

Molecular, Immunological, and Structural Characterization of Phl p 6, a Major Allergen and P-Particle-Associated Protein from Timothy Grass (*Phleum pratense*) Pollen¹

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Due to the wide distribution and heavy pollen production of grasses, ~50% of allergic patients are sensitized against grass pollen allergens. cDNAs coding for two isoforms and four fragments of a major timothy grass (*Phleum pratense*) pollen allergen, Phl p 6, were isolated by IgE immunoscreening from a pollen expression cDNA library. Recombinant Phl p 6 (rPhl p 6), an acidic protein of 11.8 kDa, was purified to homogeneity as assessed by mass spectrometry and exhibited almost exclusive α -helical secondary structure as determined by circular dichroism spectroscopy. Phl p 6 reacted with serum IgE from 75% of grass pollen-allergic patients ($n = 171$). IgE binding experiments with rPhl p 6 fragments indicated that the N terminus of the allergen is required for IgE recognition. Purified rPhl p 6 elicited dose-dependent basophil histamine release and immediate type skin reactions in patients allergic to grass pollen. A rabbit antiserum raised against purified rPhl p 6 identified it as a pollen-specific protein that, by immunogold electron microscopy, was localized on the polysaccharide-containing wall-precursor bodies (P-particles). The association of Phl p 6 with P-particles may facilitate its intrusion into the deeper airways and thus be responsible for the high prevalence of IgE recognition of Phl p 6. Recombinant native-like Phl p 6 can be used for in vitro as well as in vivo diagnoses of grass pollen allergy, whereas N-terminal deletion mutants with reduced IgE binding capacity may represent candidates for immunotherapy of grass pollen allergy with a low risk of anaphylactic side effects. *The Journal of Immunology*, 1999, 163: 5489–5496.

Type I allergy is a genetically determined hypersensitivity disease that affects >20% of the population in industrialized countries (1). As a consequence of this immunodisorder, allergic patients produce IgE Abs against per se innocuous, mostly airborne proteins from pollen, mites, moulds, and animal hair/dander. The symptoms of type I allergy (allergic rhinitis, conjunctivitis, allergic asthma, and anaphylactic shock) result from allergen-induced cross-linking of effector cell (mast cell, basophil)-bound IgE Abs and subsequent release of inflammatory mediators (2). Because ~40% of allergic individuals suffer symptoms after contact with grass pollen, research has concentrated on the characterization of relevant grass pollen allergens by protein and immunochemical methods (3). Although groups of major allergens have been identified as cross-reactive moieties that occur in most grass species (4), nothing was known concerning their nature and biological functions.

The recent application of molecular biological techniques to allergen characterization has revealed the primary structures of allergens and facilitated the production of recombinant allergens for diagnostic and therapeutic purposes (5). Components of the plant cytoskeleton (e.g., profilin) (6) as well as calcium-binding pollen proteins (7) have been identified as relevant allergens. The fact that allergic patients exhibit immediate type reactions upon contact with various unrelated allergen sources thus can be explained by the cross-reactivity of their IgE Abs with ubiquitous allergens. Evidence that group 1 grass pollen allergens belong to a family of cell wall-loosening proteins (expansins) (8) and that grass group 5 allergens may possess RNase activity (9) has restimulated ideas that the biological function of a given protein may be related to its allergenicity. The recent findings that major grass pollen allergens can either become attached to small-sized particles (e.g., group 1 allergens to diesel exhaust, Ref. 10) or may become airborne as small pollen subcompartments (e.g., group 5 allergens in amyloplasts, Ref. 11) would provide a possible mechanism explaining how certain allergens may be able to reach the deep airways of patients and elicit allergic asthma.

We isolated cDNAs coding for a major timothy grass pollen allergen, Phl p 6. Recombinant Phl p 6 (rPhl p 6) was overexpressed in *Escherichia coli* and purified to homogeneity; its secondary structure content was determined by circular dichroism (CD)³ analysis. Phl p 6 IgE epitopes and the prevalence of IgE recognition by Phl p 6 among patients allergic to grass pollen were studied. The allergenic activity of purified rPhl p 6 was analyzed by basophil histamine release assays and by skin testing of allergic patients. Surprisingly, immunogold electron microscopy localized

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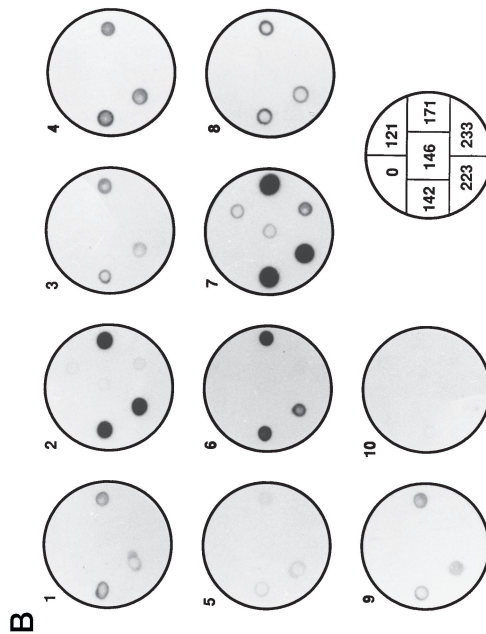
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³ Abbreviations used in this paper: CD, circular dichroism; MALDI, matrix-assisted laser desorption and ionization; TOF, time of flight.

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Phl p 6 predominantly on the polysaccharide-containing wall-precursor bodies (P-particles) of mature timothy grass pollen. This unique feature may possibly indicate that the high rate of sensitization of patients allergic to grass pollen against Phl p 6 is due to P-particle-linked intrusion of Phl p 6 into the deeper airways. The greatly reduced IgE binding capacity of N-terminally truncated rPhl p 6 indicates that N-terminal deletion variants of Phl p 6 may be considered as hypoallergenic candidate molecules for immunotherapy of grass pollen allergy.

Materials and Methods

Biological materials, patients' sera, antisera, and recombinant allergens

Pollen from timothy grass (*Phleum pratense*), rye grass (*Lolium perenne*), rye (*Secale cereale*), Kentucky blue grass (*Poa pratensis*), wheat (*Triticum sativum*), cultivated oat (*Avena sativa*), and common reed (*Phragmites communis*) were from Allergon (Välinge, Sweden). Timothy grass seeds were purchased from Austroaat (Vienna, Austria) and grown for 4 wk to obtain fresh leaves and roots. Patients allergic to grass pollen were characterized as described previously (4). The rabbit anti-celery profilin antiserum (RP1) has been described previously (12). A rabbit anti-rPhl p 6 antiserum was raised against purified rPhl p 6 using CFA (Charles River, Kisslegg, Germany). The recombinant timothy grass pollen allergens rPhl p 1, rPhl p 2, and rPhl p 5 were purified as described previously (13). Recombinant timothy grass pollen profilin was purified by poly(L-proline) affinity chromatography (6).

Isolation and characterization of cDNAs coding for Phl p 6 isoforms/fragments

A total of 350 IgE-reactive clones were isolated from an expression cDNA library constructed from mature timothy grass pollen in phage λ gt 11 (14). Six cDNAs (c121, c142, c146, c171, c223, and c233) with sequence homology to a Phl p 6-encoding cDNA (15) were subcloned into plasmid pUC18 and sequenced (16, 17). Sequences were analyzed using the McVector program (Kodak, Rochester, NY). A search for Phl p 6-homologous protein sequences was done with the FastA program (GCG package) (18) in the SwissProt database. The sequences of Hol l 5 and Hor v 5 allergens were retrieved from the European Molecular Biology Laboratory (EMBL) database. A multiple sequence alignment was produced with ClustalW (19) and edited by hand. The GDE sequence editor (S. Smith, Harvard University, Cambridge, MA) and Colormask (J. Thompson, EMBL, Heidelberg, Germany) were used to color conserved residues with related properties (19). Protein secondary structure and surface accessibility predictions were done with the PHD program on the EMBL Predict-Protein server (20).

Mapping of Phl p 6 IgE epitopes, expression, and purification of rPhl p 6

The IgE binding capacity of phage clones expressing Phl p 6 isoforms and fragments was investigated by a plaque lift assay (21). The DNA coding for the mature Phl p 6 allergen was PCR-amplified from the clone 142 DNA and subcloned into the *Nde*I/*Eco*RI site of pET-17b. rPhl p 6 was expressed in *E. coli* BL21 (DE3) in liquid culture. Cells were suspended in 25 mM

imidazole (pH 7.4) and 0.1% Triton X-100 and lysed by the addition of lysozyme (20 μ g/g cells) for 30 min at room temperature as well as by freeze-thawing cycles. DNA was digested with DNase I (0.1 mg/g cell pellet) for 20 min at room temperature. The protein extract was centrifuged for 20 min at $10,000 \times g$ (Sorvall (Wilmington, DE) RC5C, SS34 rotor) to remove insoluble materials. rPhl p 6 was enriched in a precipitate obtained by the addition of ammonium sulfate (40–60% w/v). The precipitate was dissolved in 10 mM of Tris (pH 6) and dialyzed against this buffer; after centrifugation (20 min at $10,000 \times g$, Sorvall RC5C, SS34 rotor), the precipitate was applied to a DEAE cellulose-Sepharose column (Pharmacia). Unbound proteins were eluted with 10 mM Tris (pH 6) and 4% v/v isopropanol. Fractions containing >80% pure Phl p 6 were adjusted to a pH of 8 with NaOH and subjected to a second chromatography step on a DEAE-cellulose-Sepharose column. Elution of bound proteins with a 0–0.5 M NaCl gradient at a pH of 8 yielded fractions containing pure rPhl p 6; these fractions were dialyzed against double distilled H_2O .

Matrix-assisted laser desorption and ionization (MALDI)-time of flight (TOF) and CD analysis of purified rPhl p 6

Laser desorption mass spectra were acquired in a linear mode with a TOF Compact MALDI II instrument (Kratos, Manchester, U.K.) (piCHEM, Graz, Austria). CD spectra were recorded on a Jasco (Tokyo, Japan) J-710 spectropolarimeter fitted with a Jasco PTC-348WI Peltier type temperature control system and interfaced with a Fisons Haake (Paramus, NJ) GH water bath. Far-UV CD spectra were recorded at 20°C in a 2-mm path-length quartz cuvette (Hellma, Mullheim, Baden, Germany) at a protein concentration of 7 μ M. Thermal denaturation of Phl p 6 was monitored 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 upon cooling (50°C/h) to the starting temperature (20°C). 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 is the ellipticity of the denatured protein. For rPhl p 6, Θ_U was assumed to be equal to Θ_{220} at 85°C and Θ_N was assumed to be equal to Θ_{220} at 20°C.

IgE-binding capacity of rPhl p 6, and cross-reactivity with natural Phl p 6 and other timothy grass pollen allergens

The prevalence of IgE anti-rPhl p 6 reactivity was determined in sera from 171 patients allergic to grass pollen and, for control purposes, in sera from nonatopic persons by ELISA (13). The presence of cross-reactive IgE epitopes on natural Phl p 6 and rPhl p 6 was investigated by IgE immunoblot inhibition experiments (4). A possible immunological relationship between rPhl p 6 and recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) (13) was studied by ELISA competition as described previously (4).

Histamine release experiments

Granulocytes were isolated from heparinized blood samples of individuals allergic to grass pollen; the samples contained rPhl p 6-reactive IgE Abs by dextran sedimentation (22). Cells were incubated with increasing concentrations of purified rPhl p 5, rPhl p 6, and with an anti-human IgE Ab (E124.2.8 D ϵ 2, Immunotech, Marseilles, France). Histamine released into the supernatants was measured by RIA (Immunotech).

FIGURE 1. A, Multiple sequence alignment, secondary structure, and solvent accessibility prediction of Phl p 6 variants and group 5 allergens. The amino acid sequences of the mature allergens without their leader peptides were compared. Asterisk lines represent the regions spanned by the Phl p 6 clones; isolated single letters indicate those amino acids that are different from clone 142. Each allergen sequence is preceded by its database entry code (S32101: Phl p 5A, timothy grass; HL5ALLERG: Hol l 5, velvet grass; MP91_POAPR to MP93_POAPR: Poa p 9 isoforms, Kentucky bluegrass; MP5B_LOLPR: Lol p 5b, rye grass; MP51_PHAAQ to MP54_PHAAQ: Pha a 5 isoforms, canary grass; MP5B_PHLPR: Phl p 5B, timothy grass; MP5A_LOLPR: Lol p 5a, rye grass; and HV06640: Hor v 9, barley). Dashes in the sequences indicate gaps. The sequences were colored to illustrate conserved features in addition to amino acid identity. All glycines and prolines are colored in orange and yellow, respectively. The other residues were colored according to conservation of their physicochemical properties (purple = acidic; blue = hydrophobic; light blue = hydrophobic tendency; red = basic; green = hydrophilic; white = unconserved). The bottom lines display the predicted secondary structure (SecStructPred), the predicted solvent accessibility (SolAccessPred), and a numbering reference (ruler). In the secondary structure prediction, uppercase letters are used for positions in which accuracy is >86%; H and E indicate residues in α or β conformation, respectively. In the solvent accessibility prediction, uppercase letters are used for residues in which accuracy is >69%; A and B indicate exposed and buried residues respectively. B, IgE reactivity of rPhl p 6 isoforms and fragments. Nitrocellulose filters containing proteins from recombinant λ gt 11 phage expressing two Phl p 6 isoforms (c142 and c223), Phl p 6 fragments (c121, c146, c171, c233), and, for control purposes, λ gt11 wild-type phage (0) were probed with serum IgE from nine grass pollen-allergic patients (1–9) and from one nonallergic individual (10).

Skin testing

After informed consent was obtained, skin prick tests were performed on the forearms of the individuals as described previously (23). Individuals were pricked with 20- μ l aliquots containing different concentrations (1 μ g/ml, 10 μ g/ml, and 100 μ g/ml) of purified rPhl p 6, rPhl p 5, timothy grass pollen extract, histamine, and NaCl (ALK, Horsholm, Denmark).

Analysis of the presence of Phl p 6-related allergens in other grass species and tissue-specific expression of Phl p 6

Protein extracts from pollens, leaves, and roots were obtained by homogenizing the tissues in SDS sample buffer (24). Insoluble materials were removed by centrifuging the extracts (10,000 $\times$ g for 20 min; Sorvall RC5C, SS34 rotor). Protein extracts were separated by 14% SDS-PAGE (25) and blotted onto nitrocellulose membranes (26). Nitrocellulose strips were probed with a rabbit anti-celery profilin antiserum, RP1 (12), rabbit anti-rPhl p 6 antiserum, and the latter rabbit's preimmune serum. Bound rabbit Abs were detected with a 1/1000 dilution of ^{125} I-labeled donkey anti-rabbit Ig antiserum (Amersham, Buckinghamshire, U.K.).

In situ localization of Phl p 6 by immunogold electron microscopy

Timothy grass pollen grains were anhydrously fixed as described previously (27). Ultrathin sections were incubated with equal concentrations of either rabbit anti-rPhl p 6 Ig (Ig: protein G-purified Ig fraction) or preimmune Ig. Bound rabbit Abs were detected with goat anti-rabbit IgG Abs coupled to 10-nm-diameter colloidal gold particles (Plano, Wetzlar, Germany) (27).

Results

Isolation and characterization of cDNAs coding for isoforms/fragments of Phl p 6

Six cDNA clones (c142, c223, c171, c121, c233, and c146) coding for Phl p 6 isoforms/fragments were isolated from a timothy grass pollen λ gt11 library with serum IgE from a patient allergic to grass pollen (Fig. 1A). The sequences of the described clones have been deposited in the GenBank database (accession numbers: Y16955-Y16960). The deduced amino acid sequence of Phl p 6 (clone 142) contained a 28-aa hydrophobic leader peptide. A molecular mass of 11.8 kDa and an isoelectric point of 5.5 was calculated for the mature Phl p 6 (clone 142) protein, which starts with a glycine residue and shows a high content of alanine residues (20.9%) (Fig. 1A). The computer-aided secondary structure analysis of Phl p 6 indicates a predominant helical content; the calculation of solvent accessibility predicts that many of the N-terminal amino acids are solvent exposed, whereas most of the C-terminal amino acids appeared buried (Fig. 1A). A search for sequence motifs revealed the presence of one potential N-linked glycosylation site (NAS: amino acids 15–17), one N-terminal myristoylation site (GKAT: amino acids 1–4), two cAMP-dependent protein kinase phosphorylation sites (KATT: amino acids 2–5; KYKT: amino acids 33–36), and two peroxisomal targeting sequences (GKA: amino acids 1–3; SKA: amino acids 54–56). The deduced Phl p 6 amino acid sequence displayed identity with a recently submitted Phl p 6 sequence (15) and similarities with the N-terminal portions of group 5 grass pollen allergens (Fig. 1A).

The Phl p 6 N terminus is relevant for IgE binding

Nitrocellulose-bound β -galactosidase-fused complete (c223, c142), N-terminally truncated rPhl p 6 (c171, c121, c233, and c146) and, for control purposes, β -galactosidase alone were exposed to serum IgE from nine grass pollen allergic individuals and a nonallergic person (Fig. 1B). The results obtained showed that the two complete Phl p 6 isoforms and a Phl p 6 fragment lacking only four of the N-terminal amino acids strongly bound IgE from all patients tested that were allergic to grass pollen, and that the IgE binding capacity of the partial Phl p 6 clones decreased depending upon the number of amino acids that were absent from the

proteins' N terminus. A partial clone (clone 121) lacking the N-terminal 30 amino acids had almost completely lost its IgE binding capacity (Fig. 1, A and B).

E. coli expression and purification of rPhl p 6: IgE binding capacity of purified rPhl p 6

rPhl p 6 was overexpressed in *E. coli* BL21 (DE3). A combination of several purification steps yielded pure and soluble rPhl p 6 (~5 mg protein/L *E. coli* culture), which was identified by SDS-PAGE as one of the low molecular mass timothy grass pollen allergens (Fig. 2A). MALDI-TOF analysis of purified rPhl p 6 resulted in two mass/charge peaks of 11,790 and 5,896 corresponding with the MH⁺ and M2H2⁺ species of the sample, which were in agreement with the deduced Phl p 6 molecular mass (11,789 Da).

rPhl p 6-specific IgE Abs were detected in 128 sera from 171 patients allergic to grass pollen but not in the sera from 10 non-allergic individuals. Preabsorption of sera from patients allergic to grass pollen with rPhl p 6 led to a great reduction of IgE binding to a 10–14 kDa moiety in nitrocellulose-blotted timothy grass pollen extract, indicating that rPhl p 6 and natural Phl p 6 share IgE epitopes. ELISA competition experiments demonstrated that only a small percentage (<20%) of Phl p 5-specific IgE could be pre-absorbed with rPhl p 6. IgE binding to rPhl p 1, rPhl p 2, and recombinant timothy grass profilin was not reduced after preincubating sera with rPhl p 6. These results identify Phl p 6 as a major allergen that is distinct from other grass pollen allergens.

rPhl p 6 folds in a stable all α -helical conformation

The far-UV CD spectrum of purified rPhl p 6 (Fig. 2B) indicates that the protein contains a considerable amount of α -helical secondary structure. The spectrum is characterized by two broad minima at 208 nm and 220 nm and a maximum at 191 nm. The secondary structure prediction (Fig. 1A) is in good agreement with the CD measurements, as it indicates predominant α -helical secondary structure content. The unfolding transition of rPhl p 6 is monophasic and is highly cooperative, with a melting point of 61°C. At 85°C, rPhl p 6 assumes a random coil conformation, with a typical minimum at 200 nm. rPhl p 6 shows a high degree of folding reversibility, evident from the cooling curve profile (Fig. 2C) and the far-UV spectrum recorded at 20°C after cooling from 85°C (Fig. 2B).

rPhl p 6 induces dose-dependent basophil histamine release and immediate type skin reactions in patients allergic to grass pollen

Purified rPhl p 6 induced specific and dose-dependent histamine release from basophils of a patient allergic to grass pollen (Fig. 3A). rPhl p 5, which represents a highly active grass pollen allergen (Ref. 14, R. Valenta and S. Flicker, unpublished data), induced maximal release already at a lower concentration compared with rPhl p 6. rPhl p 6, rPhl p 5, and timothy grass pollen extract induced immediate type skin reactions in four patients allergic to grass pollen, but not in the nonallergic individuals (Table I; Fig. 3B). Although no reactions to NaCl were observed, histamine induced wheal reactions in all individuals tested (Table I; Fig. 3B).

Group 6 allergens represent pollen-specific proteins

Although major groups of grass pollen allergens occur in pollens of most grass species (4), group 6 allergens were reported to occur exclusively in timothy grass (*P. pratense*) pollen (15). A rabbit anti-rPhl p 6 antiserum cross-reacted with group 5 allergens in nitrocellulose-blotted pollen extracts from various monocots (*P. pratense*, *L. perenne*, *S. cereale*, *T. sativum*, *A. sativa*, *P. communis*) between 25 and 28 kDa (Fig. 4A, lane 2). Phl p 6 or Phl p 6-related allergens at 11 kDa were detected exclusively in pollens

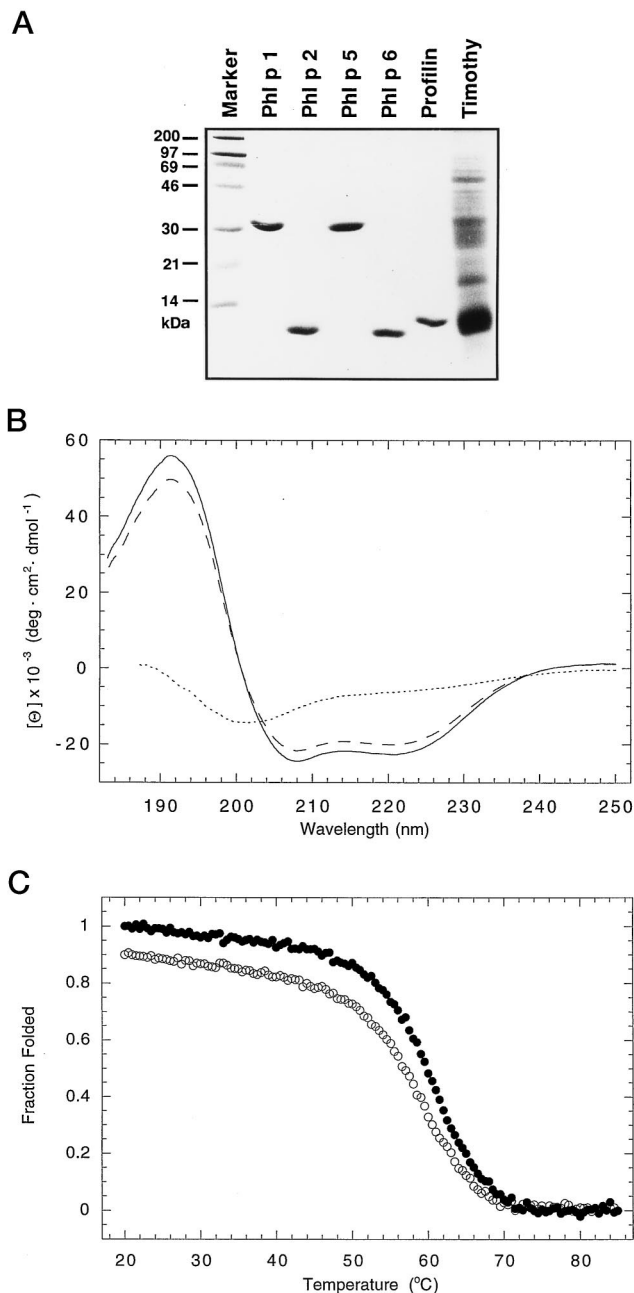


FIGURE 2. A, Purity of recombinant timothy grass pollen allergens. Coomassie brilliant blue-stained, SDS-PAGE-containing purified, recombinant timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, Phl p 6, and timothy grass pollen profilin) and natural timothy grass pollen extract (Timothy) are shown. M, molecular mass marker. B and C, CD analysis. B, Far-UV CD spectra of rPhl p 6, expressed as mean residue ellipticity (Θ) (y-axis), were recorded in the wavelength range displayed on the x-axis at 20°C (solid line), at 85°C (dotted line), and at 20°C after cooling from 85°C (dashed line). C, Thermal denaturation and cooling of purified rPhl p 6 monitored at 220 nm (x-axis, temperature in degrees Celsius; y-axis, apparent fraction of the folded protein).

from *P. pratense* and *P. pratensis*. Although a putative N-glycosylation site was found in the amino acid sequence deduced from the Phl p 6 cDNA sequence, the comparable molecular masses observed for natural Phl p 6 and rPhl p 6 exclude heavy glycosylation of natural Phl p 6 (Figs. 2A and 4A). Rabbit anti-rPhl p 6 Abs strongly reacted with Phl p 6 at 11 kDa in nitrocellulose-blotted timothy grass pollen but not with leaf or root extracts (Fig. 4B,

lane 2). Profilin was detected in all three tissues at ~14 kDa (Fig. 4B, lane 1).

Immunoelectron microscopic localization of Phl p 6 in timothy grass pollen

Using postembedding immunogold electron microscopy, rabbit anti-rPhl p 6 Abs bound to the numerous polysaccharide (P-) particles, which fill much of the interior of a mature timothy grass pollen grain (Fig. 4C). The greatest accumulation of gold particles was observed on sectioned surfaces of the P-particles, indicating that Phl p 6 is present on rather than in the P-particles. Little (cytosol, exine) or no (mitochondria, intine) anti-rPhl p 6 immunoreactivity was observed in other parts of the pollen grain. Likewise, almost no gold particles were detected in the amyloplasts. This localization pattern, taken together with our finding that a rabbit anti-rPhl p 5 antiserum failed to label the P-particles (data not shown), excludes the possibility that the immunolabeling of the P-particles resulted from the presence of cross-reactive group 5 allergens. Control experiments performed with preimmune Ig yielded only a few nonspecifically adsorbed gold particles (Fig. 4D).

Discussion

Approximately 40% of allergic patients display immediate type symptoms after contact with grass pollen (3). We have isolated cDNAs coding for isoforms and fragments of a major timothy grass pollen allergen, designated Phl p 6. Phl p 6 represents an 11.8-kDa protein allergen that is recognized by IgE Abs of 75% of patients allergic to grass pollen. The prevalence of IgE recognition of rPhl p 6 is thus in accordance with that reported earlier for natural Phl p 6, indicating that carbohydrate moieties do not play a relevant role in the recognition of Phl p 6 by IgE (28, 29). In agreement with the peptide sequence data obtained for natural Phl p 6, we found that the deduced amino acid sequence of rPhl p 6 shows a high degree of sequence homology with the N-terminal portions of group 5 grass pollen allergens, a family of 25- to 35-kDa major grass pollen allergens (29, 14). Due to the presence of an N-terminal hydrophobic leader peptide, Phl p 6 represents an independent allergen, rather than a group 5 allergen fragment. In agreement with the proposal of other authors, who analyzed a Phl p 6-encoding cDNA clone (15), we suggest that group 5 and group 6 allergens may have evolved from common ancestor genes similar to what was described for group 1 and group 2/3 grass pollen allergens (30). The assumption that Phl p 6 belongs to an independent group of grass pollen allergens is also supported by our finding that Phl p 6 shares few cross-reactive IgE epitopes with group 5 allergens and no epitopes with other grass pollen allergens. The prediction of solvent accessibility indicated that many of the Phl p 6 N-terminal amino acids are solvent exposed, whereas most of the C-terminal amino acid residues appeared to be buried. Although not proof, this finding is in agreement with data obtained from the IgE epitope mapping experiments, which indicate that the protein N terminus is critically involved in IgE recognition. However, it is equally possible that the N terminus itself represents a dominant IgE epitope, or that deletion of the N terminus affects conformational Phl p 6 IgE epitopes.

The expression of Phl p 6 in *E. coli* yielded large amounts of soluble and folded recombinant protein that contained an almost exclusive α -helical secondary structure. The α -helical fold of Phl p 6 is a further confirmation that there are no common structural features that predispose a certain protein to behave as an allergen. Although Phl p 6 is very likely an all α -helical protein, Bet v 1, the major birch pollen allergen (31) and Bet v 2, birch profilin (32) have a mixed $\alpha\beta$ fold. As revealed by CD spectroscopic analysis,

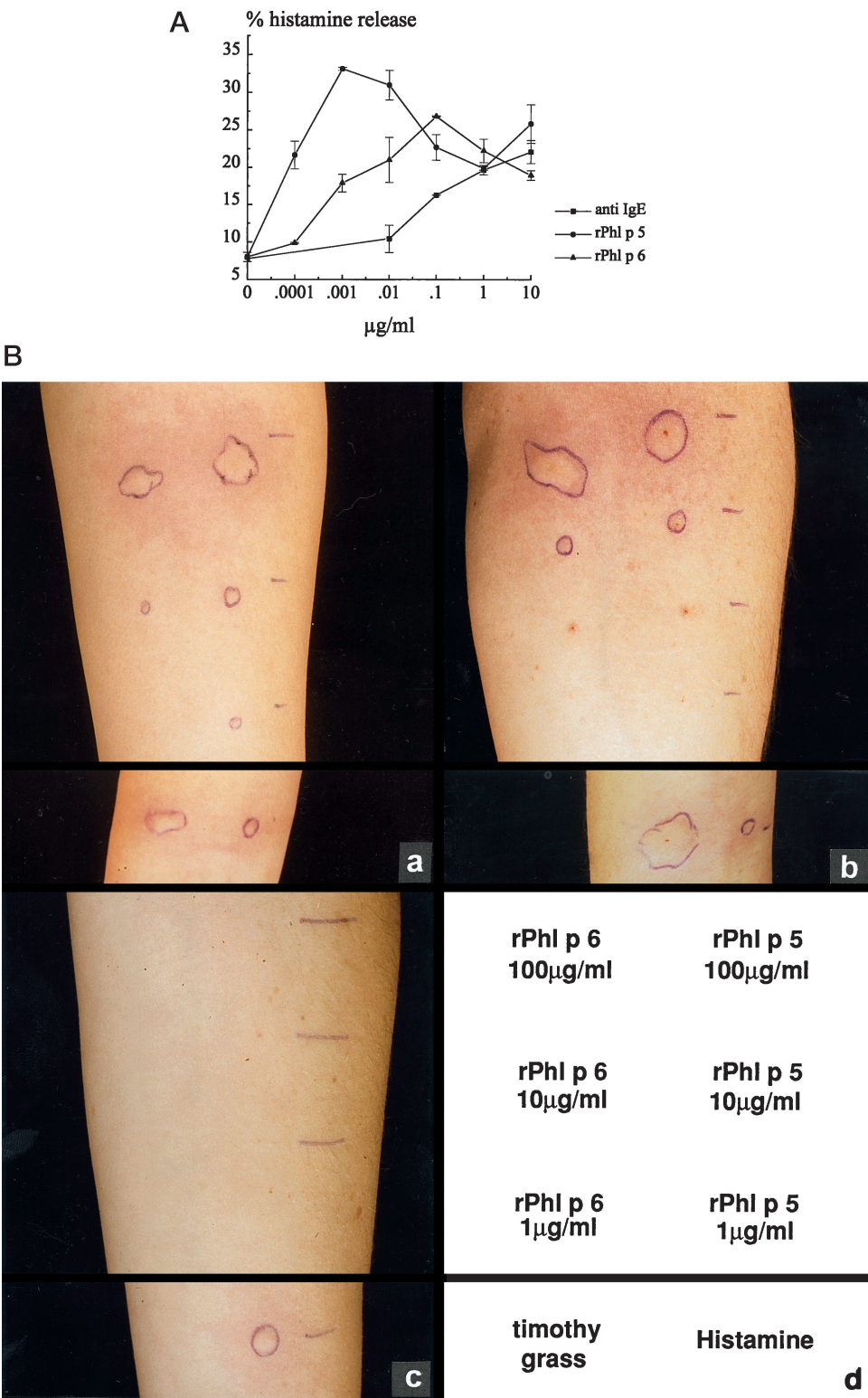


FIGURE 3. A, rPhl p 6 induces basophil histamine release. Granulocytes from a patient allergic to grass pollen were incubated with various concentrations (x-axis) of purified rPhl p 6 (triangles), Phl p 5 (points), or an anti-IgE mAb (squares). The percentage of histamine released into the supernatant is displayed on the y-axis. Results represent the means ($\pm$ SD) of triplicate determinations. B, Induction of immediate type skin reactions with rPhl p 6 in sensitized allergic patients. Two patients allergic to grass pollen (LW (a) and HP (b)) and a nonallergic individual (SV (c)) were pricked on their forearms with increasing concentrations of rPhl p 6 and rPhl p 5 as well as with histamine and timothy grass as indicated in d. The wheal area was surrounded with a ballpoint pen.

rPhl p 6 shares with other immunologically unrelated pollen allergens (e.g., Bet v 1 (33), Bet v 2 (6, 32)) the remarkable intrinsic tendency to refold into a stable conformation after denaturation. Another feature that is shared by Phl p 6 and other important plant allergens is high expression in pollen tissue. The fact that most of the plant allergens characterized so far are expressed predominantly in mature pollen may therefore be interpreted as a footprint of sensitization via the respiratory tract (34).

Table I. Immediate type skin reactivity to rPhl p 6

Individual	Phl p 6 (10 μg/ml)	Phl p 6 (100 μg/ml)	Phl p 5 (10 μg/ml)	Phl p 5 (100 μg/ml)	Timothy Grass	Histamine	NaCl
Patients allergic to grass pollen							
HP	5	16.5	5.5	13	16	5.5	0
SF	0	13	2	11	8	7	0
CS	0	12	5	8.5	12	9	0
LW	2.5	10.5	5	13	9	5.5	0
Nonallergic individuals							
SV	0	0	0	0	0	6	0
SS	0	0	0	0	0	7.5	0

By immunogold electron microscopy, Phl p 6 was primarily localized on the P-particles of mature pollen. P-particles are small polysaccharide-containing bodies that represent ≤30% of the contents of the dormant pollen grain and, during pollen germination, transfer material into the growing pollen-tube wall (35, 36). The occurrence of Phl p 6 on the P-particles may be of clinical relevance, as P-particles could act as small-sized (<2.5 μ) and there-

fore respirable allergen-carriers that bring Phl p 6 in immediate contact with the bronchial mucosa. A P-particle-linked intrusion of Phl p 6 into the deeper respiratory tract would thus explain the high prevalence (75%) of sensitization against this allergen, although only a few grass species (*P. pratense*, *P. pratensis*) contained rabbit anti-rPhl p 6-reactive moieties in the low (10–12 kDa) molecular mass range.

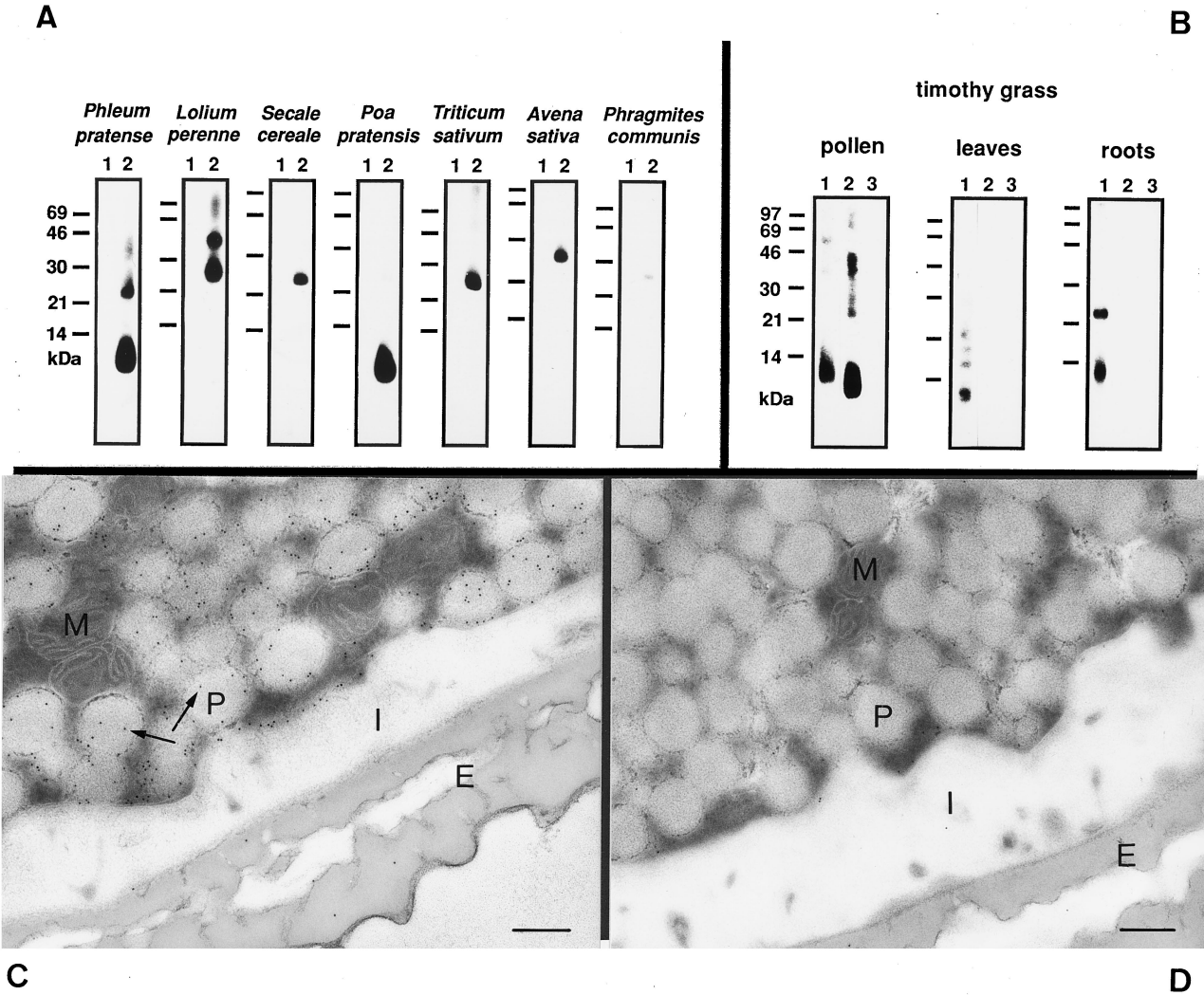


FIGURE 4. Tissue-specific expression of Phl p 6. **A**, Nitrocellulose-blotted grass pollen extracts from various monocots were probed with rabbit preimmune Ig (lane 1) or rabbit anti-rPhl p 6 Ig (lane 2). **B**, Comparable amounts of nitrocellulose-blotted protein extracts from timothy grass pollen, leaves, and roots were incubated with rabbit anti-profilin Ig (lane 1), rabbit anti-Phl p 6 Ig (lane 2), or rabbit preimmune Ig (lane 3). **C** and **D**, Ultrastructural localization of Phl p 6. Ultrathin sections of timothy grass pollen were stained with rabbit anti-Phl p 6 Ig (**C**) and with rabbit preimmune Ig (**D**). Bound rabbit Abs were detected with a gold-conjugated goat anti-rabbit Ig antiserum (gold particles = black dots). Arrows indicate Phl p 6 immunoreactivity on the P-particles. E, exine; I, intine; P, P-particle; M, mitochondria. The bars represent 0.250 μm.

The *E. coli*-expressed purified rPhl p 6 allergen reacted with IgE Abs of the majority of patients allergic to grass pollen and induced basophil histamine release as well as immediate type skin reactions. It may therefore be used for in vitro as well as in vivo (skin test) diagnoses of grass pollen allergy. Our finding that deletion of the N-terminal portion of Phl p 6 dramatically reduced the IgE binding capacity of the allergen may lead to the construction of hypoallergenic Phl p 6 deletion mutants that may be used for specific immunotherapy of grass pollen allergy with reduced anaphylactic side effects. Recently, the bacterial expression of two hypoallergenic fragments comprising amino acids 1–74 and amino acids 75–160 of the major birch pollen allergen, Bet v 1, was reported (23). The recombinant hypoallergenic Bet v 1 fragments contained most of the Bet v 1-specific T cell epitopes (23) and, upon immunization of animals, induced Abs that blocked IgE binding to the complete Bet v 1 wild-type molecule (S. Vrtala and R. Valenta, unpublished data). Our data obtained for Phl p 6 provide good evidence that it may be possible to use a similar strategy to produce a hypoallergenic N-terminally truncated Phl p 6 version that as such or in combination with a C-terminally truncated Phl p 6 molecule could be used for immunotherapy of Phl p 6-sensitized individuals.

In conclusion, we have produced a major timothy grass pollen allergen, Phl p 6, as a recombinant molecule useful for the in vitro and in vivo diagnosis of grass pollen allergy. Hypoallergenic deletion variants of Phl p 6 may be used for specific immunotherapy of grass pollen allergy.

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