

Molecular Characterization of Polygalacturonases as Grass Pollen-Specific Marker Allergens: Expulsion from Pollen via Submicronic Respirable Particles¹

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Grass pollen belong to the most important allergen sources involved in the elicitation of allergic asthma. We have isolated cDNAs coding for Bermuda grass (*Cynodon dactylon*) and timothy grass (*Phleum pratense*) pollen allergens, belonging to a family of pectin-degrading enzymes (i.e., polygalacturonases). The corresponding allergens, termed Cyn d 13 and Phl p 13, represent glycoproteins of ~42 kDa and isoelectric points of 7.5. rPhl p 13 was expressed in *Escherichia coli* and purified to homogeneity. Immunogold electron microscopy using rabbit anti-rPhl p 13 Abs demonstrated that in dry pollen group 13, allergens represent primarily intracellular proteins, whereas exposure of pollen to rainwater caused a massive release of cytoplasmic material containing submicronic particles of respirable size, which were coated with group 13 allergens. The latter may explain respiratory sensitization to group 13 allergens and represents a possible pathomechanism in the induction of asthma attacks after heavy rainfalls. rPhl p 13 was recognized by 36% of grass pollen allergic patients, showed IgE binding capacity comparable to natural Phl p 13, and induced specific and dose-dependent basophil histamine release. Epitope mapping studies localized major IgE epitopes to the C terminus of the molecule outside the highly conserved functional polygalacturonase domains. The latter result explains why rPhl p 13 contains grass pollen-specific IgE epitopes and may be used to diagnose genuine sensitization to grass pollen. Our finding that rabbit anti-rPhl p 13 Abs blocked patients' IgE binding to the allergen suggests that rPhl p 13 may be used for immunotherapy of sensitized patients. *The Journal of Immunology*, 2004, 172: 6490–6500.

Type I allergy represents a major health problem in industrialized countries, affecting almost 500 million people worldwide. Allergic patients suffer from the production of IgE Abs against otherwise harmless Ags, termed allergens, which occur in our environment. Contact with environmental allergen sources (e.g., mites, insects, animal dander, molds, plant pollen, food) induces a cascade of IgE-mediated inflammatory responses in allergic patients (1).

Due to the worldwide distribution and heavy pollen production, grasses represent the major outdoor source of inhalant allergens against which >40% of allergic patients are sensitized. In the sixties, Johnson and Marsh (2) characterized a group of major grass pollen allergens, designated group 1, in rye grass by protein chem-

ical techniques. Through the application of rDNA technology to allergen characterization in the late eighties our knowledge of the molecular nature of grass pollen allergens has dramatically increased (reviewed in Ref. 3). A panel of recombinant grass pollen allergens resembling the epitope complexity of grasses belonging to the subfamily Pooideae, the most common subfamily of grasses in the Northern hemisphere, has been produced (reviewed in Ref. 4). However, the allergens of grasses belonging to the subfamily of Chloridoideae, which are abundant in the Southern hemisphere, are less well characterized. In this context, it has been demonstrated that pollen of Bermuda grass (*Cynodon dactylon*), one of the most important allergen sources in tropical and subtropical climates, lacks major allergen groups (e.g., groups 2, 5, and 6) which are abundant in the Pooideae (5). Furthermore, it has been shown that grass pollen allergic patients from southern parts of the world exhibit an IgE reactivity profile to Chloridoideae allergens which is not represented by recombinant Pooideae allergens (6). So far, only group 1 allergens, a family of cysteine-rich 30-kDa glycoproteins, have been identified as grass pollen-specific marker allergens occurring both in Pooideae and Chloridoideae (reviewed in Ref. 4).

We have screened an expression cDNA library constructed from mature pollen of Bermuda grass, the most common member of the Chloridoideae, with serum IgE of a grass pollen allergic patient cosensitized to Pooideae and Chloridoideae to identify common antigenic determinants. The isolation and characterization of an IgE-reactive Bermuda grass pollen clone, designated Cyn d 13, allowed us to identify a family of polygalacturonases, which have been described as group 13 allergens in the Pooideae (7–9), as diagnostic grass pollen-specific marker allergens occurring in both

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Pooideae and Chloridoideae. Using the cDNA coding for Cyn d 13, the corresponding cDNA from timothy grass (*Phleum pratense*) pollen was isolated by DNA hybridization. IgE-reactive, rPhl p 13, the Cyn d 13 homologue from timothy grass, was expressed in *Escherichia coli*, and analyzed regarding its biochemical and immunological characteristics. Using rabbit Abs raised against rPhl p 13, we localized group 13 allergens in the dry pollen grain and studied the mechanisms of allergen release responsible for sensitization and triggering of the disease. The finding that group 13 allergens are only expressed in grass pollen suggests that they represent diagnostic markers for a genuine grass pollen sensitization. Biological activity of rPhl p 13, as determined by basophil histamine release, revealed that the recombinant allergen can be used for in vitro and in vivo diagnosis of group 13-sensitized grass pollen allergic patients. The fact that rabbit Abs raised against rPhl p 13 blocked the binding of patients' IgE to the allergen further indicates that the recombinant allergen may be used for immunotherapy of sensitized patients.

Materials and Methods

Biological materials, allergens, Abs, grass pollen-allergic patients

Pollen of timothy grass (*P. pratense*), birch (*Betula verrucosa*), and mugwort (*Artemisia vulgaris*) were purchased from Allergon (Välinge, Sweden). For the immunolocalization experiments, fresh timothy grass pollen was collected near Münster (Germany) and was used immediately after shedding. Fresh fruits, vegetables, and nuts (celery, kiwi, apple, peanut, carrot) were bought at the local market.

Natural group 13 pollen allergens from timothy grass (*P. pratense*), meadow fescue (*Festuca pratensis*), Kentucky bluegrass (*Poa pratensis*), perennial ryegrass (*Lolium perenne*), maize (*Zea mays*), and orchard grass (*Dactylis glomerata*) were purified as described previously (9, 10). A rabbit anti-rPhl p 13 antiserum was raised against purified rPhl p 13 using Freund's adjuvant (Charles River Breeding Laboratories, Kisslegg, Germany). For immunolocalization experiments, anti-rPhl p 13 Ig was purified from the anti-rPhl p 13 antiserum by protein G affinity chromatography (Pierce, Rockford, IL).

Sera were obtained from 100 grass pollen allergic patients by venipuncture and were stored at -20°C until use. Grass pollen allergy was diagnosed on the basis of case history, skin prick testing, and determination of specific IgE to grass pollen extracts using the CAP-FEIA System (Pharmacia, Uppsala, Sweden). IgE reactivity profiles of patients were determined by component-resolved diagnosis using recombinant timothy grass pollen allergens as described (11).

Isolation and characterization of cDNAs coding for Phl p 13

A partial cDNA clone of 460 bp which showed sequence homology with polygalacturonases from various plant species was obtained by screening of a cDNA expression library from Bermuda grass (*C. dactylon*) pollen (12) with serum IgE from a grass pollen allergic patient. To identify the cDNA coding for the cross-reactive allergen from timothy grass (*P. pratense*), a cDNA expression library constructed from mature timothy grass pollen in phage λ gt 11 (13) was screened with the partial Bermuda grass cDNA, that had been labeled with ^{32}P by random oligonucleotide priming (14). Hybridization was conducted in $6\times$ SSC, $5\times$ Denhardt's solution, 0.5% (w/v) SDS, 100 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA, and 50% (v/v) formamide at 42°C . Filters were washed at 42°C , twice for 20 min each in $2\times$ SSC, 0.1% SDS and once for 30 min in $0.5\times$ SSC, 0.1% (w/v) SDS. A total of 18 independent positive clones were isolated, subcloned into plasmid pUC18, and sequenced using a T7 Sequencing kit (Pharmacia). Comparison of the nucleotide and deduced amino acid sequences with entries in the GenBank, European Molecular Biology Laboratory, DNA Data Base in Japan, and Protein Data Bank libraries revealed that 12 of the clones showed sequence homology to plant polygalacturonases. Both DNA strands of these 12 cDNA clones were sequenced (MWG-Biotech, Ebersberg, Germany) and a multiple sequence alignment of the deduced amino acid sequences with polygalacturonases retrieved from the SwissProt database was produced with ClustalW (15). Sequence motifs were identified by comparison of the deduced protein sequences with the PROSITE database of protein families and domains using the ExPASy Molecular Biology Server (Swiss Institute of Bioinformatics, Basel, Switzerland).

Expression and purification of rPhl p 13 and two rPhl p 13 fragments

A DNA coding for the mature Phl p 13 allergen without the N-terminal signal sequence and with a C-terminal hexahistidine tag (= rPhl p 13) was obtained by PCR amplification using the full-length clone PG1 Phl as template and the following oligonucleotide primers (MWG-Biotech): 5'-primer A (5'-GGG ATT TTA ATG GGG AAG AAG GAG GAG AAG AAG G-3') containing a *MseI* site (italics) and a methionine-encoding ATG (underlined) and 3'-primer B (5'-GGG AAT GAA TTC TCA GTG GTG GTG GTG GTG GTG GGC GGC GCA AGT GTT AGC CTC GC-3') containing an *EcoRI* site (italics) and a DNA segment introducing six histidine residues (underlined) in front of the stop codon. The PCR product was cut with *MseI/EcoRI* and subcloned into the *NdeI/EcoRI* site of the expression vector pET-17b (Novagen, Madison, WI). A truncated version of PG1 Phl lacking the first 229 bp was obtained as a by-product in the PCR described above. Subcloning of this shorter fragment into pET-17b gave an expression plasmid coding for a rPhl p 13 fragment, designated rPhl p 13-1, which represented a histidine-tagged, N-terminally truncated version of PG1 Phl. A DNA fragment coding for the conserved polygalacturonase domains (aa 208–300) with a C-terminal histidine tag was PCR-amplified from the full-length PG1 Phl clone with the following oligonucleotide primers (MWG-Biotech): 5'-primer C (5'-C GCA TTA ATG GCG CCC GGG GAC AGC CCC AAC-3') containing an *AsnI* site (italics) and a methionine-encoding ATG (underlined) and 3'-primer D (5'-CGC GAA TTC CTA GTG ATG ATG ATG ATG ATG GTC CTC GTA CGA CTT GAT CCG GAG GCC G-3') containing an *EcoRI* site (italics) and a DNA segment introducing six histidine residues (underlined) in front of the stop codon. The PCR product was cut with *AsnI/EcoRI* and subcloned into the *NdeI/EcoRI* site of the expression vector pET-17b.

Recombinant proteins were expressed in liquid cultures of *Escherichia coli* BL21(DE3) after induction of protein synthesis with isopropyl β -D-thiogalactoside (0.5 mM). The majority of the histidine-tagged recombinant proteins accumulated in the inclusion body fraction. Bacterial cells were lysed and proteins were solubilized in 6 M guanidine HCl, 100 mM NaH_2PO_4 , 10 mM Tris, 1 mM PMSF (pH 8.0), and recombinant proteins were purified by Ni^{2+} metal-ion affinity chromatography (Qiagen, Hilden, Germany). Purity of the protein samples was evaluated by Coomassie brilliant blue staining (Coomassie brilliant blue R-250; Bio-Rad, Richmond, CA) (16) of SDS polyacrylamide gels (17).

Matrix-assisted laser desorption and ionization-time of flight mass spectrometry of rPhl p 13

Laser desorption mass spectra were acquired in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, U.K.; piCHEM, Graz, Austria). Samples were dissolved in 10% acetonitrile (0.1% trifluoroacetic acid) and α -cyano-4 hydroxy-cinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

Protein extracts, SDS-PAGE, and immunoblotting

Soluble proteins were extracted from pollen of timothy grass (*P. pratense*), birch (*B. verrucosa*), and mugwort (*A. vulgaris*) by continuous shaking in PBS containing 2 mM PMSF overnight at 4°C . Insoluble particles were removed by centrifugation at $17,000\times g$ for 30 min at 4°C and the amount of proteins in the supernatants was determined using a Lowry assay (Bio-Rad DC Protein Assay). Protein extracts from celery tubers (*Apium graveolens*), kiwis (*Actinidia chinensis*), apples (*Malus sylvestris*), peanuts (*Arachis hypogaea*), and carrots (*Daucus carota*) were prepared as described (18).

IgE reactivity to nitrocellulose-blotted allergens was determined by immunoblotting (19). Allergens were separated by SDS-PAGE and blotted to nitrocellulose (19). Allergen-containing membranes were probed with sera from allergic patients and, for control purposes, from nonallergic individuals, diluted 1/10 in PBST (PBS, pH 7.5, containing 0.5% v/v Tween 20). Bound IgE Abs were detected with 1/15 diluted ^{125}I -labeled anti-human IgE Abs (RAST; Pharmacia). To investigate the presence of cross-reactive IgE epitopes between rPhl p 13 and polygalacturonases from other plants, 1/10 diluted patients' sera were preincubated overnight at 4°C with 10 $\mu\text{g}/\text{ml}$ purified group 13 allergens (rPhl p 13 or natural group 13 allergens) and, for control purposes, with 10 $\mu\text{g}/\text{ml}$ BSA. Preabsorbed sera were then exposed to nitrocellulose-blotted rPhl p 13 or allergen extracts and bound IgE Abs were detected as described for immunoblotting.

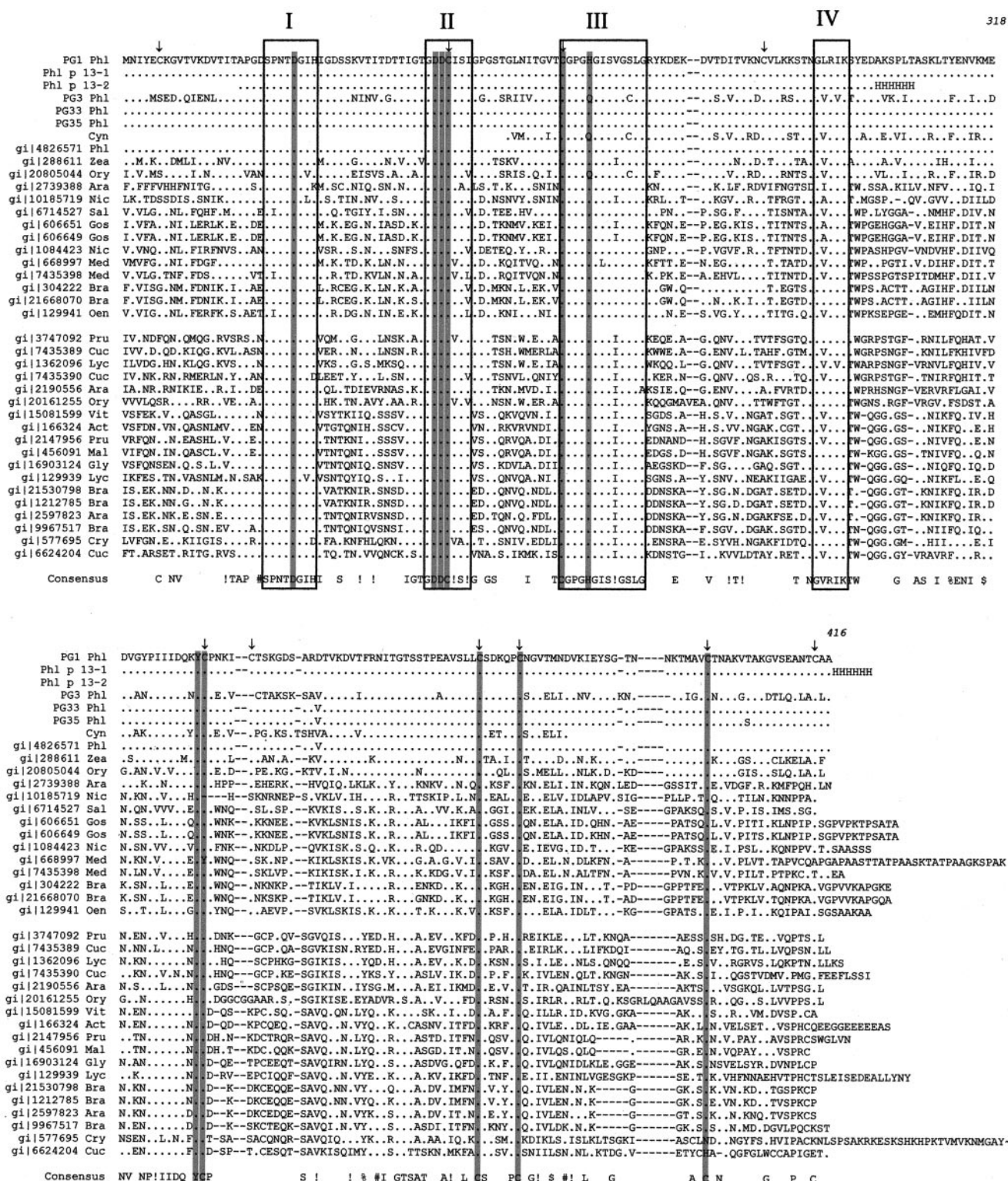


FIGURE 1. Comparison of the deduced amino acid sequence of the PG1 cDNA clone coding for Phl p 13 with cDNA clones coding for Phl p 13 isoforms or fragments (Phl p 13-1, Phl p 13-2, PG3 Phl, PG33 Phl, PG35 Phl) and the Cyn d 13 fragment from Bermuda grass (Cyn) with plant exo- and endopolygalacturonases and a plant polymethylgalacturonase (gi|577695 Cry). The four functional domains which are conserved in all polygalacturonases are indicated by boxes. Amino acids described as invariant residues in plant and fungal polygalacturonases are highlighted in gray. Sequences are preceded by their database entry codes and are grouped according to homology with PG1. gi|4826571 Phl (timothy grass), gi|288611 Zea (maize), gi|20805044 Ory (rice), gi|2739388 Ara (thale cress), gi|10185719 Nic and gi|1084423 Nic (tobacco), gi|6714527 Sal (willow), gi|606651 Gos and gi|606649 Gos (cotton), gi|668997 Med and gi|7435398 Med (alfalfa), gi|304222 Bra (rape), gi|21668070 Bra (broccoli) and gi|129941 Oen (evening primrose) represent pollen exopolygalacturonases, whereas gi|3747092 Pru and gi|2147956 Pru (peach), gi|7435389 Cuc and gi|7435390 Cuc (muskmelon), gi|1362096 Lyc and gi|129939 Lyc (tomato), gi|2190556 Ara and gi|2597823 Ara (thale cress), gi|20161255 Ory (rice), gi|15081599 Vit (wine grape), gi|166324 Act (kiwifruit), gi|456091 Mal (apple), gi|16903124 Gly (soybean), gi|21530798 Bra (turnip), gi|1212785 Bra and gi|9967517 Bra (rape) and gi|6624202 Cuc (cucumber) represent plant endopolygalacturonases. Dots represent identical amino acids and dashes indicate gaps. In the bottom line, those amino acids are listed or indicated with a symbol (!: I or V; \$: L or M; #: F or Y; #: N, D, Q, or E) which occur in at least 50% of the sequences. Cysteine residues in PG1 are marked by arrows.

pollen sections were then stained with uranyl acetate and lead citrate and analyzed in a transmission electron microscope (H 500; Hitachi, Tokyo, Japan) operated at 75 kV. For FESEM analysis of gold particle distribution, pollen preparations were dehydrated in graded series of ethanol, air-dried, coated with a 1.5 nm film of Pt/C and analyzed with an "in-lens" field emission scanning electron microscope (S-5000; Hitachi) operated in the backscattered electron mode.

Histamine release assays

Dextran sedimentation was used to isolate granulocytes from heparinized blood samples from grass pollen allergic patients. Granulocytes were incubated with increasing concentrations of rPhl p 13 and, for control purposes, with anti-human IgE Ab as described (22). Histamine liberated in the supernatants was measured by radioimmunoassay (Immunotech, Marseille, France). Results representing the mean values of triplicate determinations are expressed as the percentage of total histamine, which was determined after freeze thawing of the cells.

ELISAs

The prevalence of IgE reactivity to rPhl p 13 and to fragment aa 208–300 (rPhl p 13-2) was determined in sera from grass pollen allergic patients and, for control purposes, in sera from nonatopic individuals by ELISA. ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with the purified recombinant proteins (5 µg/ml in 0.1M sodium bicarbonate, pH 9.6) and blocked with 1% (w/v) BSA in TBS containing 0.05% (v/v) Tween 20 (TBST). Plates were incubated with sera diluted 1/5 in TBST, 0.5% (w/v) BSA and bound IgE Abs were detected with an alkaline phosphatase-coupled mouse monoclonal anti-human IgE Ab (BD Pharmingen, San Diego, CA) diluted 1/1000 in TBST, 0.5% (w/v) BSA (23) or with a HRP-coupled goat anti-human IgE Ab (1/2500 diluted in TBST, 0.5% (w/v) BSA; KPL, Gaithersburg, MD) (24).

For ELISA inhibition experiments, sera were preincubated with rPhl p 13-2 (10 µg/ml of 1/5 diluted serum) or, for control purposes, with buffer (TBST, 0.5% (w/v) BSA) and thereafter exposed to ELISA plate-bound rPhl p 13 (5 µg/ml). Bound IgE was detected using the HRP-coupled goat anti-human IgE Ab (KPL) and reduction of IgE binding to rPhl p 13 was calculated as the percentage of inhibition of IgE binding = $100 - (\text{OD}_F / \text{OD}_B) \times 100$, where OD_F and OD_B represent the extinction coefficients after preincubation with fragment rPhl p 13-2 and with buffer, respectively.

The ability of a rabbit antiserum raised against purified rPhl p 13 to inhibit the binding of patients' IgE to rPhl p 13 was examined in ELISA competition experiments as previously described (25). ELISA plate-bound rPhl p 13 (1 µg/ml) was preincubated with different concentrations of the anti-rPhl p 13 antiserum and, for control purposes, with the corresponding preimmune serum. After washing, plates were incubated with 1/5 diluted sera from grass pollen allergic patients and bound IgE was detected with HRP-coupled goat anti-human IgE Ab (KPL) (24). The percentage of inhibition of IgE binding achieved by preincubation with the anti-rPhl p 13 rabbit antiserum was calculated as follows: percent inhibition of IgE binding = $100 - (\text{OD}_s / \text{OD}_p) \times 100$, where OD_s and OD_p represent the ex-

inction coefficients after preincubation with the rabbit serum and the pre-immune serum, respectively.

Results

Sequence analysis of group 13 allergens shows that they belong to a family of pectin-degrading enzymes

Using serum IgE from a grass pollen allergic patient, we isolated a partial cDNA from a Bermuda grass pollen cDNA library, which showed sequence homology to plant exopolygalacturonases (Fig. 1, Cyn). This cDNA cross-hybridized with RNA transcripts from various grass species (data not shown) and therefore was used as a probe to isolate homologous cDNA clones from a timothy grass pollen cDNA library. The four cDNA clones obtained coded for polygalacturonase isoforms and/or fragments (Fig. 1, PG1 Phl, PG3 Phl, PG33 Phl, PG35 Phl) with significant sequence homology to a previously described timothy grass pollen allergen, Phl p 13 (gi|4826571 Phl in Fig. 1) (7) and hence were designated Phl p 13 isoallergens. The two full-length clones, PG1 Phl and PG35 Phl, were nearly identical to the previously described Phl p 13 isoform (gi|4826571 Phl in Fig. 1). PG1 Phl differed from the Phl p 13 isoform in 20 nucleotides (11 of them in the coding region) whereas PG35 Phl contained 25 nucleotide exchanges (17 in the coding region) (data not shown). However, at the amino acid level, the proteins differed from each other by only two residues. The high homology, and the even distribution of nucleotide substitutions over both the translated and the untranslated regions, suggests that PG1 Phl, PG35 Phl, and the published Phl p 13 isoform represent allelic variants of the same gene. The other two clones (PG3 Phl and PG33 Phl) coding for N-terminally truncated isoforms showed considerably lower degrees of homology to PG1 Phl and PG35 Phl as well as to each other. Although in the case of PG33 Phl, the differences were found mainly in the 3' noncoding region, PG3 Phl, represented a partial isoform with only 71% amino acid sequence homology. Thus, our data are in agreement with the described occurrence of multiple polygalacturonase gene families in various plant species (reviewed in Ref. 26).

The open reading frame of the longest Phl p 13 cDNA (PG1 Phl) coded for a protein of 416 amino acids, of which the first 22 amino acids were identified by sequence homology as a cleavable, hydrophobic N-terminal signal sequence (27). The molecular mass of the mature Phl p 13 allergen was predicted to be 41.6 kDa and an

Table I. Percentage amino acid sequence identities^a

	PG1 (%)	PG35 (%)	Phl (%)	Zea (%)	Ory (%)	Nic (%)	Sal (%)	Gos (%)	Bra (%)	Pru (%)	Cuc (%)	Lyc (%)	Ory (%)	Act (%)	Mal (%)	Gly (%)	Cry (%)
PG1	100	99	99	68	58	41	42	39	43	41	39	41	37	36	36	37	37
PG35		100	99	69	58	41	42	40	43	41	39	39	37	38	36	38	38
gi 4826571 Phl			100	73	61	42	47	41	47	41	38	42	40	39	38	40	39
gi 288611 Zea				100	56	40	44	42	44	40	38	42	34	38	38	37	34
gi 20805044 Ory					100	40	43	36	41	39	38	40	37	38	37	35	36
gi 10185719 Nic						100	38	42	43	36	35	35	38	36	38	36	32
gi 6714527 Sal							100	54	49	41	36	41	37	37	36	39	36
gi 606651 Gos								100	50	40	38	39	38	37	39	37	36
gi 304222 Bra									100	37	33	36	35	33	34	34	33
gi 3747092 Pru										100	62	62	52	41	39	41	40
gi 7435389 Cuc											100	54	45	40	37	40	37
gi 1362096 Lyc												100	49	44	39	41	41
gi 20161255 Ory													100	37	36	37	36
gi 166324 Act														100	64	61	44
gi 456091 Mal															100	61	43
gi 16903124 Gly																100	43
gi 577695 Cry																	100

^a Percentage amino acid sequence identities between exopolygalacturonases of monocotyledonous (PG1, PG35, Phl, Zea, Ory; bold) and dicotyledonous (Nic, Sal, Gos, Bra) plants, endopolygalacturonases (Pru, Cuc, Lyc, Ory, Act, Mal, Gly) and a polymethylgalacturonase (Cry). Sequence names are preceded by the database entry codes.

isoelectric point of 7.48 was calculated. A search for sequence motifs revealed the presence of a signature sequence highly conserved among polygalacturonases (aa 253–266: GVTCGPGHGISVGS), which belongs to the active site of the enzyme. Sequence analysis further identified four potential N-linked glycosylation sites (aa 94–97: NFTG; aa 250–253: NITG; aa 354–357: NITG; aa 392–395: NKTM), and an ATP/GTP-binding site (aa 52–59: GAKPDGKT).

The alignment of the deduced amino acid sequences of the four Phl p 13 clones and the incomplete Bermuda grass clone (Cyn), designated Cyn d 13, with plant exo- and endopolygalacturonases (Fig. 1), showed the highest degrees of sequence conservation in four domains I–IV. These domains are characteristic features of all

eukaryotic and prokaryotic polygalacturonases (28, 29) and are suggested for substrate binding and enzymatic activity (30–32). Several other amino acids outside of these domains implicated in enzyme activity (32, 33) are also conserved in the Phl p 13 clones (i.e., aspartic acid (D) at position 60, tryptophans (W) at positions 69, 125, 150, and tyrosine (Y) at position 330) (Fig. 1). Furthermore, several cysteine residues are maintained in all plant polygalacturonases suggesting similarities in their overall three-dimensional structures (Fig. 1, arrows).

Pollen exopolygalacturonases, including the Phl p 13 isoforms, represent a separate group among plant polygalacturonases (26) with a close evolutionary relationship (sequence

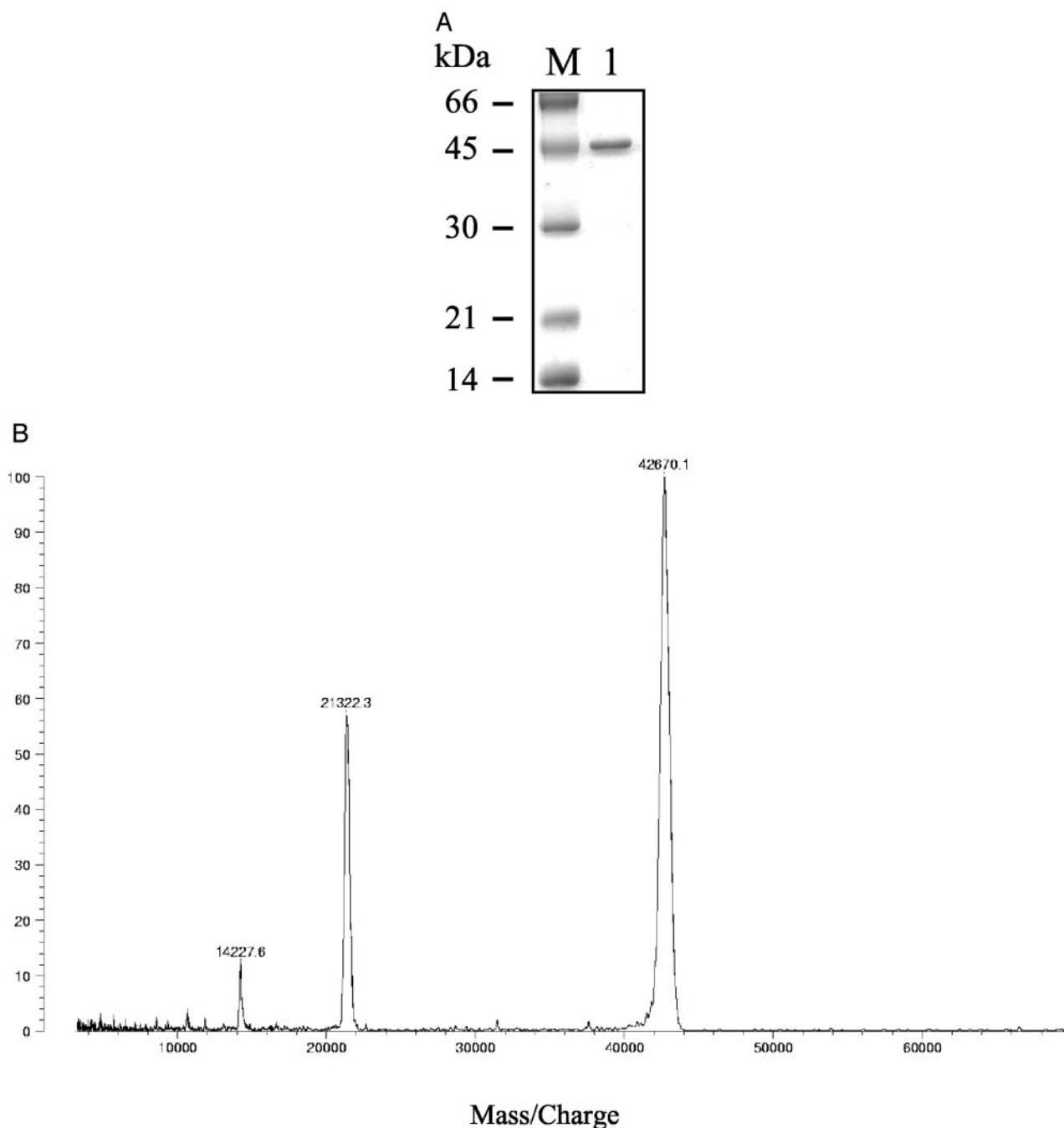


FIGURE 2. Characterization of purified rPhl p 13. *A*, Coomassie brilliant blue-stained SDS-PAGE containing purified rPhl p 13 (lane 1) and a molecular mass marker (lane M). Molecular masses are indicated in the left margin. *B*, Mass spectrometric analysis of purified rPhl p 13. The mass/charge ratio is shown on the x-axis and signal intensity is displayed on the y-axis as the percentage of the most intensive signal obtained in the investigated mass range. The peaks at 14,227.6, 21,322.3, and 42,670.1 correspond to the $M3H^+$, $M2H^+$, and MH^+ forms of rPhl p 13, respectively.

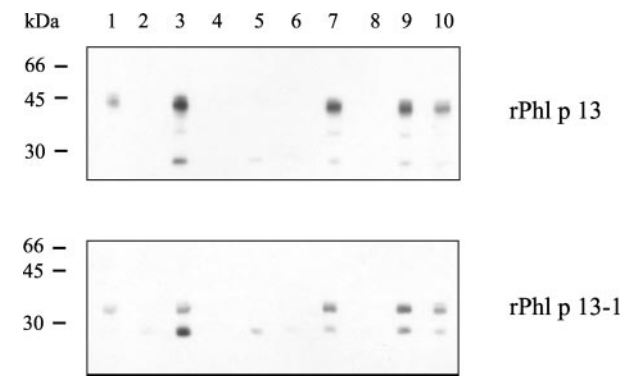


FIGURE 3. IgE reactivity of rPhl p 13 and the N-terminally truncated rPhl p 13 fragment (rPhl p 13-1). Nitrocellulose-blotted proteins were exposed to sera from grass pollen allergic patients (lanes 1–10). Molecular masses are indicated in the left margin.

homologies up to 73%) with pollen-derived exopolysaccharuronases but only up to 42% sequence homology with endopolysaccharuronases from somatic plant tissues (Table I). Pollen-specific enzymes from monocotyledonous plants showed much higher degrees of sequence homology among each other (56–73%) than with enzymes from dicotyledonous plants (36–47%).

Expression, purification, and biochemical characterization of rPhl p 13

The mature Phl p 13 allergen was obtained as a recombinant molecule by expression of the PG1 Phl cDNA without a hydrophobic leader sequence in *E. coli*. A C-terminal hexahistidine tag was added to facilitate the purification of rPhl p 13. Approximately 1.5 mg/L culture of rPhl p 13 could be purified by nickel affinity chromatography (Fig. 2A).

Mass spectrometric analysis of the purified protein revealed three mass/charge peaks at 42,670.1, 21,322.3, and 14,227.6 Da corresponding to the MH⁺, M2H⁺, and M3H⁺ forms of the protein. The comparison of the experimentally determined molecular mass (42,670 Da) (Fig. 2B) with the mass calculated according to the deduced amino acid sequence of His-tagged rPhl p 13 suggests that an authentic recombinant protein had been produced.

Phl p 13 is an important grass pollen allergen with IgE binding epitopes in the C terminus of the molecule outside the highly conserved polygalacturonase domains

The prevalence of IgE recognition of the recombinant allergen was determined by ELISA using sera from 100 grass pollen allergic

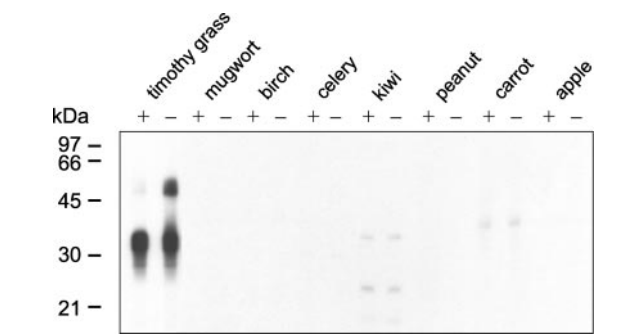


FIGURE 4. Group 13 allergens are markers for grass pollen allergy. Serum IgE from a representative Phl p 13-allergic patient was used to search for Phl p 13 cross-reactive allergens in pollen (timothy grass, mugwort, birch), fruit, vegetable, and nut extracts (kiwi, apple, celery, carrot, peanut). The serum was preadsorbed with rPhl p 13 (lanes +) or BSA (lanes –). Molecular masses are indicated in the left margin.

patients. rPhl p 13 produced in *E. coli* reacted with IgE Abs from 36% of the patients suggesting that Phl p 13 is an important allergen for grass pollen allergic patients (data not shown). A cross-reactivity analysis performed in nine Phl p 13-allergic patients with other purified timothy grass pollen allergens (rPhl p 2, rPhl p 5, rPhl p 6, nPhl p 4) indicated that Phl p 13 does not share IgE epitopes with any of the tested allergens and hence represents a high m.w. allergen distinct from other grass pollen allergens (data not shown).

To gain information about the localization of IgE epitopes on Phl p 13, we expressed in *E. coli* a recombinant, histidine-tagged PG1 Phl fragment (rPhl p 13-1), which lacked the first 106 N-terminal amino acids but contained the four domains implicated in the enzymatic activity of the protein (Fig. 1, I–IV). A comparative analysis of IgE reactivity to complete rPhl p 13 and to the rPhl p 13-1 fragment is shown in Fig. 3. Sera from all Phl p 13-allergic patients (Fig. 3, patients 1, 3, 7, 9, and 10) showed comparable IgE reactivity to complete rPhl p 13 and to the N-terminally truncated

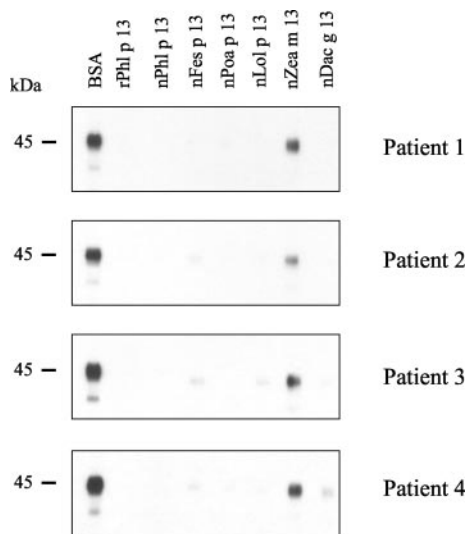


FIGURE 5. Cross-reactivity between rPhl p 13 and natural group 13 grass pollen allergens. Nitrocellulose-blotted purified rPhl p 13 was exposed to sera from four grass pollen allergic patients that had been preincubated with natural group 13 allergens from timothy grass (lanes nPhl p 13), meadow fescue (lanes nFes p 13), Kentucky blue grass (lanes nPoa p 13), ryegrass (lanes nLol p 13), maize (lanes nZea m 13), cocksfoot (lanes nDac g 13) and for control purposes, with rPhl p 13 and BSA.

Table II. Inhibition of IgE binding to Phl p 13 with a recombinant fragment containing aa 208–300 of Phl p 13 (rPhl p 13-2)^a

Patients	IgE Reactivity to rPhl p 13 after Preincubation with		% Inhibition of IgE Binding
	Buffer	rPhl p 13-2	
1	1.015	0.713	29.75
2	2.407	2.257	6.23
3	0.611	0.643	No
4	1.447	1.360	6.01
5	1.438	1.061	26.22
6	0.317	0.332	No
7	0.336	0.292	13.10
8	0.691	0.670	3.04

^a Sera from eight Phl p 13-reactive patients (1–8) were preincubated with fragment rPhl p 13-2 or with buffer and consecutively exposed to ELISA plate-bound rPhl p 13. OD values, corresponding to the amount of bound IgE, and percentage inhibition of IgE binding to rPhl p 13 achieved by preincubation with rPhl p 13-2 are displayed.

fragment suggesting that most of the major IgE epitopes are localized in the C-terminal portions of the molecule. For a more precise localization of the IgE binding epitopes, the region containing the highly conserved enzyme domains (Fig. 1, aa 208–300) was expressed as a histidine-tagged recombinant fragment (rPhl p 13-2) and its IgE binding capacity was analyzed in direct and inhibition IgE ELISA experiments (Table II). The fact that rPhl p 13-2 only weakly inhibited patients' IgE binding to rPhl p 13 indicates that the majority of IgE epitopes map to the less-conserved regions of the C terminus of the molecule.

rPhl p 13 is a diagnostic marker allergen for genuine grass pollen sensitization

Next we investigated whether rPhl p 13 shares IgE epitopes with polygalacturonases from various plants. Fig. 4 exemplifies that IgE reactivity of sera from Phl p 13-allergic patients to natural Phl p 13 (47 kDa band) is inhibited by rPhl p 13. However, many of these patients showed either no or weak IgE binding to allergens from other pollens, fruits, vegetables and nuts or their IgE reactivity was

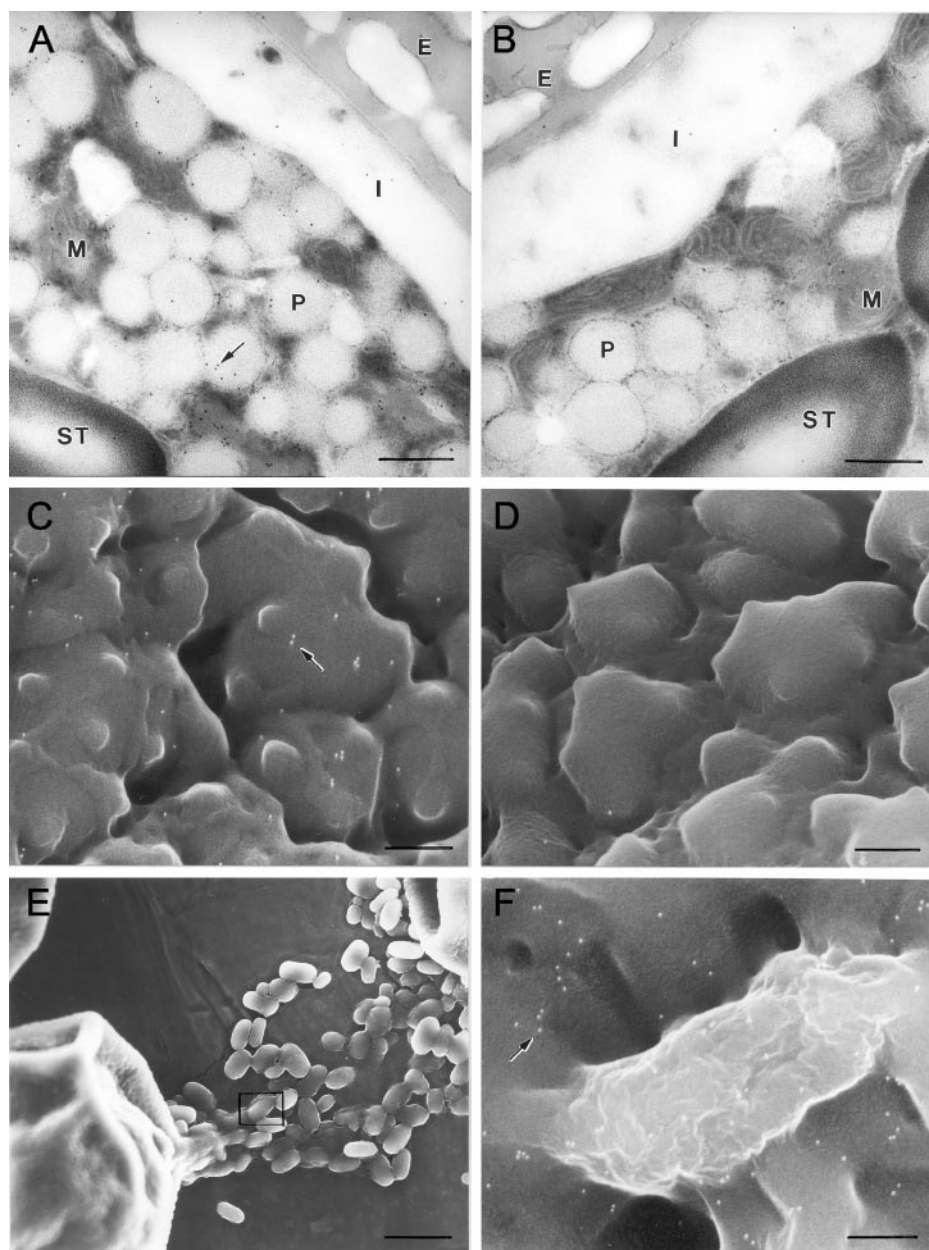
not inhibited by preadsorption of the sera with rPhl p 13. In contrast, group 13 allergens could be purified from the most common grasses (meadow fescue, Kentucky bluegrass, ryegrass, orchard grass) and inhibited IgE reactivity to purified rPhl p 13 (Fig. 5). Only group 13 allergens from maize showed weak inhibition of IgE reactivity to Phl p 13 most likely due to a lower degree of sequence homology (Table I).

Taken together these data suggested that IgE cross-reactivity is confined to group 13 allergens from grasses but does not occur between rPhl p 13 and exo- and endopolygalacturonases of other higher plants. Thus, rPhl p 13 may be considered as a diagnostic marker allergen for genuine grass pollen sensitization.

Hydrated grass pollen expels respirable submicronic particles bearing Phl p 13

In mature, dry timothy grass pollen, Phl p 13 was localized by immunogold electron microscopy using Abs raised against rPhl p 13 in the cytoplasm associated with numerous polysaccharide (P-) particles (Fig. 6A) and, occasionally on the pollen surface (Fig.

FIGURE 6. Expulsion of Phl p 13-bearing submicronic particles from hydrated timothy grass pollen. Transmission electron micrographs of ultrathin sections of dry timothy grass pollen (A and B) and scanning electron micrographs of the dry pollen surface (C and D) probed with rabbit anti-Phl p 13 Ig (A and C) or preimmune Ig (B and D). E and F, Low (E) and high (F) magnification scanning micrographs of hydrated timothy grass pollen expelling cytoplasmic material containing submicronic particles labeled with rabbit anti-Phl p 13 Ig. The rectangle in E indicates an area comparable to the area shown in higher magnification in F. Bound Phl p 13-specific Ig was detected with secondary Abs coupled to colloidal gold particles of 10 nm (gold particles appearing as black dots in A and B, white dots in C to F). In A, B, and E, bars represent 0.5 μ m, in C, D, and F bars represent 0.25 μ m. E, exine; I, intine; M, mitochondria; P, polysaccharid particle; ST, starch grain.



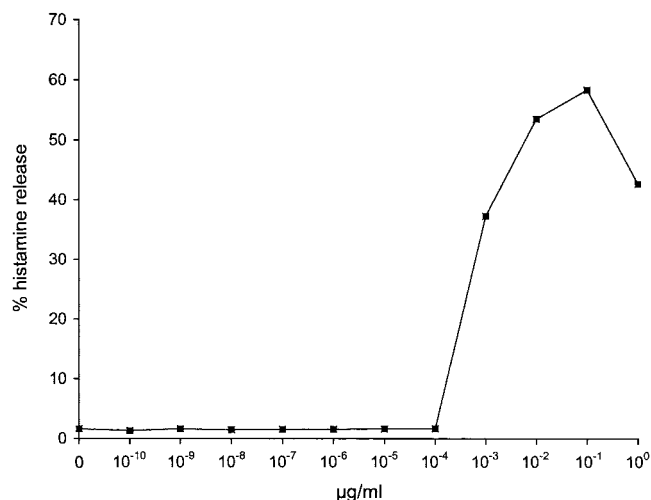


FIGURE 7. rPhl p 13 induces specific basophil histamine release. Granulocytes from a grass pollen allergic patient were incubated with various concentrations (x-axis) of the purified recombinant allergen. The percentage of histamine released into the supernatant is displayed on the y-axis.

6C). The P-particle-associated Phl p 13 labeling found in the periphery of the sectioned P-particles, indicates that Phl p 13 is localized in the outer parts or on the surface of these particles. In addition, some anti-Phl p 13 immunoreactivity was also found in the cytoplasm of the pollen grains. Control experiments with pre-immune Abs showed low background labeling (Fig. 6, B and D).

Upon hydration of grass pollen grains in rain water, a dramatic swelling of pollen grains and expulsion of cytoplasmic material containing submicronic particles through the germination pores was observed by scanning electron microscopy (Fig. 6E). This expulsion represents such a violent process that the majority of subcellular compartments and particles is destroyed. Only starch granules, covered with cytoplasmic material, remain intact (Fig. 6, E and F). A detailed analysis of the expelled cytoplasmic material with anti-Phl p 13 Abs revealed that the material contained Phl p 13, suggesting particle expulsion as a mode for Phl p 13 release (Fig. 6F). Incubation of hydrated pollen specimens with pre-immune Abs gave no significant staining (data not shown).

rPhl p 13 induces specific histamine release from basophils of grass pollen allergic patients

The allergenic activity of rPhl p 13 was studied by incubating granulocytes of a Phl p 13-allergic patient with purified rPhl p 13. As exemplified in Fig. 7, rPhl p 13 induced a specific and dose-dependent release of histamine, whereas no activation of basophils from nonallergic patients was found (data not shown).

Immunization with rPhl p 13 induces IgG Abs that block allergic patients' IgE binding to the allergen

The capacity of rabbit anti-rPhl p 13 Abs to inhibit human IgE binding to the allergen was examined in ELISA competition assays using 13 sera from Phl p 13-sensitized grass pollen allergic pa-

tients (Table III). For the majority of the patients, anti-rPhl p 13 IgG caused a significant inhibition of IgE binding to rPhl p 13, ranging between 30 and 86%, with a mean inhibition of 64%. IgE reactivity of only one of the patients was not inhibited by rabbit anti-rPhl p 13 Abs (Table III, patient 11). In a next set of experiments, we investigated whether competition of allergic patients' IgE binding depended on the titer of rabbit anti-rPhl p 13 Abs. Detailed ELISA competition experiments with sera from six of the patients were performed using anti-rPhl p 13 Abs diluted from 1/20 to 1/100,000 (Fig. 8). Competition of human IgE binding to rPhl p 13 depended on the titer of rabbit anti-rPhl p 13 Abs. Almost complete inhibition of IgE binding was observed up to a dilution of 1/100 and considerable blocking was obtained up to a dilution of 1/1,000 of the rabbit Abs. The degree of competition did not seem to depend on the levels of rPhl p 13-specific IgE Abs present in the patients' sera because comparable blocking was observed for patients' sera containing high (patient 5) or low (patient 8) Phl p 13-specific IgE levels (Table III; Fig. 8).

Discussion

Grass pollen belong to the most potent elicitors of allergic asthma. So far, only group 1 grass pollen allergens, a family of 30-kDa glycoproteins, have been identified as genuine grass pollen allergens that occur in most grass species (4). In this study, we characterized a second group of grass pollen-specific marker allergens. This allergen group was identified using serum IgE from a grass pollen allergic patient, cosensitized to the most common subfamilies of grasses (i.e., Pooideae and Chloridoideae) for the screening of a cDNA library from Bermuda grass, a member of the subtropical/tropical Chloridoideae subfamily. One cDNA clone showing cross-hybridization with RNA from pollen of timothy grass, a member of the temperate Pooideae grasses, was then used to isolate corresponding cDNAs from timothy grass pollen. The analysis of the DNA and deduced amino acid sequences of the clones from Bermuda grass and timothy grass showed that these allergens belong to a family of plant polygalacturonases. Based on sequence homology with a previously isolated timothy grass pollen cDNA clone, designated Phl p 13 (7, 9), the Bermuda grass and timothy grass pollen isoallergens we identified were termed group 13 allergens (i.e., Cyn d 13, Phl p 13).

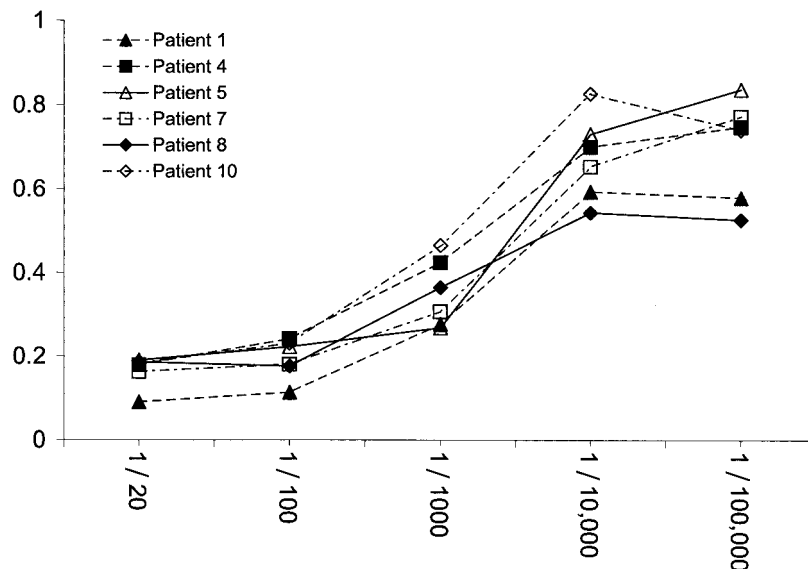
Polygalacturonases are a class of enzymes known to be involved in the degradation of pectin, which occurs as a complex polysaccharide in the primary cell wall of higher plants. These enzymes are expressed in the whole plant kingdom in a wide range of different tissues and developmental stages (reviewed in Ref. 26). Based on their mode of action, they are divided into endopolygalacturonases, which randomly cleave the backbone of the pectin molecule and exopolygalacturonases, which remove terminal sugars. Cyn d 13 and the Phl p 13 isoallergens showed significant sequence identity (~40%) with allergens of the polygalacturonase family identified in several other unrelated plants (e.g., tomato, oilseed rape, Japanese cedar) (34–36), with the highest degrees of sequence homology in the four functional polygalacturonase domains. Despite the high degree of sequence similarity among plant

Table III. Rabbit anti-rPhl p 13 Abs inhibit patients' IgE binding to Phl p 13^a

Patients	1	2	3	4	5	6	7	8	9	10	11	12	13
Preimmune serum	0.563	0.908	1.081	0.534	0.696	2.279	0.648	0.579	1.184	0.327	0.264	0.253	0.537
Anti-rPhl p 13 serum	0.174	0.132	0.189	0.222	0.200	1.605	0.232	0.271	0.215	0.130	0.454	0.122	0.196
% Inhibition of IgE binding	69	86	83	58	71	30	64	53	82	60	No	52	64

^a OD values, corresponding to the amount of bound IgE, and percentage inhibition of IgE binding are displayed.

FIGURE 8. Inhibition of patients' IgE binding to rPhl p 13 depends on the titer of rabbit anti-rPhl p 13 Abs. ELISA plate-bound rPhl p 13 was preincubated with increasing dilutions (x-axis) of rabbit anti-Phl p 13 Ig. The binding of six grass pollen allergic patients' IgE (OD values) is displayed on the y-axis.



polygalacturonases, rPhl p 13 showed no significant IgE cross-reactivity with plant allergen sources (e.g., ragweed-, birch pollen, plant-derived food) other than grass pollens. However, for grass pollen it could be demonstrated that rPhl p 13 shares cross-reactive IgE epitopes with polygalacturonases of the most common grasses including Pooideae and Chloridoideae (e.g., Bermuda grass: Cyn d 13). This interesting pattern of cross-reactivity confined to grass pollen was studied in detail by IgE epitope mapping. IgE binding and IgE inhibition experiments using the N-terminally truncated fragment rPhl p 13-1 (aa 107–416) and the fragment rPhl p 13-2, containing only the highly conserved functional polygalacturonase domains (amino acid 208 to 300), revealed that the major IgE epitopes are located in the C terminus of the molecule but outside of these functional domains. These results explain why cross-reactivity of Phl p 13-specific IgE is confined to grass pollen polygalacturonases.

Recombinant Phl p 13 was expressed as functional allergen in *E. coli*. It showed IgE reactivity comparable to the natural Phl p 13 and may hence be used as a diagnostic marker allergen, which allows one to precisely identify patients with a genuine sensitization to grass pollen. A diagnostic test based on rPhl p 13 alone will not enable one to identify all grass pollen allergic patients, but will discriminate patients who are genuinely sensitized to grass pollen from those who show grass pollen reactivity due to sensitization to highly cross-reactive allergens present in unrelated allergen sources (e.g., profilin, calcium-binding allergens) (37, 38).

Grass pollen not only represents one of the most potent and frequent allergen sources but also induces attacks of bronchial asthma, especially after heavy rainfalls (39, 40). The latter has been attributed to the release of submicronic, respirable, allergen-bearing particles from grass pollen exposed to rainwater (21). Therefore, we studied the ultrastructural localization of Phl p 13 in timothy grass pollen by immunogold electron microscopy using a rabbit antiserum raised against the rPhl p 13 allergen. In dry pollen, Phl p 13 was localized predominantly in the cytoplasm, associated with polysaccharide containing, wall-precursor (P-) particles, and occasionally on the surface of the pollen grains. High-resolution scanning electron microscopy of timothy pollen grains exposed to rainwater showed a swelling of pollen grains and their subsequent rupture led to the release of large amounts of cytoplasmic material including submicronic particles, mainly through the germination pore. The submicronic particles of respirable size and the cytoplasm released during this process were

coated with Phl p 13 and therefore may be involved in the elicitation of asthma attacks in sensitized grass pollen allergic patients.

Our results have identified rPhl p 13 as an important and grass pollen-specific marker allergen. Therefore, it may be also included in a mixture of recombinant grass pollen allergens which may be used instead of crude grass pollen allergen extracts for immunotherapy of grass pollen allergy. The possible usefulness of rPhl p 13 for immunotherapy of sensitized patients is demonstrated by immunization experiments. We found that immunization of rabbits with rPhl p 13 induced high titers of Abs which blocked the binding of allergic patients' IgE to the allergen. Such allergen-specific IgG responses may exhibit protective effects by at least three mechanisms (41). First, they may suppress allergen-induced activation of mast cells and thus immediate reactions (11). Second, they may inhibit IgE-mediated allergen presentation to T cells and the consecutive release of proinflammatory cytokines (42). Finally, it is possible that blocking Abs inhibit the production of allergen-specific IgE Abs induced in B ϵ -memory or plasma cells by allergen contact (43).

In summary, rPhl p 13 may represent a diagnostic tool to identify allergic patients genuinely sensitized to grass pollen and a relevant component to be included in a grass pollen-specific allergy vaccine.

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