

Structural investigations of the major allergen *Phl p I* on the complementary DNA and protein level

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Until now investigations of group I grass allergens have mainly been performed on ryegrass allergen (Lol p I). We studied this major allergen grass group with timothy grass pollen (Phl p I), a very common and important cause of type I allergy, to determine intraspecific and interspecific variations among different grass species. By immunoscreening a timothy grass pollen complementary DNA library we obtained three full-length clones. They revealed identical nucleotide sequences in the coding regions consisting of 262 amino acids, including a leader sequence of 23 amino acid residues. The comparison of our data with the amino acid sequences deduced from Lol p I and Hol I clones showed sequence identities of greater than 85% and homologies of greater than 90%, indicating a high degree of sequence conservation. Despite the high degree of homology, amino acid differences were in immunodominant positions, which may be responsible for the differing immune response to group I allergens of different grass species. (J ALLERGY CLIN IMMUNOL 1995;95:987-94.)

Key words: Complementary DNA cloning, grass pollen allergen, IgE binding, immunoblotting, two-dimensional gel electrophoresis

Among airborne allergens giving rise to IgE-mediated allergy, grass pollen plays a very important role. The first pollen allergen, which was intensively studied, was isolated from ryegrass (*Lolium perenne*). This *Lol p I* allergen, formerly termed Rye I,¹ reveals an IgE prevalence of 95%.² Johnson and Marsh³ demonstrated that the allergen was polymorphic, showing isoallergenic variants. The authors estimated a carbohydrate content of about 5%.⁴ Because the IgE reactivity of the components was not destroyed by deglycosylation, the allergenicity was assumed to be located on the protein core. Esch and Klapper^{5,6} were able to elucidate IgE-reactive sites after cyanogen bromide cleavage and trypsin degradation of *Lol p I* and by use of monoclonal antibodies (Mab) directed against different epitopes. Thus they defined an IgE-reactive region spanning the 23 C-terminal amino acids.

Abbreviations used

2D:	Two-dimensional
bp:	Base pair
cDNA:	Complementary DNA
DTT:	Dithiothreitol
IPTG:	Isopropyl β -D-thiogalactopyranoside
Mab:	Monoclonal antibody
PAGE:	Polyacrylamide gel electrophoresis
pI:	Isoelectric point
SDS:	Sodium dodecyl sulfate

Many research groups, including Baldo et al.⁷ and Weber,⁸ have demonstrated a high crossreactivity between components of different grass species. Grass group I allergens demonstrate similar IgE reactions with RAST inhibition⁹ and crossed immunoelectrophoresis.¹⁰ A high degree of similarity has also been demonstrated with Mab analysis and fingerprinting techniques on extracts obtained from different grasses.^{11,12} Furthermore, these similarities were confirmed by N-terminal protein sequencing.¹³ However, each test also showed a few variations. According to studies of Marsh et al.,¹⁴ timothy grass pollen exhibited less antigenic and allergenic crossreactivity with groups

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I, II, and III of rye than the respective antigens of fescue, orchard, and velvet. Because the reactivity of the patient sera and the Mab were not identical in different sorts of pollen extracts, the question arises whether the structural differences are located in IgE-reactive epitopes. Thus microheterogeneity might play a role in allergenicity and must be considered when extracts for hyposensitization therapy are selected.

To estimate the influence of isoallergenic variations and interspecific differences in the primary structure, we determined the entire allergen structure of the grass group I allergen in the widespread timothy grass pollen (*Phleum pratense*), *Phl p* I, by complementary DNA (cDNA) sequencing. Immunodominant epitopes of the well-defined *Lol p* I allergen were compared with the deduced amino acid sequence of *Phl p* I and data of group I allergens of other grasses, to estimate the influence of the amino acid variations.

METHODS

Preparation of pollen extract

One gram of timothy pollen (ARTU Biologicals, Lelystad, The Netherlands) was suspended in 55 ml of 0.1 mol/L NH_4HCO_3 buffer (pH 8.0) and incubated at room temperature for 30 minutes with agitation. After centrifugation for 30 minutes at 17000g (4°C), the supernatant was dialyzed against double-distilled water. After lyophilization, the extract was stored at 4°C until use.

Patient sera and Mab

For the identification of IgE-reactive proteins we used a mixture of sera from three grass pollen-sensitive patients (Pharmacia CAP System, CAP score 6; RAST for the quantification of specific IgE, scores according to manufacturer's manual [Pharmacia Diagnostics AB, Uppsala, Sweden]). The Mab Bo 1¹⁵ and IG 12 were obtained by the hybridoma technique with crude timothy grass pollen extract as immunogen.

Western blot analysis

Proteins were separated by discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with the buffer system of Laemmli¹⁶ with a stacking gel of 5% T and 0.75% C and a running polyacrylamide gradient gel of 15% T and 4.0% C. Samples were reduced by incubating at 95°C for 5 minutes in sample buffer containing 40 mmol/L dithiothreitol (DTT; Merck, Darmstadt, Germany). After cooling, the samples were alkylated with 60 mmol/L iodoacetamide (Merck). The proteins were separated by electrophoresis and subsequently transferred to a nitrocellulose membrane (Schleicher & Schüll, Dassel,

Germany) by semidry blotting¹⁷ for 30 minutes at 0.8 mA/cm².

Two-dimensional immunoblotting

The two-dimensional (2D) PAGE blot was carried out according to the method of Görg et al.,¹⁸ with modifications as described in detail.¹⁹ In the first dimension isoelectric focusing was performed in immobilizing pH gradients. The second dimension was run in SDS gels. For subsequent immunologic investigations thermoblotting (capillary blotting on a 50°C heating plate²⁰) was performed, whereas semidry blotting was used for biochemical studies.

Protein staining and immunologic detections

After blotting, the nitrocellulose membrane was stained by 0.1% (vol/vol) india ink (Pelikan AG, Hannover, Germany).²¹

To detect the allergens, the nitrocellulose membrane was incubated in patients' pooled serum or in supernatants of Mab. IgE reactivity was identified by mouse anti-human IgE (Immuntotech, Hamburg, Germany). Subsequently alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (Dianova, Hamburg, Germany) was added. The binding patterns were visualized by a substrate solution consisting of 0.033% (wt/vol) nitroblue tetrazolium chloride and 0.017% (wt/vol) 5-bromo-4-chloro-3-indolyl phosphate potassium salt (Serva, Heidelberg, Germany) in 0.1 mol/L tris(hydroxymethyl) aminomethane-buffered saline solution (pH 9.5).²²

Carbohydrate residues were detected by the DIG Glycan Detection Kit (Boehringer, Mannheim, Germany) and further determined by the DIG Differentiation Kit (Boehringer) by use of several lectins as described by the manufacturer.

cDNA cloning and allergen expression

A cDNA library of timothy grass pollen²³ was prepared according to the method of Gubler and Hoffmann.²⁴ To generate a double-stranded cDNA, 10 µg of timothy grass pollen messenger RNA was used with the cDNA synthesis kit (Pharmacia). cDNA was cloned into λ-ZAP Vector (Stratagene, La Jolla, Calif.) and then packaged into bacteriophages (λDNA In Vitro Packing Kit, Amersham Buchler, Braunschweig, Germany). *Escherichia coli* K12 XL-Blue (Stratagene) was transduced with in vitro packaged phages. Screening for grass group I-specific clones was performed by Mab IG 12. After induction by 2 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG) immunoscreening was carried out with the Mab IG 12 and a patient's pooled serum. Positive clones were rescreened, isolated, and amplified.

The coinfection with the helper phage Exassist (Stratagene) resulted in the in vivo excision of pBluescript SK⁻ plasmid. Double-stranded DNA was sequenced by the method of Sanger et al.²⁵ (T7 Sequencing Kit, Pharmacia) and by use of the following nested primers

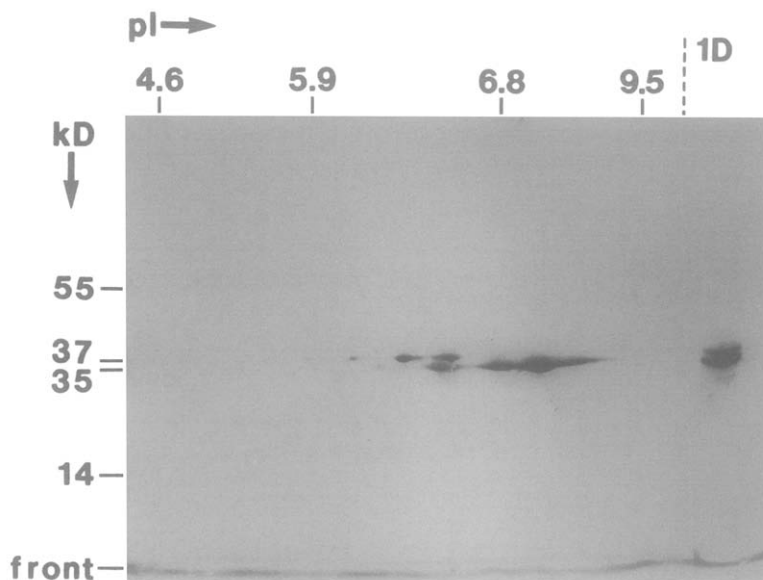


FIG. 1. 2D immunoblotting of timothy grass pollen extract showing binding reactivity of group I-directed Mab IG 12.

within the coding region 5'-AGCACCTGGTACG-GCAA-3', 5'-AACCCCAACTACCTGGC-3', 5'-AAG-GCCGACACCGCCTA-3'.

The primers were synthesized with the Cyclone Plus synthesizer (Millipore, Bedford, Mass.).

For expression of the fusion proteins transfected *E. coli* K12 SOLR cells (Stratagene) were induced by 2 mmol/L IPTG. Aliquots were taken after 0 and 2 hours of incubation and suspended in SDS sample buffer for subsequent separation of the proteins by SDS-PAGE and immunoblotting.

Computer analyses

Sequence data were analyzed on GeneWorks and PCGENE software (Intelligenetics, Geel, Belgium) and on DNAsis and Prosis Software (Pharmacia LKB Biotechnology, Uppsala, Sweden). The antigenic index was calculated according to the algorithm of Jameson and Wolf.²⁶

Nucleotide sequence accession number

The nucleotide sequence of pPHLPl.p4 has been submitted to the EMBL (European Molecular Biology Laboratory) Nucleotide Sequence Data Library with the accession number Z27090.

RESULTS

Isolation and characterization of *Phl p I* clones

The Mab IG 12 was used to screen a cDNA library of timothy grass pollen for group I clones.

Fig. 1 shows the binding pattern of the Mab IG 12 after 2D immunoblotting of timothy pollen

extract. The antibody detects two IgE-reactive components of 37 kD with pI 6.4 and 6.6 and four of 35 kD with pI 6.5, 6.8, 7.1, and 7.3. The antibody showed no crossreactivity to cell lysate of *E. coli* strain SOLR as investigated by Western blotting. The cDNA library was screened by IG 12 hybridoma supernatant without further dilution. After rescreening with patients' pooled serum and Mab IG 12, three clones were obtained. In vivo excision and plasmid preparation yielded a 1.2 kb insert for pPHLPl.p1 and 1.5 kb inserts for both pPHLPl.p4 and pPHLPl.p9.

Nucleotide sequencing (Fig. 2) revealed three full-length clones coding for complete *Phl p I* proteins. They exhibited identical sequences for the coding region consisting of 69 base pairs (bp) for the leader sequence followed by 720 bp for the mature protein. PHLPl.p1 possesses noncoding regions 17 bp upstream and 373 bp downstream of the coding region. The cDNA clones of PHLPl.p4 and PHLPl.p9 are completely identical. They differ from the PHLPl.p1 clone by size (revealing 8 bp upstream and 352 bp downstream of the coding region).

Finally, we characterized the expression products by immunoblotting. The expression of PHLPl.p1 in *E. coli* strain SOLR is shown in Fig. 3. The two other clones produced similar results, although the expression was much weaker.

Fig. 3 exhibits the reactivities of patients' pooled serum and group I-directed Mabs to timothy

1	TA	GCA	ATT	GAA	GAC	AAG	ATG	GCT	TCT	TCC	TCA	TCG	GTT	CTG	CTG	GTC
1							M	A	S	S	S	S	V	L	L	V
48	GTG	GCG	CTG	TTC	GCC	GTC	TTC	CTG	GGC	TCT	GCG	CAT	GGC	ATC	CCC	AAG
11	V	A	L	F	A	V	F	L	G	S	A	H	G	<u>I</u>	P	K
96	GTC	CCC	CCC	GGC	CCG	AAC	ATC	ACG	GCG	ACC	TAC	GGC	GAC	AAG	TGG	CTG
27	V	P	P	G	P	N	I	T	A	T	Y	G	D	K	W	L
144	GAC	GCG	AAG	AGC	ACC	TGG	TAC	GGC	AAG	CCG	ACG	GCC	GCC	GGT	CCC	AAG
43	<u>D</u>	A	K	S	T	W	Y	G	K	P	T	A	A	G	P	K
192	GAC	AAC	GGC	GGC	GCG	TGC	GGG	TAC	AAG	GAC	GTG	GAC	AAG	CGC	CCG	TTC
59	D	N	G	G	A	C	G	Y	K	D	V	D	K	P	P	F
240	AGC	GGC	ATG	ACC	GGC	TGC	GGC	AAC	ACC	CCC	ATC	TTC	AAG	TCC	GGC	CGG
75	S	G	M	T	G	C	G	N	T	P	I	F	K	S	G	R
288	GGC	TGC	GGC	TCC	TGC	TTC	GAG	ATC	AAG	TGC	ACC	AAG	CCC	GAG	GCC	TGC
91	G	C	G	S	C	F	E	I	K	C	T	K	P	E	A	C
336	TCC	GGC	GAG	CCC	GTG	GTG	GTC	CAC	ATC	ACC	GAC	GAC	AAC	GAG	GAG	CCC
107	S	G	E	P	V	V	V	H	I	T	D	D	N	E	E	P
384	ATC	GCC	GCG	TAC	CAC	TTC	GAC	CTC	TCC	GGC	ATC	GCG	TTC	GGG	TCC	ATG
123	I	A	A	Y	H	F	D	L	S	G	I	A	F	G	S	M
432	GCC	AAG	AAG	GGC	GAC	GAG	CAG	AAG	CTG	CGC	AGC	GCC	GGC	GAG	GTG	GAG
139	A	K	K	G	D	E	Q	K	L	R	S	A	G	E	V	E
480	ATC	CAG	TTC	CGC	CGC	GTC	AAG	TGC	AAG	TAC	CCG	GAG	GGC	ACC	AAG	GTC
155	I	Q	F	R	R	V	K	C	K	Y	P	E	G	T	K	V
528	ACC	TTC	CAC	GTG	GAG	AAG	GGG	TCC	AAC	CCC	AAC	TAC	CTG	GCG	CTG	CTG
171	T	F	H	V	E	K	G	S	N	P	N	Y	L	A	L	L
576	GTG	AAG	TTT	TCC	GGC	GAC	GGC	GAC	GTG	GTG	GCG	GTG	GAC	ATC	AAG	GAG
187	V	K	F	S	G	D	G	D	V	V	A	V	D	I	K	E
624	AAG	GGC	AAG	GAC	AAG	TGG	ATC	GCG	CTC	AAG	GAG	TCG	TGG	GGA	GCC	ATC
203	K	G	K	D	K	W	I	A	L	K	E	S	W	G	A	I
672	TGG	AGG	ATC	GAC	ACC	CCG	GAG	GTG	CTC	AAG	GGC	CCC	TTC	ACC	GTC	CGC
219	W	R	I	D	T	P	E	V	L	K	G	P	F	T	V	R
720	TAC	ACC	ACC	GAG	GGC	GGC	ACC	AAG	GCG	AGG	GCC	AAG	GAC	GTC	ATC	CCC
235	Y	T	T	E	G	G	T	K	A	R	A	K	D	V	I	P
768	GAG	GGC	TGG	AAG	GCC	GAC	ACC	GCC	TAC	GAG	TCC	AAG	TGA	ACA	ATC	CAT
251	E	G	W	K	A	D	T	A	Y	E	S	K	*			
816	CCA	TCA	ATG	GAT	CCT	TCA	CCA	CCC	CTA	CGT	ACC	ACT	TCC	GGC	AAG	TTC
864	AAT	TTT	GAC	TCA	ACA	AGA	TCA	CCA	ATA	AGT	TAA	CCG	GTC	GGC	CAC	ATC
912	TAT	ATA	GGC	CAG	CGC	AAG	CTC	CTA	GCA	TGT	TCA	TGC	TAC	AGA	GAG	GGG
960	AAA	TGC	GAG	AGT	AAT	AAC	TAA	TTT	GAG	GTC	GAG	ACC	TTG	CGA	CTT	GCG
1008	AGG	TGT	ATG	TAG	GAG	GCA	ACC	AAC	AAA	AAA	TGG	TTT	GTC	CTC	CGG	CCC
1056	ACA	CTC	GGA	CCG	CTT	TAA	TTG	TTT	ATG	TAT	CTA	AGA	TTG	TTC	ATG	TTT
1104	ATG	AAC	ATA	TAT	ATT	GTA	GCG	GCC	GCG	AAT	TCG	ATA	TCA	AGC	TTA	TCG
1152	ATA	CCG	TCG	ACC	TCG	AGG	GGG	GGC	CCG	G						

FIG. 2. cDNA and deduced amino acid sequence of clone PHLPI.p1. N terminus, determined already by protein sequencing,³¹ is underlined. Arrow indicates NH₂ terminus of mature protein.

pollen extract and to cell lysates of pPHLp1-transfected SOLR (SOLR/p1). For expression analysis samples of the bacterial cultures were taken 0 and 2 hours after IPTG induction. Mab IG 12 and patients' IgE identified a fusion protein of about 38 kd after 2 hours of induction, whereas no immunoreactive components were detectable at the start (0 hours). For comparison we separated timothy pollen extract: Mab IG 12 bound strongly to a 35 kd and weakly to a 37 kd protein. (Mab Bo 1 was used as a control; it specified two grass group V proteins of 32 and 38 kd that were also detectable by patients' IgE in crude pollen extract.)

Investigation of timothy grass group I for posttranslational modifications

After deduction of the primary structure of *Phl p I* from the cDNA data, we considered possibilities for posttranslational modifications. Because seven cysteine residues per molecule (amino acid residues 41, 57, 69, 72, 77, 83, and 139) were determined, there is possibly much variation in the disulfide bonding. To examine their influence on the conformation of *Phl p I* allergens, we treated timothy pollen extract before Western blotting with increasing concentrations of reducing reagent.

Fig. 4 demonstrates the molecular size variations of *Phl p I* isoallergens (identified by Mab IG 12) caused by addition of DTT (0 to 40 mmol/L). Each lane reveals two protein bands of 35 and 37 kd. Additionally, the unreduced sample shows IgE reactive components of 32 and 55 kd. These protein bands disappeared when more than 0.4 mmol/L DTT is added. These results clearly show that the minor and major shifts in the molecular size are due to variations in the protein structure caused by the cleavage of disulfide bonds.

Comparison of grass group I protein sequences of timothy grass, ryegrass, and velvet grass

Group I allergens of different grass species were compared to determine their interspecific variability. Besides our investigations concerning *Phl p I*, complete sequence data of group I allergens were available for ryegrass (*Lol p I*; including some isoallergens)^{27,28} and velvet grass (*Hol l I*)²⁹ (Fig. 5).

At the first sight there is a high protein sequence homology between the grass species of group I. We determined the following sequence identities (homologies shown in parentheses): *Phl p I* and *Hol l I*, 88.9% (93.1%); *Phl p I* and *Lol l I*, 85.9%

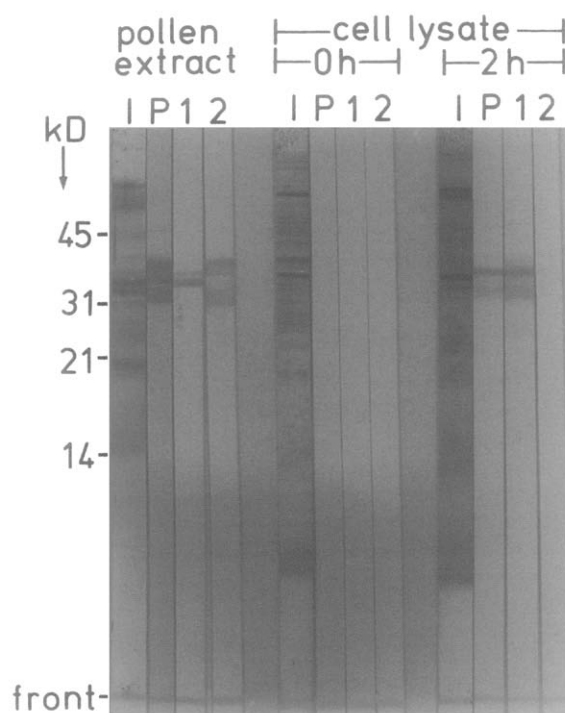


FIG. 3. Expression of clone PHLp1 in *E. coli* SOLR after IPTG treatment. Western blotting of timothy grass pollen extract and SOLR/p1 cell lysates was performed after induction for 0 and 2 hours. *I*, Protein staining by india ink; *P*, patients' pooled sera; *1*, group I-directed Mab IG 12; *2*, group V-directed Mab Bo 1.

(90.9%); and *Hol l I* and *Lol p I*, 91.6% (93.9%). The complete *Phl p I* consisting of 265 amino acid residues revealed 46 differences compared with *Lol p I* and *Hol l I*, of which 37 were similar substitutions (indicated in Fig. 5). The leader sequences of *Phl p I* and *Lol p I* were identical, whereas *Hol l I* exhibited two additional amino acids and three amino acid exchanges. The mature proteins of *Lol p I* and *Hol l I* consisted of 239 amino acid residues, whereas *Phl p I* had one fewer amino acid. Except for the highly conserved residue region 93 of the *N*-termini, amino acid variability appeared to be equally distributed along the rest of the protein. This variability is predicted to give rise to alterations in the secondary structures of the group I allergens.

DISCUSSION

Group I grass allergens are of considerable importance, because they have a high IgE prevalence² and are present in a number of grasses.³⁰ Timothy grass and ryegrass are important allergenic species and are widespread throughout northern and central Europe. According to Marsh et al.,¹⁴ these allergens show an incomplete anti-

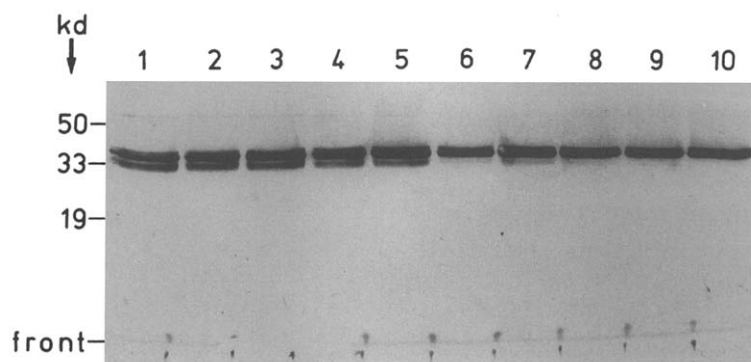


FIG. 4. Western blotting of timothy grass pollen to study influence of reduction on *Phl p I* structure. Pollen extract was treated with sample buffers differing in DTT concentrations: 1, no DTT; 2, 0.08 mmol/L; 3, 0.11 mmol/L; 4, 0.20 mmol/L; 5, 0.27 mmol/L; 6, 0.4 mmol/L; 7, 0.8 mmol/L; 8, 1.6 mmol/L; 9, 20 mmol/L; and 10, 40 mmol/L DTT (normally used).

PHL_P_1	IPKVPPGPNITATYGDKWLDKSTWYGKPTAAGPKDNGGACGYKDVKPP	50
HOL_L_1	IAKVPPGPNITATYGDWLDKSTWYGKPTGAGPKDNGGACGYKDVKPP	50
LOL_P_1	IAKVPPGPNITAEYGDKWLDKSTWYGKPTGAGPKDNGGACGYKNVDKAP	50
	* ***** **	
PHL_P_1	FSGMTGCGNTPIFKSGRGCSCFEIKCTKPEACSGEPVVVHITDDNEEPI	100
HOL_L_1	FSGMTGCGNTPIFKDGRGCSCFEIKCTKPEACSGEPVTVHITDDNEEPI	100
LOL_P_1	FNGMTGCGNTPIFKDGRGCSCFEIKCTKPEACSGEAVTVITDDNEEPI	100
	* ***** s***** *	
PHL_P_1	AAYHFDLSGIAFGSMAKKGDEQKLRSAGEVEIQFRRVKCKYPEGTKVTFH	150
HOL_L_1	APYHFDLSGHAFSGMAKKGEEQKLRSAGELELKFRRVKCKYPDGTKPTFH	150
LOL_P_1	APYHFDLSGHAFSGMAKKGEEQNVRSALELQFRRVKCKYPDGTKPTFH	150
	* ***** s** s***** s** s**	
PHL_P_1	VEKGSNPNYLALLVKF-SGDGDVVAVDIKEKGKDKWIALKESWGAIWRID	199
HOL_L_1	VEKGSNPNYLALLVKYIDGDGDVVAVDIKEKGKDKWIELKESWGAVWRVD	200
LOL_P_1	VEKASNPNYLAILVKYVDGDGDVVAVDIKEKGKDKWIELKESWGAVWRID	200
	*** ***** s*** s***** ***** s** s*	
PHL_P_1	TPEVLKGPFTVRYTTEGGTKARAKDVIPEGWKADTAYESK	239
HOL_L_1	TPDKLTGPFTVRYTTEGGTKGEAEDVIPEGWKADTAYEAK	240
LOL_P_1	TPDKLTGPFTVRYTTEGGTKSEFEDVIPEGWKADTSYSAK	240
	** s * ***** ***** s* s*	

FIG. 5. Comparison of deduced amino acid sequences of timothy grass *Phl p I* (clone PHLPI.p1), velvet grass *Hol I I* (clone Hol I I²⁹), and ryegrass *Lol p I* (clone Lol P1, precursor allergen R7^{27, 28}; positions of isoallergenic variants are underlined [position 45, N, D; position 154, A, G; position 187, I, T; position 223, F, V]). Characters below amino acid sequences indicate that position in alignment is perfectly conserved (asterisk), well conserved (s), or different (blank space). Amino acids said to be similar are A, S, and T; D and E; N and Q; R and K; I, L, M, and V; and F, Y, W.

genic identity with *Lol p I*. We determined the primary structure of *Phl p I* by cDNA sequencing and compared it with the well-defined *Lol p I*. B-cell and T-cell epitopes that have already been

determined for *Lol p I* were compared with the structures of *Phl p I* and *Hol I I* to identify common and private epitopes.

By immunoscreening a timothy grass pollen

cDNA library with the Mab IG 12 we obtained three full-length clones coding for *Phl p* I. The clones PHLPl.p4 and PHLPl.p9 are identical; they might have derived from a common precursor by amplification of the cDNA library. These two clones differ from PHLPl.p1 in both size and nucleotide sequence of the upstream noncoding region. From this we hypothesize that PHLPl.p1 and PHLPl.p4 might be derived from independent genes.

Comparing the grass group I allergens of timothy grass, ryegrass, and velvet grass, we demonstrated sequence identities of greater than 86% and homologies of greater than 90%. According to botanical taxonomy, the three grasses of the Festucoideae subfamily are classified into different tribes: perennial ryegrass, Festuceae; timothy grass, Agrostideae; and velvet grass, Aveneae.⁷ With regard to the antigenicity and concentration of grass group I components, *Lol p* I has more in common with *Hol l* I than with *Phl p* I.¹⁴

To focus our studies on allergenic relevant regions of the group I allergens, we compared the immunodominant B- and T-cell epitopes of *Lol p* I with the corresponding sequences of *Phl p* I and *Hol l* I. Esch and Klapper^{5,6} identified two B-cell epitopes on *Lol p* I spanning from position 23 to 35 and from 217 to 240. The first epitope, consisting of 13 amino acids, differs between the three group I allergens at only one residue. At this point *Lol l* I and *Phl p* I possess uncharged amino acids (glycine and alanine, respectively) and *Hol l* I has aspartic acid. When the C-terminal epitopes are compared, two segments, four amino acids in length (221 to 224 and 235 to 238 referring to *Lol p* I), were highly variable. In this region *Lol p* I differs by seven amino acid residues to *Phl p* I and by four residues to *Hol l* I. Similar variability was observed in the segments corresponding to the immunodominant T-cell epitope (position 191 to 210) of *Lol p* I: *Phl p* I differs by four amino acid residues, whereas *Hol l* I differs by one. Thus the three grass species possess nonidentical immunodominant regions of the group I allergens. These differences are not uniformly distributed along the epitope but exist as clusters. How these sequence differences affect protein folding ultimately affects the immunologic response. If the different structures are accessible to antibodies on the protein surface, then they are able to elicit different responses. If, however, the substitutions are hidden within the protein, they will have no effect.

cDNA data allow us to determine the variability of isoallergens resulting from nucleotide exchanges, but posttranslational modifications can be

determined only at the protein level. The amino acid sequence of *Phl p* I deduced from the cDNA data fits to the N-terminal protein sequences of the 37 kd isoallergens.³¹ By protein sequencing we obtained further information concerning post-translational modifications. Hydroxyproline residues were identified in positions 5 and 8. These hydroxylations were also determined for *Lol p* I, *Poa p* I, and *Sec c* I.¹³

In position 9 we determined an asparagine residue by cDNA sequencing, which corresponds to the sequence data of the *Lol p* I and *Hol l* I clones. By protein sequencing no amino acid was detectable in this position of *Phl p* I allergens, supporting the observation of Matthiesen et al.¹³ of group I allergens of several grass species. Perez et al.²⁷ assumed a glycosylation site attached to the asparagine residue in position 9 in *Lol p* I, which would explain the blank in the chromatogram and which agrees with the structural motif Asn-X-Thr for N-glycosylation.³²

Posttranslational modifications (e.g., hydroxylation and glycosylation) can increase the hydrophilicity of protein segments. According to Chou and Fasman,³³ the hydrophilicity corresponds to antigenicity. Until now no algorithms have been available to take posttranslational modifications into consideration. Using the algorithm of Jameson and Wolf,²⁶ we determined high antigenic indices (data not shown) along the whole *Phl p* I molecule except for the C-terminus of the allergen, which was identified to be IgE reactive by fragmentation experiments.^{5,6}

Johnson and Marsh⁴ reported that skin prick testing with *Lol p* I (revealing seven cysteine residues in corresponding amino acid positions) caused decreased reactions after treatment by reducing agents. To study intramolecular crosslinking via cysteine residues in *Phl p* I, the influence of a stepwise addition of DTT on the molecular size was determined. Increasing concentrations of DTT shifted a 32 kd antigen to sizes of 35 and 37 kd. This slight increase can be explained by intramolecular disruption of disulfide bonds. Because most of the cysteine residues are closely adjacent (positions 41, 57, 69, 72, 77, and 83), an unfolding would have minor effects on the increase in molecular size, as that which we observed. Therefore we assume that disulfide bonds exist between these cysteines. Additionally, a faint 55 kd protein band was detectable only under nonreducing conditions. This might be caused by crosslinking of two group I allergens. Thus the disulfide bridges are important for the allergenicity of *Phl p* I.

With regard to hyposensitization therapy, it is still uncertain whether crude extracts with a broad spectrum of allergenic sources (e.g., different grass pollen species) or with a mixture consisting of a few well-defined allergenic components have to be used. The former has the disadvantage of sensitizing patients against unrelated components, whereas the latter is potentially missing components essential for therapy. Both the study of the isoallergenic variations and the identification of IgE-reactive binding sites are necessary to answer these questions.

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