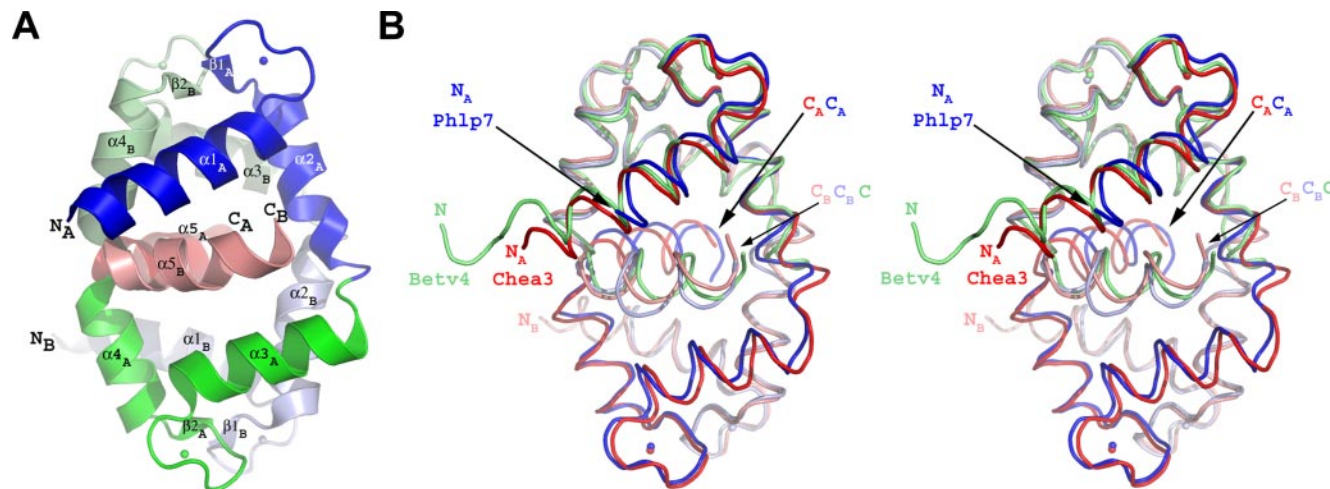


FIGURE 3. Three-dimensional structures of rChe a 3, rPhl p 7, and rBet v 4. A, Schematic representation of the crystal structure of rChe a 3 (dimer AB). Shown are EF-hand I (blue), EF-hand II (green), and the C-terminal $\alpha 5$ helix (red), with dark colors for monomer A and lighter colors for monomer 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 rPhl p 7 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.

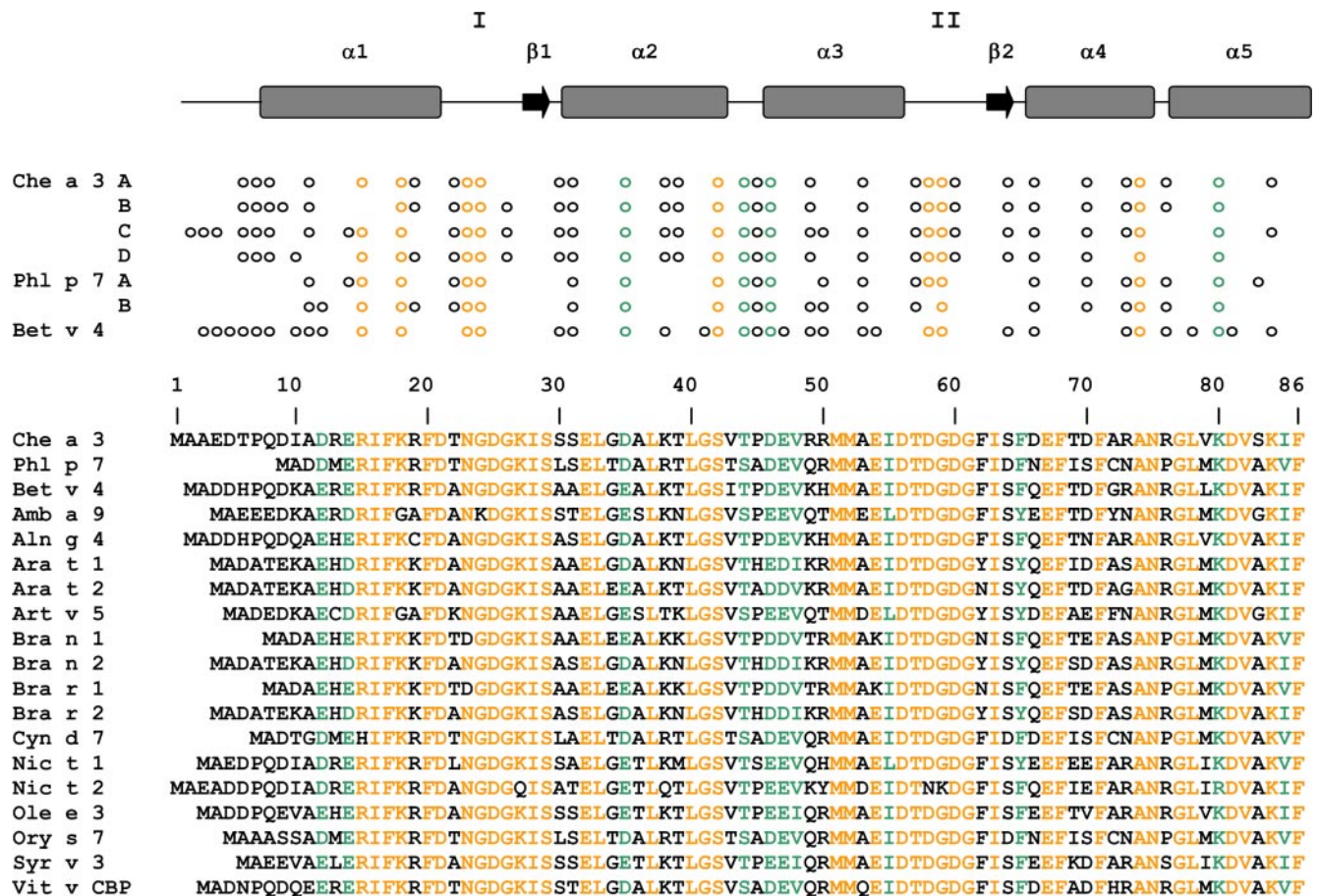


FIGURE 4. Structural comparison of Che a 3 with other polcalcins. Sequence alignment of the 19 presently known two EF-hand pollen allergen sequences (from the top: Che a 3, common lambs quarters; Phl p 7, timothy grass; Bet v 4, white birch; Amb a 9, ragweed; Aln g 4, alder; Ara t 1/2, mouse-ear cress; Art v 5, mugwort; Bra n 1/2, rape; Bra r 1/2, turnip; Cyn d 7, bermuda grass; Nic t 1/2, common tobacco; Ole e 3, common olive; Ory s 7, rice; Syr v 3, common lilac; and Vit v CBP, grape). Ninety percent conserved residues are orange; homologous exchanges (Glu/Asp, Gln/Asn, Arg/Lys, Ser/Thr, Ile/Leu/Val, Tyr/Phe) are green. Surface-exposed residues (i.e., amino acids that have a surface accessibility >50% in the three-dimensional structures) are shown above the alignment as spheres and color coded according their sequence conservation as in the alignment. The surface exposure of residues 2–5 of Che a 3 monomers A,B,D and residues 2 of Phl p 7 monomers A,B could not be determined because those residues were disordered in the crystal structures. The secondary structure plot is derived from the three-dimensional structure of rChe a 3. The helices α 1– α 4 of the N- and C-terminal EF-hands (I and II) and the C-terminal α 5-helix are represented by gray tubes and the short β -strands (β 1, β 2) by black arrows.

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 ($<10^{-2}$ μ 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).

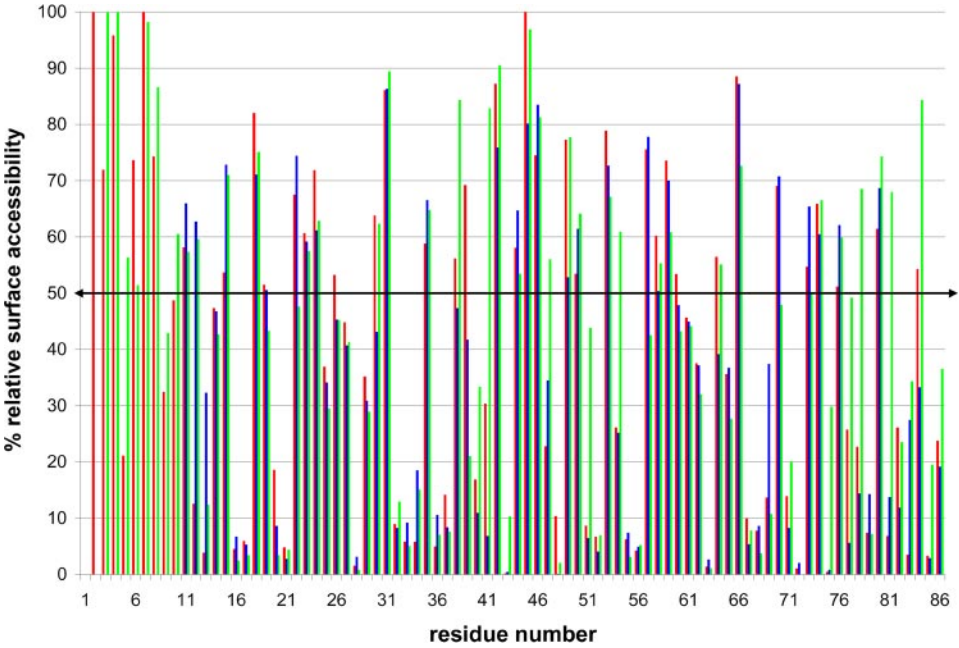
Similarity of the crystal structure of rChe a 3 with the structures of rPhl p 7 and rBet v 4

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 Å² (AB dimer) and 8507 Å² (CD dimer) (37). Like rPhl p 7 (14, 38) and rBet v 4 (15), each rChe a 3 monomer consists of five α -helices (α 1– α 5), two short β -strands (β 1, β 2), and two calcium-coordinating loops (Figs 3A and 4). Helices α 1 and α 2 compose the N-terminal EF-hand I, and α 3 and α 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 Å² (4349 Å² in the AB dimer, 4441 Å² 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 α 5

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.



helices compose the middle part. The N-terminal $\alpha 1$ 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).

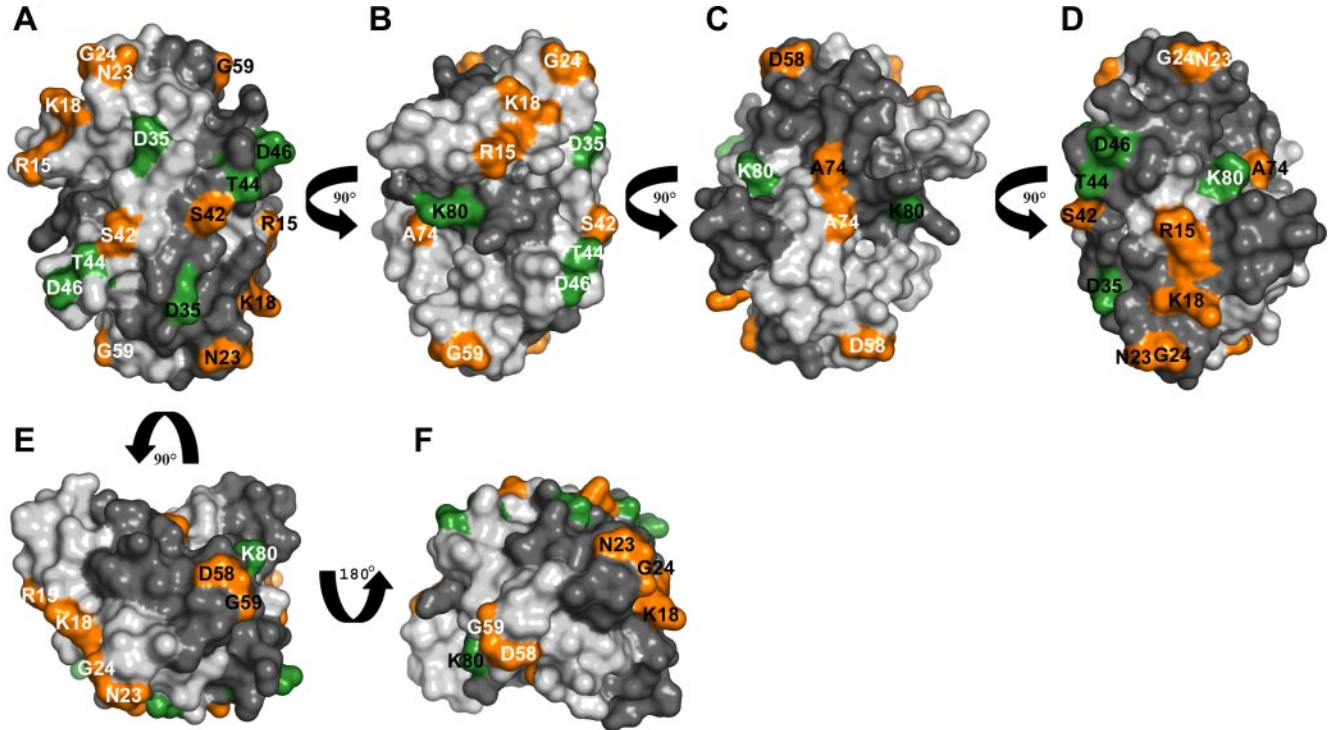


FIGURE 6. Location of putative cross-reactive residues on the molecular surface of rChe a 3. The rChe a 3 monomers are distinguished by lighter (monomer A) and darker colors (monomer B). Putative cross-reactive residues are indicated (orange, 90% conservation; green, homologous exchanges) analogously 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 $\alpha 5$ 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 ($1.4\text{-}\text{\AA}$ probe radius) of the rChe a 3 cavity is $183\text{ }\text{\AA}^3$ and $205\text{ }\text{\AA}^3$ for dimer AB and dimer CD, respectively. These values compare well with the $178\text{ }\text{\AA}^3$ 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 $\sim 3\text{ }\text{\AA}$. 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 $\alpha 5$ -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, Asp⁴⁶, 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. Asp³⁵ 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 $\alpha 4$ and $\alpha 5$ 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 charges. These clusters could be recognized by up to three different IgE Abs in the case of the monomeric polcalcin form and by four to five Abs in the case of the dimeric allergen forms that are sufficient for effective mast cell degranulation (44). Earlier mutational analysis of the highly homologous cross-reactive polcalcin from oil seed rape indeed supports our model. Mutation of the amino acid 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

H-bonds) that are surrounded by few hydrophobic contacts. Due to the lack of more high resolution allergen-Fab complex structures, it cannot be concluded that the recognition of allergen loops by Ab combining sites is a general rule for allergen epitopes, but all four patches of putative cross-reactive polcalcin residues identified in our analysis are positioned in surface-exposed loop regions. Because each of these patches consists of 2–4 hydrophilic residues they may be too small to comprise a complete Ab binding epitope, but it appears, at least according to the data of Okada (45), that they indeed contain amino acids involved in Ab reactivity. It also has to be mentioned that the mutation of other amino acid residues not predicted in our model had a dramatic influence on IgE recognition of two EF-hand allergens (39, 48). However, these mutations targeted residues in the interior of the intact allergen that most likely perturbed IgE recognition by inducing conformational changes of the allergen.

Polcalcins are the first example of a protein family where a correlation between immunological cross-reactivity and structure-derived information on conserved surface-exposed amino acids that contribute to conformational epitopes was established. We have performed our studies using a potent allergen recognized by polyclonal IgE-antibodies from allergic patients but believe that our approach may have general applicability. For this purpose, it will be important to test our idea by extensive mutagenesis studies and a parallel structural as well as a IgE-cross-reactivity analysis of the mutants. Moreover, it will be necessary to compare larger numbers of structures of homologous Ags paired with a detailed knowledge about Ab cross-reactivities. Based on this approach, it should be possible to obtain tools for the prediction of cross-reactive conformational epitopes on protein Ags.

Acknowledgments

We thank Christoph Kratky for supplying financial support and the infrastructure that made this work possible and Karl Gruber and Ulrike Wagner for helpful comments, discussions, and their commitment in data collection. We acknowledge the (ESRF) in Grenoble, France, for provision of the synchrotron radiation facilities, and we thank Dominique Bourgeois for assistance in using beamline ID14-4.

Disclosures

Rudolf Valenta is an allergy consultant for Phadia and Biomay and has also received research grants from both companies.

References

- Wuthrich, B., C. Schindler, T. C. Medici, J. P. Zellweger, and P. Leuenberger. 1996. IgE levels, atopy markers, and hay fever in relation to age, sex and smoking status in a normal adult Swiss population: SAPALDIA (Swiss Study on Air Pollution and Lung Diseases in Adults) Team. *Int. Arch. Allergy Immunol.* 111: 396–402.
- Kay, A. B. 2001. Allergy and allergic diseases: first of two parts. *N. Engl. J. Med.* 344: 30–37.
- Kay, A. B. 2001. Allergy and allergic diseases: second of two parts. *N. Engl. J. Med.* 344: 109–113.
- Midoro-Horiuti, T., E. G. Brooks, and R. M. Goldblum. 2001. Pathogenesis-related proteins of plants as allergens. *Ann. Allergy Asthma Immunol.* 87: 261–271.
- Markovic-Housley, Z., M. Degano, D. Lamba, E. von Roepenack-Lahaye, S. Clemens, M. Susani, F. Ferreira, O. Scheiner, and H. Breiteneder. 2003. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J. Mol. Biol.* 325: 123–133.
- Niederberger, V., G. Pauli, H. Gronlund, R. Froschl, H. Rumpold, D. Kraft, R. Valenta, and S. Spitzauer. 1998. Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. *J. Allergy Clin. Immunol.* 102: 579–591.
- Breiteneder, H., and C. Ebner. 2000. Molecular and biochemical classification of plant-derived food allergens. *J. Allergy Clin. Immunol.* 106: 27–36.
- Valenta, R., M. Duchene, K. Pettenburger, C. Sillaber, P. Valent, P. Bettelheim, M. Breitenbach, H. Rumpold, D. Kraft, and O. Scheiner. 1991. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253: 557–560.
- Scheurer, S., A. Wangorsch, J. Nerkamp, P. S. Skov, B. Ballmer-Weber, B. Wuthrich, D. Haustein, and S. Vieths. 2001. Cross-reactivity within the profilin panallergen family investigated by comparison of recombinant profilins from pear (Pyr c 4), cherry (Pru av 4), and celery (Api g 4) with birch pollen profilin Bet v 2. *J. Chromatogr. B. Biomed. Sci. Appl.* 756: 315–325.
- Valenta, R., B. Hayek, S. Seiberler, A. Bugajska-Schretter, V. Niederberger, A. Twardosz, S. Natter, L. Vangelista, A. Pastore, S. Spitzauer, and D. Kraft. 1998. Calcium-binding allergens: from plants to man. *Int. Arch. Allergy Immunol.* 117: 160–166.
- Tinghino, R., A. Twardosz, B. Barletta, E. M. Puggioni, P. Iacovacci, C. Butteroni, C. Afferni, A. Mari, B. Hayek, G. Di Felice, et al. 2002. Molecular, structural, and immunologic relationships between different families of recombinant calcium-binding pollen allergens. *J. Allergy Clin. Immunol.* 109: 314–320.
- Ledesma, A., R. Barderas, K. Westritschnig, J. Quiralte, C. Y. Pascual, R. Valenta, M. Villalba, and R. Rodriguez. 2006. A comparative analysis of the cross-reactivity in the polcalcin family including Syr v 3, a new member from lilac pollen. *Allergy* 61: 477–484.
- Ledesma, A., E. Gonzalez, C. Y. Pascual, J. Quiralte, M. Villalba, and R. Rodriguez. 2002. Are Ca²⁺-binding motifs involved in the immunoglobulin E-binding of allergens? Olive pollen allergens as model of study. *Clin. Exp. Allergy* 32: 1476–1483.
- Verdino, P., K. Westritschnig, R. Valenta, and W. Keller. 2002. The cross-reactive calcium-binding pollen allergen, Phl p 7, reveals a novel dimer assembly. *EMBO J.* 21: 5007–5016.
- Neudecker, P., J. Nerkamp, A. Eisenmann, A. Nourse, T. Lauber, K. Schweimer, K. Lehmann, S. Schwarzwinger, F. Ferreira, and P. Rosch. 2004. Solution structure, dynamics, and hydrodynamics of the calcium-bound cross-reactive birch pollen allergen Bet v 4 reveal a canonical monomeric two EF-hand assembly with a regulatory function. *J. Mol. Biol.* 336: 1141–1157.
- Barderas, R., M. Villalba, C. Y. Pascual, E. Batanero, and R. Rodriguez. 2004. Profilin (Che a 2) and polcalcin (Che a 3) are relevant allergens of *Chenopodium album* pollen: isolation, amino acid sequences, and immunologic properties. *J. Allergy Clin. Immunol.* 113: 1192–1198.
- Ledesma, A., M. Villalba, E. Batanero, and R. Rodriguez. 1998. Molecular cloning and expression of active Ole e 3, a major allergen from olive-tree pollen and member of a novel family of Ca²⁺-binding proteins (polcalcins) involved in allergy. *Eur. J. Biochem.* 258: 454–459.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
- Deléage, G., and C. Geourjon. 1993. An interactive graphic program for calculating the secondary structure content of proteins from circular dichroism spectrum. *Comput. Appl. Biosci.* 9: 197–199.
- Greenfield, N., and G. D. Fasman. 1969. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 8: 4108–4116.
- Otwinowski, Z., and W. Minor. 1997. Processing of x-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 276: 307–326.
- Read, R. J. 2001. Pushing the boundaries of molecular replacement with maximum likelihood. *Acta Crystallogr. D* 57: 1373–1382.
- Collaborative Computational Project, Number 4. 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D* 50: 760–763.
- Winn, M. D., M. N. Isupov, and G. N. Murshudov. 2001. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr. D* 57: 122–133.
- Jones, T. A., J. Y. Zou, S. W. Cowan, and M. Kjeldgaard. 1991. Improved methods for binding protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* 47: 110–119.
- Brünger, A. T., P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J. S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, et al. 1998. Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D* 54: 905–921.
- Emsley, P., and K. Cowtan. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D* 60: 2126–2132.
- Laskowski, R. A., M. W. MacArthur, D. S. Moss, and J. M. Thornton. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* 26: 283–291.
- Hoof, R. W., G. Vriend, C. Sander, and E. E. Abola. 1996. Errors in protein structures. *Nature* 381: 272.
- Lovell, S. C., I. W. Davis, W. B. Arendall, III, P. I. de Bakker, J. M. Word, M. G. Prisant, J. S. Richardson, and D. C. Richardson. 2003. Structure validation by α geometry: phi, psi and $C\beta$ deviation. *Proteins* 50: 437–450.
- Fraczkiewicz, R., and W. Braun. 1998. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J. Comput. Chem.* 19: 319–333.
- DeLano, W. L. 2002. *The PyMOL User's Manual*. DeLano Scientific, San Carlos, CA.
- Niederberger, V., B. Hayek, S. Vrtala, S. Laffer, A. Twardosz, L. Vangelista, W. R. Sperr, P. Valent, H. Rumpold, D. Kraft, et al. 1999. Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. *FASEB J.* 13: 843–856.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- Villalba, M., E. Batanero, R. I. Monsalve, M. A. Gonzalez de la Pena, C. Lahoz, and R. Rodriguez. 1994. Cloning and expression of Ole e I, the major allergen

- from olive tree pollen: polymorphism analysis and tissue specificity. *J. Biol. Chem.* 269: 15217–15222.
36. Barderas, R., M. Villalba, M. Lombardero, and R. Rodriguez. 2002. Identification and characterization of Che a 1 allergen from *Chenopodium album* pollen. *Int. Arch. Allergy Immunol.* 127: 47–54.
37. Connolly, M. L. 1993. The molecular surface package. *J. Mol. Graphics* 11: 139–141.
38. Verdino, P., K. Westritschnig, R. Valenta, and W. Keller. 2003. Three-dimensional structure of the panallergen Phl p 7. *Int. Arch. Allergy Immunol.* 130: 10–11.
39. Westritschnig, K., M. Focke, P. Verdino, W. Goessler, W. Keller, A. Twardosz, A. Mari, F. Horak, U. Wiedermann, A. Hartl, et al. 2004. Generation of an allergy vaccine by disruption of the three-dimensional structure of the cross-reactive calcium-binding allergen, Phl p 7. *J. Immunol.* 172: 5684–5692.
40. Valenta, R., and D. Kraft. 2001. Recombinant allergen molecules: tools to study effector cell activation. *Immunol. Rev.* 179: 119–127.
41. Valenta, R. 2002. The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* 2: 446–453.
42. Jarvinen, K. M., P. Chatchatee, L. Bardina, K. Beyer, and H. A. Sampson. 2001. IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* 126: 111–118.
43. Sampson, H. A. 2005. Food allergy: when mucosal immunity goes wrong. *J. Allergy Clin. Immunol.* 115: 139–141.
44. Gieras, A., M. Focke-Tejkl, T. Ball, P. Verdino, A. Hartl, J. Thalhamer, and R. Valenta. 2007. Molecular determinants of allergen-induced effector cell degranulation. *J. Allergy Clin. Immunol.* 119: 384–390.
45. Okada, T., I. Swoboda, P. L. Bhalla, K. Toriyama, and M. B. Singh. 1998. Engineering of hypoallergenic mutants of the *Brassica* pollen allergen, Bra r 1, for immunotherapy. *FEBS Lett.* 434: 255–260.
46. Mirza, O., A. Henriksen, H. Ipsen, J. N. Larsen, M. Wissenbach, M. D. Spangfort, and M. Gajhede. 2000. Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J. Immunol.* 165: 331–338.
47. Padavattan, S., T. Schirmer, M. Schmidt, C. Akdis, R. Valenta, I. Mittermann, L. Soldatova, J. Slater, U. Mueller, and Z. Markovic-Housley. 2007. Identification of a B-cell epitope of hyaluronidase, a major bee venom allergen, from its crystal structure in complex with a specific Fab. *J. Mol. Biol.* 368: 742–752.
48. Engel, E., K. Richter, G. Obermeyer, P. Briza, A. J. Kungl, B. Simon, M. Auer, C. Ebner, H. J. Rheinberger, M. Breitenbach, and F. Ferreira. 1997. Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains. *J. Biol. Chem.* 272: 28630–28637.