

A Hypoallergenic Vaccine Obtained by Tail-to-Head Restructuring of Timothy Grass Pollen Profilin, Phl p 12, for the Treatment of Cross-Sensitization to Profilin¹

Kerstin Westritschnig,* Birgit Linhart,* Margarete Focke-Tejkl,* Tea Pavkov,‡ Walter Keller,‡ Tanja Ball,* Adriano Mari,§ Arnulf Hartl,|| Angelika Stöcklinger,|| Sandra Scheiblhofer,|| Josef Thalhamer,|| Fatima Ferreira,|| Stefan Vieths,# Lothar Vogel,# Alexandra Böhm,† Peter Valent,† and Rudolf Valenta^{2*}

Profilins are highly cross-reactive allergens in pollens and plant food. In a paradigmatic approach, the cDNA coding for timothy grass pollen profilin, Phl p 12, was used as a template to develop a new strategy for engineering an allergy vaccine with low IgE reactivity. Non-IgE-reactive fragments of Phl p 12 were identified by synthetic peptide chemistry and restructured (rs) as a new molecule, Phl p 12-rs. It comprised the C terminus of Phl p 12 at its N terminus and the Phl p 12 N terminus at its C terminus. Phl p 12-rs was expressed in *Escherichia coli* and purified to homogeneity. Determination of secondary structure by circular dichroism indicated that the restructuring process had reduced the IgE-reactive α -helical contents of the protein but retained its β -sheet conformation. Phl p 12-rs exhibited reduced IgE binding capacity and allergenic activity but preserved T cell reactivity in allergic patients. IgG Abs induced by immunization of mice and rabbits with Phl p 12-rs cross-reacted with pollen and food-derived profilins. Recombinant Phl p 12-rs, rPhl p 12-rs, induced less reagenic IgE to the wild-type allergen than rPhl p 12. However, the rPhl p 12-rs-induced IgGs inhibited allergic patients' IgE Ab binding to profilins to a similar degree as those induced by immunization with the wild type. Phl p 12-rs specific IgG inhibited profilin-induced basophil degranulation. In conclusion, a restructured recombinant vaccine was developed for the treatment of profilin-allergic patients. The strategy of tail-to-head reassembly of hypoallergenic allergen fragments within one molecule represents a generally applicable strategy for the generation of allergy vaccines. *The Journal of Immunology*, 2007, 179: 7624–7634.

Immunoglobulin E-mediated allergies affect >20% of the population in industrialized countries (1). The symptoms of allergy (e.g., rhinoconjunctivitis, asthma, food allergy, dermatitis, and anaphylactic shock) are caused by IgE recognition of per se harmless Ags (i.e., allergens) (2). In allergic patients, allergen contact causes the immediate release of inflammatory mediators from mast cells as well as chronic T cell- and eosinophil-mediated tissue inflammation (3). Allergic tissue inflammation can

be mitigated using anti-inflammatory drugs and immunosuppressive agents. However, only allergen-specific immunotherapy, the administration of gradual increasing quantities of the disease-eliciting allergens, may be considered as causative treatment (4). Allergen-specific immunotherapy has long-lasting clinical effects and may prevent the progression of mild forms of allergy to severe manifestations (5, 6). Due to extensive work conducted in the field of molecular allergen characterization, the structures of many common and important allergens have been revealed by cDNA cloning and recombinant DNA technology (reviewed in Ref. 7). Recombinant allergens and their epitopes are already used in diagnostic tests to analyze and monitor the patient's sensitization profile and allow the precise selection of patients for allergen-specific immunotherapy (8, 9).

To reduce allergenic side effects, several immunotherapy studies have been conducted using T cell epitope containing peptides with no or low IgE reactivity (10–12). More recently, genetically modified hypoallergenic recombinant allergen derivatives have been engineered (reviewed in Ref. 13). The advantages of genetically modified allergens are that they exhibit strongly reduced allergenic activity, combine most of the allergen-derived T cell peptides within one molecule, and induce protective Ab responses upon immunization that can antagonize IgE-mediated effects. The latter has been demonstrated recently in a clinical immunotherapy study conducted in birch pollen-allergic patients with genetically modified derivatives of the major birch pollen allergen, Bet v 1 (14–16).

In the current study we exemplify the development of a new strategy for the engineering of a recombinant hypoallergenic allergen derivative for vaccination against allergy to profilin. Profilins (12–14 kDa) are ubiquitous actin-binding proteins that occur

*Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, Center for Physiology and Pathophysiology, and †Division of Hematology and Hemostaseology, Department of Internal Medicine I, Vienna General Hospital, Medical University of Vienna, Vienna, Austria; ‡Institute of Chemistry, University of Graz, Graz, Austria; §Allergy Unit, National Health Service, Rome, Italy; ||Institute of Physiology and Pathophysiology, Paracelsus Private Medical University, Salzburg, Austria; ||Department of Molecular Biology, University of Salzburg, Salzburg, Austria; and #Paul-Ehrlich-Institut, Langen, Germany

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² Address correspondence and reprint requests to Dr. Rudolf Valenta, Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, Center for Physiology and Pathophysiology, Vienna General Hospital (Allgemeines Krankenhaus Wien), Medical University of Vienna, Währinger Gürtel 18–20, A-1090, Vienna, Austria. E-mail address: Rudolf.valenta@meduniwien.ac.at

in a variety of eukaryotic organisms and thus represent highly cross-reactive allergens. They have been identified as clinically relevant allergens in tree, grass, and weed pollens as well as in plant-derived food and, accordingly, have been designated pan-allergens (17–20).

By competitive IgE inhibition studies we identified profilin from timothy grass pollen, Phl p 12 (21), as the member of the profilin family that carries the majority of IgE epitopes of plant profilins. Non-IgE-reactive fragments of Phl p 12 were determined by epitope mapping based on synthetic peptides. In accordance with the epitope mapping, the Phl p 12-encoding cDNA was used as a template to generate a restructured (rs)³ recombinant Phl p 12 derivative, designated rPhl p 12-rs, that represents a tail-to-head recombination of the allergen's C-terminal and N-terminal part within one single molecule. We report the molecular and immunological characterization of the restructured profilin, rPhl p 12-rs, and demonstrate that vaccination with this derivative induces IgG Abs that cross-react with profilins from various pollens and plant food and protects against profilin-induced allergic reactions.

Materials and Methods

Purification of pollen and plant food profilins

Recombinant profilins from timothy grass pollen (rPhl p 12; Ref. 21), birch pollen (rBet v 2; Ref. 22), mugwort pollen (rArt v 4; Ref. 23), lychee (rLit c 4), cashew nut (rAna c 1), carrot (rDau c 4), hazelnut (rCor a 2), and banana (rMus xp 1; Ref. 19) were expressed in *Escherichia coli* and purified using poly-L-proline-loaded agarose columns (Amersham Bioscience) as described (22).

Natural (n) timothy grass pollen profilin (nPhl p 12) was purified from pollen grains by affinity chromatography using poly-L-proline-loaded agarose (Amersham Bioscience) as described (17).

Characterization of profilin-allergic patients

Patients suffering from polysensitization to pollens from various unrelated plants (i.e., trees, grasses, weeds) and plant-derived food were diagnosed according to previously defined criteria (24). Profilin-allergic patients were identified by measuring serum IgE Abs specific for timothy grass pollen profilin (Phl p 12) and birch pollen profilin (rBet v 2) by ImmunoCAP RAST (Phadia), dot blot, or ELISA analysis as described (25).

IgE ELISA competition experiments

ELISA inhibition experiments were performed using ELISA plate-bound rPhl p 12 (coating concentration: 5 µg/ml). Sera from four profilin-allergic patients (dilution 1/5) were preadsorbed with rPhl p 12, rBet v 2, or rArt v 4 (concentration: 0.1, 1, and 10 µg/ml, respectively) and then exposed to plate-bound rPhl p 12. Bound IgE Abs were detected with an alkaline-phosphatase-conjugated monoclonal anti-human IgE Ab (BD Pharmingen). All determinations were carried out in duplicate and results are displayed as means ± SD.

Peptide synthesis

Five peptides spanning almost the entire sequence of the timothy grass pollen profilin Phl p 12 (peptide 1: aa 1–23; peptide 2: aa 25–50; peptide 3: aa 51–77; peptide 4: aa 79–108; and peptide 5: amino acids 109–131; Table I) were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) strategy with 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation (0.1-mmol small scale cycles) on the Applied Biosystems peptide synthesizer model 433A. Preloaded polyethylene glycol/polystyrene resins (0.15–0.2 mmol/g loading; PerSeptive Biosystems) were used as the solid phase to build up the peptides. Chemicals were purchased from Applied Biosystems. A cysteine residue was added to each peptide either on the N- or C-terminal end 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 of triisopropylsilan (Fluka), and 9.5 ml of trifluoroacetic acid, for 2 h and precipitated in *tert*-butyl methyl ether (Fluka). The identity of the peptides was checked by mass spectrometry, and they were purified to >90% purity by preparative HPLC (piChem).

Expression and purification of a recombinant “tail-to-head” derivative from timothy grass pollen profilin, rPhl p 12-rs

A Phl p 12 derivative that starts with the C-terminal portion of Phl p 12 (aa 78–131) at the N terminus and contains the N-terminal portion (aa 1–77) at the C terminus was constructed by an overlapping PCR technique. The cDNA coding for the C-terminal portion of Phl p 12 (aa 78–131) (21) was amplified with the primer pair MDE-1 (5'-CATATGGAACCCGCGCG GTCATC-3') and MDE-2 (5'-GTACGTCTGCCACGCCATCATGCCTT GTTCAAC-3') to insert an *NdeI* site (underlined) in the 5' end. The cDNA coding for the N-terminal portion (aa 1–77) (21) was amplified using the primer pair MABC-1 (5'-GTTGAACAAGGCATGATGTCGTGGCA GACG3'-) and MABC-2 (5'-GAATTCTTAATGGTGATGGTGATGGTG ACCCTGGATGACCATGTA-3') to place a DNA segment (italics) coding for a C-terminal hexahistidine tail at the 3' end. The inserted *EcoRI* site is underlined.

The two PCR products were combined in a final PCR using the primer pair MDE-1 and MABC-2 to generate the DNA coding for the restructured “tail-to-head” Phl p 12 derivative that was designated rPhl p 12-rs. The Phl p 12-rs-encoding cDNA was cloned into the plasmid pSTBlue-1 (Stratagene) and both DNA strands were sequenced (MWG Biotech).

Subsequently, the Phl p 12-rs-encoding cDNA was cut out of the above construct with *NdeI* and *EcoRI* and subcloned into the *NdeI* and *EcoRI* site of expression plasmid pET17b (Novagen), and the DNA sequence of both strands was again confirmed (MWG Biotech).

rPhl p 12-rs was expressed in *E. coli* BL21 (DE3) (Stratagene) in liquid culture. *E. coli* were grown to an OD₆₀₀ of 0.4 in Luria broth medium containing 100 mg/L ampicillin. The expression of Phl p 12-rs was induced by adding isopropyl-β-thiogalactopyranoside to a final concentration of 1 mM and further culturing the cells for an additional 4 h at 37°C. *E. coli* cells from a 500-ml culture were harvested by centrifugation and resuspended in buffer A (100 mM NaH₂PO₄, 10 mM Tris, and 8 M urea (pH 7.5)). After centrifugation at 20,000 rpm for 30 min the supernatant was allowed to bind to a nickel column (Qiagen), unbound material was washed out with buffer A (pH 6.3), and pure recombinant Phl p 12-rs protein could be eluted using buffer A (pH 4.9). Purified rPhl p 12-rs was refolded by stepwise dialysis against buffer A with a gradient from 6 to 0 M urea. A last dialysis step was done against PBS.

Protein purity was confirmed by SDS-PAGE. MALDI-TOF mass spectrometry (piChem) was used to determine the exact mass of the protein, and quantification was performed with the Micro BCA kit (Pierce).

Circular dichroism spectroscopy (CD), secondary structure determination, and thermal stability determination

CD measurements recorded at 20°C were performed on a Jasco J-810 spectropolarimeter, and thermal denaturation measurements were done on a Jasco J-715 spectropolarimeter as described (26). Far-UV CD spectra of rPhl p 12, rPhl p 12-rs, and nPhl p 12 were recorded from 260 to 197 nm using 0.1- or 0.2-cm path length cuvettes, and those of peptides were recorded from 250 to 180-nm using 0.02-cm path length cuvettes. Each spectrum resulted from an average of three scans, with 0.2-nm resolution at a scan speed of 20 nm/min. In the experiments comparing rPhl p 12, rPhl p 12-rs, and the peptides, the proteins/peptides were dissolved in PBS as follows: rPhl p 12, 0.2 mg/ml; rPhl p 12-rs, 0.2 mg/ml; peptide 2, 0.6 mg/ml; peptide 3, 1.6 mg/ml; peptide 4, 1.2 mg/ml; and peptide 5, 0.4 mg/ml. The CD spectrum of peptide 1 could not be recorded because of poor solubility. The comparison of rPhl p 12 and nPhl p 12 was performed with nPhl p 12 (concentration: 0.3 mg/ml) and rPhl p 12 (concentration: 0.8 mg/ml). Results are expressed as the molar mean residue ellipticity (θ) at a given wavelength.

The secondary structure contents of the recombinant proteins were calculated with CDFIT, a secondary structure estimation program provided by Jasco using the reference set as described (27).

Thermal denaturation curves for the two recombinant proteins were measured using 0.02- and 0.1-cm water jacket cylindrical cells thermostated by an external computer-controlled water bath. The data were recorded in a temperature range of 20°C to 95°C every 10 degrees by a step scan procedure with a heating rate of 60°C/h and a scan speed of 50 nm/min. The thermal denaturation curves were calculated and fitted to a sigmoidal function, and the transition temperature (*T_m*) was determined from the point of inflection using the program Origin 5.0 (MicroCal).

Model building of Phl p 12

A model for Phl p 12 was generated using the automated protein modeling server (28). The model is based on highly homologous profilin structures of

³ Abbreviations used in this paper: rs, restructured; rPhl, recombinant profilins from timothy grass pollen; CD, circular dichroism; HAS, human serum albumin; n, natural; RBL, rat basophil leukemia.

Table I. Amino acid sequences and characteristics of Phl p 12, Phl p 12-rs, and Phl p 12-derived peptides

Protein Name	Amino Acid Sequence ^a	No. of Amino Acids	PI ^b	Molecular Mass (kDa)	Overall Fold (α -Helix/ β -Sheet)
Phl p 12	MSWQTYVDEHLMCEIEGHHLASAAILGHDGTVWAQSAFDPQFKPEEIT GIMKDFDEPHLAPTGMFVAGAKYMIQGE EPGAVIRGKKGAGGITI KKTGQALVVGIYDEPMTPGQCNMVERLGDYLVQGM	131	4.92	14	84% (45% α -helix/26% β -sheet)
Phl p 12-rs	MEPGAVIRGKKGAGGITIKKTGQALVVGIYDEPMTPGQCNMVERLGD YLVQGM MSWQTYVDEHLMCEIEGHHLASAAILGHDGTVWAQSAF DPQFKPEEITGIMKDFDEPHLAPTGMFVAGAKYMIQGHHHHHH	138	5.68	15.1	37% (5% α -helix/21% β -sheet)
Phl p 12 peptide 1	MSWQTYVDEHLMCEIEGHHLASAC	24	4.77	2.8	Not soluble
Phl p 12 peptide 2	ILGHDGTVWAQSAFDPQFKPEEITGIC	27	4.31	2.9	Random coil
Phl p 12 peptide 3	MKDFDEPHLAPTGMFVAGAKYMIQGC	28	5.36	3	Random coil
Phl p 12 peptide 4	CEPGAVIRGKKGAGGITIKKTGQALVGIYDE	32	9.05	3.1	Random coil (16% β -sheet)
Phl p 12 peptide 5	PMTPGQCNMVERLGDYLVQGM	24	4.14	2.6	Random coil

^a Boldfaced, underlined sequences represent peptides 4 and 5 (see Fig. 2B).

^b Isoelectric point.

Hevea brasiliensis profilin (29), the birch pollen profilin (30), and the profilin from *Arabidopsis thaliana* (31). Loop regions were built automatically and the model was energy minimized with the program Gromos96 (32).

Allergic patients' IgE reactivity and T cell proliferation

Direct binding of IgE to the Phl p 12-derived peptides, rPhl p 12 and rPhl p 12-rs, was investigated by nondenaturing dot blot experiments. Two-microliter aliquots of the peptides rPhl p 12 and rPhl p 12-rs (concentration: 0.5 μ g/ μ l) and human serum albumin (HSA; used as control protein) were dotted onto nitrocellulose strips. The strips were exposed to patients' sera and bound IgE Abs were detected with ¹²⁵I-labeled anti-human IgE Abs (Phadia) (17).

Heparinized venous blood samples were collected from four profilin-allergic grass pollen allergic patients. PBMC were stimulated with rPhl p 12 or rPhl p 12-rs (0.5 μ g/ml), and, for control purposes, with 4 U of IL-2 per well (positive control; Boehringer-Mannheim), HSA (0.5 μ g/ml), or medium alone (negative controls) in triplicate. After 6 days, proliferative responses were measured by [³H]thymidine incorporation and expressed as stimulation indices (33).

ELISA inhibition of IgE binding to rPhl p 12

Sera from profilin-allergic patients were diluted 1/5 in PBS containing 0.05% (v/v) Tween 20 and 0.5% (w/v) BSA. Serum dilutions were incubated with rPhl p 12, rPhl p 12-rs, or BSA (10 μ g/ml serum dilution) overnight at 4°C and then allowed to react with ELISA plate-bound rPhl p 12 (coating concentration: 5 μ g/ml) as described (34). After overnight incubation 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 containing 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 of inhibition of IgE binding was calculated as follows: percentage of inhibition = $100 \times [(A - B)/A]$, where A represents the OD values obtained after incubation of serum with BSA and B represents the OD values after the incubation of serum with rPhl p 12 or rPhl p 12-rs, respectively.

Basophil activation experiments

Granulocytes were isolated from heparinized blood samples of profilin-sensitized patients ($n = 2$) by dextran sedimentation (35). After isolation, cells were incubated with various concentrations of rPhl p 12, rPhl p 12-rs, or a monoclonal anti-human IgE Ab (Immunotech) in histamine release buffer (Immunotech) at 37°C for 30 min. Thereafter, cell-free supernatants were recovered and subjected to histamine measurement by a radioimmunoassay (Immunotech). Total histamine was determined after freeze-thawing of cell samples. Histamine release (mean values of duplicate determinations) is expressed as a percentage of the total histamine (35).

In addition, RBL-2H3 cells (a rat basophil leukemia (RBL) cell line (RBL-703/21) transfected with the cDNA coding for the human high affinity IgE receptor chain; Ref. 36) were sensitized with human IgE from different profilin-allergic patients ($n = 6$). RBL-2H3 cells were plated in 96-well tissue culture plates (1×10^5 /well) and passive sensitization was performed by incubating cells with sera from six profilin-allergic patients and, for control purposes, with serum from one nonallergic individual at a final dilution of 1/10 overnight (36). Unbound Abs were removed by washing the cell layer three times 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 cell degranulation was induced by cross-linking the receptor-bound Phlp12-specific IgE with 0.1, 1, 10, and 100 ng/ml rPhlp12 or rPhlp12-rs, respectively. The release of β -hexosaminidase from activated RBL cells was measured as described (37).

Cross-reactivity of Phl p 12-rs-specific IgG Abs

Rabbits were immunized with rPhl p 12 or rPhl p 12-rs using Freund's complete (first immunization) and incomplete adjuvant (first booster injection after 4 wk, second booster injection after 7 wk) (Charles River). Rabbits were bled 8 wk after the first immunization.

Cross-reactivity of rabbit IgG Abs with various profilins was investigated by immunoblotting. Approximately 3 μ g/cm gel of recombinant pollen profilins (rPhl p 12, rBet v 2, and rArt v 4) and plant food profilins (rLit c 4, rAna c 1, rDau c 4, rCor a 2, and rMus xp 1) were separated by preparative electrophoresis on a 14% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes (Schleicher & Schuell) (38). The reactivity of rabbit IgG Abs raised against rPhl p 12 or rPhl p 12-rs with blotted profilins was visualized with 125 iodine-labeled donkey anti-rabbit IgG Abs (Amersham Biosciences) as described (17). Patients' IgE reactivity to blotted profilins was detected with 125 iodine-labeled anti-human IgE Abs (Pharmacia) (17).

Titration of rabbit IgG raised against rPhl p 12 and rPhl p 12-rs to ELISA plate-bound pollen and plant food profilins (5 μ g/ml) was done with dilutions (1/2000 to 1/64,000) of rabbit antisera. Profilin-specific IgG Abs were detected using HRP-labeled donkey anti-rabbit IgG antiserum (Amersham Biosciences).

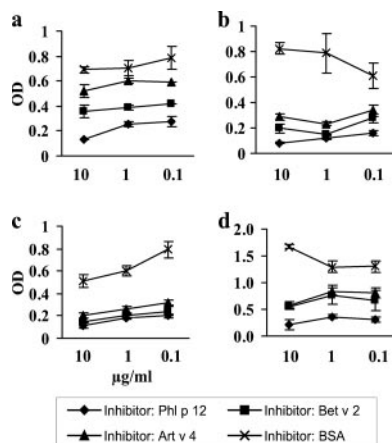


FIGURE 1. Inhibition of IgE binding to rPhl p 12 with pollen profilins. Sera from four profilin-sensitized patients (a–d) were preincubated with rPhl p 12 (◆), rBet v 2 (■), rArt v 4 (▲), or BSA (×). Concentrations of inhibitors are shown on the x-axis. Mean OD values from duplicate determinations $\pm$ SD corresponding to the amount of bound IgE are shown on the y-axis.

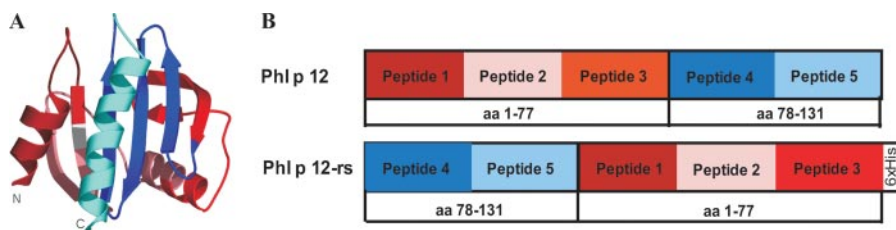


FIGURE 2. A, Ribbon representation of the structure of the timothy grass pollen profilin Phl p 12 modeled according to the three-dimensional structures of birch pollen, *A. thaliana*, and *H. brasiliensis* profilin. The N- and C-terminal helices involved in IgE recognition of profilins are indicated. Peptides without allergenic activity are colored in red, pink, and magenta (N-terminal part) and dark and light blue (C-terminal part). B, Linear representation of the Phl p 12 wild-type protein and the restructured tail-to-head derivative (Phl p 12-rs). The N- and C-terminal parts of Phl p 12 and the corresponding peptides are colored as in A. 6×His, Hexahistidine tag.

Inhibition of patients' IgE binding to pollen and plant-derived food profilins by IgG

ELISA plates (Nunc Maxisorp) were coated with rPhl p 12, rBet v 1, rDau c 4, rCor a 2, rMus xp 1, and rAna c 1 (1 μ g/ml) and preincubated with a 1/50 dilution of the anti-Phl p 12 antiserum, the anti-Phl p 12-rs antiserum, and, for control purposes, with the corresponding preimmune sera. After washing, plates were incubated with 1/3 diluted sera from eight profilin-sensitized patients and bound IgE Abs were detected with a HRP-labeled anti-human IgE antiserum from goat (Kirkegaard & Perry Laboratories) diluted 1/2500. The percentage of inhibition of IgE binding achieved by preincubation with the anti-Phl p 12 or anti-Phl p 12-rs antiserum was calculated as follows: percentage of inhibition of IgE binding = $100 - \text{OD}_p / \text{OD}_i \times 100$, where OD_i and OD_p represent the extinctions after preincubation with the rabbits' immune sera and the corresponding preimmune sera, respectively.

Immunization of mice

Six-week-old female BALB/c mice (Charles River) were immunized s.c. with either 10 μ g of rPhl p 12, 10 μ g of rPhl p 12-rs, or a equimolar mixture of the five peptides (2 μ g of each peptide) adsorbed to aluminum hydroxide (39). Sera were obtained via bleeding from the tail vein and stored at -20°C until use. Measurements of IgE and IgG1 specific for rPhl p 12, rPhl p 12-rs, and the peptides were done as described (39).

The question of whether immunization of mice with the different immunogens (i.e., rPhl p 12, rPhl p 12-rs, and the peptides) induces an allergic immune response against the Phl p 12 wild-type allergen (i.e., the in vivo allergenicity of the immunogens) was studied by RBL assay. RBL cells were loaded with sera from immunized mice and the presence of Phl p 12-specific reaginic Abs was demonstrated by inducing degranulation with the Phl p 12 allergen as described (40).

Inhibition of RBL cell degranulation with rPhl p 12-rs-specific IgG

The ability of rPhl p 12- and rPhl p 12-rs-specific IgG to inhibit rPhl p 12-induced degranulation of RBL cells was determined as follows. RBL cells loaded with profilin-specific mouse-IgE obtained by sensitization of mice with rPhl p 12 were exposed to rPhl p 12 (1 ng/ml) that had been preincubated in Tyrode's buffer with 0, 2, 5, 7.5, or 10% (v/v) of rPhl p 12- or rPhl p 12-rs-specific rabbit Abs or the corresponding preimmune sera for 2 h at 37°C (26). Similarly, RBL-2H3 (RBL-703/21) cells transfected with cDNA coding for the human high affinity IgE receptor chain (36) were passively sensitized with serum from three profilin-allergic patients as described above in this section in the paragraph *Basophil activation experiments*. In these experiments, the ability of rPhl p 12- and rPhl p 12-rs-specific IgG to inhibit Phl p 12-induced RBL degranulation was determined by the preincubation of rPhl p 12 in Tyrode's buffer with 10% (v/v) heat-inactivated (56°C) rPhl p 12- or rPhl p 12-rs-specific rabbit antiserum or the corresponding preimmune sera for 2 h at 37°C .

The reactants were then added to the RBL cells and the release of β -hexosaminidase was measured as described (37). Results are reported as fluorescence units and the percentage of total β -hexosaminidase release after lysis of cells with 1% Triton X-100.

Results

Phl p 12 contains the majority of IgE epitopes among pollen profilins

The ELISA IgE inhibition experiments in Fig. 1 demonstrate that, for sera from four representative profilin-allergic patients, rPhl p

12 contains the majority of IgE epitopes when compared with Bet v 2 and Art v 4. Using three different concentrations of rPhl p 12, rBet v 2, or rArt v 4 (0.1, 1, and 10 μ g/ml), rPhl p 12 inhibited IgE reactivity to rPhl p 12 best, whereas rBet v 2 and rArt v 4 were less effective.

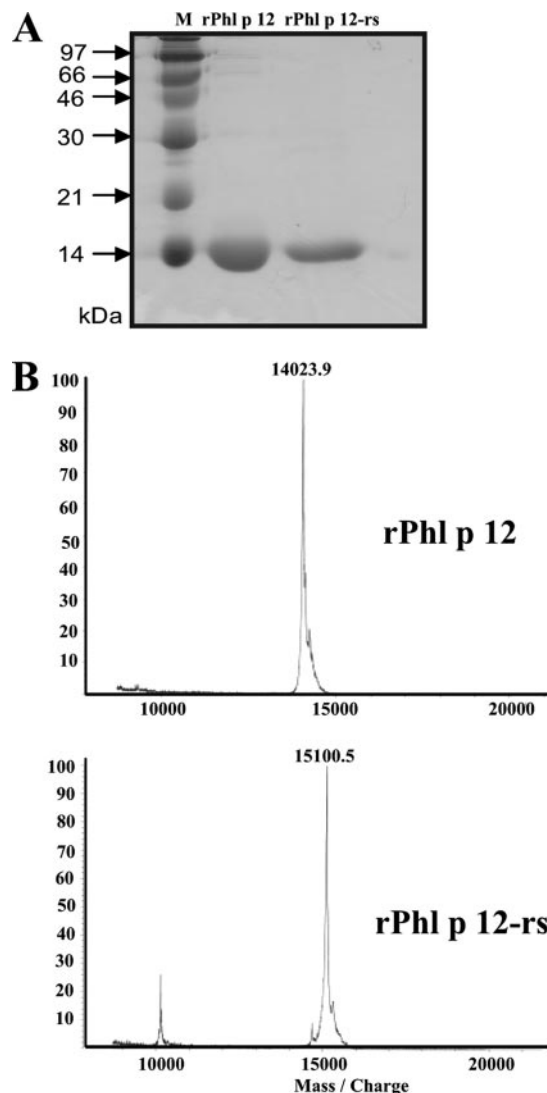


FIGURE 3. A, Coomassie-stained SDS PAGE. A molecular mass marker (lane M) and purified rPhl p 12 and rPhl p 12-rs were loaded. Molecular masses (kDa) are indicated on the left margin. B, Mass spectrometry analysis of rPhl p 12 and rPhl p 12-rs. The x-axis shows the mass/charge ratio, and signal intensity is displayed on the y-axis as a percentage of the most intensive signal obtained in the investigated mass range.

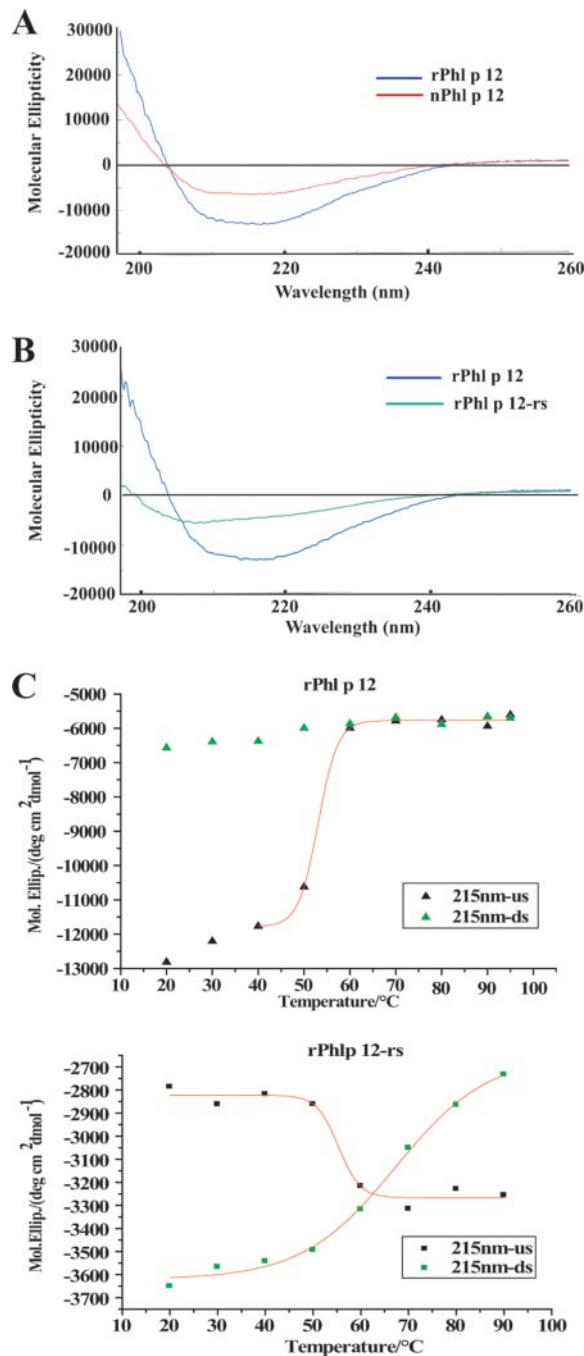


FIGURE 4. A and B, Comparison of rPhl p 12 with nPhl p 12 (A) and rPhl p 12 with rPhl p 12-rs (B) by CD. The mean-residue molar ellipticity in degree cm² dmol⁻¹ (y-axis) was recorded for a range of wavelengths (x-axis). C, Temperature-dependent changes of ellipticity at 215 nm (y-axis) plotted as an average of three adjacent wavelengths were determined for rPhl p 12 and rPhl p 12-rs over a temperature range of 20°C to 90/95°C (x-axis). Up-scan (us; black symbols) indicates molar ellipticity during increase of temperature; down-scan (ds; green symbols) indicate molar ellipticity while cooling down.

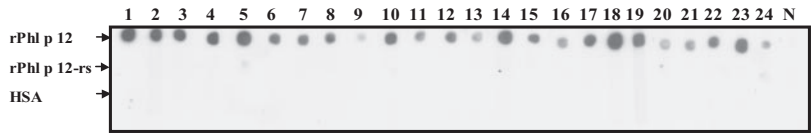


FIGURE 5. IgE reactivity of nitrocellulose-dotted rPhl p 12 and rPhl p 12-rs. Dotted proteins as well as HSA (negative control) were exposed to sera from 24 profilin-allergic patients (lanes 1–24). Lane N represents serum from a nonallergic person. Bound IgE Abs were detected with anti-human IgE Abs and visualized by autoradiography.

Table II. ELISA inhibition of patients' IgE binding to Phl p 12^a

Patient	Optical Density			Percentage (%) Inhibition of IgE binding to Phl p 12 by	
	BSA	Phl p 12	Phl p 12-rs	Phl p 12	Phl p 12-rs
1	0.66	0.08	0.48	87.5	29.3
2	0.59	0.09	0.39	84.7	33.7
3	0.52	0.12	0.32	76.1	38.4
3	0.85	0.15	0.68	86.5	20.2
4	0.86	0.08	0.64	90.6	25.3
5	1.53	0.13	1.05	91.4	31.4
6	0.88	0.12	0.52	85.9	40.8
Mean				86.0	31.0

^a Sera from six profilin-allergic patients were preadsorbed with BSA, rPhl p 12, and rPhl p 12-rs. OD values corresponding to IgE antibodies bound to coated rPhl p 12 are shown. Percentages of inhibition of IgE binding to rPhl p 12 are displayed for the six sera and the mean inhibitions were calculated.

Genetic engineering of rPhl p 12-rs, a tail-to-head derivative of timothy grass pollen profilin

The strategy for converting the timothy grass pollen profilin Phl p 12 into a hypoallergenic vaccine is based on the recombination of low allergenic fragments in the form of a restructured protein, termed Phl p 12-rs. In a first step, Phl p 12-derived peptides of a length between 22 and 29 aa spanning the whole Phl p 12 sequence were synthesized (Table I and Fig. 2, A and B). Because these isolated peptides lacked IgE reactivity, two larger Phl p 12 fragments, one comprising the three N-terminal peptides (peptides 1–3; i.e., amino acids 1–77 of Phl p 12) and a second representing the C-terminal peptides 4 and 5 (i.e., amino acids 78–131), were produced as a tail-to-head fusion protein containing the C-terminal fragment on its N terminus and the N-terminal fragment on its C terminus (Fig. 2B). The peptides are indicated in a model of the Phl p 12 structure that was generated according to the three-dimensional structure of the birch pollen profilin Bet v 2, the *A. thaliana* profilin, and the *H. brasiliensis* latex profilin Hev b 8 (Fig. 2A) (29, 30). Because the IgE epitopes of the highly cross-reactive birch profilin primarily map to the N- and C-terminal portions of Bet v 2 (30), it was expected that the restructuring will lead to a strong reduction of IgE recognition.

Purification of rPhl p 12-rs and rPhl p 12

rPhl p 12 was expressed in *E. coli* and purified by poly-L-proline affinity chromatography as described (22). rPhl p 12 accumulated in the soluble cytoplasmic fraction of *E. coli* yielding ~5 mg/L culture. The restructured Phl p 12, rPhl p 12-rs, was found in the insoluble inclusion body fraction of *E. coli* (~1 mg/l culture) and hence required preparation under denaturing conditions followed by the subsequent refolding of the protein. It had lost the characteristic affinity of profilins to poly-L-proline and therefore was purified to homogeneity by nickel affinity chromatography via a hexahistidine tag that had been added to its C terminus (Fig. 2B).

Table III. Human T cell Proliferation^a

Patient	Stimulation Index		
	rPhl p 12	rPhl p 12-rs	IL-2
1	3.8	3.7	25.1
2	4.2	6	11.6
3	7.9	5.8	32.7
4	4.4	5.5	9.5
Mean	5.1	5.2	

^a PBMC from four profilin-allergic patients were stimulated with rPhl p 12, rPhl p 12-rs, or IL-2 and stimulation indices are displayed. Mean stimulation indices are shown for rPhl p 12 and rPhl p 12-rs.

Fig. 3A shows the purified rPhl p 12 and rPhl p 12-rs in a Coomassie-stained SDS-PAGE. Mass spectrometry confirmed the molecular mass predicted for purified rPhl p 12-rs (15.1 kDa), whereas the experimental mass from rPhl p 12 was 14.0 kDa, indicating the cleavage of the N-terminal methionine (Fig. 3B).

CD analysis demonstrates that the overall structure and refolding behavior of rPhl p 12-rs is different from that of rPhl p 12

The CD spectrum of rPhl p 12 at 20° shows a folded protein with a mixed $\alpha\beta$ fold (Fig. 4, A and B). The calculation of the

secondary structure content revealed 45% α -helical and 26% β -sheet content of rPhl p 12 with an overall fold of 84% (Table I). The comparison of the CD spectra of recombinant Phl p 12 and natural Phl p 12 showed that they were of comparable shape, indicating that both protein preparations exhibit similar secondary structures (Fig. 4A).

The rPhl p 12-rs preparation contained only ~37% folded protein. The folded portion resembled mainly a β -sheet structure (31%) with a strongly reduced α -helical portion (5%) (Fig. 4B and Table I).

The thermal stability and the refolding capacity of rPhl p 12 and rPhl p 12-rs were determined by a step-scan procedure (Fig. 4C). The unfolding temperatures (T_m transition temperatures) of rPhl p 12 (53.1°C) and rPhl p 12-rs (55.3°C) were very similar. For the rPhl p 12-rs protein a second transition occurred above 90°C (data not shown). rPhl p 12 did not refold upon cooling, whereas rPhl p 12-rs showed a gradual increase of the CD signal at 215 nm upon cooling from 90°C to 20°C with a marked transition temperature of 67.3°C (Fig. 4C).

For peptide 1 no CD measurements could be performed because of the bad solubility of the peptide. Peptides 2, 3, and 5 presented random coil spectra (pronounced minimum at 200 nm), and only peptide 4 exhibited some residual β -sheet conformation (Table I).

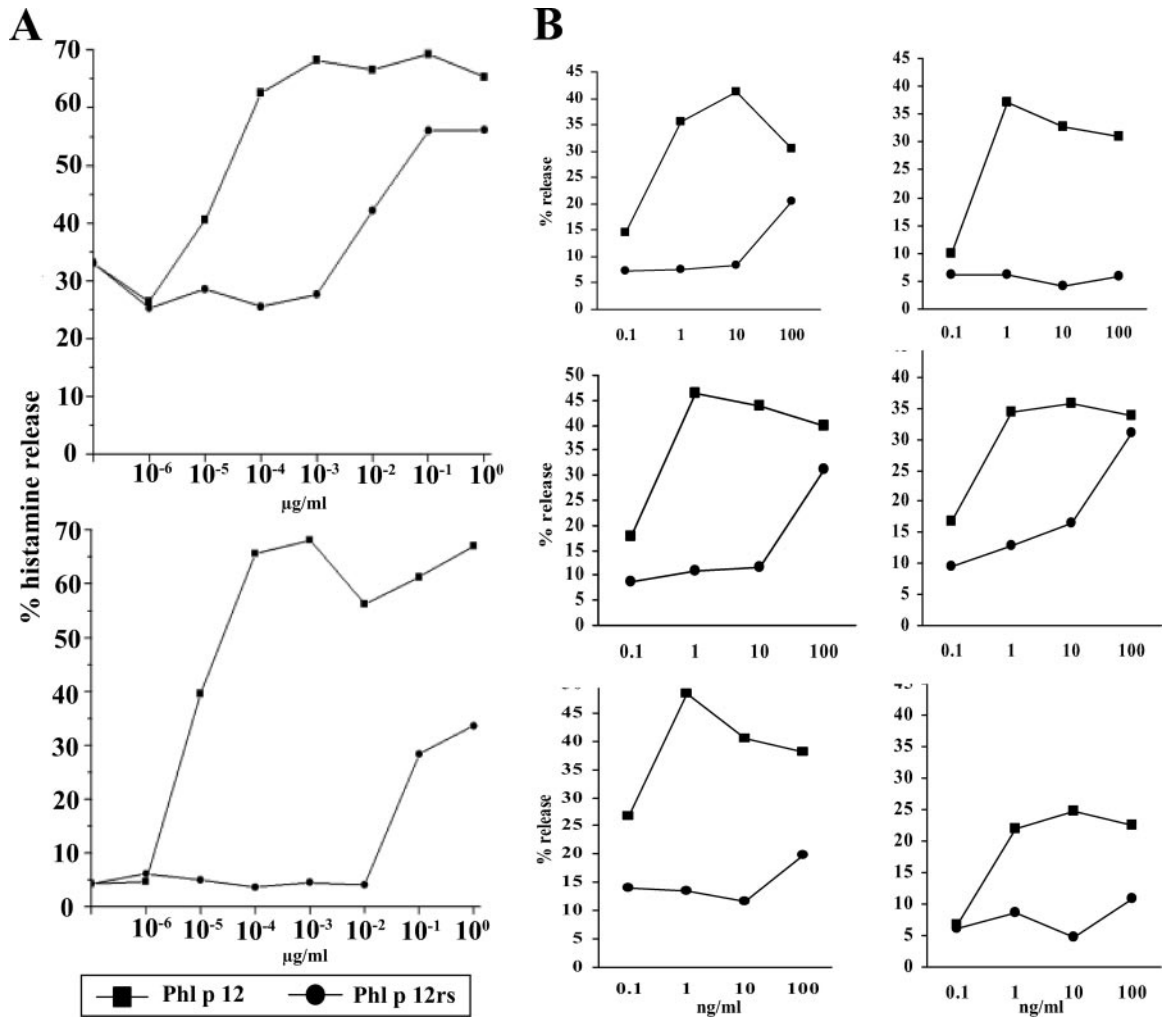


FIGURE 6. A, Induction of basophil histamine release in two profilin-allergic patients. Patients' granulocytes were incubated with various concentrations (x-axis) of rPhl p 12 (■) and rPhl p 12-rs (●). B, Induction of β -hexosaminidase release from a RBL cell line expressing the human Fc ϵ RI. RBL cells were passively sensitized with IgE Abs from six profilin-allergic patients and incubated with increasing concentrations (x-axis) of rPhl p 12 (■) and rPhl p 12-rs (●). The results show the percentages of total mediator released into the supernatants (y-axis).

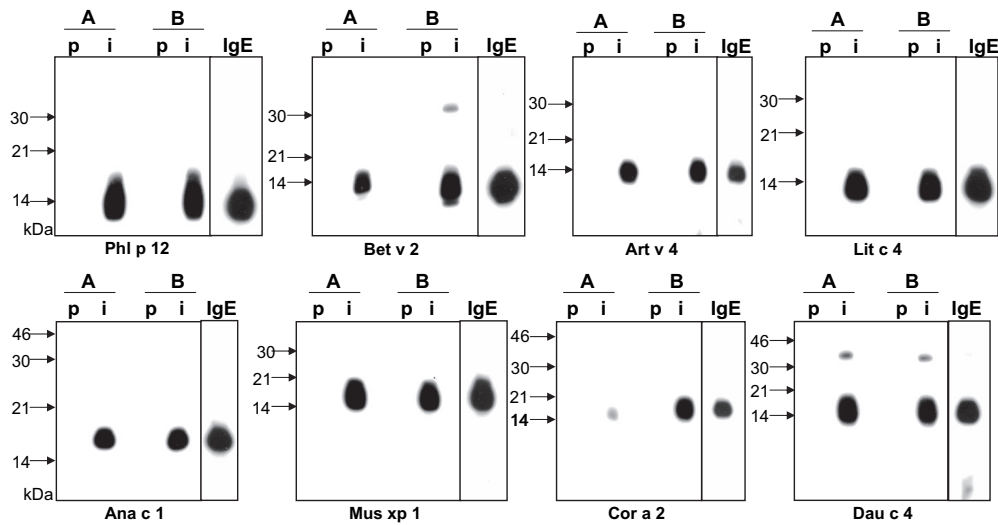


FIGURE 7. Cross-reactivity of Abs raised against rPhl p 12 and rPhl p 12-rs with various pollen and plant food profilins. The preimmune (*lanes p*) and immune sera (*lanes i*) of rabbits immunized with rPhl p 12 (*A*) and rPhl p 12-rs (*B*) as well as serum IgE from a profilin-allergic patient (*IgE*) were exposed to nitrocellulose-blotted recombinant profilins from timothy grass pollen (Phl p 12), birch pollen (Bet v 2), mugwort pollen (Art v 4), lychee (Lit c 4), cashew nut (Ana c 1), banana (Mus xp 1), hazelnut (Cor a 2), and carrot (Dau c 4). Molecular masses (kDa) are shown in the *left margins*.

Reduced IgE binding capacity but preserved T cell reactivity of rPhl p 12-rs

A nondenaturing dot blot analysis of IgE reactivity to rPhl p 12 and rPhl p 12-rs was performed with sera from 24 profilin-allergic patients. Each of these sera showed IgE reactivity to rPhl p 12, but only two sera showed very weak IgE reactivity to rPhl p 12-rs (Fig. 5). The IgE reactivity of the soluble proteins was compared by using IgE ELISA inhibition experiments (Table II). Sera from six profilin-sensitized patients were preadsorbed with rPhl p 12, rPhl p 12-rs, or BSA and the remaining IgE reactivity to plate-bound rPhl p 12 was quantified (Table II). Preadsorption of sera with rPhl p 12-rs led to an inhibition of IgE reactivity to rPhl p 12 ranging

from 20 to 40% with mean inhibition of 31.2%, whereas rPhl p 12 strongly inhibited IgE reactivity (range: 76–91%; mean inhibition: 86%). Both IgE binding assays thus confirm the reduced IgE reactivity of rPhl p 12-rs compared with that of rPhl p 12.

Although IgE recognition of rPhl p 12-rs was impaired, T cell epitopes appeared to be preserved. rPhl p 12-rs induced a comparable proliferation in PBMC from four profilin-allergic patients as that of rPhl p 12 (Table III). The proliferation induced with HSA was in the range of that observed with medium alone (negative controls; stimulation index: 1) (data not shown). IL-2 (positive control) induced proliferations ranging from 9.5 to 32.7 (stimulation index values), indicating the responsiveness of T cells (Table III).

FIGURE 8. Titration of antisera raised by immunization with rPhl p 12 and rPhl p 12-rs for reactivity with various pollen and plant food profilins. ELISA plate bound recombinant profilins from timothy grass pollen (Phl p 12), birch pollen (Bet v 2), mugwort pollen (Art v 4), lychee (Lit c 4), cashew (Ana c 1), banana (Mus xp 1), hazelnut (Cor a 2), and carrot (Dau c 4) were incubated with different dilutions (1/2000 to 1/64,000) of rabbit antisera raised against rPhl p 12 and rPhl p 12-rs. OD values corresponding to the amounts of bound Abs are displayed for each serum dilution.

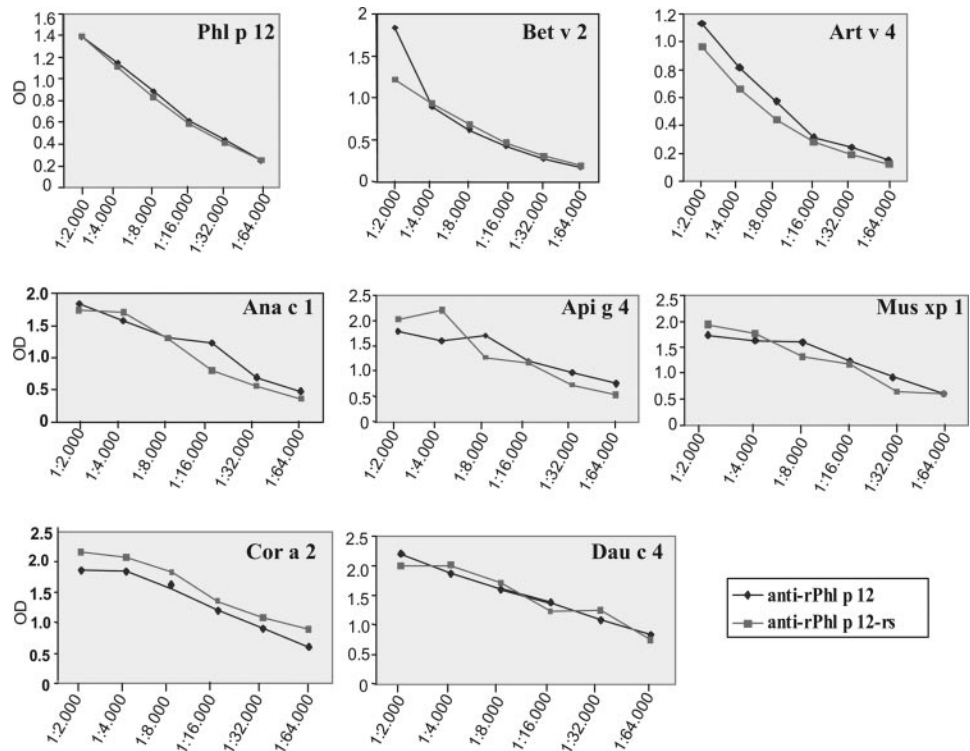
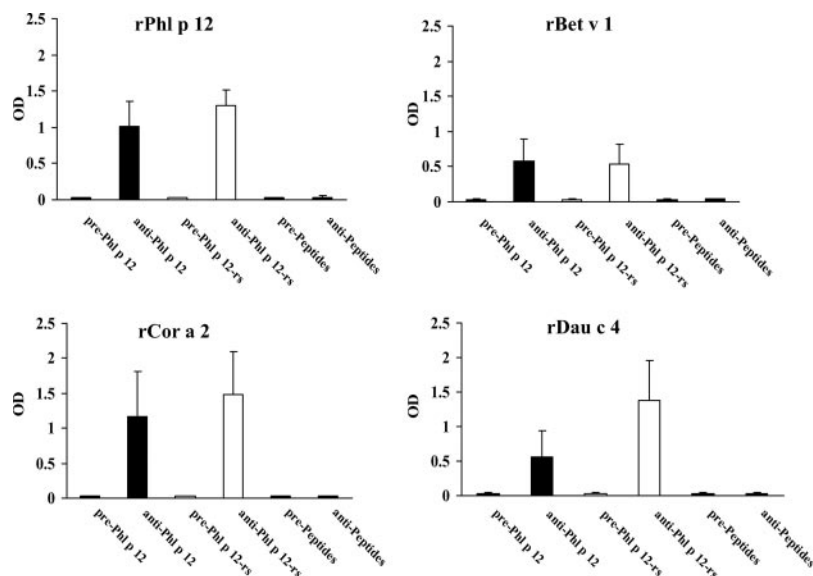


FIGURE 9. Induction of profilin-specific IgG1 Abs in mice. IgG1 levels (mean OD values $\pm$ SD; y-axes) specific for rPhl p 12, rBet v 2, rCor a 2, and rDau c 4 are displayed for the preimmune (pre-) and immune (anti-) sera of mice that had been immunized with rPhl p 12, rPhl p 12-rs, or with Phl p 12-derived peptides.



Reduced allergenic activity of rPhl p 12-rs as demonstrated by basophil histamine release experiments

Basophil granulocytes from two Phl p 12-allergic patients were exposed to various concentrations of rPhl p 12 and rPhl p 12-rs to study their allergenic activity (Fig. 6A).

rPhl p 12 induced strong and dose-dependent histamine release in basophils from both patients, yielding maximal histamine release at concentrations of 10^{-3} μ g/ml, whereas a similar maximal histamine release could not be induced by rPhl p 12-rs up to the highest concentration of 1 μ g/ml, indicating a >100 -fold reduction of allergenic activity compared with that of rPhl p 12 (Fig. 6A).

In a separate set of experiments a RBL cell line transfected with the human Fc ϵ RI was used. RBL cells were loaded with serum IgE Abs from six profilin-sensitized patients and stimulated with increasing concentrations of rPhl p 12 and rPhl p 12-rs. A 100-fold reduction, at the least, in the allergenic activity of rPhl p 12-rs compared with that of rPhl p 12 was observed for three sera, and a >10 -fold reduction of allergenicity was observed for the other three sera (Fig. 6B).

rPhl p 12-rs has reduced *in vivo* allergenicity but induces IgG Abs that cross-react with pollen and plant food profilins

Immunization of rabbits with rPhl p 12-rs induced IgG Abs that reacted with rPhl p 12 as well as with profilins from birch pollen (rBet v 2), mugwort pollen (rArt v 4), and the lychee (rLit c 4), cashew nut (rAna c 1), banana (rMus xp 1), hazelnut (rCor a 2), and carrot (rDau c 4) (Fig. 7). It appeared that certain profilins (e.g., Bet v 2 and Cor

a 2) were even more strongly recognized by rPhl p 12-rs-induced IgG Abs than by IgG Abs induced with rPhl p 12 (Fig. 7). The specificity of the profilin-specific IgG reactivity is demonstrated by the lack of reactivity of the rabbits' preimmune sera. As exemplified for a profilin-allergic patient, we found that patients' IgE Abs cross-reacted with all of the profilins tested (Fig. 7). The magnitude of the IgG Ab responses induced with rPhl p 12 and rPhl p 12-rs was compared by ELISA titration experiments showing that immunization with the wild-type as well as the restructured protein induced comparable titers of IgG Abs specific for pollen and plant food profilins (Fig. 8).

Similar results were obtained when mice were immunized with rPhl p 12-rs adsorbed to aluminum hydroxide (Fig. 9). rPhl p 12-rs induced IgG1 reactivity to rPhl p 12, rBet v 2, rCor a 2, and rDau c 4 that was of comparable magnitude as the reactivity induced by rPhl p 12. Phl p 12-derived synthetic peptides did not induce relevant IgG1 responses (Fig. 9).

Next, we mapped the epitope specificity of the murine IgG1 response and found that rPhl p 12-rs-induced IgG Abs recognized similar peptides as IgG Abs obtained by immunization with rPhl p 12. Immunization with Phl p 12-derived peptides induced IgG1 only against one of the five peptides tested (Table IV).

Finally we analyzed the *in vivo* allergenicity of rPhl p 12-rs (i.e., the capacity of rPhl p 12-rs to induce an allergic immune response

Table IV. Recognition frequency of Phl p 12-derived peptides in immunized mice^a

Peptide	Recognition frequency of Phl p 12-derived peptides among mice immunized with		
	rPhl p 12	rPhl p 12-rs	Peptides
1	2	2	0
2	1	2	0
3	1	3	5
4	0	3	0
5	1	2	0

^a Groups of mice ($n = 5$) were immunized with rPhl p 12 or rPhl p 12-rs or with Phl p 12-derived peptides. Displayed is the number of mice from each group mounting IgG1 antibodies to peptides spanning the Phl p 12 sequence.

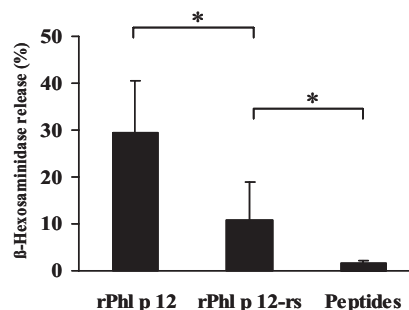


FIGURE 10. Reduced allergenicity of rPhl p 12-rs and Phl p 12-derived peptides. Rat basophils were loaded with IgE from mice immunized with rPhl p 12, rPhl p 12-rs, or Phl p 12-derived peptides and then exposed to rPhl p 12. The percentages of β -hexosaminidase release are displayed (y-axis). Statistically significant differences ($p < 0.05$) between the groups regarding release are indicated by an asterisk.

Table V. Inhibition of allergic patients' IgE binding to various profilins by IgG Abs^a

Patient	Percentage Inhibition of IgE binding to											
	Phl p 12		Bet v 2		Cor a 2		Mus xp 1		Dau c 4		Ana c 1	
	a-Phl p 12	a-Phl p 12-rs	a-Phl p 12	a-Phl p 12-rs	a-Phl p 12	a-Phl p 12-rs	a-Phl p 12	a-Phl p 12-rs	a-Phl p 12	a-Phl p 12-rs	a-Phl p 12	a-Phl p 12-rs
1	91	82.7	88.3	84.4	61.3	58.7	70.6	46.4	82.7	83.3	70	71.7
2	82.5	72.8	74.9	77.6	73.3	60.3	74.4	50.8	75.5	83.2	62.3	45.1
3	89.4	72.3	75.4	76.4	58.8	61.6	74.4	33.5	65.4	63.1	61.6	56
4	77.4	72	69	69.6	56.00	52	57.2	39.8	65	66.8	71.5	41.5
5	86.3	65.2	35	66.4	68.6	52	75.5	27.2	97.5	93.8	59.5	56.2
6	71.2	84.7	54.4	72	57	62.7	73.9	25.6	58.5	68.3	44.3	42.6
7	83.6	70.4	60.2	70.8	69.7	59.7	72.5	35.6	72.4	71.3	28.1	32.2
8	89.1	57.4	61	79	57.8	57.8	73	29.6	70	67	ND	ND
Mean	83.8	72.3	64.8	74.5	62.3	58.1	71.4	36.1	73.3	74.6	56.8	53.6

^a The inhibition of IgE binding to recombinant profilins from grass (Phl p 12), birch pollen (Bet v 2), and plant foods (hazelnut, Cor a 2; banana, Mus xp 1; carrot, Dau c 4; and cashew nut, Ana c 1) achieved with rPhl p 12- and rPhl p 12-rs-induced IgG (a-Phl p 12 or a-Phl p 12-rs) is shown for eight patients. The average inhibition of IgE binding is displayed. ND, Not done.

against the wild-type allergen). rPhl p 12-rs- and Phl p 12-derived peptides induced significantly less reagenic IgE Abs specific for the Phl p 12 wild-type allergen than rPhl p 12 upon the immunization of mice (Fig. 10).

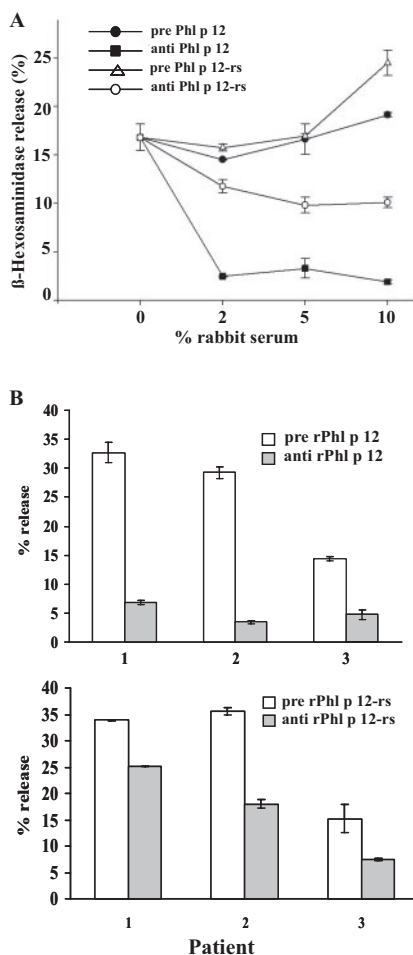


FIGURE 11. Inhibition of Phl p 12-induced basophil degranulation by anti-Phl p 12- and anti-rPhl p 12-rs-induced IgG. *A*, Rat basophils had been loaded with rPhl p 12-specific mouse IgE and exposed to rPhl p 12 preincubated with increasing concentrations (x-axis) of rabbit preimmune (pre) and immune (anti) sera. *B*, A rat basophil cell line transfected with the human FcεRI had been loaded with human IgE from three profilin-sensitized patients and exposed to rPhl p 12 preincubated with 10% v/v of rabbit preimmune (pre) and immune (anti) sera. The percentages of β-hexosaminidase release are displayed (y-axis).

rPhl p 12-rs induced Abs inhibit patient's IgE binding to pollen and plant food profilins to a similar extent than rPhl p 12-induced Abs

The question of whether Phl p 12-rs induced IgG Abs block allergic patients' IgE binding to profilins from several allergen sources was investigated by ELISA. The mean inhibition of IgE binding to timothy grass pollen profilin achieved with Phl p 12-induced Abs and Phl p 12-rs-induced Abs was comparable with 83.8 and 72.3%, respectively (Table V). IgE binding to the birch pollen profilin Bet v 2 was inhibited even stronger with Phl p 12-rs-specific Abs (mean inhibition: 74.5%) than with Phl p 12-induced Abs (mean inhibition: 64.8%). IgE binding to plant food profilins were inhibited with both antisera to a very similar degree (Cor a 2: 62.3% average inhibition with anti-Phl p 12-IgG, 58.1% with anti-Phl p 12-rs-IgG; Dau c 4: 73.3% average inhibition with anti-Phl p 12-IgG, 74.6% with anti-Phl p 12-rs-IgG; Ana c 1: 56.8% average inhibition with anti-Phl p 12-IgG, 53.6% with anti-Phl p 12-rs-IgG). Only IgE binding to banana profilin, Mus xp 1, was less inhibited with anti-Phl p 12-rs-induced IgG (36.1%) than with anti-Phl p 12-induced IgG (71.4%) (Table V).

IgG Abs induced by immunization with Phl p 12-rs inhibit Phl p 12-induced basophil degranulation

The biological relevance and possible protective activity of IgG Abs induced by immunization with rPhl p 12-rs were investigated in a defined cellular model system using RBL cells that were loaded with profilin-specific IgE. Preincubation of rPhl p 12 with increasing concentrations (2–10% (v/v)) of rabbit anti-Phl p 12-rs Abs and rabbit anti-Phl p 12 Abs led to a dose-dependent inhibition of Phl p 12-induced mediator release from RBL cells loaded with IgE Abs from mice that were sensitized to Phl p 12 (Fig. 11A). No inhibition of basophil degranulation was observed when the allergen was preincubated with the same concentration of the corresponding preimmune Ig.

These results were confirmed when RBL cells expressing the human FcεRI receptor were passively sensitized with serum IgE Abs from three profilin-allergic patients (Fig. 11B). Again, anti-Phl p 12 Abs and, to a lower degree, anti-rPhl p 12-rs Abs inhibited rPhl p 12-induced degranulation (Fig. 11B).

Discussion

Patients sensitized to profilins, a family of highly conserved cytoskeletal proteins, may show allergic reactions to pollens and foods from a variety of plants (17–24, 41–46). We report the engineering and characterization of a vaccine for the treatment of those patients who exhibit broad pollen and plant food cross-reactivities due to profilin sensitization. The timothy grass pollen

profilin Phl p 12 was identified as a template for the vaccine, because IgE cross-inhibition studies have shown that it contains the majority of relevant IgE epitopes among plant profilins. Our finding was in accordance with previous studies suggesting that grass pollen profilins may be the primary sensitizing molecules in most profilin-allergic individuals (44, 45).

A new strategy based on “tail-to-head” fusion of Phl p 12-derived low-allergenic fragments was used to generate a restructured Phl p 12, termed rPhl p 12-rs. rPhl p 12-rs showed a strong reduction of IgE reactivity, which appeared to be due to changes in the profilin secondary structure. The three-dimensional structure of the birch profilin Bet v 2 has been solved by x-ray crystallography and IgE epitopes have been mapped (29), primarily to the N-terminal and C-terminal α -helices. We thus assumed that a head-to-tail restructuring of Phl p 12 would severely compromise the α -helical contents and the IgE-reactivity of this molecule. Indeed, the restructured rPhl p 12-rs exhibited a reduced α -helical content and a strong reduction of allergenic activity, whereas a considerable portion of β -sheet conformation was preserved. It is possible that the reduction of the overall fold of rPhl p 12-rs also contributed to its reduced IgE reactivity. However, it should be noted in this context that rPhl p 12 did not refold after heating but still showed IgE reactivity after a boiling and denaturing SDS-PAGE, which speaks against the fact that denaturation has caused reduced IgE reactivity. Moreover, rPhl p 12-rs did not only present an altered fold but, in contrast to rPhl p 12, regained fold after thermal denaturation. We therefore think that the reduction of the IgE reactivity of rPhl p 12-rs is most likely due to the change of the α -helical elements rather than to an overall reduction of fold.

rPhl p 12-rs induced >100-fold less histamine release from basophil granulocytes of profilin-allergic patients than rPhl p 12 and 10- to 100-fold reduced degranulation in a humanized RBL cell line that had been loaded with IgE from six profilin-allergic patients, indicating that it will induce fewer and lesser therapy-related side effects mediated by IgE.

rPhl p 12-rs and the Phl p 12-derived peptides induced lower allergenic immune responses to the wild-type allergen than rPhl p 12 upon immunization of the mice and thus exhibited reduced in vivo allergenicity. However, only rPhl p 12-rs immunization, but not peptide immunization, induced robust Phl p 12-specific IgG responses. In addition, immunization with rPhl p 12-rs induced IgG Abs that cross-reacted with a broad variety of pollen and plant food profilins. Both, the degree of cross-reactivity and the titer of these IgG Abs were similar to those induced by immunization with the rPhl p 12 wild-type allergen. Another important result was that the IgG Abs induced by immunization with rPhl p 12-rs were able to inhibit allergic patients' IgE binding to profilins both from pollens and from plant-derived food. Furthermore, rPhl p 12-rs-induced IgG Abs specifically inhibited profilin-induced degranulation of basophils. We thus anticipate that vaccination with rPhl p 12-rs will induce a similar kind of counterimmune response as has been observed when birch pollen allergic patients were treated with genetically modified variants of the major birch pollen allergen, Bet v 1. In this vaccination study, actively treated patients developed a strong IgG response against natural Bet v 1 and cross-reactive allergens that specifically suppressed allergen-induced basophil degranulation, nasal inflammation, and boosting of the IgE memory responses (14–16).

The strategy of generating recombinant hypoallergenic allergen derivatives for allergy vaccination by “tail-to-head” fusion of allergen fragments has not been described previously. We believe that it may have several advantages over previously published strategies (e.g., T cell peptides, fragmentation, mutations, and deletions).

For example, the approach of using allergen-derived, T cell epitope-containing peptides is hampered by the fact that a large number of different peptides must be included in a therapeutic vaccine to cover the T cell epitope repertoire of the complete allergen. The latter approach is primarily thought to modulate T cell responses but does not induce protective IgG responses. In contrast, the rPhl p 12-rs molecule contains the full primary sequence of the Phl p 12 wild-type molecule and thus the corresponding T cell epitopes. Furthermore it seems to contain enough Phl p 12-derived sequences (i.e., B cell epitopes) to induce protective Ab responses against the wild-type allergen upon immunization. We consider the possibility that vaccination with rPhl p 12-rs will induce a T cell response against “neoepitopes” possibly created through the reorganization of the molecule as an unlikely event, because the sequence derived from the fusion of the fragments only matched profilin sequences in the databases.

The reduction of IgE reactivity by mutations and deletions normally requires multiple mutations and may lead to the loss of sequences necessary for the induction of T cell or Ab responses, whereas the restructuring does not require extensive alterations or deletions. Fragmentation of allergens has a disadvantage in that it delivers several recombinant allergen fragments or peptides that need to be produced separately, whereas restructuring a molecule overcomes this problem.

In conclusion, we have developed a new strategy to engineer a restructured hypoallergenic variant of timothy grass pollen profilin that may be used for the treatment of profilin-allergic patients suffering from broad cross-reactivities. The approach of reassembling hypoallergenic allergen variants by the “tail-to-head” approach may be applied to the engineering of similar vaccines for many other allergen sources.

Disclosures

The authors have no financial conflict of interest.

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