

cDNA Cloning of a Major Allergen from Timothy Grass (*Phleum pratense*) Pollen; Characterization of the Recombinant *Phl p V* Allergen¹

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ABSTRACT. We isolated a cDNA encoding a major grass pollen allergen from a timothy grass (*Phleum pratense*) pollen expression cDNA library using allergic patients' IgE. The complete cDNA encoded an allergen that binds IgE from about 80% of grass pollen-allergic patients. Significant sequence homology was found to other major grass pollen allergens from Kentucky bluegrass (*Poa pratense*) as well as from rye grass (*Lolium perenne*) which originally were believed to form different identities. Using different monoclonal and polyclonal antibodies raised against group V allergens we identified the recombinant protein as a group V allergen from timothy grass, *Phl p V*. In IgE-binding studies it is demonstrated that the *rPhl p V* allergen can be used to block binding of patients' IgE to natural group V isoallergens on two-dimensional immunoblots. IgE inhibition experiments show that up to 60% of grass pollen-specific IgE can be preadsorbed with the *rPhl p V* allergen from patients' sera. The purified *rPhl p V* induced specific histamine release of blood basophils from grass pollen-allergic patients. This emphasizes the usefulness of the *rPhl p V* for diagnostic and therapeutic purposes and corroborates the view that specific diagnosis and therapy of type I allergy could be performed with a limited panel of relevant recombinant allergens. *Journal of Immunology*, 1993, 151: 4773.

Type I allergic reactions represent a health problem of increasing importance in industrialized countries. In up to 20% of the population, IgE-mediated symptoms (allergic rhinitis, conjunctivitis, and bronchial asthma) are caused by airborne allergens from plant pollens, mites, moulds, and cat and dog dander, which cross-link mast cell-bound specific IgE (1). Although ex-

tensive cross-reactivities of patients' IgE with allergens from different sources are known, the panel of allergens seemed to be very complex and heterogenous. However, the characterization of allergens by immunochemical, proteinchemical and recently by molecular cloning techniques (2) indicates that because of structural and immunochemical similarities in the most important allergens, a limited panel of relevant allergens can be determined for diagnostic and therapeutic purposes. Excellent examples for the structural and immunologic relationship of major allergens are the tree pollen allergens (3, 4, 5) that also occur in plant derived food (6, 7); and the family of profilins, which could be identified as plant pan-allergens in almost all plant species (8–12).

In case of grass pollen allergy, the panel of allergens seemed to be more complex because of the occurrence of different isoallergenic variants (13). However, three recom-

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binant grass pollen allergens could be defined that are sufficient for diagnosis of grass pollen allergy (14). Now the molecular cloning and functional characterization of one of these three grass pollen allergens is described. We isolated this particular allergen from a timothy grass (*Phleum pratense*) pollen cDNA library using grass pollen-allergic patients' IgE. The sequence similarities with grass pollen allergens from different grass species and the identity with the N-terminal amino acid sequence of *Phl p V* together with the immunologic characterization of the recombinant allergen, using defined antibodies allowed us to identify the recombinant allergen as a group V allergen. The recombinant timothy grass pollen allergen that we designated *Phl p V* bound IgE of about 80% of all grass pollen-allergic patients and up to 60% of the IgE directed against total grass pollen allergens in individual sera. Binding of patients' IgE to naturally occurring group V isoforms could be blocked by preincubation with the one recombinant allergen. We purified the recombinant allergen- β -galactosidase fusion protein to homogeneity and showed that it released histamine from blood basophils of grass pollen-allergic patients in a dose-dependent and specific manner. The *rPhl p V* allergen is therefore characterized as an important diagnostic and possibly therapeutic tool for grass pollen allergy.

Materials and Methods

Characterization of allergic and control individuals

Allergic and nonallergic control individuals were characterized by case history, radioallergosorbent test, and skin prick test. In addition, sera were tested by IgE-immunoblots for reactivity to natural timothy grass pollen proteins and with recombinant timothy grass pollen allergens as described (14). Sera from nonallergic individuals or individuals with no grass pollen allergy according to the above criteria were used for control purposes.

Construction of a cDNA library from pollen of timothy grass; IgE-immunoscreening

Total RNA was extracted from timothy grass pollen (Allergon AB, Engelholm, Sweden) and poly-A⁺ RNA was isolated by oligo-dT cellulose affinity chromatography (Sigma, St. Louis, MO) (15). A cDNA synthesis kit (Amersham, London, UK) was used to construct blunt ended cDNA. After methylation with *EcoRI* methylase (Promega, Madison, WI), *EcoRI* linkers (Boehringer Mannheim, FRG) were added to the cDNA. The linkered cDNA was then digested with *EcoRI* (Boehringer Mannheim). cDNA and linkers were separated by a Nick column (Pharmacia, Uppsala, Sweden). The cDNA was then ligated into λ gt11 arms (Pharmacia) and packaged in vitro using an in vitro packaging kit (Amersham, London, UK).

200,000 plaque-forming units were immunoscreened

with serum IgE from a grass-pollen allergic patient as described (16).

Characterization of the IgE-binding clones

60 out of 300 positive clones were tested for the binding of serum IgE from 30 different grass-pollen allergic patients as described (14). Phage DNA was isolated from one clone designated c95, which bound serum IgE from about 80% of the patients. A *KpnI-SacI* fragment, containing the cDNA insert together with two flanking portions of λ gt11 DNA was ligated into the plasmid pUC 18, and transformed into *E. coli* XL 1 blue. Both DNA strands were sequenced according to the dideoxy chain-termination method (17) using synthetic oligonucleotide primers. The obtained DNA sequence and deduced amino acid sequence were compared with published grass pollen allergen sequences from Kentucky blue grass (18), rye grass (19), and partial amino acid sequences from group V allergens of different grass species (20, 21).

Expression of *Phl p V* as IgE-binding recombinant β -galactosidase fusion protein in *E. coli*

Lysogenic *E. coli* Y1089, which had been infected with the recombinant phage, were grown at 32°C to an optical density 600 of 0.5. After 20 min at 42°C, the β -galactosidase-*Phl p V* fusion protein synthesis was induced by adding IPTG (Isopropyl- β -thiogalactoside) to a final concentration of 10 mM. *E. coli* cells were grown at 37°C for 1 h, harvested and washed two times with 0.9% sodium chloride before protein extraction.

Testing of *rPhl p V* using monoclonal and polyclonal antibodies against different grass pollen allergens

Proteins from *E. coli* Y1089 containing the β -galactosidase-*Phl p V* fusion protein were separated by a 7.5% polyacrylamide gel (22) and blotted onto nitrocellulose (23). Nitrocellulose strips were incubated with a rabbit antibody raised against *Dac g V*, a monoclonal anti group V antibody 4B1 (24), a rabbit anti-*Lol p I* antibody, and the mAb 2C7, raised against group V allergens (25). The rabbit antibodies were diluted 1:1000 and antibody 2C7 1:100 in phosphate buffer (50 mM sodium phosphate, pH 7.5, 0.5% Tween 20, 0.5% BSA, 0.05% Na₂S₂O₃). Bound rabbit antibodies were detected with a 1:1000 ¹²⁵I-labeled donkey anti-rabbit antibody (Amersham, London, UK) and mouse mAb with a 1:2000 rabbit anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark) and a 1:1000 ¹²⁵I-labeled donkey anti-rabbit antibody (Amersham). In all experiments protein extracts from *E. coli* Y1089 infected with nonrecombinant λ gt11 phages were included as negative controls.

Table 1

Percentage of IgE directed against native grass pollen allergens which can be absorbed by preincubation of grass pollen-allergic patients' sera (A-S, $n = 19$) with native recombinant *Phl p V*

Patient designation	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Percentage inhibition	23%	64%	25%	11%	31%	62%	27%	13%	33%	48%	26%	34%	16%	30%	56%	42%	20%	32%	27%

IgE-inhibitions with native *rPhl p V* and denatured two-dimensional separated timothy grass pollen iso-allergens

Two-dimensional gel electrophoresis and blotting of timothy grass pollen proteins was performed as described (4). A serum of a grass-pollen allergic patient, with reactivity to group V isoallergens was diluted 1:10 in phosphate buffer and preadsorbed to plaquelifts containing native *rPhl p V* or λ gt11 proteins (negative control) overnight at 4°C. Nitrocellulose sheets, containing two-dimensional separated timothy grass pollen allergens were incubated with the preincubated sera that were further diluted 1:2 and bound serum IgE was detected as described (26). In a control experiment that was performed in parallel the preadsorbed serum was shown to contain no detectable IgE against *rPhl p V*.

Determination of the percentage of grass pollen-specific IgE directed against native *rPhl p V*

To determine the percentage of grass pollen-specific IgE that can be adsorbed by *rPhl p V*, IgE-inhibition studies with native allergens were done. In brief, patients' sera were incubated with native natural timothy grass pollen allergens, with native *rPhl p V* and with native control proteins from *E. coli*, respectively. The remaining nonadsorbed grass pollen-specific IgE was then bound to dot blotted nondenatured natural timothy grass pollen allergens and detected with ^{125}I -labeled anti-human IgE. Native timothy grass pollen protein extracts were obtained by aqueous protein extraction as described below. Native *rPhl p V* and *E. coli* control proteins were prepared by resuspending bacteria in phosphate buffer that was then subjected to three freeze-thaw cycles. Supernatants containing *rPhl p V* or *E. coli* control proteins were obtained by centrifugation of the homogenate at 20,000 rpm, in a bench centrifuge at 4° for 15 min. Serum dilutions and amounts of allergens that assured Ag excess were determined by testing serial dilutions of patients' sera for IgE-binding to nondenatured dot blotted natural or recombinant allergen extracts.

For IgE-inhibitions, sera from 19 grass-pollen allergic patients and from 2 nonallergic persons were diluted 1:15 in phosphate buffer and preincubated with the native allergens in plastic tubes at 4°C overnight. The preadsorbed sera were then again diluted 1:2 and incubated with nondenatured dot blotted natural timothy grass pollen allergens or *rPhl p V*. The amount of bound IgE was determined with ^{125}I -labeled rabbit anti-human IgE and

counted in a γ -counter (LKB, Uppsala, Sweden). All determinations were done in duplicates and results in Table I reflect mean values.

Preparation of timothy grass pollen allergens and purification of the *rPhl p V*- β -galactosidase fusion allergen

Three grams of timothy grass pollens were shaken in 40 ml of distilled water for 20 min at 4°C. The protein extracts were then centrifuged at 4°C at 65,000g for 30 min. Supernatants were then dialyzed against distilled water at 4°C and lyophilized. The recombinant β -galactosidase *Phl p V* fusion allergen was prepared by affinity chromatography using ProtoSorb lacZ immunoaffinity adsorbent (Promega) according to the manufacturer's advice. In brief, an affinity column was prepared with 1 ml ProtoSorb lacZ immunoaffinity matrix and equilibrated with 5 ml 50 mM Tris buffer, pH 7.3. Lysogenic *E. coli* Y1089 infected with the recombinant λ phages or with nonrecombinant λ gt11 phages as a negative control were resuspended in TEP buffer (100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and homogenized with an ultraturrax. The obtained crude protein extracts containing the β -galactosidase-*Phl p V* fusion protein or β -galactosidase were centrifuged at 65,000g for 15 min. The proteins of the supernatant were then precipitated with three volumes of saturated ammonium sulfate at 4°C and then pelleted at 65,000g for 20 min. The pellet was then dissolved in TEP buffer at approximately 20 mg/ml protein and diluted with Tris buffer (50 mM Tris-HCl, pH 7.3) to approximately 4 mg/ml protein. The diluted protein extract was then loaded onto the equilibrated column, which was then washed with 15 ml TN buffer (50 mM Tris-HCl, pH 7.3, 0.2% NP-40). Recombinant β -galactosidase *Phl p V* fusion protein or β -galactosidase was then eluted with three 1-ml aliquots of elution buffer (0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 10.8) followed by 1 ml of TBS (50 mM Tris-HCl, pH 7.3, 150 mM NaCl). The purified proteins were then dialyzed against distilled water, lyophilized and checked for purity by SDS-PAGE.

In vitro histamine release assays

Blood basophils from grass pollen-allergic patients, a patient with house dust mite allergy without grass pollen allergy and a normal nonallergic individual were prepared as

FIGURE 1. Complete cDNA sequence and deduced amino acid sequence of *Phl p V*. The open reading frame of timothy grass pollen allergen *Phl p V* comprises 936 nucleotides including the start codon. The first 25 amino acids that are underlined represent a leader peptide typical for secreted proteins. According to N-terminal protein sequences the mature protein starts with an alanine at position 26. The sequence data were submitted to EMBL/Genbank and are available under accession number x74735

GAATTC	CCCCATATCTTCCACCTACGGTACAAACACAGCAAGCAGCA	-49
ATGGCGGTGCACCACTGTGGCGCTGTTCTCGCCGTGGCCCTCGTGGCGGGCCCG		60
<u>M A V H O Y T V A L F L A V A L V A G P</u>		
GCCGCTCTACGCGGCGGATCTCGGTTACGGCCCCGCCACCCAGCTGCCCGGGCCGCC		120
<u>A A S Y A A D L G Y G P A T P A A P A A</u>		
GGCTACACCCCGCCACCCCGCGCCCGGCGGCGGCGCCAGCAGGTAAGGCGACG		180
G Y T P A T P A A P A E A A P A G K A T		
ACCGAGGAGCAGAAGCTGATCGAGAAGATCAACGCCGGCTTCAAGGCGGCCCTGGCCGCC		240
T E E Q K L I E K I N A G F K A A L A A		
GCCGCGGCGTCCAGCCAGCGGACAAGTACAGGACGTTCTGTCGCAACCTTCGGCGCGGCC		300
A A G V Q P A D K Y R T F V A T F G A A		
TCCAACAAGGCTTCGCGGAGGGCTCTCGGGCGAGCCCAAGGGCGCGCCGAATCCAGC		360
S N K A F A E G L S G E P K G A A E S S		
TCCAAGGCCGCTCACCTCCAAGCTCGACGCCGCTACAAGCTCGCTTACAAGACAGCC		420
S K A A L T S K L D A A Y K L A Y K T A		
GAGGCGCGACGCTGAGGCCAAGTACGACGCTACGTCGCCACCTAAGCGAGGCGCTC		480
E G A T P E A K Y D A Y V A T L S E A L		
CGCATCATCGCCGCCACCTCGAGGTCCACGCCGTCAAGCCCGCGGCCGAGGAGGTCAAG		540
R I I A G T L E V H A V K P A A E E V K		
GTATCCCCCGCGGAGCTGCAGGTCTCGAGAAGGTGACGCCGCTTCAAGGTGCT		600
V I P A G E L Q V I E K V D A A F K V A		
GCCACCGCCCAACGCCGCCGCCCAACGACAAGTTCACCGTCTTCGAGGCCGCTTC		660
A T A A N A A P A N D K F T V F E A A F		
AACGACGCCATCAAGCGAGCACGGGCGGCGCTACGAGAGCTACAAGTTCATCCCCGCC		720
N D A I K A S T G G A Y E S Y K F I P A		
CTGGAGGCCCGCTCAAGCAGGCCTACGCCGCCACCGTCGCCACCGCGCGGAGGTCAAG		780
L E A A V K Q A Y A A T V A T A P E V K		
TACTGTCTTTGAGACCGCACTGAAAAAGGCCATCACCGCATGTCGAGGCACAGAAG		840
Y T V F E T A L K K A I T A M S E A Q K		
GCTGCCAAGCCCGCGCGCTGCCACCGCCACCGCAACCGCGCGCTTGGCGCGGCCACC		900
A A K P A A A A T A T A T A A V G A A T		
GGCGCCGCCACCGCGCTACTGGTGGCTACAAAGTCTGATCAACTCGCGAATAGTATACA		960
G A A T A A T G G Y K V *		
TCAATCAGCACATATACGACCGTACGTATGTGTGCATGCATTGCCGTGCGTCGAGCAA		1020
TTTTGCTGATAATTAATCTTGAGTTTTCGTTGCATGCATCCGCGCTTATAGAGCGCA		1080
TGCACGCATTGCCATTGTGTATAATAATCAAATATCTGCAATTCAATTATGAAAAAAA		1140
AAAAAAAA		1149

described (27) and incubated either with natural grass pollen allergens from timothy grass, with the purified recombinant β -galactosidase-*Phl p V* fusion protein, with purified β -galactosidase as a negative control, or with anti-IgE mAb E-124-2-8 as a positive control. Histamine release was measured in the cellfree supernatants by radio immunoassay (Immunotech, Marseille, France).

Results

Isolation of a cDNA encoding the *Phl p V* allergen with grass pollen-allergic patients' IgE

A λ gt11 expression library was constructed from mature timothy grass pollen. 300 IgE-binding clones were isolated with serum IgE from a patient with reactivity to most grass pollen allergens. Sixty clones were enriched and probed with serum IgE from 30 different grass pollen-allergic pa-

tients and antibodies raised against group I or group V grass pollen allergens, and antibodies raised against plant profilins. 13 out of the 60 clones encoded group I allergens, 8 clones encoded group V allergens, 2 clones encoded profilin from timothy grass pollen, and 37 clones could not be identified with the antibodies used. The cDNA of clone 95 coded for a recombinant timothy grass pollen allergen that bound serum IgE from about 80% of all tested patients and reacted with all anti-group V antibodies.

DNA sequence analysis of clone 95

Sequence comparison with pollen allergens of other grass species and with the partial protein sequences of *Phl p V* isoallergens. The cDNA sequence and the deduced amino acid sequence of clone 95 are shown in Fig. 1. The coding region of the complete cDNA including the start codon

Phl p V-ISOVARIANTS:

c-Phl p V ADLGYPATPAAPAAGYTPATPAAP
 1-Phl p V .N...A.....G.....
 2-Phl p V A.....G.....
 3-Phl p V S.A.....G.E.....
 4-Phl p V G....A.....G.....

FIGURE 2. Alignment of the amino acid sequence deduced from the *Phl p V* cDNA (c-*Phl p V*) with N-terminal partial amino acid sequences of several *Phl p V* isoforms (1–4). Identical amino acid residues are indicated by points. The N-terminal partial amino acid sequences were published by Matthiesen et al. (20) and by Roberts et al. (21).

comprises 936 nucleotides and shows in the codon usage a prominent use of G and C (71% of the nucleotides). A protein of a calculated molecular weight of 31 kDa could be deduced. The N-terminal sequence of 25 amino acids corresponding to a polypeptide of 2.5 kDa (underlined in Fig. 1) is typical for eukaryotic signal peptide sequences and is highly homologous to a similar signal peptide found in the deduced amino acid sequences of Lol p Ib (19) and the *Poa p IX* isoallergens (18). The mature *Phl p V* protein starts with amino acid 26, an alanine. The first 25 amino acids of the mature protein could be compared with the partial amino acid sequences obtained from *Phl p V* isoallergens by protein sequencing (20, 21) and were found to be almost identical. The sequence comparison is displayed in Figure 2 and showed that only two out of 25 amino acids were different between the cloned *Phl p V* and the closest related *Phl p V* isoallergen. Figure 3 shows the comparison of the deduced amino acid sequence of *Phl p V* and a homologous *Poa p IX* allergen (18). *Phl p V* shares 280 identical amino acid residues with *Poa p IX*, which is a 90% sequence identity spanning the complete molecule. A similar comparison with the deduced amino acid sequence of *Lol p Ib* (19) gave a 35% sequence identity. The almost complete sequence identity with the protein sequences obtained from *Phl p V* isoallergens indicates that the cDNA of clone 95 encodes *Phl p V* and together with the related allergens from Kentucky bluegrass (*Poa p IX*) and rye grass (*Lol p Ib*) belongs to the group V family of grass pollen allergens. A comparison of the obtained nucleotide sequence and deduced amino acid sequence of *Phl p V* with the EMBL and Swissprot data base showed no significant homologies with other sequences.

Expression of the r*Phl p V* allergen in *E. coli*-testing of the recombinant allergen with patients' IgE and group I or group V-specific antibodies. To test the r*Phl p V* with well characterized mono- and polyclonal antibodies raised against natural group V and group I grass pollen allergens, we expressed the cDNA as a β -galactosidase fusion protein in lysogenic *E. coli* Y1089. For control experiments we used protein extracts from lysogenic *E. coli* Y1089 infected with nonrecombinant λ gt11 phage and infected with phage containing the cDNA coding for *Phl p I*. Three group

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MAVHQYTVLFLAVALVAGPAASYADLGYG.....PATPAAPAAGYT 50
|||||
MAVHQYTVLFLAVALVAGPAASYADVGYGAPATLATPATPAAPAAGYT 50

PATPAAPAEAPAGKATTEEOKLIEKINAGFKAAALAAAGVQADKYRTF 100
|||||
...PAAPAGAAP...KATTEOKLIEKINAGFKAAVAAAAGVPAVDKYRTF 100

VATFGAASNKFAEGLSGEPKGAEESSKAAALTSKLDAAAYKLAYKTAEGA 150
|||||
VATFGTASNKFAEALSTEPKGAASNAVLTSKLDAAAYKLAYKSAEGA 150

TPEAKYDAYVATLSEALRIIAGTLEHVAVKPAEEVKVIPAGELQVIEKV 200
|||||
TPEAKYDAYVATLSEALRIIAGTLEHVAVKPAEEVKVIPAGELQVIDKV 200

DAAFKVAATAANAAPANDKFTVFEAFNDAIKASTGGAYSYKFIPALEA 250
|||||
DAAFKVAATAANAAPANDKFTVFEAFNDAIKASTGGAYQSYKFIPALEA 250

AVKQAYATVATAPEVKYTVFETALKGATAMSEAQKAAPAAAATATAT 300
|||||
AVKQSYATVATAPEVKYTVFETALKGATAMSEAQKAAPAAAATATAT 300

AAVGAATGAATAAT.....GGYKV 338
|||||
SAVGAATGAVGAATGAATAAAGGYKTGAATPTAGGYKV 338

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FIGURE 3. Comparison of the deduced amino acid sequence of *Phl p V* (upper line) with the deduced amino acid sequence of *Poa p IX* (KBG 41) from Silvanovich et al. (18) (lower line). Vertical bars indicate sequence identities, points represent gaps.

V-specific antibodies (4B1, 2C7, and a rabbit anti-group V antibody) and as negative control, a rabbit anti-group I antibody were tested with the r*Phl p V*. All group V-specific antibodies bound to the β -galactosidase-*Phl p V* fusion allergen at around 140 kDa (asterisk Fig. 4). No binding could be detected with the group I-specific antibody. The same panel of antibodies was used to test β -galactosidase-fused *Phl p I* and β -galactosidase. None of the antibodies reacted with β -galactosidase, whereas the anti-group I and anti-group V antibodies bound specifically to the respective recombinant allergens.

r*Phl p V* inhibits binding of patients' IgE to naturally occurring group V isoallergens. Group V isoallergens from timothy grass (*Phleum pratense*) pollen proteins were separated by two-dimensional gel electrophoresis, blotted to nitrocellulose, and localized with anti-group V antibodies (data not shown). To show whether the r*Phl p V* allergen shares IgE epitopes with acidic and basic group V isoallergens, IgE inhibition experiments were done. Figure 5A shows that IgE-binding to two-dimensionally separated group V isoallergens, pH 5 to >7, was blocked by preadsorption of the serum from a grass pollen-allergic individual with r*Phl p V*, whereas the addition of *E. coli* control proteins had no effect on the IgE-binding (Fig. 5B).

Determination of the percentage of grass pollen-specific IgE directed against r*Phl p V*. To determine the percentage of grass pollen-specific IgE directed against r*Phl p V*, fluid phase IgE-inhibition studies were performed. Patients' sera were preincubated with native natural timothy grass pollen proteins, native r*Phl p V* or native *E. coli* proteins (negative control). The percentage of nonadsorbed grass pollen-specific IgE was then determined. r*Phl p V* was found to

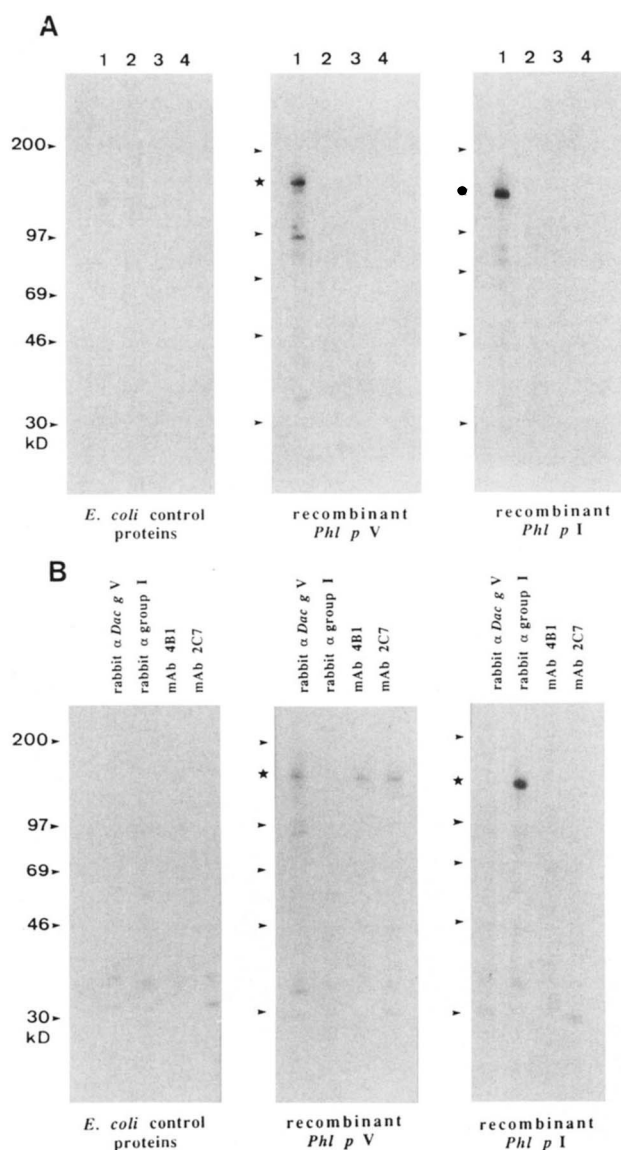


FIGURE 4. Testing of *rPhl p V*, *E. coli* β -galactosidase (negative control) and *rPhl p I* with patients' serum IgE (Figure 4A) and antibodies raised against natural grass pollen allergens (Figure 4B). In Figure 4A lane 1 represents a grass pollen-allergic patient with reactivity to group I and group V allergens, lane 2, a birch pollen-allergic individual without grass pollen allergy, lane 3 serum from a nonallergic individual and lane 4 the buffer control without addition of serum. In Figure 4B, rabbit α *Dac g V*, mAb 4B1, and mAb 2C7 represent anti-group V antibodies. A rabbit α group I antibody was used as a control.

bind up to 60% of grass pollen-specific IgE when compared with natural timothy grass pollen proteins. Table I summarizes the results from the IgE-inhibition studies done with sera from 19 patients. Two control sera from nonallergic individuals showed no detectable IgE reactivity with grass pollen allergens. The results obtained from these experiments indicate that the *rPhl p V* binds a considerable

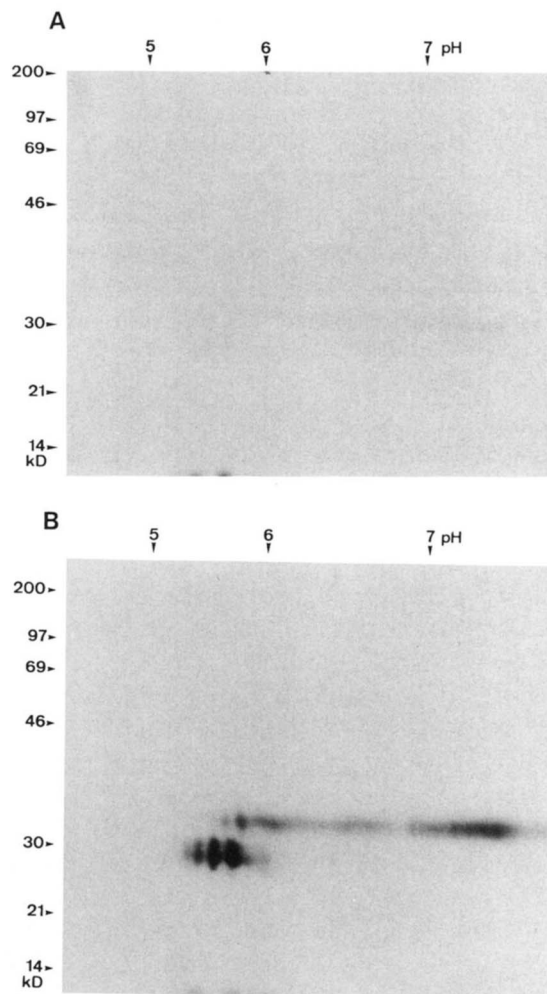


FIGURE 5. Two-dimensional IgE-immunoblot inhibition. Figure 5A shows a two-dimensional IgE-immunoblot of timothy grass pollen proteins, where the serum was preincubated with *rPhl p V*. In Figure 5B, the serum was preincubated with control *E. coli*/phage proteins. pH-values are indicated on top of the figures.

proportion of the grass pollen-specific IgE (average 33%) present in allergic patients' sera.

Purification of the *rPhl p V* allergen

We expressed the *rPhl p V* as a fusion protein with β -galactosidase in lysogenic *E. coli* Y1089 and purified it by affinity chromatography on immobilized anti- β -galactosidase antibodies. Using this method β -galactosidase was also purified to ensure that the β -galactosidase portion of the fusion allergen does not bind IgE or release histamine from patients' basophils in control experiments. The purity of the proteins was more than 95% as estimated by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

rPhl p V elicits specific and dose-dependent histamine release

To test whether the purified *rPhl p V* fusion allergen is capable of releasing specifically histamine from grass pollen-allergic patients basophils, the purified recombinant fusion allergen and purified β -galactosidase as control protein were used in histamine release experiments with blood basophils of grass pollen-allergic patients and individuals with other allergies. All patients had been characterized by case history, radioallergosorbent test, skin-prick tests, and in addition were tested for IgE binding to natural as well as recombinant timothy grass pollen allergens (14). The results of the histamine release tests are shown in Figure 6. All individuals (2, 3, and 4 - Fig. 6A) with IgE antibodies against *Phl p V* showed dose-dependent histamine release, as is shown for patient 2 in Figure 6B. No histamine release was observed with purified β -galactosidase. Basophils from control individual No. 1 with house dust mite allergy and no grass pollen allergy gave no histamine release with natural or recombinant timothy grass allergens. Incubation of patients' basophils with anti-IgE antibodies elicited histamine release in all individuals. The purified *rPhl p V* fusion allergen behaved comparably to the natural grass pollen allergens in the histamine release assays.

Discussion

In this paper we report the molecular cloning of a cDNA coding for a major allergen from timothy grass pollen and the characterization of the recombinant allergen using grass pollen-allergic patients' IgE. The clone described, encodes an allergen, which is the target for IgE antibodies of about 80% of all grass pollen-allergic patients and was selected from 60 immunopositive clones based on the differential testing with serum IgE from 30 different grass pollen-allergic individuals. According to the IgE-binding patterns and the use of several antibodies specific for group I, group V grass pollen allergens and profilin, 23 out of the 60 clones could be specified into group I allergens: *Phl p I* (Laffer S., manuscript in preparation), profilin from timothy grass (Valenta R., manuscript in preparation), and group V allergens: *Phl p V*. These three recombinant allergens were sufficient to diagnose grass pollen allergy (14). DNA sequence analysis of a complete cDNA from the group V-positive clones and comparison of the deduced amino acid sequence revealed a high degree of sequence identity with the *Poa p IX* pollen allergens from Kentucky bluegrass (*Poa pratense*) (18) and a lower degree of sequence identity with *Lol p Ib* from rye grass (*Lolium perenne*), which may be a result of uncertainties in the cDNA sequence of *Lol p Ib* (19). Almost complete sequence identity was found with the very limited partial protein sequences from timothy grass group V isoallergens (20) and group V allergens from several grass species (21). This high degree of sequence identity

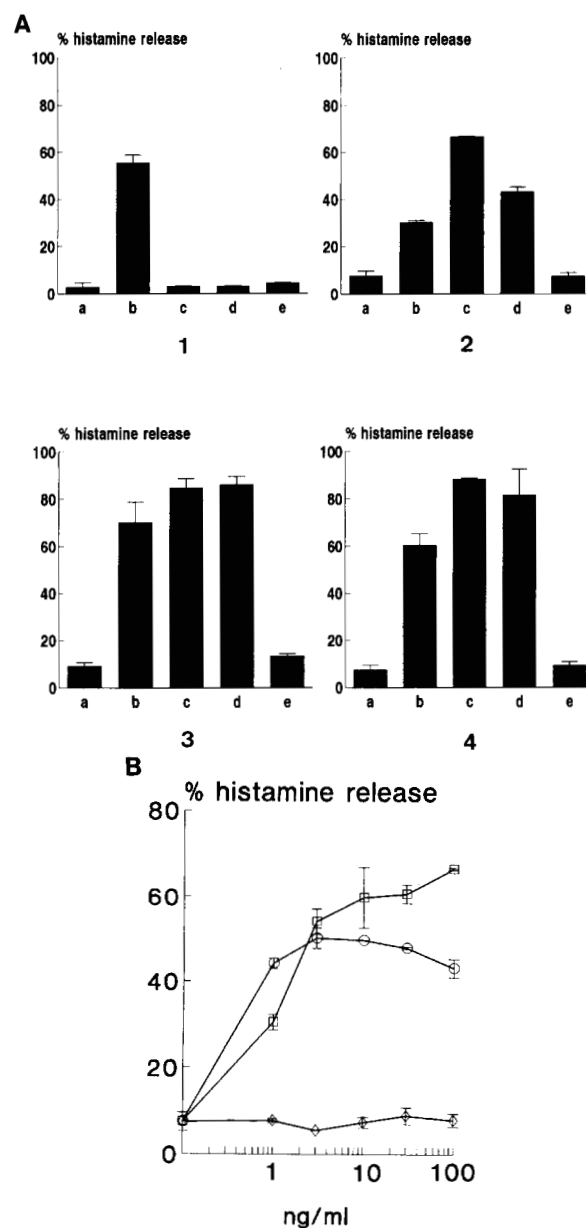


FIGURE 6. A: Histamine releases performed with *rPhl p V* and natural grass pollen allergens. Bar diagrams showing the histamine release obtained from basophils upon stimulation with histamine release buffer (a), anti-human IgE (b), total natural timothy grass pollen allergens (c), *rPhl p V* (d) or β -galactosidase (e). Patient 1 represents a mite-allergic individual with no grass pollen allergy, and patients 2 through 4 are grass pollen-allergic individuals. All values represent means of triplicate determinations at the optimal dose. B: Dose dependency of the histamine release induced in basophils of patient 2. Purified *rPhl p V* (○) and total natural timothy grass pollen allergens (□) induced dose-dependent histamine release whereas β -galactosidase (◇) did not.

between the important allergens from different grass species provides a molecular basis for the extensive cross-reactivity of grass pollen-allergic patients IgE with corresponding allergens in different grass species (28). Further

testing of the recombinant allergen with several group V-specific antibodies identified the recombinant allergen as a group V allergen from timothy grass, *Phl p V*. In addition to the molecular characterization of the cDNA encoding *Phl p V*, we used the recombinant allergen in several IgE-binding tests. It was shown that the recombinant allergen shares IgE epitopes with the naturally occurring isoallergens and is able to trigger specific histamine release from patients' basophils. When tested with sera from 98 grass pollen-allergic patients, 80% showed IgE binding to the r*Phl p V* (14), which therefore represents a major grass pollen allergen. IgE inhibition studies performed with the r*Phl p V* show that in individual patients up to 60% of the IgE reacting with grass pollen proteins can be preadsorbed with the r*Phl p V*. These data together with studies showing the cross-reactivity of patients' IgE with group V allergens from several grass species (28) indicate the therapeutic potential of the r*Phl p V*, if used for hyposensitization treatment. The fact that group V allergens of timothy grass pollen, like the closely related family of the *Poa p IX* allergens, occur as different isoallergenic variants led us to investigate whether the naturally occurring isoallergenic variants of timothy grass pollen share IgE-epitopes with the r*Phl p V* molecule. Two-dimensional IgE inhibition studies show that binding of patients IgE to group V isoallergens of different pI can be blocked by preincubation of the serum with the single r*Phl p V*. The fact that the r*Phl p V* carries IgE epitopes of nitrocellulose-blotted group V isoallergens and accounts for a considerable proportion of grass pollen-specific IgE in individual patients is of relevant importance for the application of this molecule for diagnostic and possibly therapeutic purposes. If the amount of specific IgE is correlated at least to some extent with clinical symptoms it might be assumed that successful immunotherapy with the r*Phl p V* could lead to a significant improvement of symptoms in grass pollen-allergic patients.

In addition to the IgE-binding studies, the functional properties of the r*Phl p V* were investigated by in vitro histamine release tests with basophils of grass pollen-allergic patients. The purified r*Phl p V* released histamine comparably to natural grass pollen allergens in a specific and dose-dependent manner from blood basophils of grass pollen-allergic patients. Prerequisites for allergen-specific diagnosis and therapy of type I allergic diseases with recombinant molecules are that a limited panel of relevant allergens can be defined that account for a high percentage of specific IgE. As described here for a major grass pollen allergen, *Phl p V*, the structural similarity of allergens from different sources forms the molecular basis for the concept of using a small panel of model allergens for diagnostic and therapeutic purposes. The r*Phl p V* allergen may be a good example for a recombinant allergen with a high IgE-binding capacity that may therefore serve as an excellent diagnostic and therapeutic tool for grass pollen allergy.

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