

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

VERENA NIEDERBERGER,^{*,†} BRIGITTE HAYEK,[‡] SUSANNE VRTALA,[‡] SYLVIA LAFFER,[‡] ANNA TWARDOSZ,[‡] LUCA VANGELISTA,^{||} WOLFGANG R. SPERR,[§] PETER VALENT,[§] HELMUT RUMPOLD,[†] DIETRICH KRAFT,[‡] KLAUS EHRENBERGER,^{*} RUDOLF VALENTA,^{‡,1} AND SUSANNE SPITZAUER[†]

^{*}Department of Otorhinolaryngology, [†]Institute of Medical and Chemical Laboratory Diagnostics,

[‡]Institute of General and Experimental Pathology, AKH, University of Vienna, Austria; ^{||}European Molecular Biology Laboratory, Heidelberg, Germany; and [§]Department of Internal Medicine I, Division of Hematology, AKH, University of Vienna, Austria

ABSTRACT Type I allergy, an immunodisorder that affects almost 20% of the population worldwide, is based on the immunoglobulin E (IgE) recognition of *per se* innocuous antigens (allergens). Pollen from wind-pollinated plants belong to the most potent allergen sources. We report the isolation of a cDNA coding for a 8.6 kDa two EF-hand calcium binding allergen, Phl p 7, from a timothy grass (*Phleum pratense*) pollen expression cDNA library, using serum IgE from a grass pollen allergic patient. Sequence analysis identified Phl p 7 as a member of a recently discovered subfamily of pollen-specific calcium binding proteins. Recombinant Phl p 7 was expressed in *Escherichia coli* and purified to homogeneity as determined by mass spectroscopy. Approximately 10% of pollen allergic patients displayed IgE reactivity to rPhl p 7 and Phl p 7-homologous allergens present in pollens of monocotyledonic and dicotyledonic plants. Circular dichroism analysis of the calcium-bound and apo-rPhl p 7 indicated that differences in IgE recognition may be due to calcium-induced changes in the protein conformation. The fact that patients mount IgE antibodies against different protein conformations is interpreted as a footprint of a preferential sensitization against either form. The biological activity of rPhl p 7 was demonstrated by its ability to induce basophil histamine release and immediate type skin reactions in sensitized individuals. In conclusion, IgE binding to Phl p 7 represents an example for the conformation-dependent IgE recognition of an allergen. Recombinant Phl p 7 may be used for diagnosis and perhaps treatment of a group of patients who suffer from allergy to pollens of many unrelated plant species.—Niederberger, V., Hayek, B., Vrtala, S., Laffer, S., Twardosz, A., Vangelista, L., Sperr, W. R., Valent, P., Rumpold, H., Kraft, D., Ehrenberger, K., Valenta, R., Spitzauer, S. 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 (1999)

Key Words: Type I allergy • allergen • EF-hand protein • conformational epitopes • circular dichroism spectroscopy • cross-reactivity • pollen-specific expression

ALMOST 20% OF the population in industrialized countries suffer from Type I allergy, a genetically determined immunodisorder that is based on the formation of immunoglobulin E (IgE)² antibodies against *per se* innocuous antigens (1, 2). Immediate type symptoms (allergic rhinitis, conjunctivitis, and asthma) result from the cross-linking of effector cell-bound IgE by allergens and the subsequent release of biological mediators (e.g., histamine, leukotrienes) (3). A detailed knowledge concerning the molecular nature of the disease eliciting antigens is relevant for diagnosis, prevention, and therapy of Type I allergy. Recent progress in the field of molecular allergen characterization by cDNA cloning techniques has revealed the primary structure of many relevant allergens, allowed to investigate their B cell and T cell epitopes, and provided recombinant allergens for diagnostic and therapeutic purposes (4, 5). Structural similarities among allergens were found to form the molecular basis for immunological cross-reactivity and explain why allergic patients

¹ Correspondence: Molecular Immunopathology Group, Institute of General and Experimental Pathology, AKH Medical School, University of Vienna, Waehringer Guertel 18–20, A-1090 Vienna, Austria. E-mail: A5311daa at awiuni11.edvz.univie.ac.at

² Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; IgE, immunoglobulin E; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

can exhibit clinical symptoms after contact with various unrelated allergen sources (6, 7). Cross-reactive plant allergens include the family of profilins, representing ubiquitous cytoskeletal proteins, which bind to actin (8, 9), phosphatidylinositides (e.g., PIP2) (10), and VASP (11). IgE recognition of profilins may thus be responsible for allergic symptoms after contact with pollens from unrelated plant species (trees, grasses, weeds) (12), plant-derived food (fruits, vegetables, spices) (13, 14), latex products (15), and perhaps for triggering and maintaining profilin-specific antibody formation by autoimmune mechanisms (16). Another group of cross-reactive plant allergens is exemplified by Bet v 1, the major birch pollen allergen (17). Bet v 1-homologous proteins contain a p-loop as a typical sequence motif (18, 19) and are thought to act as pathogenesis-related plant proteins with RNase activity (20). They represent cross-reactive pollen allergens in trees of the *Fagales* order (21) and in plant-derived food (fruits, vegetables, spices) (22).

Recently calcium binding plant proteins have been discovered as relevant cross-reactive allergens (23). According to number and structure of their calcium binding domains (EF-hands) as well as to interdomain sequence similarities, calcium binding proteins can be divided into more than 30 subfamilies (24). Calcium binding proteins may simply buffer and translocate calcium or, due to calcium-induced conformational changes, interact with ligands in a calcium-dependent manner and thus act as cellular messengers (25). Calcium binding plant allergens described so far include Bet v 3, a three EF-hand pollen-specific birch allergen (23), Jun o 1, a four EF-hand cypress pollen allergen (26), and a novel subfamily of two EF-hand pollen allergens recently described for birch (Bet v 4) (27, 28), alder (Aln g 4) (29), olive (Ole e 3) (30), Bermuda grass (Cyn d 7) (31, 32), and rape (33). While there is some evidence that IgE antibody recognition of these allergens can be modulated by the presence or absence of protein-bound calcium, the molecular mechanisms for this behavior have been elusive.

Here we report the isolation and characterization of a cDNA coding for Phl p 7, a two EF-hand calcium binding timothy grass pollen allergen. We expressed recombinant Phl p 7 in *Escherichia coli* and purified the recombinant protein to homogeneity. The expression of Phl p 7 in tissues of timothy grass and its elution kinetics from hydrated pollen were studied as well as its cross-reactivity with homologous allergens in tree, grass, and weed pollens. To determine the allergenic potential of recombinant Phl p 7, basophil histamine release assays and skin prick tests were performed. The possible molecular mechanisms of the calcium-dependent modulation of IgE recognition of apo- and calcium-bound Phl p 7 were

studied by circular dichroism analysis and molecular modeling.

MATERIALS AND METHODS

Biological materials, phage, *E. coli* strains, plasmids, recombinant proteins

Timothy grass (*Phleum pratense*), Bermuda grass (*Cynodon dactylon*), rye (*Secale cereale*), ragweed (*Ambrosia elatior*), and birch (*Betula verrucosa*) pollen were purchased from Allergon (Välinge, Sweden). Timothy grass and wheat (*Triticum sativum*) seeds were bought from Austroaat (Vienna, Austria). Timothy grass seeds were grown for 4 wk to obtain fresh leaves and roots. Leaves and roots were washed and frozen at -70°C until use. EcoRI-cut dephosphorylated λ gt 11 phage DNA was obtained from Amersham (Buckinghamshire, U.K.). *E. coli* strain Y1090 was purchased from Amersham, *E. coli* XL1-Blue was from Stratagene (La Jolla, Calif.), and *E. coli* BL21 (DE3) was from Novagen (Madison, Wis.). Plasmid pUC19 and pET 17 b were obtained from Boehringer (Mannheim, Germany) and Novagen, respectively. Recombinant Bet v 1, the major birch pollen allergen, was supplied from BIOMAY (Linz, Austria). Recombinant Bet v 4 was expressed as β -galactosidase fusion protein in *E. coli* Y1089, as described (27).

Antibodies, sera, patients

The rabbit anti-celery root profilin antiserum RP1 is described (13, 8). A rabbit antiserum was raised against purified recombinant Aln g 4, the Phl p 7-homologous alder pollen allergen, using Freund's adjuvans (Charles River, Kisslegg, Germany) (29). Human sera were collected from 102 grass pollen allergic individuals by venipuncture and stored at -20°C until use. Patients were characterized by case history indicative for grass pollen allergy, by a positive timothy grass pollen RAST (radioallergosorbent test) result, and by skin prick testing as described (34). Individuals with and without rPhl p 7-specific IgE antibodies were tested in basophil histamine release and skin test experiments after informed consent was obtained.

Isolation and characterization of a Phl p 7-encoding cDNA

An expression cDNA library constructed from mature timothy grass pollen in phage λ gt11 was screened with serum IgE from a grass pollen allergic patient as described (35). The 350 IgE-reactive clones were tested with antibodies/antisera with specificity for group 1, group 2/3, group 5 allergens, and profilin as well as with sera from grass pollen allergic patients with defined IgE reactivity profile to obtain phage clones expressing not-yet-described timothy grass pollen allergens. cDNAs of the remaining clones were obtained from phage liquid lysates by polymerase chain reaction (PCR) amplification using λ gt11 forward and reversed primers containing internal *Bam*HI sites (italics): λ gt11 forward 5'-CGG GAT CCC GGT TTC CAT ATG GGG ATT GGT GGC 3'; λ gt11 reversed 5'-CGC GGA TCC CGT TGA CAC CAG ACC AAC TGG TAA TG 3' (MWG, Ebersberg, Germany). The PCR products were digested with *Bam*HI, gel-purified, subcloned into the *Bam*HI site of plasmid pUC 19, and transformed in *E. coli* XL-1 blue. Plasmid DNA was purified using Quiagen tips (Quiagen, Hilden, Germany) and the 5' DNA sequences of the allergen-encoding cDNAs were determined with the λ gt11 forward primer using the chain termination method (36), ^{35}S -dCTP (NEN, Stevenage, U.K.), and a T7 sequencing kit (Pharmacia,

Uppsala, Sweden). All molecular biological manipulations followed established protocols (37). The obtained DNA sequences were compared with the DNA sequences deposited in GenBank using the BLAST server. The 5' DNA sequence of clone 144 that showed significant sequence homology with cDNAs coding for calcium binding proteins was selected for detailed characterization.

To avoid the introduction of sequence errors by PCR, original phage DNA was isolated from clone 144 using a plate lysate method (37). The phage DNA was cut with *KpnI*/*SacI* to obtain the allergen-encoding cDNA flanked on each side by ~1000 base pairs of λ gt11 DNA. The clone 144 *KpnI*/*SacI* fragment was subcloned into the *KpnI*/*SacI* site of plasmid pUC19 and both DNA strands were sequenced using the λ gt11 primers as well as sequence specific internal primers (MWG).

Analysis of the clone 144 DNA and deduced amino acid sequence, calculations of molecular mass, pI, secondary structure, and surface accessibility were performed with the McVector Program (Kodak, Rochester, N.Y.). DNA and deduced amino acid sequence of clone 144 were compared with the sequences deposited in GenBank using the program BLASTN and BLASTP via the BLAST server.

***E. coli* expression and purification of rPhl p 7**

The coding region of clone 144 was amplified by PCR using the following primers: Phl p 7-5': 5' GGG AAA TTC CAT ATG GCG GAC GAC ATG GAG AGG 3' and Phl p 7-3': 5' CCG GAA TTC ATC AGA AGA CCT TGG CGA CGT C 3'. The primers contained a *NdeI* site (italics) and an *EcoRI* site (italics), respectively. The PCR product was cut with *NdeI*/*EcoRI*, gel-purified, and subcloned into the *NdeI*/*EcoRI* site of plasmid pET 17b. *E. coli* BL 21 (DE 3) colonies expressing the rPhl p 7 allergen were identified with serum IgE from a Phl p 7-allergic individual by colony screening. Plated *E. coli* were induced to synthesize recombinant proteins by overlay with nitrocellulose filters soaked in 10 mM IPTG. Filter-bound *E. coli* were disrupted by freeze thawing and colonies expressing rPhl p 7 were identified with serum IgE from a Phl p 7-allergic patient. Bound IgE antibodies were detected with an alkaline phosphatase-labeled mouse monoclonal anti-human IgE antibody (Pharmingen, San Diego, Calif.). The DNA sequence of the IgE-reactive pET 17b clone used for protein expression was confirmed by sequencing. Recombinant Phl p 7 was overexpressed in *E. coli* BL21 (DE3). *E. coli* were grown to an OD₆₀₀ of 0.4 in LB-medium containing 100 mg/l ampicillin. The expression of recombinant protein was induced by adding isopropyl- β -thiogalactopyranoside to a final concentration of 1 mM and culturing for an additional 4 h at 37°C. *E. coli* cells from a 600 ml culture were harvested by centrifugation, resuspended in 15 ml PBS containing 5 mM phenylmethylsulfonyl fluoride (PMSF), and homogenized using an ultraturrax (Ika, Heidelberg, Germany). A fraction containing soluble proteins was obtained after centrifugation of the homogenate at 10,000 rpm (Sorval RC5C, SS34 rotor) for 30 min at 4°C. Recombinant Phl p 7 was enriched in the soluble protein fraction after precipitation and removal of contaminating proteins by boiling and centrifugation (10,000 rpm, 30 min, 4°C, Sorval RC5C, SS34 rotor). A similar enrichment of rPhl p 7 in the soluble protein fraction was obtained after addition of 70% w/v ammonium sulfate to the soluble *E. coli* fraction. The soluble rPhl p 7-enriched protein fraction obtained after centrifugation (18,000 rpm, Sorvall SS34, 4°C, 30') was dialyzed against water, lyophilized, resuspended in 50 ml buffer I (25 mM Imidazole, 1 mM β -mercaptoethanol, pH=7.4), and applied to a DEAE anion exchange column (Pharmacia). rPhl p 7 was eluted by a NaCl gradient (buffer I containing 500 mM NaCl) at ~200 mM NaCl. Fractions

containing pure rPhl p 7 were pooled, dialyzed against water, and lyophilized. rPhl p 7 samples were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining as well as by mass spectroscopy.

Protein extracts, SDS-PAGE, immunoblotting

Pollen protein extracts were prepared by homogenizing 1.5 g pollen in 50 ml of distilled water containing 5 mM PMSF with an ultraturrax (IKA, Heidelberg, Germany) and extraction of the homogenate for 3 h at 4°C under continuous shaking. Homogenates were centrifuged at 20,000 *g* for 30 min at 4°C to remove insoluble particles. Supernatants were lyophilized and checked for quantity and quality of proteins by SDS-PAGE (38) and Coomassie blue staining (39) as well as by immunoblotting. The presence of Phl p 7 and profilin in the supernatant and pellet fraction of hydrated timothy grass pollen grains was studied by immunoblotting as described (40). For the analysis of the tissue-specific expression of Phl p 7, pollen, leaves, and roots were homogenized in SDS sample buffer with an ultraturrax. The homogenate was boiled and insoluble materials were removed by centrifugation (10,000 *g*, 20 min, RT). Total extracts from *E. coli* before and after induction of protein expression were obtained in the same way. Approximately 100 μ g/cm gel of total protein extracts and 5 μ g/cm gel of purified proteins were separated by preparative 15% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) (41). Sera from allergic patients and (for control purposes) from nonatopic individuals, were diluted 1:10 in buffer A [50 mM Na phosphate, pH 7.5, 0.5% w/v bovine serum albumin (BSA), 0.5% v/v Tween 20, 0.05% Na₂S₂O₃] and exposed to nitrocellulose-blotted proteins. Bound IgE was detected with ¹²⁵I-labeled anti-human IgE antibodies as described (12) and visualized by autoradiography using KODAK X-OMAT films and intensifying screens (Kodak, Heidelberg, Germany). Rabbit antisera were diluted 1:2000 in buffer A and probed with nitrocellulose-blotted proteins. Bound rabbit antibodies were detected with ¹²⁵I-labeled donkey anti-rabbit antibodies (Amersham) and visualized by autoradiography.

MALDI-TOF (matrix-assisted laser desorption and ionization-time-of-flight analysis) of purified rPhl p 7

Laser desorption mass spectra were acquired in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, U.K.), operating at 20 kV acceleration voltage and equipped with a nitrogen UV laser (337 nm, pulse duration 3 ns) (piCHEM, Research and Development, Graz, Austria). The *m/z* values were calibrated externally. Samples were dissolved in 10% acetonitrile (0.1% TFA). Alpha-cyano-4 hydroxy-cinnamic acid was used as a matrix dissolved in 60% acetonitrile (0.1% TFA). For sample preparation, a 1:1 mixture of protein solution and matrix solution was deposited onto the target and air dried.

IgE absorption experiments

The share of IgE epitopes between natural allergens in timothy grass (*Phleum pratense*), Bermuda grass (*Cynodon dactylon*), birch (*Betula verrucosa*), and ragweed (*Ambrosia elatior*) pollen extracts as well as rBet v 4 (27) was studied by immunoabsorption experiments as described (12). Sera from allergic patients containing rPhl p 7-specific IgE antibodies and from a nonatopic individual were preabsorbed with 10 μ g/ml rPhl p 7 or, for control purposes, with 10 μ g/ml BSA overnight at 4°C. Preabsorbed sera were then exposed to

TABLE 1. Induction of immediate type skin reactions with rPhl p 7

Individual	rPhl p 7				Timothy	Histamine
	1 µg/ml	5 µg/ml	10 µg/ml	20 µg/ml		
A	5	6.5	7.5	10	4	6.5
B	6.5	7.5	10	10.5	10	7.5
C	7.5	11	11.5	13.5	12	6
N	—	—	—	—	—	5

nitrocellulose-blotted natural pollen extracts or rBet v 4 and bound IgE antibodies were detected with ^{125}I -labeled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden). The reduction of IgE binding to rBet v 4 after preabsorption of the sera with rPhl p 7 was quantified by γ counting as described (21).

Basophil histamine release experiments

Granulocytes were isolated from heparinized blood samples of three pollen allergic patients containing rPhl p 7-specific IgE and (for control purposes) from a nonatopic individual by dextran sedimentation (42, 43). Cells were incubated with increasing concentrations (0.0001 µg/ml, 0.001 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml) of purified rPhl p 7 and (for control purposes) with monoclonal anti-human IgE antibodies. Histamine released in the supernatant was measured by radioimmunoassay (Immunotech, Marseille, France). Total histamine was determined after freeze thawing of the cells. Results are displayed as mean values of triplicate determinations and represent the percentage of total histamine.

Skin prick testing

After informed consent had been obtained, three pollen allergic patients and (for control purposes) a nonatopic individual were skin prick tested on their forearms with 20 µl aliquots of solutions containing different concentrations (1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml) of purified rPhl p 7 and (for control purposes) with timothy grass pollen extract, histamine, and sodium chloride (ALK, Horsholm, Denmark), as described (44). The skin reactions were recorded 20 min after testing by photography and by transferring the ball point pen-surrounded wheal area with scotch tape to paper. The mean wheal diameters displayed in Table 1 were determined as follows: $D_m = (D_1 + D_2)/2$. D_1 and D_2 represent the maximal longitudinal and transversal diameter of the wheal reaction in mm, respectively.

Calcium-dependent IgE binding of rPhl p 7

Equal amounts (5 µg/dot) of purified rPhl p 7 were dotted onto nitrocellulose membranes of equal size (20 mm x 6 mm) and were exposed to four sera from pollen-allergic patients containing rPhl p 7-reactive IgE-antibodies in the presence (buffer A containing 0.1 mM CaCl_2) or absence of calcium (buffer A containing 5 mM EGTA), as described (23). Bound IgE antibodies were detected with ^{125}I -labeled anti-human IgE-antibodies (RAST, Pharmacia). The amount of bound IgE was quantified by γ counting of the nitrocellulose strips (Wizzard, Automatic Gamma Counter, Wallac, Uppsala, Sweden) (21).

Circular dichroism spectroscopical analysis of purified rPhl p 7

Circular dichroism (CD) spectra were recorded on a Jasco J-710 spectropolarimeter fitted with a Jasco PTC-348WI peltier type temperature control system and interfaced with a Fisons HAAKE GH water bath. The instrument was calibrated with a 0.1% aqueous solution of d-10-camphor-sulfonic acid. Results were expressed as the mean residue ellipticity ($[\Theta]$) at a given wavelength. Far-ultraviolet CD spectra were recorded at 20°C, 95°, and 98°C in a 2 mm path-length quartz cuvette (Hellma, Mullheim, Baden, Germany) at a protein concentration of 10 µM. Spectra were recorded with 0.1 nm resolution and resulted from averaging 10 scans. The final spectra were corrected by subtracting the corresponding base line spectrum obtained under identical conditions. All measurements were performed in MilliQ water, pH 7.2. Thermal denaturation of Phl p 7 was monitored using a 2 mm cuvette (Hellma) by recording the ellipticity during temperature increase (50°C/h) at 220 nm. The reversibility of the unfolding process was checked by measuring the restoration of the CD signal after cooling (50°C/h) to the starting temperature (20°C). Measurements were performed in MilliQ water pH 7.2 at a protein concentration of 10 mM. Results were expressed as the mean residue ellipticity (Θ) at a given wavelength. The fraction of folded protein was calculated as $F=1-U$, where $U = (\Theta_{220} - \Theta_N)/(\Theta_U - \Theta_N)$. Θ_N is the ellipticity of the protein in the native state and Θ_U that of the denatured protein. For rPhl p 7, Θ_U was assumed to be equal to Θ_{220} at 85°C and Θ_N to Θ_{220} at 20°C.

Molecular modeling of the open and closed states of Phl p 7

The ribbon representation of the apo- and calcium-bound form of Phl p 7 was prepared by homology modeling using the WHAT IF program (45) and is displayed with MOLMOL (46). The coordinates of *Drosophila melanogaster* calmodulin were used as a template.

RESULTS

Isolation and characterization of a cDNA coding for a two EF-hand calcium binding timothy grass pollen allergen, Phl p 7

One of 350 IgE-reactive phage clones (clone 144) isolated from a timothy grass pollen expression cDNA library contained an open reading frame of 237 nucleotides that coded for a protein with a calculated molecular mass of 8.6 kDa and a pre-

Phl p 7	M A D D M E R I F K R F	D T N G D G K I S L S E L	T D A L R T L G S T	35
Cyn d 7	K T - - - - - T G - - - H - - - -	- - - - - A - - - - -	- - - - -	
Aln g 4	- - - D H P Q D Q A E H - - - - - C -	- A - - - - - A - - - - -	G - - - K - - - V	
Bet v 4	- - - D H P Q D K A E R - - - - -	- A - - - - - A A - - -	G E - - K - - - I	
Bra n 1	- - - A E H - - - - - K -	- D - - - - - A A - - -	E E - - K K - - V	
Bra n 2	- - - . A T E K A E H D - - - - - K -	- A - - - - - A - - - - -	G - - - K N - - V	
Bra r 1	- - - A E H - - - - - K -	- D - - - - - A A - - -	E E - - K K - - V	
Bra r 2	- - - . A T E K A E H D - - - - - K -	- A - - - - - A - - - - -	G - - - K N - - V	

Phl p 7	S A D E V Q R M M A E I	D T D G D G F I D F N E F	I S F C N A N P G L M K D V A K V F	78
Cyn d 7	- - - - -	- - - - - D - - - - -	- - - - -	
Aln g 4	T P - - - K H - - - - -	- - - - - S - Q - - -	T N - A R - - R - - V - - - - I -	
Bet v 4	T P - - - K H - - - - -	- - - - - S - Q - - -	T D - A R - - R - - L - - - - I -	
Bra n 1	T P - D - T - - - - K -	- - - - - N - S - Q - - -	T E - A S - - - - -	
Bra n 2	T H - D I K - - - - -	- - - - - Y - S Y Q - - -	S D - A S - - R - - - - - I -	
Bra r 1	T P - D - T - - - - K -	- - - - - N - S - Q - - -	T E - A S - - - - -	
Bra r 2	T H - D I K - - - - -	- - - - - Y - S Y Q - - -	S D - A S - - R - - - - - I -	

Figure 1. Comparison of the deduced amino acid sequence of Phl p 7 with those of other two-EF hand calcium binding pollen allergens. Amino acids that are identical to the Phl p 7 sequence are indicated by dashes, points indicate gaps. The calcium binding domains are framed. The cDNA and deduced amino acid sequence of Phl p 7 have been deposited in the EMBL data base under the accession number Y17835.

dicted pI of 3.99. A motif search revealed the presence of two typical calcium binding domains (EF-hands) in the deduced amino acid sequence of clone 144 (**Fig. 1**). EF-hands are helix-loop-helix motifs that usually pair together to bind to calcium through four carboxylate or carboxamide groups and a single backbone carbonyl oxygen placed in the loop with a specific spacing. These residues that represent the marker motif of the EF-hand family are found in the clone 144 deduced amino acid sequence with no diversity from the consensus. Comparison of the amino acid sequence deduced from clone 144 with those deposited in GenBank and the known allergen-sequences revealed best homology with a group of low molecular weight, two EF-hand calcium binding pollen allergens recently described for birch (Bet v 4) (27, 28), alder (Aln g 4) (29), Bermuda grass (Cyn d 7) (31, 32), and rape (Bra r 1, Bra r 2, Bra n 1, Bra n 2) (33) (**Fig. 1**). On the basis of the high sequence homology of the clone 144-encoded timothy grass pollen allergen with the Bermuda grass pollen allergen, Cyn d 7, and the fact that no group 7 timothy grass pollen allergen has been identified so far, we designated the gene product Phl p 7 and submitted it as such to GenBank (accession number: Y17835). The deduced amino acid sequence of Phl p 7 was most closely related to that of Cyn d 7 (93.6% sequence identity). The sequence identity of Phl p 7 with the other two

EF-hand pollen allergens was 64.1% (Aln g 4), 62.8% (Bet v 4), 69.2% (Bra n 1, Bra r 1), and 61.5% (Bra n 2, Bra r 2).

***E. coli* expression and purification of rPhl p 7**

Recombinant Phl p 7 was expressed in *E. coli* BL21 (DE3) using a T7 RNA polymerase-driven expression system. SDS-PAGE analysis of *E. coli* extracts before (**Fig. 2A**: lane 1) and after induction of protein synthesis (**Fig. 2A**: lane 2) indicated expression of a band of ~6 kDa apparent molecular mass in both samples. As observed for rAln g 4, the Phl p 7-homologous allergen from alder pollen (29), rPhl p 7, showed an apparent molecular mass of 6 kDa (**Fig. 2A**), lower than deduced from their amino acid sequences. Phl p 7 and Aln g 4 are acidic proteins and most likely possess high electrophoretic mobility in SDS-PAGE. A considerable amount of rPhl p 7 remained in the soluble fraction (**Fig. 2A**: lanes 3 and 4). As reported for many other calcium binding proteins (24), rPhl p 7 was further enriched in the soluble fraction after boiling and centrifugation (**Fig. 2A**: lane 5). Likewise, rPhl p 7 was enriched and still soluble after addition of 70% w/v ammonium sulfate (**Fig. 2A**: lane 6). The solution obtained was desalted and passed over a DEAE anion exchange column, and pure/soluble rPhl p 7 could be recovered (**Fig. 2A**: lane 7).

MALDI-TOF analysis of purified rPhl p 7 yielded

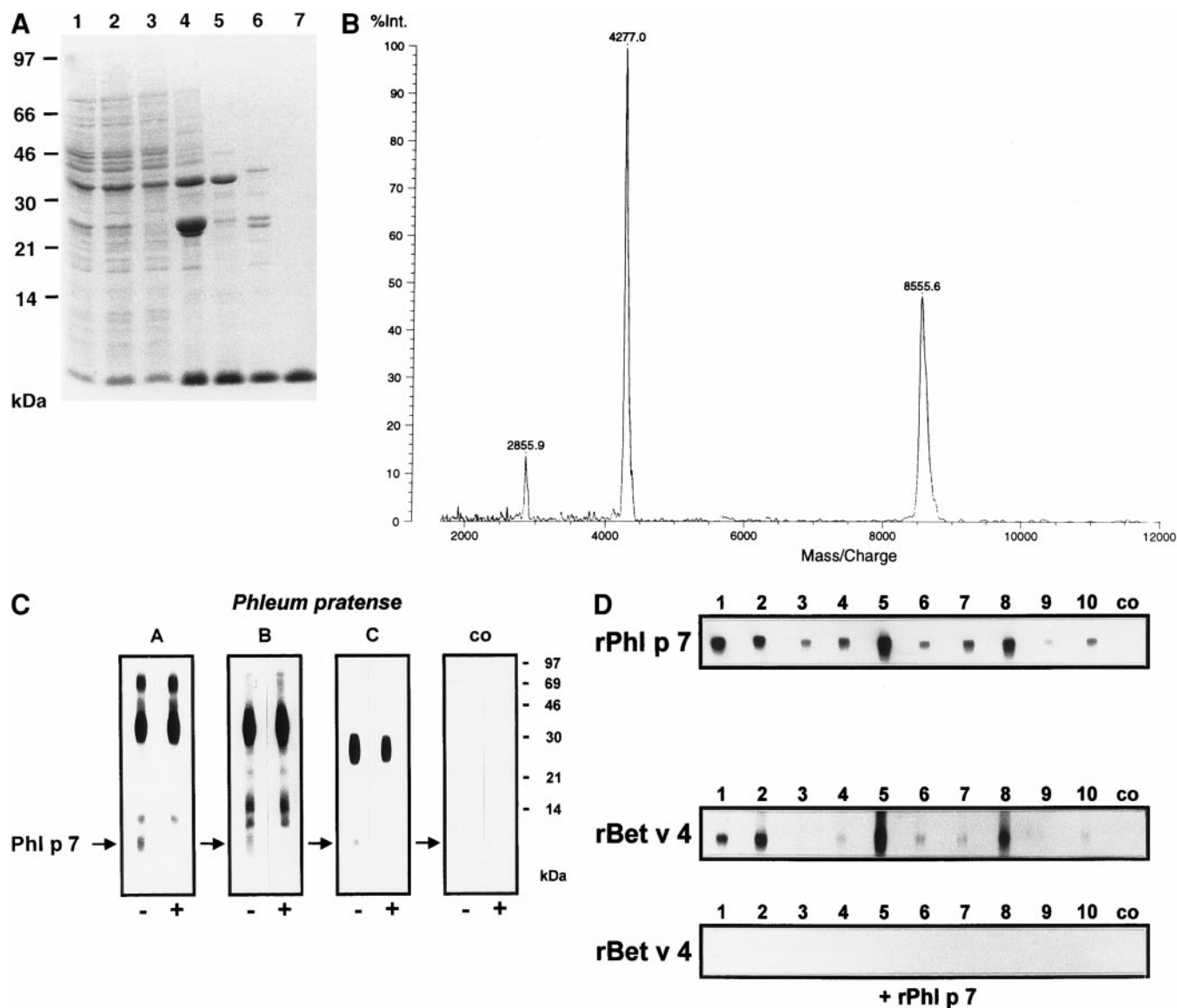


Figure 2. A) *E. coli* expression and purification of rPhl p 7. Coomassie-stained SDS-PAGE containing total *E. coli* protein extract before induction with IPTG (lane 1), total *E. coli* protein extract after induction with IPTG (lane 2), the insoluble pellet fraction (lane 3), the soluble fraction (lane 4) of the induced *E. coli* protein extract, soluble proteins yielded after boiling of the soluble *E. coli* fraction (lane 5), and proteins remaining soluble in 70% w/v ammonium sulfate (lane 6). Lane 7 contains 10 μ g of rPhl p 7 purified by DEAE chromatography from the material in lane 6. Molecular weights are displayed on the left margin. B) Mass spectroscopical analysis of purified rPhl p 7. The mass/charge ratio is shown on the x axis. The signal intensity is shown on the y axis as percentage of the most intensive signal obtained in the investigated mass range. The peaks at 8555.6, 4277.0 and 2855.9 correspond to the MH⁺, M2H⁺, and M3H⁺ species of rPhl p 7, respectively. C) rPhl p 7 inhibits IgE binding to natural Phl p 7. Nitrocellulose-blotted timothy grass pollen extract was exposed to sera from three grass pollen allergic patients containing rPhl p 7-specific IgE antibodies (A–C) and to serum from a nonatopic individual (co). The sera were preabsorbed with BSA (lanes –) or rPhl p 7 (lanes +). The arrows indicate the positions of natural Phl p 7. Molecular masses (kDa) are shown on the right margin. D) rPhl p 7 binds IgE antibodies and inhibits IgE reactivity to rBet v 4. Serum IgE reactivity of 10 Phl p 7-allergic patients (1–10) and one nonatopic person (co) to nitrocellulose-blotted rPhl p 7 and rBet v 4 are shown in the upper parts of the figure. Preincubation of the sera with rPhl p 7 (+ rPhl p 7) inhibited IgE binding to rBet v 4 (lower part of the figure).

three mass/charge peaks of 2855.9 Da, 4277.0 Da and 8555.6 Da corresponding to the M3H⁺, M2H⁺, and MH⁺ species of the protein, respectively (Fig. 2B). The molecular mass determined by mass spectrometry is thus in good agreement with the molec-

ular mass calculated from the Phl p 7 deduced amino acid sequence (8.6 kDa).

Purified rPhl p 7 inhibited IgE binding to a 6 kDa moiety, presumably representing natural Phl p 7, present in nitrocellulose-blotted timothy grass pollen

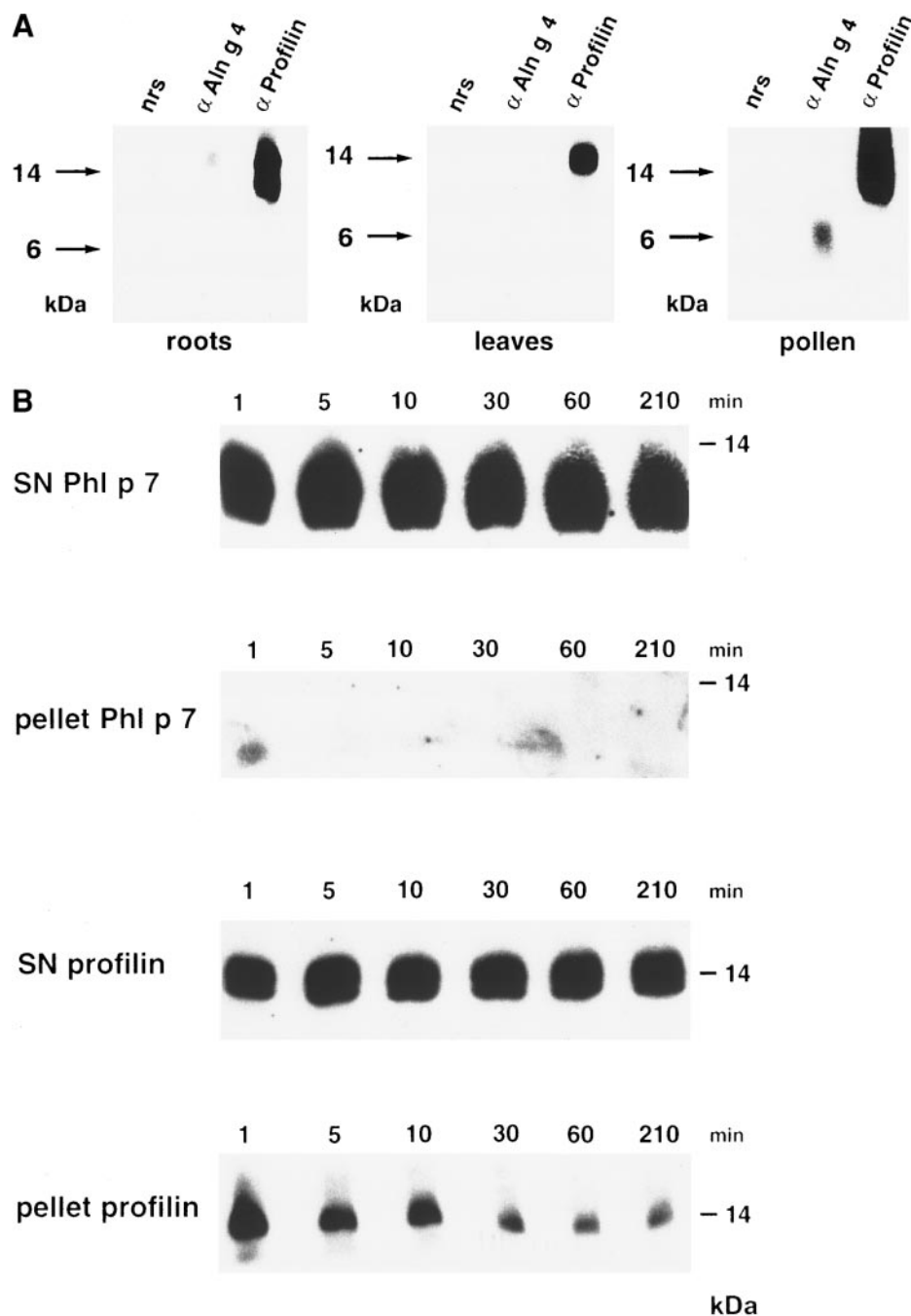


Figure 3. Expression of Phl p 7 in tissues of timothy grass (*Phleum pratense*) and pollens of various plants. Rapid elution of Phl p 7 from hydrated timothy grass pollen. A) Comparable amounts of nitrocellulose-blotted timothy grass root, leaf, and pollen extracts were probed with normal rabbit serum (lanes: nrs), a rabbit anti-Aln g 4 antiserum (lanes: α Aln g 4), and a rabbit anti-profilin antiserum (lanes: α profilin). B) Nitrocellulose-blotted supernatant (SN) and pellet fractions obtained after various times (1 min, 5 min, 10 min, 30 min, 1 h, 3.5 h) of timothy grass pollen hydration were analyzed for the presence of Phl p 7 and profilin by immunoblotting. Molecular weights are indicated on the right side.

extract (Fig. 2C) and specifically bound IgE antibodies of 10 sensitized grass pollen allergic patients, but not of a nonallergic individual (Fig. 2D). Preincubation of the rPhl p 7-reactive sera with rPhl p 7 yielded according to γ counting a reduction of IgE reactivity to rBet v 4 of 77.2% for one serum (Fig. 2D: serum 1) and between 90.1%–99.2% for the other 7 Bet v 4-reactive sera (Fig. 2D: sera 2, 4–8, 10).

Phl p 7 represents a two EF-hand calcium binding protein that is highly expressed in pollen

To study the expression of Phl p 7 in various tissues of timothy grass, we used a rabbit antiserum that was

raised against purified recombinant Aln g 4, a highly homologous allergen (sequence identity of 64.1%) (GenBank accession number: Y17713). The rabbit anti-Aln g 4 antiserum (lane 2) but not the rabbits' preimmune serum (lane 1) reacted with nitrocellulose-blotted purified rPhl p 7 (data not shown). Comparable amounts of nitrocellulose-blotted timothy grass pollen, leaf, and root proteins were exposed to rabbit anti-Aln g 4 antiserum and (for control purposes) to the rabbits' preimmune serum and a rabbit anti-profilin antiserum. Whereas anti-profilin immunoreactivity was detected in all three tissues at 14 kDa (Fig. 3A: α profilin), Phl p 7 was detected in pollen but not in root and leaf extracts (Fig. 3A: α

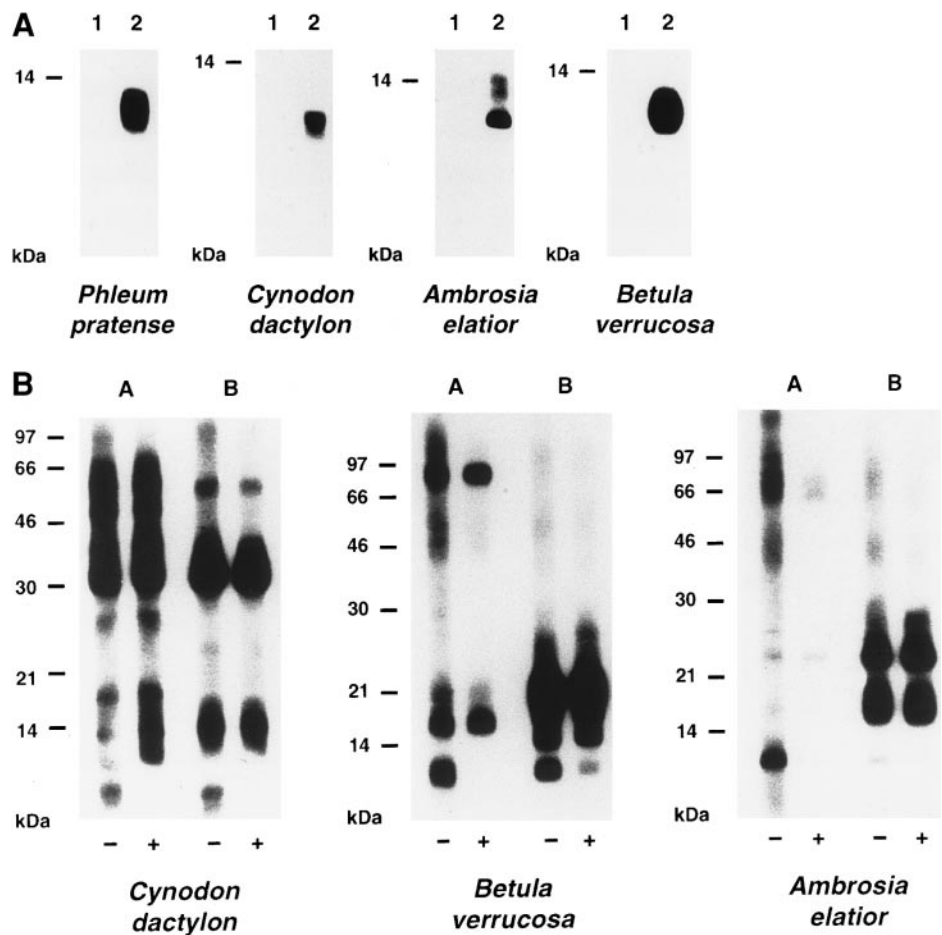


Figure 4. Recombinant Phl p 7 shares epitopes with natural pollen allergens from Bermuda grass, birch, and ragweed. *a*) Nitrocellulose-blotted grass- (*Phleum pratense*, *Cynodon dactylon*), weed (*Ambrosia elatior*), and tree (*Betula verrucosa*) pollen extracts were incubated with rabbit preimmune serum (lanes 1) and the rabbit anti-Aln g 4 antiserum (lanes 2). *b*) Sera from two rPhl p 7-reactive pollen allergic individuals (A, B) were preabsorbed with BSA (lanes: -) or rPhl p 7 (lanes: +) and exposed to nitrocellulose-blotted Bermuda grass (*Cynodon dactylon*), birch (*Betula verrucosa*), and ragweed (*Ambrosia elatior*) pollen extracts. Bound IgE antibodies were detected with ^{125} I-labeled anti-human IgE antibodies and visualized by autoradiography.

Aln g 4). The rabbits' preimmune serum showed no significant binding to the three extracts in the molecular weight range of Phl p 7 (Fig. 3A: nrs). Taken together, these data indicate that Phl p 7 represents a protein that is highly expressed in mature pollen and could not be detected in leaf or root tissues.

Rapid and complete elution of Phl p 7 after hydration of timothy grass pollen

It has been demonstrated that many relevant pollen allergens represent intracellular proteins that become rapidly eluted after pollen hydration (47, 40, 48). The analyses of nitrocellulose-blotted supernatant and pellet fractions obtained after hydration of timothy grass pollen for different time intervals with a rabbit anti-Aln g 4 as well as with a rabbit anti-profilin antiserum are displayed in Fig. 3B. Both Phl p 7 and profilin were detected in the supernatant fraction obtained after 1 min of pollen hydration (Fig. 3B). The profilin content in the pollen grain-containing pellet fraction decreased continuously until 30 min of hydration, although substantial amounts of profilin remained detectable in the pollen grains even after 3.5 h of hydration (Fig. 3B). In contrast, we found that Phl p 7 was completely eluted within the first minutes of pollen hydration

(Fig. 3B). No Phl p 7 immunoreactivity was detected in the pellet fractions analyzed after 5 min, 10 min, 30 min, 1 h, and 3.5 h of pollen hydration. Our results thus demonstrate that after a few minutes of hydration, Phl p 7 is completely eluted out of the pollen grains.

Phl p 7 is a cross-reactive pollen allergen

Ten out of 102 sera from grass pollen allergic patients contained rPhl p 7-reactive IgE antibodies (Fig. 2D). All patients with rPhl p 7-specific IgE antibodies suffered from allergy to pollen of other grass species, trees, and weeds. Therefore, we investigated whether rPhl p 7 shares epitopes with allergens in pollen of Bermuda grass (*Cynodon dactylon*), birch (*Betula verrucosa*), and ragweed (*Ambrosia elatior*).

A rabbit anti-rAln g 4 antiserum (Fig. 4a: lanes 2), but not the rabbit preimmune serum (Fig. 4a: lanes 1), cross-reacted with two EF-hand allergens in nitrocellulose-blotted natural pollen extracts from timothy grass (*Phleum pratense*), Bermuda grass (*Cynodon dactylon*), ragweed (*Ambrosia elatior*), and birch (*Betula verrucosa*) at 6–8 kDa. These results suggest that Phl p 7-homologous proteins are expressed in mature pollen of a variety of higher plants, including monocotyledonic and dicotyledonic species.

The presence of common IgE epitopes on Phl p 7 and possible homologous pollen allergens was studied by IgE inhibition experiments. Preabsorption of two sera from rPhl p 7-reactive patients (Fig. 4b: A, B) with rPhl p 7 (lanes +), but not with BSA (lanes -), led to a complete (sera A, B: *Cynodon*, *Ambrosia*; serum A: *Betula*) or significant (serum B: *Betula*) reduction of IgE binding to moieties of 6–8 kDa. The reduced anti-IgE reactivity of certain high molecular weight components (40–97 kDa) in birch (serum A) and ragweed pollen (sera A and B) extracts could be caused by the presence of two EF-hand allergen oligomers or independent high molecular weight allergens that share IgE epitopes with rPhl p 7. The enhanced anti-IgE reactivity of 14–21 kDa moieties in Bermuda grass pollen extract after preincubation of serum A with rPhl p 7 may result from the binding of IgE-rPhl p 7 immunocomplexes to these components.

rPhl p 7 induces basophil histamine release and immediate type skin reactions in pollen allergic patients

The biological relevance of rPhl p 7-specific IgE reactivity was investigated by basophil histamine release experiments (Fig. 5) and skin testing (Table 1). rPhl p 7 induced histamine release from basophils of three pollen allergic patients containing rPhl p 7-reactive IgE antibodies (Fig. 5A, data not shown), but not from basophils of a nonallergic individual (Fig. 5B). Basophils from all four donors released histamine after exposure to anti-IgE antibodies (Fig. 5A, B; data not shown). The same three allergic patients (A, B, C) and the nonallergic individual (N) who were tested in basophil release experiments were skin pricked with four concentrations of rPhl p 7 and (for control purposes) with timothy grass pollen extract, histamine, and isotonic sodium chloride solution (Table 1). rPhl p 7 and timothy grass pollen extract induced immediate type skin reactions in all three pollen allergic patients (A–C) but not in the nonallergic individual (N) (Table 1). Histamine elicited wheal reactions in all four individuals (Table 1).

Calcium-dependent IgE recognition of rPhl p 7

Exposure of purified nitrocellulose-bound rPhl p 7 under native conditions to serum IgE antibodies of Phl p 7 allergic patients in the presence of Ca^{2+} (lanes: +) or EGTA (lanes: -) resulted in significant variations of polyclonal serum IgE recognition of rPhl p 7 (Table 2). Due to the polyclonal nature of the IgE response in allergic individuals (i.e., the formation of IgE antibodies against many epitopes), calcium-dependent modulation of IgE recognition varied. As exemplified in Table 2 for four Phl p 7-reactive sera, certain patients

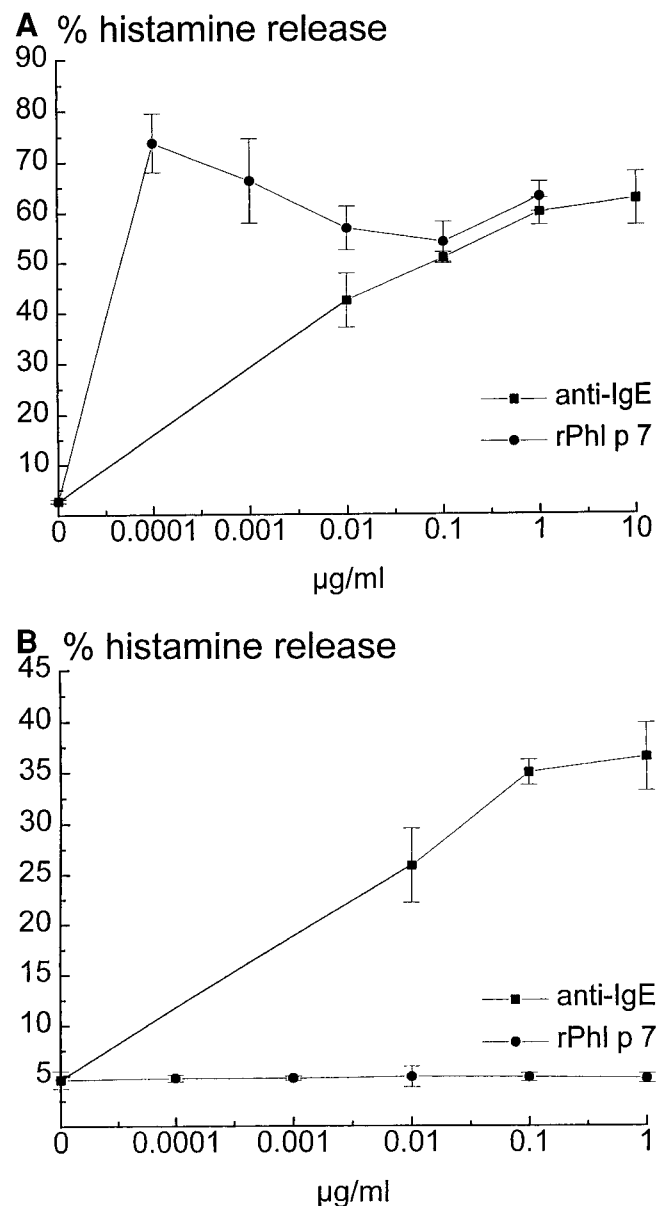


Figure 5. Purified rPhl p 7 induces basophil histamine release. A) Basophils from a Phl p 7-allergic patient; B) from a nonatopic individual were incubated with various concentrations (x axis) of purified rPhl p 7 (points) or monoclonal anti-IgE antibodies (squares). The percentage of total histamine released into the supernatant is displayed on the y axis. Results represent the means $\pm$ SD of triplicate determinations and error bars are displayed.

showed stronger IgE binding to the calcium-bound form of rPhl p 7 (Table 2; serum 1: +89%; serum 2: +112%). However, we also found that IgE antibodies of certain patients displayed reduced IgE reactivity to the calcium-bound-form of rPhl p 7 (Table 2; serum 3: -36%; serum 4: -43%). The IgE reactivity of the tested sera thus exemplified that the polyclonal IgE antibody response in Phl p 7 allergic patients can be predominantly directed against epitopes exposed on either the calcium-bound or apo form of the allergen.

TABLE 2. Different IgE binding capacity of the apo- and calcium-bound form of rPhl p 7

sera	cpm		%
	EGTA	Calcium	
1	703	1328	+89%
2	489	1038	+112%
3	328	209	-36%
4	3557	2011	-43%

Circular dichroism analysis of rPhl p 7

The far-ultraviolet circular dichroism spectrum of purified rPhl p 7 (Fig. 6A) recorded at 20°C indicates that the protein contains a considerable amount of α -helical secondary structure. The spectrum is characterized by a broad minimum at 208 nm, a shoulder at 222 nm, and a maximum at 186 nm. Thermal unfolding of rPhl p 7 was monitored as change in ellipticity at 220 nm and expressed as mean residue ellipticity (θ) (Fig. 6D). In the presence of 5 mM EGTA, the unfolding transi-

tion of rPhl p 7 is monophasic and highly cooperative with a melting point of 72°C; at 95°C, calcium-free rPhl p 7 assumes a random coil conformation, with a typical minimum at 200 nm. rPhl p 7 shows a high degree of folding reversibility evident from the far-UV spectra recorded at 20°C, after cooling from 98°C and 95°C (Fig. 6B, C), respectively, in the absence or presence of EGTA. From the thermal denaturation profiles and far-UV spectra at 98°C (no EGTA) and 95°C (5 mM EGTA), it is immediately evident that the thermal stability of rPhl p 7 depends on the presence of protein-bound calcium. Calcium-bound rPhl p 7 does not unfold completely and, even at 98°C, retains most of its native-like conformation (Fig. 6B, D).

DISCUSSION

We report the isolation and *E. coli* expression of a cDNA coding for a pollen-specific two EF-hand calcium binding allergen from timothy grass (*Phleum pratense*) pollen. Phl p 7 represents a 8.6 kDa acidic

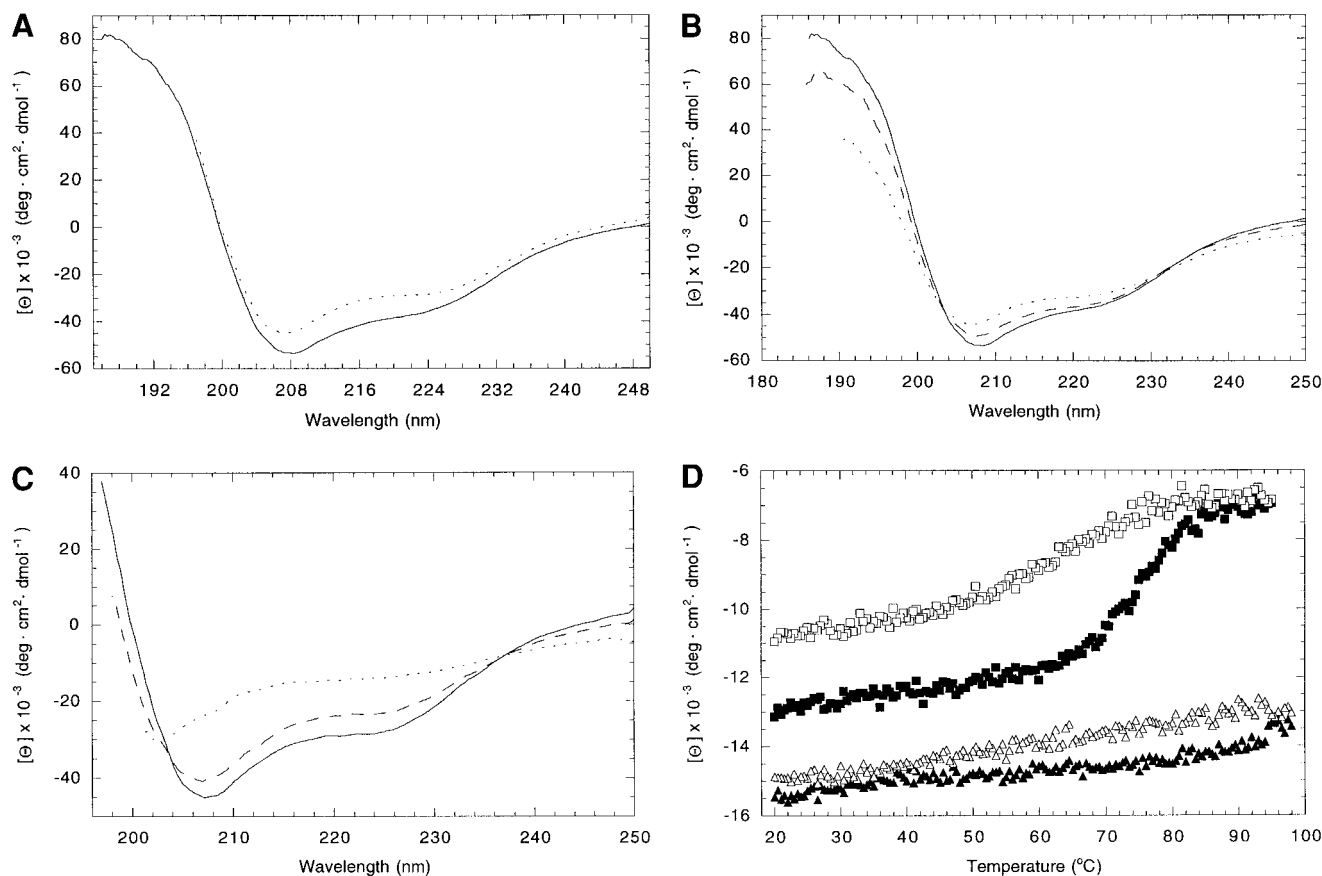


Figure 6. Far-ultraviolet circular dichroism analysis of rPhl p 7. A) Calcium-bound rPhl p 7 (continuous line) and apo-rPhl p 7 in the presence of 5 mM EGTA (dotted line) at 20°C. B) Calcium-bound rPhl p 7 at 20°C (continuous line), at 98°C (dotted line) and at 20°C after cooling from 98°C (dashed line). C) rPhl p 7 in the presence of 5 mM EGTA at 20°C (continuous line), at 95°C (dotted line), and at 20°C after cooling from 95°C (dashed line). All spectra are expressed as mean residue ellipticity (θ) (y axis) at a given wavelength (x axis). D) Thermal denaturation (black triangles) and refolding (white triangles) of calcium-bound rPhl p 7. Thermal denaturation (black squares) and refolding (white squares) of rPhl p 7 in the presence of 5 mM EGTA. The mean residue ellipticity (θ) at 220 nm (y axis) is displayed for a given temperature range (x axis).

protein that contains two canonical EF-hand domains with no diversity from the EF-hand consensus (24). Comparison of the Phl p 7 amino acid sequence with those sequences submitted to the databases revealed that Phl p 7 belongs to a novel subfamily of recently described two EF-hand calcium binding pollen allergens that are preferentially expressed in pollen of higher plants. Our contention that Phl p 7 and the related two EF-hand allergens represent a distinct and novel subfamily of calcium binding proteins is supported by structural, immunological, and biological considerations. 1) There are only a few examples of other two EF-hand calcium binding proteins (e.g., S-100-like proteins), all of which differ from Phl p 7 and the Phl p 7-related allergens in their interdomain sequences (24, 25). 2) It has been demonstrated that the Phl p 7-related two EF-hand allergens do not share epitopes with already known EF-hand allergens (e.g., Bet v 3, parvalbumin) (23, 49). 3) Unlike plant calmodulins, Phl p 7-related allergens were expressed in pollen but not in leaves or roots.

The pollen-specific expression of Phl p 7 may be relevant for two reasons. First, because it is well established that pollen germination and pollen tube growth are Ca^{2+} -dependent processes (50). Pollen calcium has also been attributed to be of importance for the reorientation of pollen tube growth (51) and the self-incompatibility response (52). Regarding pollen tube growth, it has become apparent that the cytoplasmic Ca^{2+} gradient is strongly correlated with tube growth (53) and there is a steep, tip-directed gradient of Ca^{2+} (54); recently it has been shown by calcium imaging that calcium pulses are coincident with peak growth rates (55, 56). The importance of calcium levels for pollen germination and pollen tube growth must therefore require the presence of pollen proteins that tightly control and regulate calcium metabolism. Phl p 7 and other pollen-specific calcium binding proteins, e.g., Bet v 3, which are abundantly expressed in plant pollens but not in other plant tissues, may therefore represent such regulatory proteins. Within minutes of pollen hydration, Phl p 7 is completely eluted from the pollen grain, a process that likely will influence the balance between intracellular and extracellular calcium concentrations and thus affect pollen germination and tube growth.

Second, pollen-specific expression of Phl p 7-related allergens is of interest regarding their role as highly cross-reactive pollen allergens. Specific or highly up-regulated expression in pollen, but not somatic tissues, represents a major feature of prominent plant allergens (6). In the present study it is demonstrated that Phl p 7 and related two EF-hand allergens represent highly cross-reactive allergens in pollens of trees, grasses, and weeds. Approximately

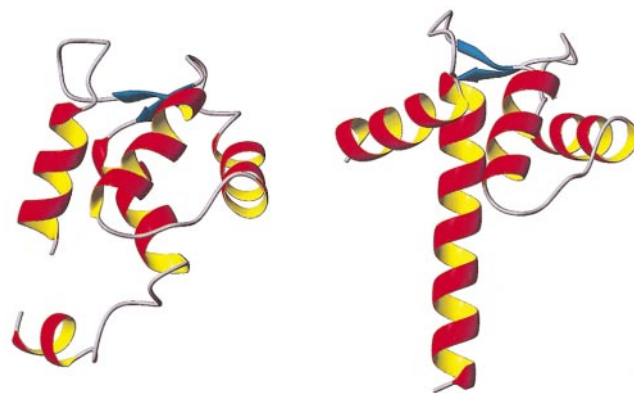


Figure 7. Ribbon representation of the apo- (left) and calcium-bound (right) form of Phl p 7 as modeled from the coordinates of *Drosophila melanogaster* calmodulin. Red indicates the helices (left helix: NH_2 terminus; right helix: COOH terminus) and light blue the calcium binding β -sheets.

10% of pollen allergic individuals react with the two EF-hand pollen allergens and therefore suffer from allergic symptoms after contact with pollens of many unrelated plant species. Although recognized at a rather low prevalence (10% of pollen allergic patients), we found that rPhl p 7 exhibited an extremely potent allergenic activity. rPhl p 7 induced basophil histamine release and immediate type skin reactions at extremely low concentrations and thus represents a biologically very active allergen.

The allergenic activity of rPhl p 7 may be related to its proper folding. According to CD analysis, rPhl p 7 was folded and contained mostly α -helical secondary structure elements. Proper folding represents a prerequisite for an allergen to interact with and to cross-link effector cell-bound IgE in solution, and thus induce the release of biological mediators. The CD measurements also revealed that calcium-bound rPhl p 7 had a far greater thermal stability than the EGTA-treated apoform. Calcium-bound rPhl p 7 thus shares the property of great thermal stability with many other calcium binding proteins (57, 24), a fact that allowed us to enrich rPhl p 7 in the soluble fraction of *E. coli* extracts by boiling of the homogenate. Increased stability due to calcium binding against, for example, proteolysis, may be of relevance to protect the protein under physiological (e.g., pollen hydration and germination) as well as pathological conditions, i.e., when Phl p 7 becomes eluted from the pollen grain during contact with the mucosa of allergic patients. It has been found that rapid elution from pollen grains after hydration as well as high stability and refolding capacity represent major features of prominent allergens (40, 58; Vangelista and Valenta, unpublished results). The latter two properties may be the reason why Phl p 7 is able to elicit and to trigger IgE responses in allergic patients.

CD spectroscopical analysis also revealed significant structural differences of calcium-bound and apo-rPhl p 7, a property that in the case of calcium sensor proteins (e.g., calmodulin) allows for the calcium regulation of interactions with various ligands (24, 25). Although we have no evidence so far for physiological ligands of Phl p 7 and related two EF-hand proteins, we found that IgE antibodies of allergic patients bound to rPhl p 7 in a calcium-dependent manner. The ribbon representations built for the apo- and calcium-bound form of Phl p 7 suggest that the molecule can occur in two conformations, referred to as 'closed' and 'open', which correspond to the calcium-free (apoform) and calcium-bound states (59) (Fig. 7). The apoform (closed form) (Fig. 7, left) differs from the calcium-bound form (open form) (Fig. 7, right) by the relative orientation of the helices. Whereas in the closed state the four helices form a very compact structure, calcium binding would push apart the two helices of each EF-hand motif and thus allow exposure of hydrophobic residues that become available for interaction with target peptides (e.g., IgE antibodies). The different IgE recognition of the apo- and calcium-bound Phl p 7 may therefore be explained by a preferential sensitization either to conformational epitopes present on the calcium-bound form of rPhl p 7 or to epitopes present on the calcium-free (apo-) form of the allergen. This hypothesis, that IgE recognition of allergens can mirror the contact with certain allergen conformations/states during early sensitization, is supported by data on the IgE recognition of other prominent plant allergens. IgE epitopes of profilin, a highly conserved actin binding protein, were mapped to the regions involved in the binding to natural ligands (e.g., actin); it was therefore concluded that patients were sensitized preferentially against the ligand-free profilin (60, 61). Likewise, it has been reported that IgE antibodies can distinguish between different conformations/states of the major birch pollen allergen, Bet v 1 (58). The fact that patients exhibited reduced IgE binding to certain Phl p 7 conformations may represent a basis for the generation of 'hypoallergenic' allergen variants for specific immunotherapy. The latter concept has been used to generate recombinant fragments (44) and mutants (62) of the major birch pollen allergen Bet v 1 with reduced anaphylactic activity. Similar results may be obtained if the calcium binding sites of Phl p 7 are destroyed by site-directed mutagenesis.

In conclusion, Phl p 7 represents a member of a novel family of pollen-specific two EF-hand calcium binding proteins and a prominent cross-reactive pollen allergen containing calcium-modulated conformational IgE epitopes. In its recombinant form, it may be used to diagnose and treat a group of atopic

patients who display allergic symptoms on contact with pollens of many unrelated plant species. **[F]**

We thank Dr. Annalisa Pastore for her suggestions and for molecular modeling as well as Dr. Philip Deviller, Lyon, France, for the rabbit anti-celery profilin antiserum. This study was supported by grant Y078GEN of the Austrian Science Fund, by the ICP grant of the Austrian Ministry of Research and Transport, and by a grant from Pharmacia and Upjohn, Uppsala, Sweden.

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Received for publication July 31, 1998.

Revised for publication December 16, 1998.