

Generation of an Allergy Vaccine by Disruption of the Three-Dimensional Structure of the Cross-Reactive Calcium-Binding Allergen, Phl p 7¹

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The grass pollen allergen, Phl p 7, belongs to a family of highly cross-reactive calcium-binding pollen allergens. Because Phl p 7 contains most of the disease-eliciting epitopes of pollen-derived calcium-binding allergens, hypoallergenic variants were engineered according to the x-ray crystal structure of Phl p 7 for allergy vaccination. In three recombinant variants, amino acids essential for calcium binding were mutated, and two peptides comprising the N- and C-terminal half were obtained by synthetic peptide chemistry. As determined by circular dichroism analysis and size exclusion chromatography coupled to mass spectrometry, recombinant mutants showed altered structural fold and lacked calcium-binding capacity, whereas the two synthetic peptides had completely lost their structural fold. Allergic patients' IgE Ab binding was strongest reduced to the variant containing two mutations in each of the two calcium-binding sites and to the peptides. Basophil histamine release and skin test experiments in allergic patients identified the peptides as the vaccine candidates with lowest allergenic activity. Immunization of rabbits with the peptides induced IgG Abs that blocked allergic patients' IgE binding to Phl p 7 and inhibited allergen-induced basophil degranulation. Our results indicate that disruption of an allergen's three-dimensional structure represents a general strategy for the generation of hypoallergenic allergy vaccines, and demonstrate the importance of allergen-specific IgG Abs for the inhibition of immediate allergic symptoms. *The Journal of Immunology*, 2004, 172: 5684–5692.

Type I allergy is a genetically determined hypersensitivity disease affecting >25% of the population in the industrialized world (1). Allergic patients are characterized by their intrinsic tendency to form IgE Abs against otherwise harmless Ags (i.e., allergens) (2). IgE recognition of allergens induces a cascade of inflammatory reactions via the activation of various effector cells (3–5). The molecular and structural characterization of the disease-eliciting allergens in the most common allergen sources (e.g., pollen, mites, animals, food, molds, insects) has led to the identification of highly cross-reactive allergens (6–8). Thus, patients mounting IgE Abs against cross-reactive allergens exhibit allergic reactions to a broad variety of allergen sources containing these allergens (9, 10).

During the last few years, distinct proteins containing two calcium-binding domains (i.e., EF-hands) have been identified as a

family of highly cross-reactive allergens in pollens of the most common allergenic plants (grasses, trees, and weeds) (7, 11–15). The two EF-hand allergens represent proteins with a molecular mass of ~8–9 kDa, which rapidly elute from pollen grains and induce severe allergic reactions in sensitized patients (7, 10, 16). Due to IgE cross-reactivity, patients allergic to two EF-hand allergens exhibit broad sensitization to pollens of most plant species (10, 17). It has been shown that the two EF-hand allergen from timothy grass pollen, Phl p 7, contains the majority of IgE epitopes of pollen-derived calcium-binding allergens (17), and its three-dimensional structure has been solved recently (18). We have therefore selected Phl p 7 as a paradigmatic allergen for the development of vaccines for the treatment of allergies to calcium-binding allergens.

Allergen-specific immunotherapy is based on the administration of increasing doses of disease-eliciting molecules to allergic patients to induce allergen-specific nonresponsiveness (19). Although allergen-specific immunotherapy is the only causative treatment of IgE-mediated allergies, it suffers from the disadvantage that the application of allergens can induce anaphylactic side effects. Furthermore, it is based on vaccines made from relatively undefined allergen extracts, which cannot be tailored according to the patient's individual IgE reactivity profile (16).

It has been shown that rPhl p 7 can be used to diagnose patients allergic to calcium-binding allergens, who are suitable for specific immunotherapy with this protein (20). Based on the finding that IgE recognition of Phl p 7 and related calcium-binding allergens depends on the presence of protein-bound calcium (7, 11, 15) and on the intact three-dimensional structure of Phl p 7 (18), we have investigated different strategies for the development of hypoallergenic Phl p 7 derivatives for immunotherapy. Three rPhl p 7 mutants containing mutations in the calcium-binding domains and

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Table I. Characteristics of Phl p 7, two Phl p 7-derived synthetic peptides, and three Phl p 7 mutants

	Borders Amino Acid	Amino Acid Exchange	Number of Amino Acids	Determined Molecular Mass (kDa)	Isoelectric Point (pI)	Structural Integrity
Phl p 7	1–78		78	8.555	3.99	+
Peptide 1						
P1	2–37		37	3.932	4.40	–
Peptide 2						
P2	37–78		42	4.702	3.71	–
Mutant						
MD1.6	1–78	EF-hand 1: E24→A24				
Mutant						
M2A	1–78	EF-hand 2: D59→A59	78	8.56	4.08	+
		EF-hand 1: D17→A17				
		E24→A24	78	8.516	4.17	+
		EF-hand 2: D59→A59				
Mutant						
M4	1–78	EF-hand 1: D17→A17				
		E24→A24				
		EF-hand 2: D52→A52	78	8.472	4.28	+
		D59→A59				

two synthetic peptides comprising the N- and C-terminal half of the molecule were produced and characterized regarding structural and immunological properties.

Materials and Methods

Generation, expression, and purification of rPhl p 7 and Phl p 7 mutants

rPhl p 7 mutants were obtained by introducing point mutations into the cDNA of Phl p 7 using a Chameleon double-stranded site-directed mutagenesis kit (Stratagene, East Kew, Australia) using the following oligonucleotide primers: M1.1, 5'-ATCTCTCTGTGCGCGTGACGGAC-3'; M1.6, 5'-ATCGACTTCAACGCGTTCATCTCC-3'; M2A, 5'-GACACGAACGGTGCCGGGAAGATC-3'; M4, 5'-GACACCGACGGCGCGGCTTCATC-3'; in combination with selection primers (Sel-PvuII, 5'-CGCGCGAGGGATCTGCGGTAAAGC-3'; Sel-BstI, 5'-CGCATAGTTAAGCCAGTCCACACTCCGC-3'; Sel-Eco 47 III, 5'-GGGTCAATGCCAG

AACTTCGTTAATAC-3'; Sel-XhoI, 5'-GGCGGCCGAGCGAGCAGATCCGGCTGC-3') changing restriction sites in plasmid pET 17b. The presence of the point mutations was confirmed by dsDNA sequencing of each plasmid construct (MWG Biotech, Kisslegg, Germany). A graphical representation of the mutational strategies was prepared according to the three-dimensional structure of Phl p 7 with MOLSCRIPT (21) and Raster3D (22).
rPhl p 7 as well as rPhl p 7 mutants were expressed in *Escherichia coli* BL21 (DE3) (Stratagene) in liquid culture. *E. coli* were grown to an OD₆₀₀ of 0.4 in Luria-Bertani medium containing 100 mg/L ampicillin. The expression of recombinant proteins was induced by adding isopropyl-β-thiogalactopyranoside to a final concentration of 1 mM and further culturing for additional 4 h at 37°C. *E. coli* cells from a 500-ml culture were harvested by centrifugation, resuspended in 10 ml of PBS, and homogenized using an ultraturax (Ika, Heidelberg, Germany). Fractions containing soluble proteins were obtained after centrifugation of the homogenates at 10,000 rpm (RC5C, SS34 rotor; Sorvall, Bad Homburg, Germany) for 30 min at 4°C. Enrichment of the proteins in the soluble fraction was achieved

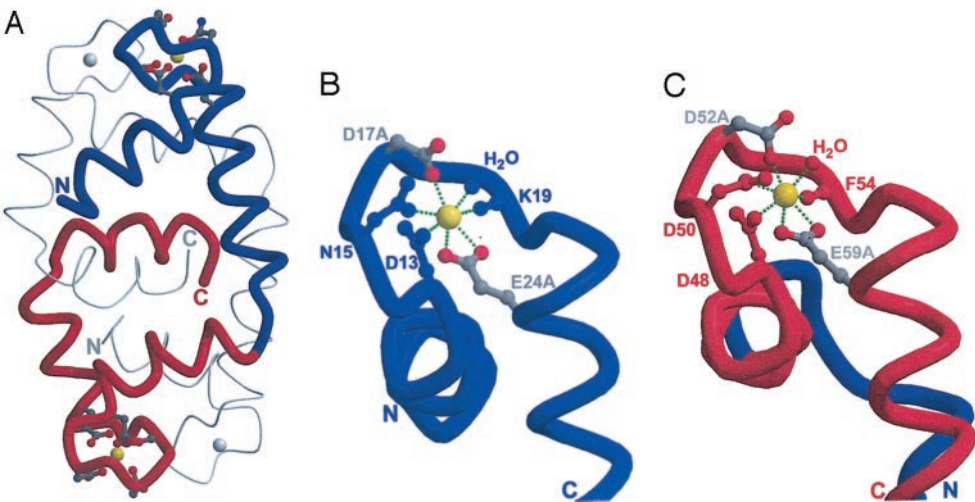
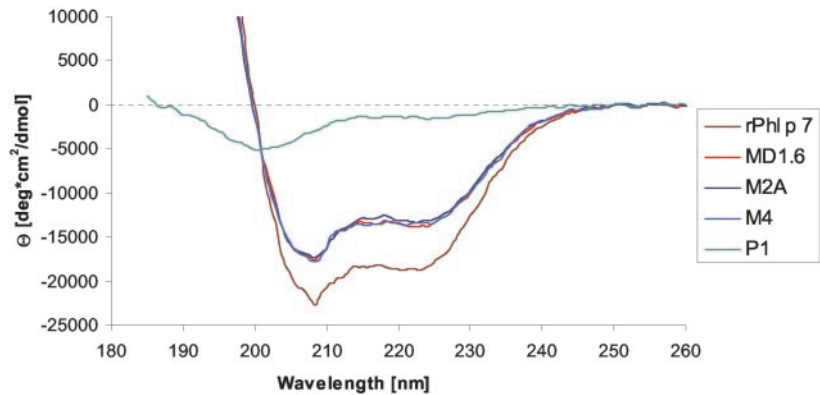


FIGURE 1. A–C, Representation of the three-dimensional structure of Phl p 7, the position of two Phl p 7-derived peptides, and of the mutational strategies. A, Tube representation of the intertwined Phl p 7 dimer. One of the two intertwined monomers has been colored to illustrate the two peptides (blue, N-terminal peptide; red, C-terminal peptide). N and C termini are labeled, and the second monomer is pictured in light gray. The side chains of the calcium-coordinating residues in the canonical EF-hand motifs are shown in a ball-and-stick representation (see B and C), and the calcium ions are highlighted in yellow in one molecule. B, Close-up view of the N-terminal EF-hand. The polypeptide backbone is shown as a blue tube; N and C termini are labeled. The calcium ion (yellow) in the middle of the loop is coordinated 7-fold by oxygen atoms of asparagines (N15) or aspartic acids (D13, D17), a backbone carbonyl oxygen (K19), a water molecule, and the oxygen atoms of a highly conserved bidentate glutamic acid (E24). The residues D17 and E24 mutated to alanine in M4 are pictured with gray carbon and red oxygen atoms, whereas the nonmutated residues are shown in blue. The interactions between the calcium ion and the coordinating oxygen atoms are represented by green dotted lines. C, Close-up view of the C-terminal EF-hand. The calcium ion is pictured in yellow. The calcium coordination also follows the canonical EF-hand motif and is rather identical with the N-terminal calcium-binding site. The polypeptide backbone, as well as the nonmutated calcium-coordinating side chains of the two aspartic acids D48 and D50, the water molecule, and the backbone carbonyl oxygen of F54 are all shown in red. The residues D52 and E59, which were mutated to alanine in M4, are represented with gray carbon and red oxygen atoms. Coordination between oxygen atoms and the calcium ion is symbolized with green dots.

FIGURE 2. CD spectra. The mean residue ellipticity (θ) (y-axis) of rPhl p 7, the Phl p 7 mutants (MD1.6, M2A, M4), and the N-terminal peptide (P1) is shown for a range of wavelengths (x-axis).



by addition of 70% w/v ammonium sulfate to the soluble *E. coli* fraction and removal of precipitated contaminating proteins by centrifugation (18,000 rpm, Sorval SS34, 4°C, 30 min). The supernatants containing soluble rPhl p 7 or Phl p 7 mutants were dialyzed against water, lyophilized, resuspended in 50 ml of buffer A (25 mM imidazole, 1 mM 2-ME, pH 7.4), and applied to a DEAE anion exchange column (Pharmacia, Uppsala, Sweden). Fractions with pure rPhl p 7 or Phl p 7 mutants were eluted with a NaCl gradient (buffer A containing 500 mM NaCl) at ~200 mM NaCl and dialyzed against water. The purity of the proteins was confirmed by SDS-PAGE, and concentrations in the samples were determined with a Micro BCA kit (Pierce, Rockford, IL) using BSA as a standard. The Phl p 7-ho-

mologous allergen from alder, rAln g 4, was expressed in *E. coli* and purified, as described (15).

Size exclusion chromatography coupled to inductively coupled plasma mass spectrometry (ICPMS)

Volumes of 5 μ l of either 5 mg ml⁻¹ of Phl p 7 or 4 mg ml⁻¹ of mutant M4 dissolved in double-distilled water were incubated overnight with 15 μ l of buffer B (20 mM Tris-HCl, 50 mM NH₄Cl, pH 7.0). A total of 5 μ l of each of these protein samples was applied to a Superdex 75 PC 3.2/30 column (Amersham, Buckinghamshire, U.K.), equilibrated with buffer B, and isocratically eluted at a flow rate of 0.1 ml min⁻¹. The monitoring was

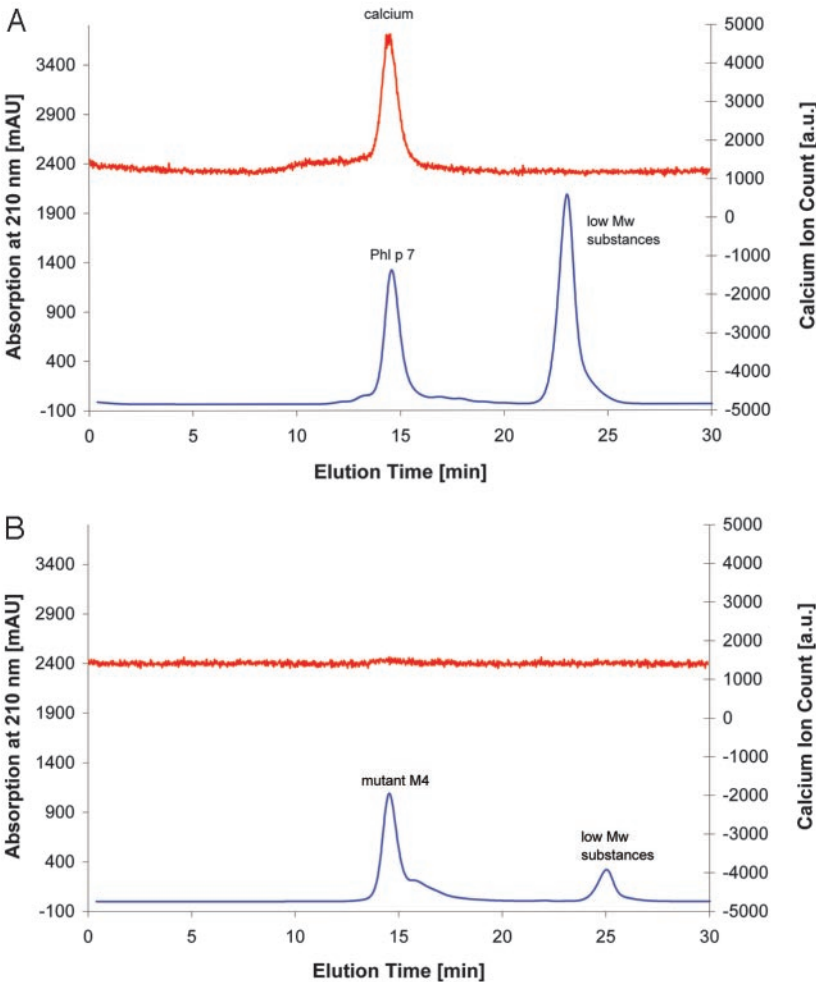


FIGURE 3. A and B, Size exclusion chromatography (SEC) coupled to ICPMS for Phl p 7 (A) and M4 (B). The UV absorption traces at 210 nm (mAU: milli absorption units) are shown in blue (y-axis: Phl p 7, M4, low m.w. substances), and the corresponding ion counts (a.u.: arbitrary units) for calcium are represented in red (y-axis) for a given elution time (x-axis).

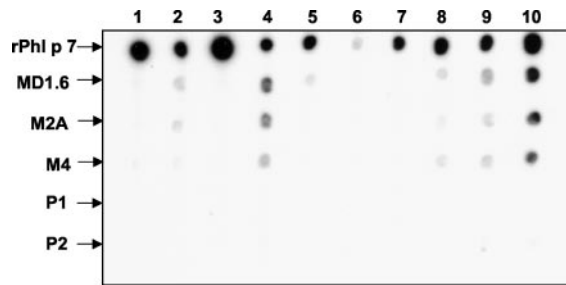


FIGURE 4. IgE reactivity of nitrocellulose-dotted rPhl p 7, Phl p 7 mutants (MD1.6, M2A, M4), and peptides (P1, P2). Dotted proteins and peptides were exposed to sera from 10 Phl p 7-allergic patients (lanes 1–10). Bound IgE Abs were detected with anti-human IgE Abs.

performed via UV absorption at 210 nm, and the column was calibrated using the Amersham Pharmacia Low Molecular Weight Gel Filtration Calibration Kit. The Agilent 1100 HPLC system was coupled to an Agilent 7500c ICPMS (Agilent Technologies, Palo Alto, CA), in which calcium (^{43}Ca , ^{44}Ca , ^{48}Ca) and sulfur (^{34}S) isotopes were simultaneously monitored. The sulfur signal was used as an internal standard for the elution of proteins.

Peptide synthesis

Two peptides, comprising the N-terminal or the C-terminal half of Phl p 7 wild type (aa 2–37 or 37–78, respectively), were synthesized using Fmoc (9-fluorenylmethoxycarbonyl) strategy with 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation (0.1 mmol small-scale cycles) on the Applied Biosystems peptide synthesizer

Table II. Serum IgE reactivity of rPhl p 7, Phl p 7-derived peptides (P1, P2), and the mutant M4

Patient	IgE Binding (cpm)			
	rPhl p 7	P1	P2	M4
1	402.8	12.6	13.0	64.2
2	197.5	6.5	32.1	22.5
3	1602.7	27.7	63.4	100.2
4	413.9	16.3	36.5	98.6
5	399.6	6.5	34.5	21.4
6	74.6	10.2	7.0	11.2
7	266.7	5.5	11.1	34.3
8	243.0	16.8	33.0	51.4
9	242.4	11.7	23.9	61.2
10	538.6	12.7	11.2	80.2
11	285.5	12.7	24.8	18.4
12	237.9	16.1	28.6	61.2
13	2278.4	13.6	16.9	72.7
14	310.7	17.5	17.7	29.5
15	1631.2	45.5	147.8	401.9
16	220.5	30.6	22.2	100.8
17	506.5	18.6	20.0	15.6
18	569.8	12.9	17.5	22.2
19	135.4	10.3	12.5	19.3
20	170.6	24.3	16.8	22.0
21	325.2	33.6	18.8	153.7
22	254.5	18.0	20.2	12.2
23	65.9	19.2	27.1	30.9
24	46.0	31.9	30.8	34.5
25	154.4	28.4	40.3	36.7
26	157.4	39.0	25.2	39.1
27	295.8	25.3	40.3	42.0
28	311.5	38.5	76.8	140.8
29	229.0	40.5	76.8	140.3
30	161.5	42.0	36.4	34.4
Mean	426.3	21.9	32.1	65.8
Mean reduction of IgE binding		89.0%	86.1%	77.3%
SD +/-		13.4%	13.5%	18.1%

model 433A (Foster City, CA). Preloaded polyethyleneglycol polystyrene resins (0.15–0.2 mmol/g loading) (PerSeptive Biosystems, Warrington, U.K.) were used as solid phase to build up the peptides. Chemicals were purchased from Applied Biosystems. Coupling of amino acids was confirmed by conductivity monitoring in a feedback control system. One cysteine residue was added to each peptide to facilitate coupling of the peptides to carriers. Peptides were cleaved from the resins with a mixture of: 250 μl of distilled water, 250 μl triisopropylsilan (Fluka, Buchs, Switzerland), and 9.5 ml trifluoroacetic acid for 2 h, and precipitated in tert-butylmethylether (Fluka). The identity of the peptides was checked by mass spectrometry, and they were purified to >90% purity by preparative HPLC (piChem, Graz, Austria).

Matrix-assisted laser desorption and ionization-time of flight mass spectrometry and circular dichroism (CD)³ analysis of purified rPhl p 7, Phl p 7 mutants, and peptides

Laser desorption mass spectra of rPhl p 7 and Phl p 7 mutants were acquired in a linear mode with a time of flight compact matrix-assisted laser desorption and ionization II instrument (Kratos, Manchester, U.K.) (piChem). CD measurements of proteins and peptides dissolved in MilliQ water at concentrations of 2.3×10^{-5} M (rPhl p 7), 1.5×10^{-5} M (MD1.6, M2A, M4), or 6.3×10^{-5} M (P1) were conducted on a Jasco J-715 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a 0.1-cm path-length cell equilibrated at 20°C. Spectra were recorded with 0.5 nm resolution at a scan speed of 100 nm/min and resulted from averaging three

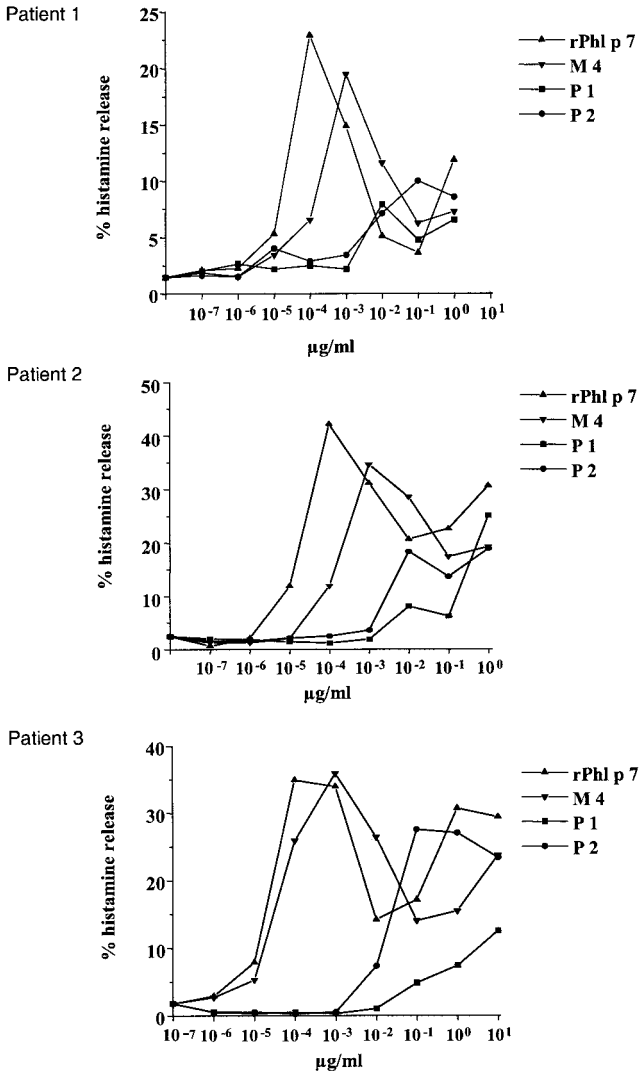


FIGURE 5. Induction of basophil histamine release in three Phl p 7-allergic patients. Patients' granulocytes were incubated with various concentrations (x-axis) of rPhl p 7, Phl p 7-derived peptides (P1, P2), or the Phl p 7 mutant (M4). The percentage of total histamine released into the supernatant is displayed on the y-axis.

Table V. Inhibition of allergic patients' IgE binding to rPhl p 7 by rabbit Abs

Patient	Percentage of Inhibition				
	Anti-P1-KLH	Anti-P2-KLH	Anti-M4	Anti-M4-KLH	Anti-rPhl p 7
1	0	64.8	0	35.8	82
2	0	42.2	2.7	18.9	83.7
3	0	42.2	19.9	4	79
4	9.4	39.1	0	35.2	64.1
5	17.4	22.1	19	20	56.6
6	0	21.4	9.3	12.3	44
7	9.8	7.4	0	10.3	26
Mean	5.2	34.1	7.2	19.5	62.2

Rat basophil leukemia (RBL) cell degranulation experiments

RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 cells/well), incubated for 24 h at 37°C using 7% CO₂. Passive sensitization was performed with mouse sera containing two EF-hand allergen-reactive IgE at a final dilution of 1/30 for 2 h. Unbound Abs were removed by washing the cell layer twice in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM D-glucose, 12 mM NaHCO₃, 10 mM HEPES, and 0.1% w/v BSA, pH 7.2). RBL cells, preloaded either with Phl p 7- or Aln g 4-specific mouse IgE, were exposed to rPhl p 7 (0.1 µg/ml) or rAln g 4 (0.01 µg/ml). The allergens were preincubated in Tyrode's buffer with 0, 2, 5, 7.5, or 10% v/v of rabbit antisera for 2 h at 37°C, as follows: 1) equal volumes of sera from rabbits obtained before or after immunization with the two KLH-coupled peptides (P1, P2); 2) serum from a rPhl p 7-immunized rabbit or the corresponding preimmune serum. Preincubated allergens were added to the RBL cells for 30 min in a humidified atmosphere at 37°C, and their supernatants were analyzed for β-hexosaminidase activity by incubation with 80 µM 4-methylumbelliferyl-N-acetyl-β-D-glucosamide (Sigma-Aldrich, Vienna, Austria) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C. The reaction was stopped by addition of 100 µl of glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7), and the fluorescence was measured at λ_{ex}: 360/λ_{em}: 465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as fluorescence units, and percentage of total β-hexosaminidase released after lysis of cells with 1% Triton X-100.

Results

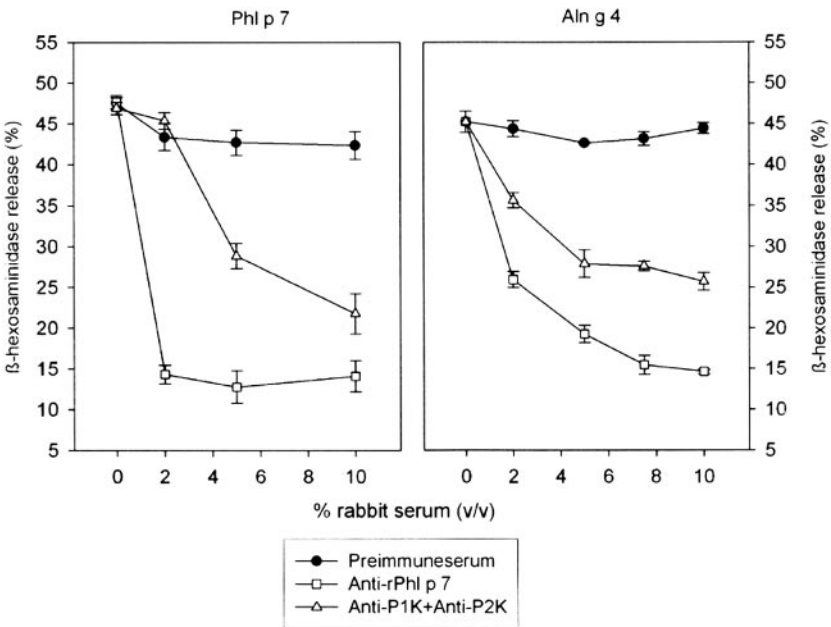
Biochemical and structural characterization of Phl p 7 derivatives

To reduce the allergenic activity of rPhl p 7, two different strategies were pursued. First, we synthesized two peptides comprising the N-terminal (P1: aa 2–37) and C-terminal (P2: aa 37–78) half of Phl p 7 with the aim to disrupt the three-dimensional structure of Phl p 7, which is composed of an intertwined dimer (Table I; Fig. 1A). The second strategy of introducing point mutations into the calcium-binding domains of Phl p 7 was based on the observation that depletion of protein-bound calcium reduced the IgE-binding capacity of Phl p 7 (7). Three rPhl p 7 mutants were engineered (Table I). In mutant MD1.6, the amino acids (E24, D59) that provide two oxygen atoms for the coordination of calcium binding and thus act as bidentate ligands were exchanged (Fig. 1, B and C; Table I). Mutant M2A differs from MD1.6 by the exchange of an additional amino acid (i.e., D17) involved in calcium binding in the first EF-hand domain (Fig. 1B; Table I). Finally, mutant M4 was generated by exchanging an additional amino acid (D52) in the second EF-hand motif of M2A (Fig. 1C; Table I).

The comparison of the rPhl p 7 wild-type protein with the three mutants by CD analysis showed that the mutants still contained considerable amount of α-helical structure (Fig. 2). Their spectra were characterized by minima at 224 and 208 nm and a strong maximum below 200 nm. The α-helical content was identical for the mutants, whereas the native rPhl p 7 showed a significantly larger α-helical signal. This finding is indicative for a loosening up of the α-helical core structure due to the mutations in the calcium-binding sites. The N-terminal peptide, P1, exhibited a random coil secondary structure, with the characteristic minimum at 200 nm. The C-terminal peptide, P2, showed reduced solubility, indicating that truncation of rPhl p 7 protein has led to the disruption of the three-dimensional structure.

The question as to whether the mutations in the calcium-binding domains had indeed abolished the calcium-binding capacity of the mutants was studied by size exclusion chromatography coupled to ICPMS (Fig. 3). The fact that rPhl p 7 coeluted with a strong calcium peak, whereas M4 showed a similar elution profile without

FIGURE 6. Inhibition of rPhl p 7 (left) or rAln g 4 (right)-induced basophil degranulation by anti-rPhl p 7 and anti-peptide IgG. Rat basophils had been loaded with Phl p 7 (left)- or Aln g 4 (right)-specific mouse IgE.



calcium peak demonstrates that the mutant did not contain protein-bound calcium. The additional, later-eluting peaks in the UV absorption traces correspond to low m.w. substances (e.g., salts), which elute beyond the separation range of the column and were monitored due to adsorption at 210 nm (Fig. 3).

Phl p 7 mutants and peptides exhibit reduced IgE-binding capacity

Dot blot analysis of sera from 10 Phl p 7-sensitized patients showed that the IgE-binding capacity of the three mutants (MD1.6, M2A, M4) was strongly reduced, whereas the two peptides showed no detectable IgE reactivity at all (Fig. 4). A detailed analysis of the IgE-binding capacity of M4 and the two peptides (P1, P2) was performed for additional 30 Phl p 7 allergic patients (Table II). The quantification of IgE Abs bound to rPhl p 7 and the three derivatives showed a mean reduction of IgE-binding capacity of 89 and 86.1% for peptides 1 and 2, respectively, and of 77.3% for mutant M4 (Table II). The reduction of IgE-binding capacity of Phl p 7 derivatives was also confirmed by IgE ELISA competition assays (29) using fluid phase-added modified allergens to compete IgE binding to solid phase-bound Phl p 7 (data not shown).

Basophil histamine release and skin test experiments demonstrate strongest reduction of allergenicity for the Phl p 7-derived peptides

Basophils from three Phl p 7-allergic patients were exposed to various concentrations of rPhl p 7, M4, and the two peptides (P1, P2) (Fig. 5). rPhl p 7 induced strong and dose-dependent histamine release in basophils from all three patients, yielding maximal histamine release at a concentration between 10^{-5} and 10^{-4} μ g/ml. Compared with rPhl p 7 wild type, M4 exhibited not more than 10-fold reduced allergenic activity (Fig. 5). The strongest reduction of allergenic activity was noted for the Phl p 7-derived peptides, which exhibited a 1,000- to 10,000-fold reduced allergenic activity compared with rPhl p 7 wild type (Fig. 5). KLH-coupled peptides that were used for immunization studies also exhibited a >1,000-fold reduction of allergenic activity when compared with rPhl p 7 wild type (data not shown).

The in vitro results were confirmed by skin-prick test experiments in three Phl p 7-allergic patients and a nonatopic individual (Table III). Each of the Phl p 7-allergic patients showed pronounced immediate type skin reactions to rPhl p 7 already at 2 μ g/ml. The mean wheal diameters induced by M4 were only moderately reduced compared with the rPhl p 7 wild type (Table III). The latter results were confirmed by a skin test titration performed in 1:2 steps in another Phl p 7-allergic patient (Table III: individual no. 5). However, the two peptides did not induce immediate skin reactions in any of the patients even when amounts equimolar to the rPhl p 7 wild type were administered. All allergic patients displayed immediate skin reactions to timothy grass pollen extract and histamine. The nonatopic person showed no reaction to timothy grass pollen extract, rPhl p 7, M4, and to the peptides (Table III).

Hypoallergenic Phl p 7 derivatives induce IgG Abs that cross-react with Phl p 7 and Phl p 7-related allergens

The mutant M4 as well as the KLH-coupled peptides (P1, P2) induced IgG Abs that reacted with the rPhl p 7 wild-type allergen and the cross-reactive allergen from alder pollen, rAln g 4 (Table IV). The Ab responses induced with uncoupled and KLH-coupled M4 as well as those induced with KLH-coupled P1 were weaker than those induced with the rPhl p 7 wild-type protein and KLH-coupled P2. Uncoupled P1 and P2 induced lower rPhl p 7-reactive IgG than the coupled peptides (data not shown).

IgG Abs induced by immunization with hypoallergenic Phl p 7 derivatives inhibit allergic patients' IgE binding to Phl p 7

The strongest inhibition of patients' IgE binding to rPhl p 7, ranging between 26 and 83.7% (62.2% mean inhibition) was observed with anti-rPhl p 7 Abs (Table V). Likewise, we observed considerable reduction of anti-Phl p 7 IgE reactivity, ranging from 7.4 to 64.8% (34.1% mean inhibition) with Abs raised against the Phl p 7-derived peptide P2. Only low inhibition of IgE binding was observed with IgG Abs obtained after immunization with the other Phl p 7 derivatives (mean inhibitions: anti-P1, 5.2%; anti-M4, 7.2%; anti-KLH-M4, 19.5%) (Table V).

IgG Abs induced by immunization with hypoallergenic Phl p 7 peptides inhibit basophil degranulation

The biological relevance and possible protective activity of peptide-induced IgG Abs were investigated in a defined cellular model system using RBL cells that were loaded with allergen-specific IgE. Preincubation of rPhl p 7 with increasing concentrations (2–10% v/v) of a mixture of rabbit anti-P1 and anti-P2 Abs and with rabbit anti-rPhl p 7 Abs led to a dose-dependent inhibition of rPhl p 7-induced mediator release from RBLs that had been preloaded with Phl p 7-specific mouse IgE (Fig. 6, left). The inhibition of Phl p 7-induced release obtained at a concentration of 10% serum added was 70.7% (i.e., reduction from 5844 ± 541 U to 1716 ± 357 U) with anti-Phl p 7 IgG and 53.8% with anti-peptide IgG (i.e., reduction from 5799 ± 42 U to 2676 ± 208 U). Similar results were obtained when RBLs were preloaded with Aln g 4-specific mouse IgE and then stimulated with rAln g 4 that had been preincubated with increasing concentrations of peptide-specific rabbit IgG (Fig. 6, right). The inhibition of Aln g 4-induced release obtained at a concentration of 10% serum added was 67.6% (i.e., reduction from 5078 ± 248 U to 1643 ± 47 U) with anti-Phl p 7 IgG and 40.6% with anti-peptide IgG (i.e., reduction from 4856 ± 527 U to 2884 ± 210 U). No inhibition of basophil degranulation was observed when the allergen was preincubated with the same concentrations of preimmune Ig. No relevant degranulation (i.e., <200 U) was observed when allergens were omitted from the basophils (data not shown).

Discussion

In this work, we report the generation and characterization of an allergy vaccine with profoundly reduced allergenic activity for the treatment of allergic patients sensitized to calcium-binding pollen allergens. Calcium-binding molecules occur as cross-reactive allergens in pollens of the most common allergenic plants and are responsible for pollen polysensitization in allergic patients (10–15). Because the calcium-binding allergen from timothy grass pollen, Phl p 7, contains most of the cross-reactive IgE epitopes (17), and the three-dimensional structure of this protein has been solved recently (18), it was selected as candidate molecule for vaccine development. rPhl p 7 represents an extremely potent allergen, presumably because its allergenic activity may be related to its stable fold and the formation of dimers leading to a doubling of IgE epitopes that may give rise to potent cross-linking of effector cell-bound IgE Abs (18).

One strategy for the development of Phl p 7 derivatives with reduced allergenic activity was therefore based on the disruption of the allergen's three-dimensional structure by synthesizing peptides comprising the N-terminal or C-terminal half of Phl p 7. The second approach for the generation of hypoallergenic Phl p 7 derivatives was based on the previous finding that depletion of protein-bound calcium by EGTA (7) and mutations within the calcium-binding sites of related allergens led to a reduction of IgE

reactivity (11, 30). The recombinant mutants of Phl p 7 produced in the present study also exhibited considerably reduced IgE reactivity. Mutant M4, which contained two point mutations in each of the two calcium-binding sites, had lost its calcium-binding capacity completely, but still exhibited its dimeric overall fold with a considerable amount of α -helical secondary structure.

The in vitro and in vivo allergenic activity of calcium-binding allergens other than Phl p 7 and of mutants derived from these allergens has not yet been studied in detail. We therefore compared the allergenic activities of the rPhl p 7 wild-type allergen, the mutant M4, and the two Phl p 7-derived peptides by basophil histamine release and skin tests in allergic patients. We found that only complete disruption of the Phl p 7 structure, as was achieved by the peptide strategy, was sufficient to achieve a profound (i.e., >1000-fold) reduction of allergenic activity.

Although each of the Phl p 7 peptides was unfolded, they induced Phl p 7-specific Abs after immunization of rabbits. Similar observations have been made for recombinant fragments of the major birch pollen allergen, Bet v 1 (31), and the major timothy grass pollen allergen, Phl p 1 (28). For the Phl p 7-derived peptides as well as for the latter allergen derivatives, it could be shown that the derivative-induced IgG Abs not only recognized the wild-type allergens, but also inhibited the binding of allergic patients' IgE Abs. Results obtained with blocking rabbit IgG Abs may in fact be applicable for immunotherapy. We found that immunization of mice with hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, using schemes that are used for immunotherapy of allergic patients, yielded comparable results as the immunization of rabbits (31, 32). The ability of unfolded hypoallergenic allergen derivatives to induce IgG Abs that inhibit the binding of patients' IgE directed to conformational epitopes may be explained in two ways. One possibility is steric hindrance of IgE binding due to binding of peptide-induced Abs in close proximity to the IgE-reactive epitopes. A second possibility would be that peptide-induced Abs are directed against continuous portions within discontinuous IgE epitopes, and thus prevent IgE binding. Furthermore, it is possible that binding of peptide-specific Abs may induce conformational changes in the wild-type allergens, leading to loss of IgE epitopes.

The biological relevance and protective activity of the peptide-induced Abs were demonstrated by their ability to inhibit immediate allergic reactions (i.e., basophil degranulation). It may thus be expected that immunotherapy with the Phl p 7-derived peptides will induce IgG Abs that inhibit immediate allergic reactions induced by Phl p 7 and related calcium-binding allergens. Furthermore, such IgG Abs may inhibit IgE-mediated allergen presentation and T cell activation (33) as well as the boosting of specific IgE production induced by allergen exposure (34).

The Phl p 7 derivatives developed by us induce IgG Abs that inhibit a considerable proportion, but not all IgE reactivities against the Phl p 7 wild-type allergen. This potential disadvantage may be overcome by choosing immunization schemes that induce higher titers of blocking Abs, and is certainly outweighed by the strong reduction of allergenic activity, and thus, the increased safety of the derivative-based vaccine.

Because both Phl p 7-derived peptides together represent the complete primary sequence of Phl p 7, they contain the relevant T cell epitopes of Phl p 7, and hence may be also suitable for alternative immunotherapy strategies aimed at the induction of T cell regulation, T cell tolerance, or induction of regulatory T cells (35–37).

In conclusion, we have developed a hypoallergenic candidate vaccine suitable for the treatment, and perhaps prevention of allergies to calcium-binding allergens, and provide evidence that the

disruption of the three-dimensional structure of a given allergen is a general strategy for the generation of allergy vaccines with reduced allergenic side effects.

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