

Characterization of Phl p 4, a major timothy grass (*Phleum pratense*) pollen allergen

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Background: Group 4 grass pollen allergens represent glycoproteins with a molecular weight of 50 to 60 kD, which are present in many grass species. Almost 75% of patients allergic to grass pollen display IgE reactivity to group 4 allergens, which hence can be regarded as major grass pollen allergens.

Objective: In this study attempts were made to obtain information regarding the immunologic properties, localization, and occurrence of Phl p 4 and related allergens.

Methods: Phl p 4 was detected in timothy grass pollen extracts by immunoblotting with serum IgE and monoclonal antibodies and was localized in pollen by immunoelectron microscopy. A peptide sequence from Phl p 4 was obtained by amino acid sequencing. The resistance of Phl p 4 against trypsin was analyzed after trypsin treatment of timothy grass pollen extracts with serum IgE and monoclonal antibodies. Cross-reactivities between Phl p 4 and Amb a 1, the major allergen of ragweed, were studied by using monoclonal antibodies and by IgE-inhibition studies.

Results: Phl p 4 was characterized as a trypsin-resistant major timothy grass pollen allergen. By immunoelectron microscopy Phl p 4 was localized in the exine, cytoplasm, and amyloplast of timothy grass pollen. Significant sequence similarities of a Phl p 4 10 amino acid peptide with Amb a 1, the major ragweed allergen, could be found. The immunologic similarity of Phl p 4 and Amb a 1 was confirmed by cross-reactivity of monoclonal antibodies and patients' IgE.

Conclusion: Phl p 4 represents a trypsin-resistant major timothy grass pollen allergen with immunologic similarities to the major ragweed allergen Amb a 1 and therefore must be considered an important cross-reactive component in grass pollen and weed pollen allergy. (*J Allergy Clin Immunol* 1996;98:189-98.)

Key words: Grass pollen allergy, Phl p 4, trypsin resistance, monoclonal antibodies, cross-reactivity, Amb a 1, ragweed allergy, pollen-specific expression

Grass pollen allergy has a high prevalence in Europe, America, and Australia where up to 70% of patients with type I allergy display IgE reactivity to grass pollen allergens.¹ Group 1, group 2/3, and group 5 grass pollen allergens were identified as major allergens that are present in most grass species.²⁻⁴ Almost 95% of patients allergic to grass

Abbreviations used

mAb:	Monoclonal antibody
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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pollen show IgE specific for group 1 allergens, 80% react with group 5 allergens, and 60% to 70% display IgE reactivity to group 2/3 grass pollen allergens.⁵ Although these major grass pollen allergens are well described and the corresponding complementary DNAs have been isolated and expressed in *Escherichia coli* as functional allergens,⁶⁻¹³ little is known about group 4 grass pollen allergens. Group 4 grass pollen allergens were described as glycoproteins with molecular weight

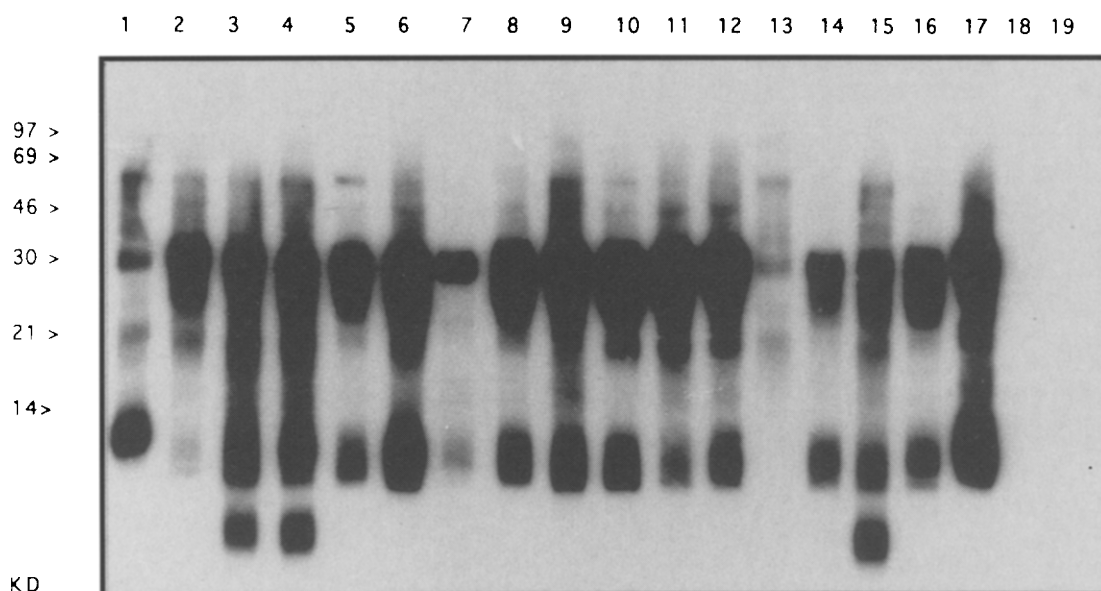


FIG. 1. IgE reactivity of sera from patients allergic to grass pollen to nitrocellulose-blotted timothy grass pollen extract. Sera from 17 patients allergic to grass pollen (lanes 1 to 17) were probed for IgE reactivity with nitrocellulose-blotted timothy grass pollen extract. The serum from a nonallergic individual was included as a control (lane 18), and in lane 19 buffer instead of serum was used (buffer control). Molecular weights are indicated at left.

of 50 to 60 kD, which are present in different grass species (e.g., ryegrass, Bermuda grass, timothy grass).¹⁴⁻¹⁸ According to IgE-immunoblotting studies done with timothy grass pollen extract, up to 75% of patients allergic to grass pollen were found to display IgE reactivity to group 4 allergens.⁵ Group 4 grass pollen allergens therefore belong to the major grass pollen allergens. On the other hand, IgE adsorption studies done with a combination of four recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5, and recombinant timothy grass profilin) have indicated that rather low levels of timothy grass pollen-specific IgE are directed against group 4 and other grass pollen allergens.¹⁹

To obtain information regarding group 4 allergens, immunoblot analyses with sera from patients allergic to grass pollen and monoclonal antibodies (mAbs) specific for Phl p 4¹⁸ were done. In addition, the localization of Phl p 4 in timothy grass pollen was studied by immunoelectron microscopy. Digestion of timothy grass pollen extracts with trypsin revealed a remarkable resistance of Phl p 4 against proteolysis. The amino acid sequence of a decapeptide derived from Phl p 4 showed significant homology with the major ragweed allergens, Amb a 1 and Amb a 2.^{20, 21} Immunologic similarity of Phl p 4 and Amb a 1 was demonstrated by cross-reactivity of Phl p 4-specific mAbs and IgE inhibition studies.

METHODS

Allergen extracts and mAbs

Timothy grass (*Phleum pratense*) pollen and ragweed (*Ambrosia trifida*) pollen were purchased from Allergon (Välinge, Sweden). Two grams of pollen was extracted in 50 ml of distilled water for 2 hours at room temperature. The protein extracts were then centrifuged at 30,000 g for 30 minutes at 4° C, and supernatants were lyophilized and stored at -20° C until use.²² The mAbs with specificity for group 4 allergens were described.¹⁸

Characterization of patients allergic to grass pollen

Sera from allergic patients were characterized by RAST, skin testing, and case history regarding grass pollen (timothy grass) and weed pollen (mugwort, ragweed) allergy as previously described.⁵ In addition, sera were tested for reactivity with recombinant timothy grass pollen allergens Phl p 1, Phl p 2, and Phl p 5 and birch profilin to determine each patient's allergogram.⁵

Trypsin digestion of grass pollen extracts

Approximately 0.5 µg of timothy grass pollen extract was dissolved in 100 µl Tris-buffered saline (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.5) and digested with 10 µg of trypsin (Boehringer, Mannheim, Germany) at 37° C for 1 hour. The reaction was stopped by addition of 60 µl of sodium dodecylsulfate sample buffer²³ and boiling for 5 minutes.

Forty microliters of the mixture and of a sample that was prepared in the same way without addition of trypsin

were loaded onto a 9% sodium dodecylsulfate–polyacrylamide gel.²³ Gels were either stained with Coomassie Blue (Bio-Rad, Richmond, Calif.)²⁴ to visualize proteins or blotted onto nitrocellulose²⁵ for antibody detection.

Determination of the amino acid sequence of a Phl p 4 peptide

Timothy grass pollen extract was separated by preparative sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 9% gel to obtain optimal separation of proteins in the 40 to 60 kd molecular weight range. Proteins were then transferred to Immobilon-polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.) by electroblotting.²⁵ Immobilon membranes were stained with Coomassie Blue according to the manufacturer's advice, and the position of group 4 allergens was marked. According to the amino acid analysis, 111 pmol of the protein was subjected to a digestion procedure. As controls, a piece of blank polyvinylidene difluoride membrane and transferrin were digested as well. The membranes were incubated in 100 μ l of digest buffer (1% hydrogenated Triton X-100, 10% CH₃CN, 100 mmol/L Tris, pH 8.0) by adding lysyl endopeptidase (Boehringer) to a final concentration of 12 μ g/ml for 24 hours at 37° C. After the digestion, the sample was separated on a Vydac C18 2.1 column (Millipore Corp.). Peak 90, which on the basis of the high-pressure liquid chromatography profile appeared homogenous, was sequenced by using a Beckman Analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The digestion, peptide purification, and sequencing were done as a commercial service at the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, Conn.).

Purification of Phl p 4 by ion-exchange chromatography and preparative Western blotting

Phl p 4 was purified by ion-exchange chromatography as described by Fahlbusch et al.²⁶ and by preparative Western blotting. For the latter, timothy grass pollen extract (0.5 mg/cm gel) was separated by 9% preparative SDS-PAGE²³ and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany).²⁵

Immunoelectron microscopic localization of Phl p 4 in timothy grass pollen

Timothy grass pollen grains were exposed to acrolein vapor for 7 days at room temperature, then dehydrated in dimethoxypropane and absolute ethanol for 4 hours each and embedded in Lowicryl K4M²⁷ with three intermediate steps of ethanol-Lowicryl (2:1, 1:1, 1:2) between. The temperature was lowered to –35° C, and polymerization was done by UV irradiation. Ultrathin sections were cut and then incubated on drops of the following solutions: (1) 5% bovine serum albumin in phosphate-buffered saline (PBS) for 5 minutes, (2) mAb

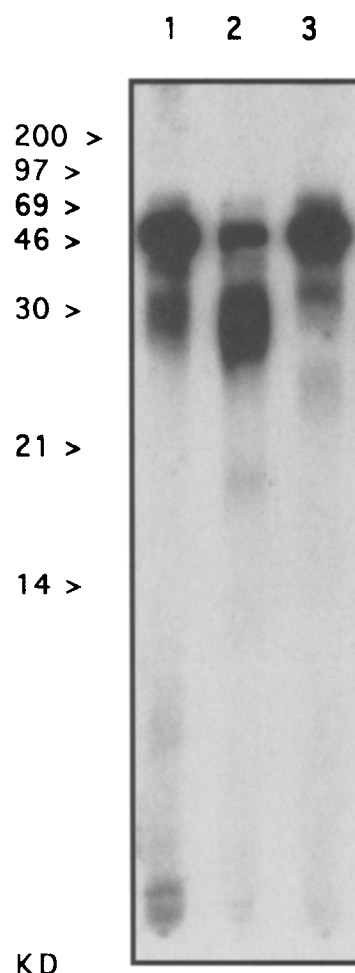


FIG. 2. Reactivity of Phl p 4–specific mAbs with nitrocellulose-blotted timothy grass pollen extract. Three murine mAbs specific for Phl p 4 (lane 1, 3C4; lane 2, 2D8; lane 3, 5H1) were probed with nitrocellulose-blotted timothy grass pollen extract.

3C4, diluted 1:160 in PBS, for 2 hours, and (3) PBS and Tris, three times for 5 minutes each. Incubation was done in a moist chamber at room temperature with PBS (pH 7.4) and Tris buffer consisting of 20 mmol/L Tris, 20 mmol/L NaN₃, 225 mmol/L NaCl, and 1% wt/vol bovine serum albumin (pH 8.2). In control experiments, the specific primary antibody was replaced by another mAb of irrelevant specificity. Moreover, in another control experiment, the gold-coupled secondary antibody was applied to the sections without prior incubation with the primary antibody as described by Grote et al.²⁷

Immunoblotting with serum IgE and mAbs

Pollen extracts (0.5 mg/cm gel) were separated by preparative 12% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell). Strips of about 5 mm in width were cut from the membranes and incubated two times for 5 minutes and once for 30 minutes in buffer A (50 mmol/L Naphosphate: pH 7.5, 0.5% vol/vol Tween

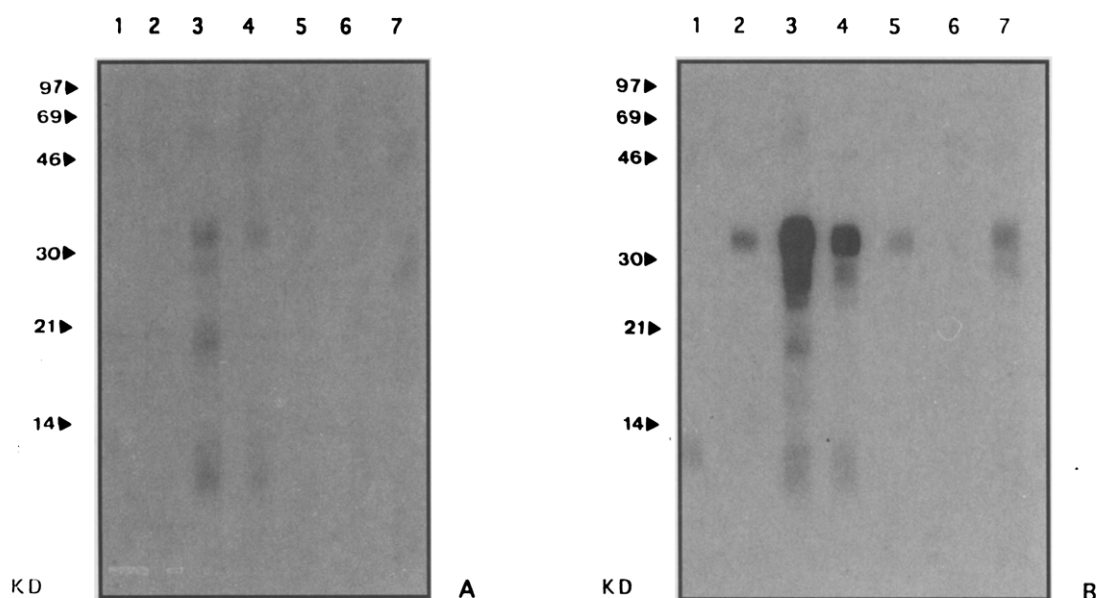


FIG. 3. Preadsorption of sera with a mixture of recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin reduces IgE binding to nitrocellulose-blotted timothy grass pollen extract. Sera from seven patients allergic to grass pollen (*lanes 1 to 7*) were preadsorbed with a mixture of recombinant allergens (**A**) or with a comparable amount of a control protein (dog albumin, negative control; **B**) and then incubated with nitrocellulose-blotted timothy grass pollen extract.

20, 0.5% wt/vol bovine serum albumin, 0.05% wt/vol NaN_3). Nitrocellulose strips were incubated with sera from allergic patients (diluted 1:10 in buffer A) or with hybridoma supernatants (diluted 1:5 in buffer A) overnight at 4°C. Strips were then washed as described previously. Bound IgE was detected with 1:10 diluted iodine 125-labeled anti-IgE antibodies (Pharmacia, Uppsala, Sweden). Bound murine mAbs were detected with a 1:500 diluted ^{125}I -labeled sheep anti-mouse antiserum (Amersham, Buckinghamshire, U.K.) or with an alkaline phosphatase-coupled rabbit anti-mouse antiserum (Dianova, Hamburg, Germany). For the detection of bound radiolabeled antibodies, Kodak X-OMATS film and intensifying screens (Kodak, Heidelberg, Germany) were used.

IgE absorption experiments

IgE inhibitions were done as described by Vrtala et al.¹³ In brief, serum samples from seven patients allergic to timothy grass pollen were depleted of Phl p 1, Phl p 2, Phl p 5, and profilin-specific IgE by preincubation with 10 µg of each recombinant allergen (recombinant birch profilin, which shares most IgE epitopes with timothy grass profilin, was used²⁸) per milliliter of 1:10 diluted serum. Purified natural Phl p 4 was used for preadsorptions at a concentration of 5 µg/ml of 1:10 diluted serum. After preincubation, the serum dilutions were diluted 1:2 and incubated with the blotted extracts, and bound IgE was detected.

RESULTS

IgE reactivity of sera from patients allergic to grass pollen

Fig. 1 shows the IgE binding of sera from 17 patients allergic to grass pollen to nitrocellulose-blotted timothy grass pollen extract. All patients' sera showed IgE reactivity to proteins of approximately 30 kD, which contain group 1 and group 5 allergens; 16 sera showed reactivity to proteins of 10 to 14 kD, which represent group 2/3 and timothy grass profilin; and three sera displayed reactivity to proteins of about 8 kD, which may represent calcium-binding allergens from timothy grass.²⁹ Fourteen sera bound to proteins of approximately 22 kD, which may represent group 6 allergens³⁰ or cleavage products of group 5 allergens. Fourteen sera reacted with proteins ranging from 46 to 90 kD, which were described as group 4 allergens.¹⁴⁻¹⁸ According to the percentage of sera that reacted with 60 kD proteins in Fig. 1 (82%) and in previous studies done with 98 sera (75%),⁵ group 4 allergens may be considered major grass pollen allergens. The typing with recombinant allergens (see Methods) showed that the serum from patient 1 contained no IgE specific for Phl p 1, Phl p 2, and Phl p 5. The band at 14 kD is due to a reactivity with timothy grass pollen profilin.

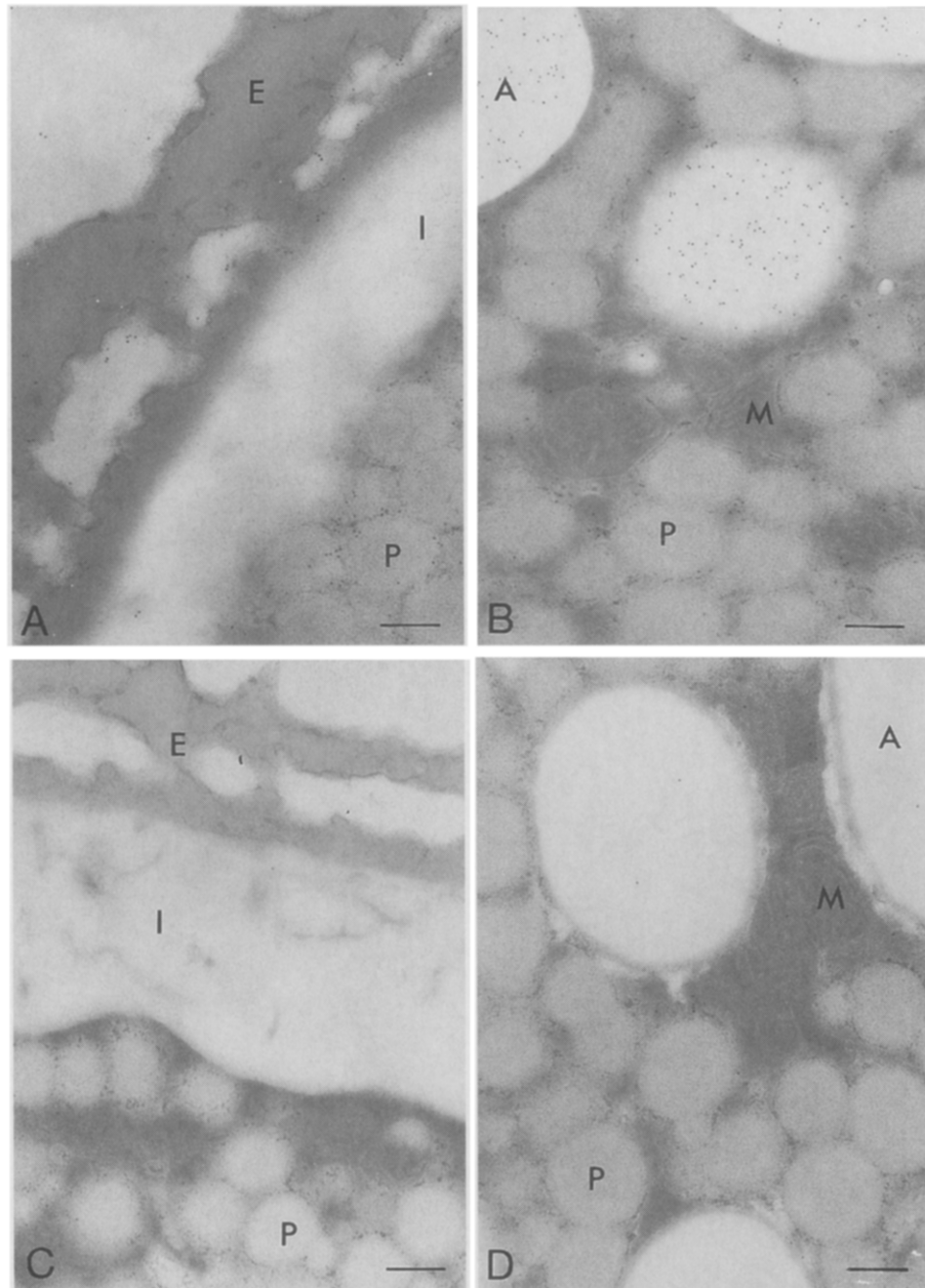


FIG. 4. Immunelectron microscopic localization of Phl p 4 in timothy grass pollen. Ultrathin sections of timothy grass pollen grains after postembedding labeling with primary antibodies and gold-conjugated secondary antibodies. **A and B,** Sections were incubated with monoclonal anti-Phl p 4 antibody 3C4. **A,** Antibody binding sites were found within the exine part of the pollen wall (slightly tangentially cut) and in the cytoplasmic matrix. **B,** Phl p 4 is localized between the P-particles and within the amyloplasts. **C and D,** An antibody with irrelevant specificity was used as a control. The cell wall (cross section) and the cytoplasm showed a few nonspecifically adsorbed gold particles. *Bar* represents 0.2 μ m. *A,* Amyloplast; *E,* exine; *I,* intine; *M,* mitochondrion; *P,* polysaccharide particle. (Original magnification $\times 50,000$.)

Phl p 4	I V A L P X G M L K	
Amb a 1.3	V K V - - G - - I -	aa 173-182
Amb a 1.2	- K V C - G - - I -	
Amb a 1.1	V K V N - G - L I -	
Amb a 2	V R V - - G - R I -	
Zm58.2	S K - H S G - - I R	
lat59	- - E G N G - - V R	
lat56	Y C T Y R S - L - R	
G10	- - P T - G - L - R	

FIG. 5. Sequence comparison of a decapeptide sequence of Phl p 4 with homologous peptides from the major ragweed allergen Amb a 1 and pollen-specific proteins. A decapeptide sequence obtained from Phl p 4 was compared with known protein sequences from the major ragweed allergens Amb a 1 and Amb a 2, a pollen-specific protein from *Zea mays* (Zm58), anther-expressed proteins from tomato (lat59, lat56), and a pollen-specific tobacco protein (G10). Identical amino acid residues are indicated by a *dash*. The amino acid in position X of the Phl p 4 peptide could not be determined.

Murine mAbs specific for Phl p 4 detect proteins of different molecular weight in timothy grass pollen extract

Three different murine mAbs specific for Phl p 4 (3C4, 2D8, 5H1) were tested for reactivity to nitrocellulose-blotted timothy grass pollen extract, as shown in Fig. 2. All three mAbs bound strongly to a protein of about 55 to 60 kd. Additional binding to proteins of 69 kd and between 30 and 40 kd was observed. The mAbs in lanes 2 and 3 demonstrated bands at 19 to 20 kd and 24 to 25 kd. The binding pattern that was observed for the Phl p 4-specific mAbs was very similar to the IgE binding pattern observed with serum 1 in Fig. 1, which did not contain IgE specific for Phl p 1, Phl p 2, and Phl p 5.

Depletion of Phl p 1, Phl p 2, Phl p 5, and profilin-specific IgE leads to a strong reduction of IgE binding to nitrocellulose-blotted timothy grass pollen extract

Fig. 3 shows the IgE binding of seven sera to nitrocellulose-blotted timothy grass pollen extract after adsorption with a combination of recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin (Fig. 3, *A*) and without preadsorption (Fig. 3, *B*). The weak IgE binding to group 4 allergens at 55 to 60 kd (lanes 3, 4, 6, and 7) was not significantly affected by the preadsorption, whereas strong reduction of the IgE binding to group 1 and 5 allergens at 30 kd was observed. At 1:20 serum

dilutions, the binding to group 4 allergens was already very weak compared with the strong binding to group 1 and group 5 allergens, indicating that low levels of timothy grass pollen-specific IgE are directed against Phl p 4 and allergens other than those of groups 1 and 5.

Localization of Phl p 4 in timothy grass pollen by immunoelectron microscopy

After incubation of timothy grass pollen sections with the mAb 3C4, Phl p 4 was found in the pollen wall and in the interior of the grain (Fig. 4, *A* and *B*). In the pollen wall, it was predominantly the exine part with its bacular cavities that was decorated with gold particles (Fig. 4, *A*). In the cytoplasmic interior of the grain, antibodies to group 4 allergen bound to the amyloplasts and the cytoplasmic matrix, as previously demonstrated for group 5 allergens.^{11, 27} There was no specific labeling of the numerous polysaccharide inclusions (P-particles) or cell organelles, such as mitochondria (Fig. 4, *B*). Control experiments replacing the group 4-specific antibody with an antibody of irrelevant specificity showed a low degree of background staining in all pollen compartments (Fig. 4, *C* and *D*). Incubation of the sections with the gold-coupled secondary antibody alone likewise yielded a small number of nonspecifically bound gold particles (data not shown).

A peptide derived from Phl p 4 showed significant sequence homology with the Amb a 1/2 major ragweed allergen family

A decapeptide sequence of Phl p 4, which was obtained by amino acid sequencing, could be compared with the National Center for Biological Information's databases (i.e., Protein Identification Resource, Genpept, and Swiss Protein databases). Fig. 5 shows significant sequence similarities of the Phl p 4 peptide with peptides from the major allergen family of ragweed (Amb a 1, Amb a 2).^{20, 21} Additional homologies were found with pollen-specific, expressed proteins from *Zea mays* (Zm58),³¹ tomato (lat59, lat56),³² and tobacco pollen (G10).³³ The peptides were located at comparable portions in the mature proteins, and all homologous proteins had a comparable molecular weight and were described to be homologous to pectate lyases from the plant pathogen *Erwinia*.³⁴ All Amb a 1 homologous proteins were reported to be highly expressed in pollen tissue and occurred in monocot plants (maize), as well as in dicot plants (ragweed, tomato, and tobacco).

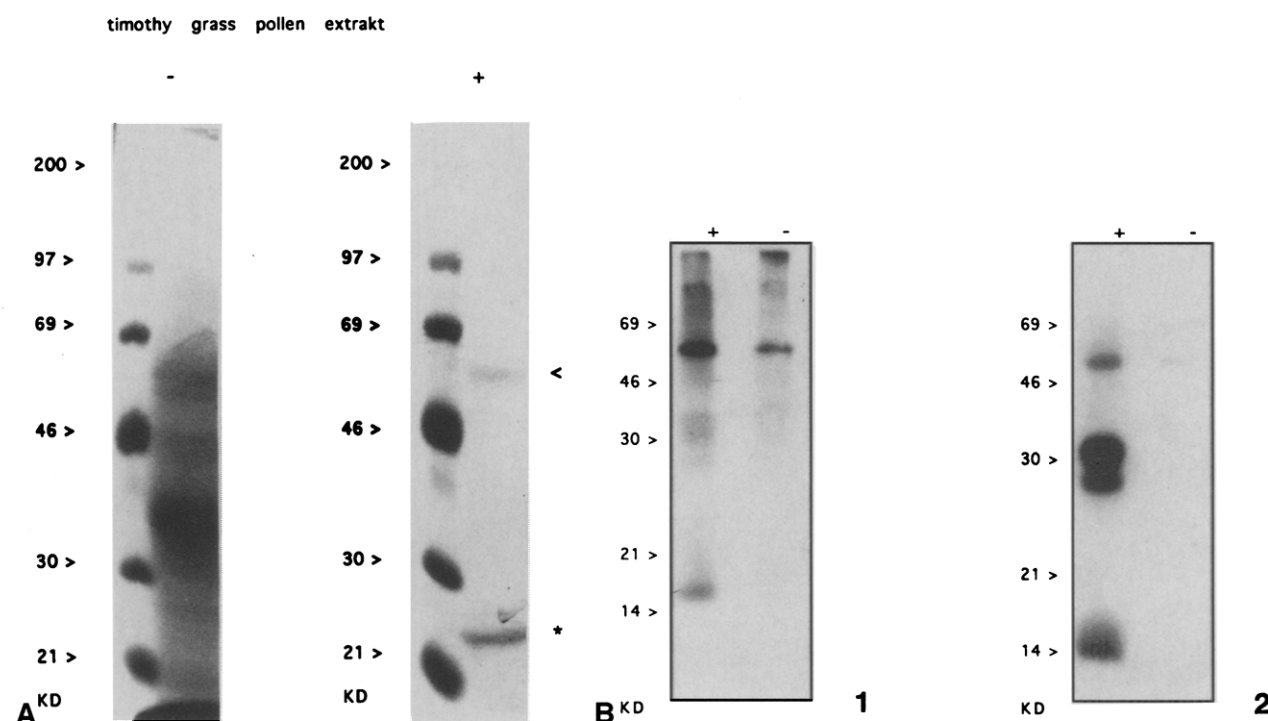


FIG. 6. A, Coomassie Blue-stained SDS-PAGE containing trypsin-digested and untreated timothy grass pollen extract. Timothy grass pollen extract was digested with trypsin (+) or untreated (–) and separated by 9% SDS-PAGE. The gel was then stained with Coomassie Blue to visualize proteins. Arrow indicates the position of a 55 to 60 kD trypsin-resistant protein, and asterisk indicates trypsin. **B,** IgE reactivity of sera from patients allergic to grass pollen with trypsin-digested and untreated timothy grass pollen extract. Sera from two patients (1 and 2) were used to detect nitrocellulose-blotted timothy grass pollen extract, which was digested with trypsin (+) or which had been untreated (–).

Phl p 4 represents a trypsin-resistant allergen

Difficulties in obtaining sufficient amounts of tryptic peptides of Phl p 4 for amino acid sequencing indicated trypsin resistance of Phl p 4, as observed for Art v 1.³⁵ To determine whether Phl p 4 might represent a trypsin-resistant allergen, timothy grass pollen extract was digested with trypsin and probed with sera from patients allergic to grass pollen. The Coomassie Blue-stained SDS-PAGE in Fig. 6, A, shows that after cleavage of timothy grass pollen extract with trypsin, only one prominent band of about 55 to 60 kD remained undigested. The additional band at 25 kD represented trypsin (indicated with an asterisk in Fig. 6, A). Fig. 6, B, shows the IgE reactivity of sera from two patients with trypsinized and untreated timothy grass pollen extract. Patient 1 had IgE antibodies specific for Phl p 4 and profilin. Trypsin digestion of timothy grass pollen extract abolished IgE binding to profilin at 14 kD, whereas IgE binding to Phl p 4 at 55 to 60 kD was still observed. In the case of patient 2 who displayed IgE

reactivity against Phl p 1, Phl p 2, Phl p 4, and Phl p 5, the IgE binding to group 1, 2, and 5 allergens was abolished after trypsinization of timothy grass pollen extract, whereas binding to Phl p 4 was still detectable. Similar experiments were done with mAbs specific for Phl p 1, Phl p 5, and Phl p 4; the outcome indicated that in contrast to the other allergens, Phl p 4 represented a trypsin-resistant allergen (data not shown).

Phl p 4 is immunologically related to the Amb a 1/2 family of major ragweed allergens

Possible immunologic similarities of Phl p 4 and Amb a 1 were investigated with mAbs and IgE-adsorption studies. In a first experiment it was demonstrated that two of three Phl p 4-specific murine mAbs cross-reacted with proteins of a comparable molecular weight in ragweed pollen extract (Fig. 7). Because proteins with significant sequence homology to Amb a 1 were also described to be present in maize pollen, cross-reac-

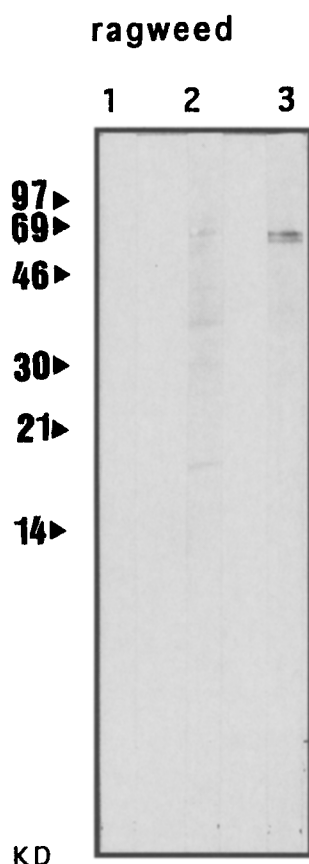


FIG. 7. Cross-reactivity of Phl p 4-specific mAbs with ragweed pollen extract. Three murine mAbs specific for Phl p 4 (lane 1, 2D8; lane 2, 3C4; lane 3, 5H1) were probed with nitrocellulose-blotted ragweed pollen extract.

tivity of the Phl p 4-specific mAbs was tested with nitrocellulose-blotted maize pollen extract; the outcome indicated that the mAbs 2D8 and 5H1 bound to maize proteins of corresponding molecular weight (data not shown). It is hence likely that Phl p 4, Amb a 1, and their homologous proteins in maize (Zm58) and perhaps tomato (lat59) may represent a family of cross-reactive plant allergens.

To further substantiate the immunologic relationship of Phl p 4 and Amb a 1, IgE inhibition experiments were done with sera from patients with grass and weed pollen allergy. The presence of common IgE epitopes was investigated by using Phl p 4, which had been purified by chromatography or by preparative Western blotting. Fig. 8 shows that preincubation of sera from two patients with purified Phl p 4 substantially reduced IgE binding to proteins of 55 to 60 kD (patient 1) and to proteins of about 30 kD (patient 2) in ragweed pollen, whereas preincubation with an unrelated allergen, dog albumin (negative control),³⁶ did not affect the IgE binding. A similar inhibition of IgE binding to ragweed pollen extract was observed

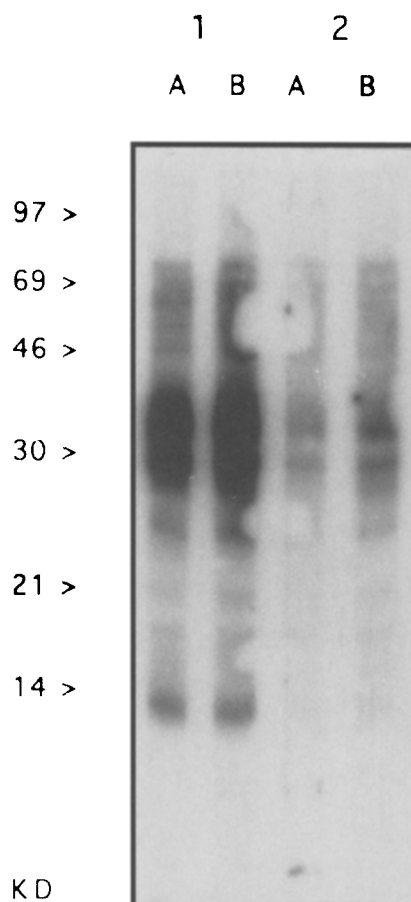


FIG. 8. Inhibition of patients' IgE binding to nitrocellulose-blotted ragweed pollen extract with purified Phl p 4. Sera from two patients allergic to grass pollen (1 and 2) were preadsorbed with purified Phl p 4 (lane A) or dog albumin (lane B) and then incubated with nitrocellulose-blotted ragweed pollen extract.

when the same sera were preadsorbed with Phl p 4, which had been isolated by preparative Western blotting (data not shown).

DISCUSSION

Grass pollen allergens belong to the most potent elicitors of type I allergy in Europe, America, and Australia. The major grass pollen allergens—described as group 1, 2/3, and 5 allergens—have been well characterized by protein and immunochemical techniques and can be produced as functional recombinant allergens in *E. coli*. Much less is known about group 4 allergens, which occur as 50 to 60 kD proteins in different grass species and represent targets for IgE antibodies of up to 75% of patients allergic to grass pollen.⁵ So far, studies have only described the presence of group 4 allergens in different grasses.¹⁴⁻¹⁸

When Phl p 4 was used as a representative allergen, the characterization of group 4 allergens was extended by proteinchemical, immunochemi-

cal, and immunohistologic techniques. This study reveals a remarkable resistance of Phl p 4 against trypsin digestion and an as yet unknown immunologic similarity of this allergen with the Amb a 1/2 major ragweed allergen family. The identification of Phl p 4 as a cross-reactive allergen in grasses and weeds might explain clinical symptoms of patients with grass pollen allergy on contact with weeds. In fact, all those patients whose sera reacted with group 4 allergens in the immunoblot shown in Fig. 1 had a positive case history, skin reaction, and serology test result indicative of weed pollen allergy.

On the one hand, IgE-immunoblotting experiments indicated that the 50 to 60 kd band, presumably representing Phl p 4, is recognized by the majority of sera from patients with grass pollen allergy. On the other hand, IgE-inhibition experiments showed that rather low amounts of grass pollen-specific IgE antibodies reacted with Phl p 4 and allergens other than Phl p 1,⁸ Phl p 2,⁹ Phl p 5,¹³ or profilin.²⁸

The rather low capacity of Phl p 4 to mount IgE responses is not due to the presence of low amounts of this allergen in grass pollen. Coomassie Blue staining of timothy grass pollen extracts identified Phl p 4 as an abundant protein. When immunelectron microscopy was used, dense labeling of Phl p 4 was found in the pollen grain sections. The allergen was localized in the cytoplasm as previously demonstrated for group 1 allergens, but the majority of the allergen was detected in the amyloplast as demonstrated for group 5 allergens.²⁷ Additional staining was observed in the inner part of the exine.

A significant sequence homology of a Phl p 4 decapeptide sequence and the major allergen family of ragweed, Amb a 1/2,^{20, 21} was found. Homologies spanning the complete sequences of the Amb a 1/2 allergen family have already been described for a number of pollen-specific proteins from tobacco,³³ tomato,³² and maize.³¹ The peptides displaying sequence similarities with the Phl p 4 peptide were located at corresponding regions in the proteins, and all homologous proteins had a comparable molecular weight of 50 to 60 kd, which corresponds to the molecular weight observed for Phl p 4 in SDS-PAGE. Recently, the complete cDNA and deduced amino acid sequence of Cry j 1, the major allergen from Japanese cedar pollen, was shown to be highly homologous to the Amb a 1/2 ragweed allergen family.³⁷

Despite these numerous reports describing sequence similarities of the Amb a 1/2 major allergen family with pollen-specific proteins and the major allergen of Japanese cedar pollen, Cry j 1, it is not

known whether these sequence similarities may be responsible for immunologic cross-reactivities. Using Phl p 4-specific mAbs and IgE-inhibition experiments, we attempted to determine whether Phl p 4 might be immunologically related to the Amb a 1/2 allergens. Cross-reactivity of the mAbs with ragweed pollen extract, as well as IgE inhibition studies with Phl p 4, proved the presence of common epitopes.

Experiments showing that Phl p 4 represented a trypsin-resistant protein are of interest because they indicate that protease-resistant IgE epitopes are not exclusively due to carbohydrate moieties but can also be formed by the protein backbone of an allergen. This is of relevance in view of previous studies describing protease-resistant cross-reactive components in pollen and vegetable foods.³⁸ It is therefore not unlikely that Phl p 4 and Amb a 1/2 homologous allergens constitute a family of cross-reactive plant allergens as described previously for profilins.³⁹⁻⁴² The presence of common IgE epitopes of Phl p 4 with ragweed allergens may provide an explanation for the clinical observation that patients allergic to grass pollen frequently also have allergy to weed pollen.

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