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Molecular characterization of *Phl p* II, a major timothy grass (*Phleum pratense*) pollen allergen

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Grass pollen allergens belong to the most important and widespread elicitors of pollen allergy. Using serum IgE from a grass pollen allergic patient, a complete cDNA encoding a group II allergen was isolated from a timothy grass (*Phleum pratense*) pollen expression library. The deduced amino acid sequence of the *Phl p* II allergen shows an average sequence identity of 61% with the protein sequences determined for group II/III allergens from rye grass (*Lolium perenne*) and a sequence identity of 43% with the C-terminal portion of group I grass pollen allergens from different species. A hydrophobic leader peptide similar to leader peptides found in other major grass pollen allergens heads the deduced amino acid sequence, indicating that group II/III grass pollen allergens belong to a family of secreted proteins. Serum IgE specific for *Phl p* II, detected the protein exclusively in pollen and not in other plant tissues. The recombinant *Phl p* II was expressed in *Escherichia coli* and showed similar IgE-binding capacity as the natural allergen.

Type I allergy; cDNA cloning; Grass pollen allergen; *Phl p* II

1. INTRODUCTION

Type I allergies represent a major health problem in industrialized countries where up to 20% of the population suffer from allergic rhinitis, conjunctivitis, and bronchial asthma. Grass pollen allergens belong to the most potent elicitors of Type I allergy. Approximately 50% of pollen allergic individuals are sensitized against grass pollen of which group I ([1,2] Laffer et al., unpublished data), group V [3,4,5] and group II/III allergens [6,7,8] are the major allergens. Although group I and group V allergens have similar molecular masses between 28 and 34 kDa, these proteins possess different antigenicities and primary structures [5]. Group II/III allergens have molecular masses between 10 and 12 kDa and have been extensively investigated by immunochemical [9,10] and proteinchemical techniques [6,7,8]. Significant protein sequence homology between group II/III allergens from rye-grass pollen (*Lolium perenne*) and the deduced amino acid sequences of group I allergens from other grass species suggest that group II/III allergens are closely related to group I allergens or may even represent cleavage products thereof [2]. Despite this structural similarity with group I allergens and additional data indicating that group I and group II/III allergens share T-cell epitopes [11], much evidence has been accumulated that group I and group II/III allergens possess different IgE-binding capacities and IgE-epitopes [9,10].

Using serum IgE from a grass pollen allergic individual, a complete cDNA coding for a group II/III allergen was isolated from a timothy grass pollen expression cDNA library. The cDNA encodes a protein of 10.7 kDa deduced molecular mass with an average sequence identity of 61% with group II and group III allergens from rye grass (*Lol p* II, *Lol p* III) [6,7]. Since sequence homology was found to groups II and III, the recombinant timothy grass pollen allergen was designated as *Phl p* II.

The cDNA coding for *Phl p* II was expressed in *Escherichia coli* as a β-galactosidase fusion protein. Using nitrocellulose sectors containing the recombinant *Phl p* II it was shown in a previous study that 62% of grass pollen allergic patients displayed IgE reactivity with the recombinant allergen [12]. Conflicting data are available regarding cross-reactivities of antibodies [9,13] and cross-reactivities at the T-cell level [11]. Cross-reactivity at the T-cell level was found between group I and group II allergens and the genetic restriction of the human immune response was found to be similar for group I and group II/III allergens. When we used recombinant *Phl p* II and recombinant *Phl p* I in IgE-adsorption experiments, no significant cross-reactivity of patients' IgE with group I and group II allergens could be found.

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with the accession number X75925.

2. MATERIALS AND METHODS

2.1. *Biological materials*

Pollen from timothy grass (*Phleum pratense*) was purchased from Allergon, Välinge, Sweden. Timothy grass seeds (Timothe Emma SK-45) were purchased from Austrosaat, Vienna, Austria. Seeds were grown at room temperature for 3 weeks to obtain fresh grass. Leaves and roots were washed in water and separated before freezing in liquid nitrogen.

2.2. *Allergic patients sera*

Grass pollen allergic individuals, non-allergic individuals and individuals with allergies other than to grass pollen were selected by case history, RAST (radio allergosorbent test), skin prick test, IgE-immunoblots and by testing with recombinant timothy grass pollen allergens as described [12]. Sera with specificity for group I and group II/III grass pollen allergens could be selected using recombinant *Phl p* V [5] and recombinant *Phl p* I (Laffer et al., unpublished data).

2.3. *Preparation of protein extracts and gel ectrophoresis*

Natural pollen allergens were prepared by incubating 2 g of pollen for 1 h in 50 ml of distilled water at room temperature with shaking. Extracts were then centrifuged for 1 h at 20,000 × *g* at 4°C to remove insoluble particles. Supernatants were then dialysed overnight against distilled water and lyophilized. Protein extracts for expression studies of group II/III allergens in different tissues were obtained by grinding timothy grass tissues (pollen, seeds, leaves and roots) under liquid nitrogen and then further homogenized in SDS-sample buffer [14] with an ultraturrax. The samples were then boiled for 10 min in SDS-sample buffer and supernatants recovered after centrifugation in a Heraeus Minifuge RF at 10,000 × *g* for 10 min. Quantity and quality of the protein extracts were checked by 12% SDS-PAGE and Coomassie blue staining.

Recombinant *Phl p* II was expressed in *E. coli* Y1089 as a β-galactosidase fusion protein after infection with λgt11 phage harbouring the cDNA coding for *Phl p* II [15]. *E. coli* expressing the allergen fusion protein were lysed in SDS-sample buffer prior to denaturing protein gel electrophoresis [14]. Natural protein extracts were subjected to 12% SDS-PAGE, whereas β-galactosidase fusion allergens were separated by 7.5% SDS-PAGE and blotted to nitrocellulose [16].

Native *Phl p* II fusion allergen used for IgE-inhibition experiments was obtained by phage infection of *E. coli* Y1090 on LB-plates containing 100 μg/ml ampicillin. The fusion proteins were induced by overlaying plates with nitrocellulose filters soaked in 10 mM IPTG.

E. coli Y1090 infected with λgt11 phage without insert served as a control. Filters containing the native *Phl p* II fusion allergen were used for IgE-adsorption.

2.4. *Isolation of clone 53 coding for* Phl p *II from a timothy grass pollen (*Phleum pratense*) cDNA library*

60 immunopositive phages were isolated from a timothy grass pollen expression library using serum IgE from a grass pollen allergic patient as described [5]. Clone 53 which bound IgE from patients with reactivity to group II/III grass pollen allergens was selected and tested for IgE binding with sera from 98 grass pollen allergic patients [12].

DNA was prepared from λgt11 phage using a plate lysate method (Amersham, Buckinghamshire, UK). Lambda DNA was digested with *Kpn*I and *Sac*I and due to a *Sac*I site in the insert at position 176 (Fig. 1, 'italics') two DNA fragments containing λgt11 flanking sequences were obtained. A 1.3 kb *Kpn*I/*Sac*I and a 1.2 kB *Sac*I fragment were isolated by preparative agarose gel electrophoresis, subcloned into plasmid pUC18 and transformed into *E. coli* XL-1 blue using the calcium chloride method [17].

Plasmid DNA was isolated from *E. coli* and checked by restriction analysis. *E. coli* containing the correct insert were grown and plasmid DNA was isolated with Quiagen tips (Quiagen, Düsseldorf, Germany). The DNA sequence of both strands was determined by the chain termination method [18] using a T7 sequencing kit (Pharmacia, Uppsala, Sweden), M13 forward and reversed primers (Boehringer, Mannheim, Germany), as well as λgt11 forward and reverse sequencing primers (Clontech, Palo Alto, USA) and [α-^{35}S]dATP (NEN, Stevenage, UK).

2.5. *Expression of the cDNA coding for* Phl p *II in* E. coli*; IgE inhibition experiment*

E. coli Y1089 were infected with lambda phage to express recombinant *Phl p* II as a β-galactosidase fusion protein [15]. Infected *E. coli* were then harvested by centrifugation and washed twice in 0.9% sodium chloride before protein extraction. Protein extracts were separated by 12% SDS-PAGE and blotted to nitrocellulose as described in section 2.3. IgE-immunodetection was done as described [5].

Serum of a grass pollen allergic patient with strong IgE-reactivity to group I and group II/III allergens but not to group V allergens was selected to test IgE-inhibition [12]. The serum was preadsorbed against native nitrocellulose-bound recombinant *Phl p* II, *Phl p* I or β-galactosidase (control) as described [5]. The preadsorbed serum was then used to detect nitrocellulose blotted natural timothy grass pollen allergens.

2.6. *Expression of* Phl p *II in different tissues of timothy grass pollen*

To investigate the expression of the *Phl p* II allergen in different tissues of timothy grass, protein extracts were prepared from pollen, seeds, roots, and leaves. The tissues were homogenized under liquid nitrogen, transferred to SDS-sample buffer and further homogenized with an ultraturrax and boiled. Insoluble particles were removed by centrifugation in a cooled Heraeus Minifuge RF at 10,000 × *g*. Supernatants were applied to 12% SDS-PAGE and, following protein estimation by Coomassie blue staining, were transferred to nitrocellulose [16]. Nitrocellulose strips which had been cut from one sheet were probed with a monoclonal anti-chicken gizzard actin antibody [19] to ensure that comparable amounts of protein were transferred. Bound mouse anti-actin antibodies were detected with a ^{125}I-labeled sheep anti-mouse antibody (Amersham, Buckinghamshire, UK). Strips cut

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ttg gat atc aac ccg tat cga tcc                                    -24

ATG TCC ATG GCG TCC TCC TCA AGC AGC AGC TTG CTG GCC ATG GCG  45
met ser met ala ser ser ser ser ser ser leu leu ala met ala

GTG CTG GCG GCG CTG TTT GCC GGC GCG TGG TGC GTC CCG AAG GTG  90
val leu ala ala leu phe ala gly ala trp cys val pro lys val

ACG TTC ACG GTG GAG AAG GGG TCC AAC GAG AAG CAC CTG GCG GTG 135
thr phe thr val glu lys gly ser asn glu lys his leu ala val

CTG GTG AAG TAC GAG GGG GAC ACC ATG GCG GAG GTG GAG CTC CGG 180
leu val lys tyr glu gly asp thr met ala glu val glu leu arg

GAG CAC GGC TCC GAC GAG TGG GTC GCC ATG ACC AAG GGG GAG GGC 225
glu his gly ser asp glu trp val ala met thr lys gly glu gly

GGC GTG TGG ACG TTC GAC AGC GAG GAG CCG CTC CAG GGG CCC TTC 270
gly val trp thr phe asp ser glu glu pro leu gln gly pro phe

AAC TTC CGG TTC CTC ACC GAG AAG GGC ATG AAG AAC GTC TTC GAC 315
asn phe arg phe leu thr glu lys gly met lys asn val phe asp

GAC GTC GTC CCA GAG AAG TAC ACC ATT GGG GCC ACC TAC GCG CCA 360
asp val val pro glu lys tyr thr ile gly ala thr tyr ala pro

GAA GAG TAG                                                        369
glu glu  *


cca tcg gtc cat cca cat gca tga tga tcc ttc cat cca tct gat  45

tta gtt cga ttt tcc ttg tgt ttt gga acg aat tgt tgc aaa tta  90

cat gtt caa aga cat atg ttg cac gaa att ttt tac taa aaa      132
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Phl p* II. The complete cDNA sequence of *Phl p* II is displayed. The deduced amino acid sequence is shown below the nucleotide sequence and the region coding for a N-terminal hydrophobic leader peptide is underligned. The internal *Sac*I site is printed in italics.

from the same preparative sheets were subsequently incubated with serum IgE from a patient specific for group I and group II/III grass pollen allergens.

3. RESULTS

3.1. *Nucleotide and deduced amino acid sequence of* Phl p *II*

The complete cDNA and deduced amino acid sequence of *Phl p* II is displayed in Fig. 1. The ATG start codon of the cDNA is followed 3′ by a stretch of 78 nucleotides coding for a polypeptide of a calculated molecular mass of 2.56 kDa displaying high homology to eukaryotic leader peptides and 35% sequence homology to published *Lol p* I and *Phl p* I leader peptides ([2], Laffer et al., unpublished data). The mature *Phl p* II protein, with amino-terminal valine, is encoded by 288 nucleotides and has a calculated molecular mass of 10.7 kDa corresponding to molecular masses of natural group II/III grass pollen allergens of 10–12 kDa as estimated by SDS-PAGE. *Phl p* II also shows 65% and 58% sequence identities to *Lol p* II and *Lol p* III, respectively (Fig. 2A). Significant sequence homology was also found with a partial amino acid sequence deduced from an incomplete and uncharacterized cDNA clone thought to code for the group II allergen, *Dac g* II, from cocksfoot grass (*Dactylis glomerata*) pollen [13,20].

The deduced amino acid sequence of *Phl p* II shows a comparable sequence identity with protein sequences derived from group II and group III rye grass allergens [6,7]. It seems likely therefore that group II/III allergens belong to a family of isoallergens, an observation supported by the various iso-proteins with different pIs seen when timothy grass pollen allergens are subjected to two-dimensional electrophoresis (data not shown). Fig. 2B shows the alignment of the deduced *Phl p* II amino acid sequence with the deduced amino acid sequence for *Lol p* I and *Phl p* I. Comparing *Phl p* II with the C-terminal portion of several group I grass pollen allergens revealed average sequence identities of 43%.

The antigenic index determined for *Phl p* II and *Phl p* I [21] as well as the prediction of T-helper cell epitopes [22] for *Phl p* II and the *Phl p* I C-terminus indicates that both groups of allergens may share the same epitopes (data not shown).

3.2. *Expression of* Phl p *II in different tissues of timothy grass*

Major grass pollen allergens such as group I and group V are specifically expressed in pollen but not in other plant tissues [1,3]. These results are based on Northern blot hybridization studies using cDNAs coding for these allergens as probes. The availability of a patient's serum containing IgE specific for group I and

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Lol p II                                          AAPVEFTVEKGSDEKNLALSIKYNKEGDSMAEVELKEHGS
                                                  | ||||||| || ||   ||  ||| |||||| ||||
Phl p II      MSMASSSSSSLLAMAVLAALFAGAWCVPKVTFTVEKGSNEKHLAVLVKY--EGDTMAEVELREHGS  40
                                                  ||  |||||| | |    ||   ||| |||||| |||
Lol p III                                         TKVDLTVEKGSDAKTLVLNIKYTRPGDTLAEVELRQHGS

Lol p II      NEWLALKKNGDGVWEIKSDKPLKGPFNFRFVSEKGMRNVFDDVVPADFKVGTTYKPE
               || |  |    ||   |  || |||||||  |||| ||||||||     | || ||
Phl p II      DEWVAMTKGEGGVWTFDSEEPLQGPFNFRFLTEKGMKNVFDDVVPEKYTIGATYAPEE          98
               ||  |||  |  |   |  || || |||||  |||||||  | |   | | || ||
Lol p III     EEWEPMTK-KGNLWEVKSAKPLTGPMNFRFLSKGGMKNVFDEVIPTAFTVGKTYTPEYN

Lol p I     ... DTKPTFHVEKASNPNYLAILVKYVDGDGDVVAVDIKEKGKDKWIELKESW
                  | || ||| ||   || ||||  ||     |   | | | |
Phl p II        VPKVTFTVEKGSNEKHLAVLVKY-EGD-TMAEVELREHGSDEWVAMTKGE  50
                  |||| ||||||   || ||||  ||     |   | | | |
Phl p I     ... GTKVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW

Lol p I         GAVWRIDTPDKLTGPFTVRYTTEGGTKSEFEDVIPEGWKADTSYSAK
                | ||  |    | |||  |  || | |  | || ||       |
Phl p II        GGVWTFDSEEPLQGPFNFRFLTEKGMKNVFDDVVPEKYTIGATYAPEE    98
                |  |  |    | |||  |  || | |    || ||       |
Phl p I         GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
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Fig. 2. (A) Comparison of the deduced amino acid sequence of *Phl p* II with group II/III and group I grass pollen allergen sequences. The comparison of the deduced amino acid sequence of *Phl p* II with the amino acid sequences from *Lol p* II and *Lol p* III shows an average sequence identity of 61%. Vertical bars represent identical amino acids. The signal peptide of *Phl p* II is underlined. (B) Alignment of the deduced amino acid sequence of *Phl p* II with the C-terminal portions of *Phl p* I and *Lol p* I allergens. A sequence identity of 43% was found with both group I grass pollen allergens.

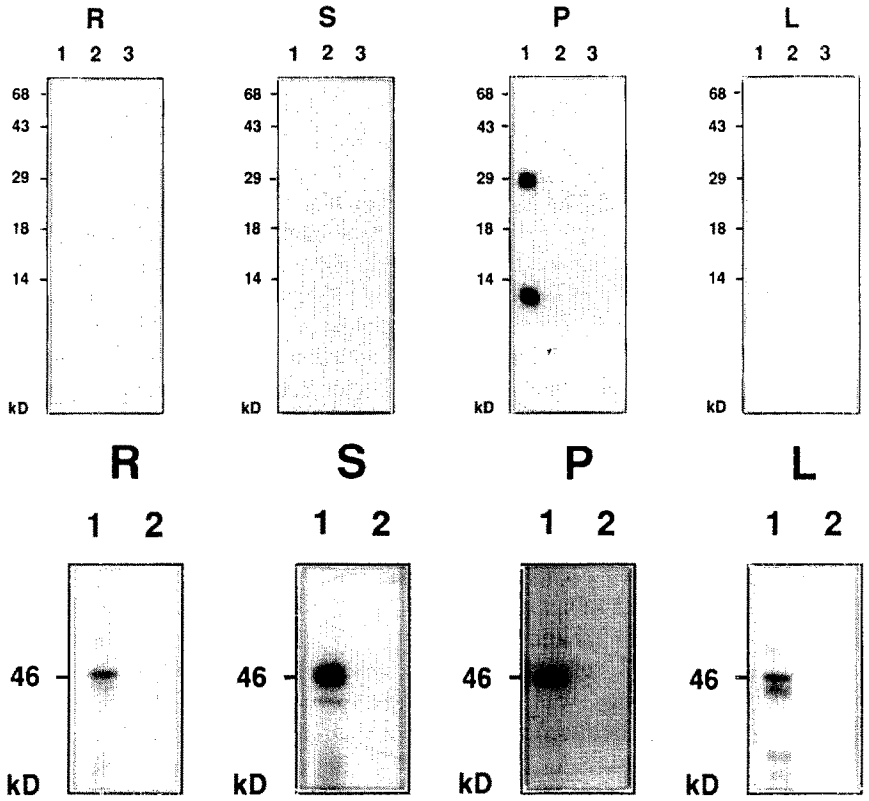

Fig. 3. (A,B) Expression of *Phl p* II in different tissues of timothy grass. Immunoblots of protein extracts from different timothy grass tissues were probed with serum IgE specific for group I and group II/III pollen allergens (Fig. 3A) or with a monoclonal anti-actin antibody (Fig. 3B). Protein extracts were prepared from roots (R), seeds (S), pollen (P), and leaves (L). In Fig. 3A nitrocellulose strips were incubated with serum IgE specific for group I and group II/III allergens (lane 1), with serum from a non-allergic control individual (lane 2) or buffer without addition of serum (lane 3). Group I and group II/III allergens are found exclusively expressed in pollen at 32 kDa and 10–12 kDa. In Fig. 3B nitrocellulose strips from the same preparative sheet as in Fig. 3A were incubated with a monoclonal anti-actin antibody (lane 1) or with buffer (lane 2). Actin can be detected in all of the protein extracts prepared whereas group I and group II/III allergens are exclusively expressed in pollen.

group II allergens enabled us to test for the presence of group I and group II allergens in different tissues of timothy grass at the protein level. A monoclonal antibody raised against chicken gizzard actin (Chemicon, Temecula, USA) with specifity for plant actins [19,23] was used as a control. Using IgE specific for group I and group II/III allergens, we found both allergens in pollen but not in leaves, roots or seeds (Fig. 3A) whereas comparable amounts of actin were detected in all tissues (Fig. 3B).

3.3. *IgE-binding capacity of recombinant* Phl p *II*

λgt11 phage harbouring the cDNA coding for *Phl p* II were used to infect lysogenic *E. coli* Y1089 to produce a β-galactosidase fusion allergen. Serum IgE of a patient with reactivity to group II allergens bound strongly to the fusion allergen (Fig. 4, lane 1) at around 120 kDa whereas a control serum from a patient with birch pollen allergy but no reactivity to grass pollen allergens (lane 2) and a serum from a non-allergic individual (lane 3) did not bind. In a previous study, nitrocellulose sectors containing the plaque lifted fusion protein were probed with serum IgE from 98 grass pollen allergic patients [12] of which 62% showed reactivity with clone 53 expressing the *Phl p* II allergen.

3.4. *Cross-reactivity of patients' IgE antibodies with* Phl p *II and* Phl p *I*

The sequence similarity of group II allergens with the C-terminal portion of group I grass pollen allergens prompted us to investigate cross-reactivities of patient's IgE between group I and group II allergens. We used serum IgE from a patient with specificity to group I and group II grass pollen allergens and recombinant *Phl p* I and recombinant *Phl p* II in IgE-adsorption studies. Preincubation of the serum with recombinant *Phl p* I inhibited IgE binding to natural *Phl p* I but did not affect IgE-binding to natural group II/III allergens. When recombinant *Phl p* II was used for preadsorption of the serum, only binding to group II allergens was inhibited whereas no effect was observed on binding to group I allergens. Preincubation with β-galactosidase did not affect IgE-reactivity to group I or group II allergens (Fig. 5). The results indicate that despite the sequence similarity between group I and group II/III allergens, distinct IgE epitopes can be found.

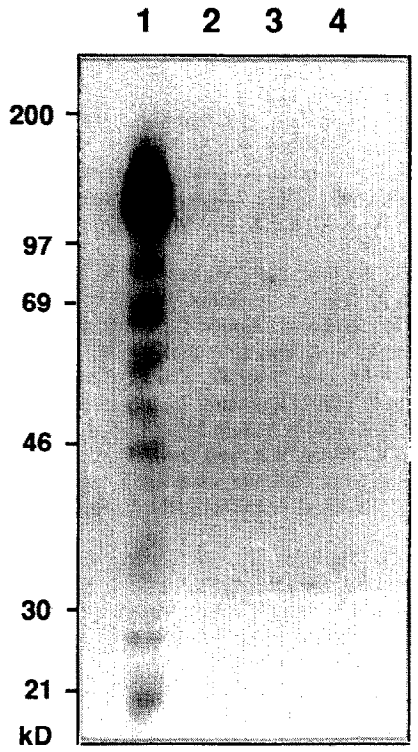

Fig. 4. Expression of recombinant *Phl p* II as a β-galactosidase fusion protein in *E. coli*. Recombinant *Phl p* II was expressed as a β-galactosidase fusion protein upon infection of *E. coli* Y1089 with λgt11 phage harbouring the cDNA for *Phl p* II. Nitrocellulose strips containing the recombinant fusion allergen were probed with serum IgE from a patient allergic to group II/III allergens (lane 1), with serum from a patient with birch pollen allergy without specific IgE against grass pollen allergens (lane 2), with serum from a non-allergic individual (lane 3) and with buffer (lane 4).

4. DISCUSSION

Grass pollen allergens belong to the most potent and frequent elicitors of type I allergy. As yet three major groups of allergens with different antigenicities have been described which can be found in the pollen of most grass species and share IgE-epitopes ([5], Laffer et al., unpublished data). Two of these major allergens, group I ([1,2], Laffer et al., unpublished data) and group V [3,4,5] have been characterized by cDNA cloning techniques. The amino acid sequence of group II/III allergens, which bind the IgE of more than 62% of grass pollen allergic patients, has been determined [6,7,8]. Although group II/III allergens belong to the most extensively investigated grass pollen allergens as yet no complete cDNA coding for a group II allergen was available.

We report here the complete cDNA sequence and subsequent expression in *E. coli* of a group II/III allergen from timothy grass pollen (*Phleum pratense*). The cDNA was isolated from a timothy grass pollen expression cDNA library using allergic patients' IgE and encodes a mature protein of 10.7 kDa deduced molecular mass. Since a similar degree of sequence homology was found with both *Lol p* II and *Lol p* III, we designated the recombinant timothy grass pollen allergen *Phl p* II. Group II/III allergens of different grass species can be resolved into several isoelectric spots by two-dimensional gel electrophoresis (Laffer et al., unpublished data) suggesting that group II/III allergens occur as isoallergenic variants. Despite the sequence homology of *Phl p* II to group I grass pollen allergens, IgE-adsorption experiments showed no significant cross-reactivities, corroborating earlier observations that group I and group II/III allergens possess different IgE epitopes [9,10]. Clone 53 expressing *Phl p* II was tested for its IgE-binding capacity using sera from 98 grass pollen allergic patients in a previous study [12] and 62% showed IgE-reactivity with recombinant *Phl p* II. This may therefore represent a useful tool in the diagnosis and treatment of grass pollen allergy.

Compared to tree pollen allergens such as *Bet v* I [24] which is highly homologous to a group of pathogenesis-related proteins and birch profilin [19,25,26,27], an actin-binding protein [19,23], nothing is known regarding any possible biological or biochemical functions of grass pollen allergens. The only common property of major grass pollen allergens is their rapid availability on

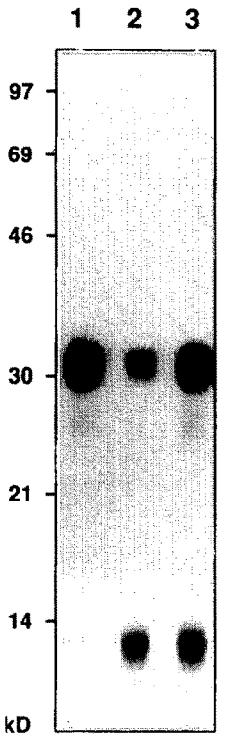

Fig. 5. Different IgE-epitopes of group II and group I timothy grass pollen allergens. To investigate cross-reactivities of patients' IgE with group I and group II/III allergens, IgE-inhibition studies were done. Serum IgE from a patient with specificity for group I and group II/III allergens was used to detect nitrocellulose blotted natural timothy grass pollen allergens. The serum was preabsorbed with filters containing recombinant *Phl p* II (lane 1), recombinant *Phl p* I (lane 2) or β-galactosidase (control) (lane 3). Preincubation with recombinant *Phl p* II inhibits binding to natural group II/III allergens at 10–12 kDa but not to group I allergens at around 32 kDa.

contact of pollen with aqueous solutions [28]. In common with group I and group V grass pollen allergens, a signal peptide was found in the deduced amino acid sequence of *Phl p* II. The signal peptide of 26 amino acids is more closely related to the leader peptides of group I grass pollen allergens ([1,2], Laffer et al., unpublished data) than to group V sequences as would be expected from immune localization data showing different localizations for group V and group I allergens [3,29]. Although we do not yet have experimental evidence regarding the biological function of major grass pollen allergens, the presence of leader peptides indicates that the major grass pollen allergens are all secreted. Major grass pollen allergens such as group I and group V are specifically expressed in pollen [1,3]. *Phl p* II was also detected exclusively in pollen.

We conclude that major grass pollen allergens belong to a family of secreted proteins which are exclusively expressed in monocot pollen and share a high degree of sequence homology in different grass species. This provides an explanation for the clinically observed phenomenon that most grass pollen allergic patients cross-react with the homologous allergens from different grass species. The recombinant *Phl p* II allergens may be useful to explore the biological function of grass pollen allergens and in addition may represent a potent tool in diagnosis and therapy of grass pollen allergy.

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