

Major allergen *Phl p* Va (timothy grass) bears at least two different IgE-reactive epitopes

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Abstract

It is established that most grass pollen allergens consist of several isoforms of which the function is mainly still unknown. A number of these allergens belonging to group V have been cloned, sequenced, and expressed. Antigenic sites and IgE-reactive epitopes of the major allergen *Phl p* Va, are unknown. We have identified the complete cDNA sequence of a *Phl p* Va isoallergen by immunoscreening of a timothy grass pollen cDNA library and mixed oligonucleotide primed amplification of N-terminal cDNA. Additionally, we found an incomplete isoallergenic cDNA clone of the same protein. Immunoreactivity of the fusion proteins with patients' sera and monoclonal antibodies showed that the clones represent group Va allergens. Comparison of deduced amino acid sequences with published sequences of *Lol p* V and *Poa p* IX revealed a homology of 81.1% and 86.9%, respectively. With affinity-purified IgE antibodies recognizing the recombinant fusion protein, we can demonstrate the existence of a common group V IgE-reactive epitope. By construction of both an N-terminal and a C-terminal peptide of the complete *Phl p* Va and cross-inhibition, we identified at least two different IgE epitopes. Eleven patients showed variable IgE immunoreactivities to both IgE-reactive epitopes. (J ALLERGY CLIN IMMUNOL 1994;94:173-81.)

IPTG	Isopropyl- β -D-thiogalactopyranoside
mAb	Monoclonal antibody
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Key words: [Timothy grass pollen](#), [major allergen](#), [group V allergens](#), [isoallergens](#), [Phl p Va](#), [cDNA cloning](#), [polymerase chain reaction](#), [fusion protein](#), [epitopes](#)

Since the dawn of recombinant cDNA technology in allergen characterization, a number of major allergens have been cloned, sequenced, and expressed.¹ Among grass pollen allergens, the most extensive data concerning deduced primary structures were revealed on *Lolium perenne*,²⁻⁵ *Poa pratense*,⁶⁻⁷ and *Phleum pratense*.⁸ Other grass pollen allergens have been cloned,⁸⁻⁹ but nucleotide sequences have not yet been published.

Protein chemistry of allergen extracts has shown that group-specific allergens consist of a variety of isoforms,^{10, 11} which can be deduced from isogenes as shown in tree pollen allergens.¹² These isoforms express different immunogenic structures, which may lead to a substantial variability in IgE-dependent immunoreactivities of allergic patients.¹³

This heterogeneity in specific IgE reactivity seems to be a common feature of individuals with grass pollen allergy,¹⁴ and has a substantial influence on the response of patients to specific immunotherapy.¹⁵ Whether one can find individual differences in IgE response to single epitopes on one allergenic molecule remains to be determined.

In this article we focus our interest on the identification of the primary structure of one major group V timothy grass pollen isoallergen. Furthermore, we present data showing that this isoallergen expresses a common IgE epitope of group V allergens in grasses and evidence that it bears at least two different IgE-reactive epitopes, which are not generally recognized by the sera of all allergic patients.

Methods

Extraction of allergens

Allergens were extracted from 1 gm of timothy grass pollen (*Phleum pratense*, batch No.: 24W28-7c; ARTU Biologicals N.V., Lelystad, Holland) in 55 ml of 0.1 mol/L HN_4HCO_3 buffer (pH 8) for 30 minutes at room temperature. After centrifugation for 30 minutes at 17000g (4° C), the supernatant was dialyzed against double-distilled water, lyophilized, and dissolved in appropriate sample buffer for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE, Western blot, and immunologic detection

SDS-PAGE was done according to the method of Laemmli,¹⁶ generally followed by electroblotting onto nitrocellulose membrane¹⁷ on a Fastblot (Biometra, Göttingen, Germany). For protein staining and immunologic detection, we used India ink and antibodies in a procedure as previously described.¹¹ For identification and estimation of the degree of IgE immunoreactivities in Western blots, intensity of allergen pattern was classified from 0 to 4.¹⁴

Isolation of poly-A RNA

We extracted RNA from 10 gm of timothy grass pollen with a slight modification of the method of Walsh et al.⁸ Pollen was ground in liquid nitrogen and broken with pestle and mortar, suspended in 50 ml of extraction buffer (400 mmol/L NaCl; 50 mmol/L Tris/HCl, pH 9; 5 mmol/L pH 8; 1% SDS; 10 mmol/L DTT, 10 mmol/L heparin; 1 mmol/L aurintricarboxylic acid) and 25 ml phenol/chloroform/isoamyl alcohol (24 : 24 : 1) followed by homogenization with an Ultra-Turrax (Janke & Kunkel, Ika-Werk, Hamburg, Germany) and centrifugation. The supernatant was extracted twice with phenol/chloroform/isoamyl alcohol, and twice with chloroform : isoamyl alcohol (24 : 1). RNA was precipitated with 2 mmol/L LiCl (final concentration). Poly-A RNA was selected on Oligo-dT cellulose (Sigma Chemical Co., St. Louis, MO.) as described by Aviv and Leder.¹⁸

Construction of cDNA library

Construction of a cDNA library was carried out according to the method of Gubler and Hoffmann.¹⁹ For generating double-stranded cDNA, 10 µg of timothy grass pollen mRNA was used with the cDNA synthesis kit (Pharmacia, Uppsala, Sweden). Complementary DNA 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* K 12XL1-Blue (Stratagene) were transduced with in vitro packaged phages, which resulted in a plaque forming unit per milliliter of 3.2×10^7 in the primary library containing 90% recombinant clones.

Monoclonal antibody, immunoscreening, and sequencing

For immunoscreening of the cDNA library we used the monoclonal antibody (mAb) Bo1,²⁰ which recognized *Phl p* Va and *Phl p* Vb from crude pollen extract. Monoclonal antibodies BG6 and Bo9²¹ identify only *Phl p* Va and *Phl p* Vb, respectively. Both are markers for internal group V allergen differentiation in timothy grass pollen.

Positive clones were plaque purified by several rounds of immunoscreening. pBluescript plasmid was excised in vivo from isolated phages by coinfection with helper phage R408 (Stratagene) and plated, and plasmids were amplified from single colonies, followed by plasmid preparation (Plasmid purification kit; Qiagen, Chatsworth, Calif.). Insert analysis was carried out by *EcoR* I and *Not* I digestion and electrophoretic separation on 1% agarose gel. Double-stranded plasmid DNA was sequenced in both directions according to the dideoxy method of Sanger et al.²² with a T7Sequencing Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) either by “walking” the cDNA sequence with sequence-specific oligonucleotide primers or on restriction fragments subcloned into pBluescript plasmid.

Mixed oligonucleotide-primed amplification of N-terminal cDNA

One microgram of mRNA was reverse-transcribed to cDNA with Superscript reverse transcriptase (Gibco-BRL, Gaithersburg, Md.). Ten nanograms of cDNA was subjected to mixed oligonucleotide primed amplification of N-terminal cDNA, as described by Lee et al.²³ with degenerate oligonucleotide primers synthesized on a Cyclone Plus DNA Synthesizer (Millipore Corp., Bedford, Mass.) by the phosphoramidite method. The sequences of the primers were deduced from both ends of the N-terminal amino acid sequence of *Phl p* Va isoallergen:¹¹

5' end: ATATGGATCCGCGNGAYYTNGGNTAYGGNNCCNGC

3' end: ATATAAGCTTGCNGCNGGNGTNGCNGGNGTTRTA

Polymerase chain reaction (PCR) was performed with a Perkin-Elmer Cetus Thermal Cycler (Perkin-Elmer Corp., Norwalk, Conn.) at 94° C for 2 minutes, 55° C for 2 minutes, and 72° C for 3 minutes for 30 cycles with Taq DNA polymerase (AmpliTaq, Perkin-Elmer Corp.) or Taq polymerase (Pharmacia, Uppsala, Sweden). PCR products were excised from nondenaturing polyacrylamide gel, electroeluted, cloned into M13 mp18 and mp19 phagemid vectors, and sequenced.

The nucleotide sequence of this 5' end anchor was then used for construction of a new 5' end primer: ATATTCTAGAGCCACCCCAGCTGCCCG. For cloning of the full-length coding sequence of a *Phl p* Va isoallergen, this 5' end primer was used in an anchor PCR together with a 3' end primer: ATATAAGCTTCGACGTAGGCGTCGTACTT, known from the incomplete cDNA sequence of clone PHLP5A1913, which was isolated by immunoscreening as described above.

FIG. 1. Nucleic acid and deduced amino acid sequence of *Phl p* Va isoallergen. The N-terminus, which was cloned by anchored PCR, is underlined. The dashed line indicates the known internal amino acid sequence determined by Edman degradation.¹¹ The *Xho* I restriction site used for subcloning and a probable polyadenylation site are shown.

1	GCC	GAT	CTA	GGC	TAC	GGC	CCG	GCC	ACC	CCA	GCT	GCC	CCG	GCC	GCC	GGC
1	A	D	L	G	Y	G	P	A	T	P	A	A	P	A	A	G
49	TAC	ACC	CCC	GCC	ACC	CCC	GCC	GCC	CCG	GCC	GGA	GCG	GAC	GCA	GCA	GGT
17	Y	T	P	A	T	P	A	A	P	A	G	A	D	A	A	G
97	AAG	GCG	ACG	ACC	GAG	GAG	CAG	AAG	CTG	ATC	GAG	AAG	ATC	AAC	GCC	GGC
33	K	A	T	T	E	E	Q	K	L	I	E	K	I	N	A	G
145	TTC	AAG	GCG	GCC	CTG	GCG	CGC	CGG	CTC	CAG	CCA	GCG	GAC	AAG	TAC	AGG
49	F	K	A	A	L	A	R	R	L	Q	P	A	D	K	Y	R
193	ACG	TTC	GTC	GCA	ACC	TTC	GGC	CCG	GCC	TCC	AAC	AAG	GCC	TTC	GCG	GAG
65	T	F	V	A	T	F	G	P	A	S	N	K	A	F	A	E
241	GGC	CTC	TCG	GGC	GAG	CCC	AAG	GGC	GCC	GCC	GAA	TCC	AGC	TCC	AAG	GCC
81	G	L	S	G	E	P	K	G	A	A	E	S	S	S	K	A
289	GCG	CTC	ACC	TCC	AAG	CTC	GAC	GCC	GCC	TAC	AAG	CTC	GCC	TAC	AAG	ACA
97	A	L	T	S	K	L	D	A	A	Y	K	L	A	Y	K	T
337	GCC	GAG	GGC	GCG	ACG	CCT	GAG	GCC	AAG	TAC	GAC	GCC	TAC	GTC	GCC	ACC
113	A	E	G	A	T	P	E	A	K	Y	D	A	Y	V	A	T
385	CTA	AGC	GAG	GCG	CTC	CGC	ATC	ATC	GCC	GGC	ACC	CTC	GAG	GTC	CAC	GCC
129	L	S	E	A	L	R	I	I	A	G	T	L	E	V	H	A
433	GTC	AAG	CCC	GCG	GCC	GAG	GAG	GTC	AAG	GTC	ATC	CCC	GCC	GCC	GAG	CTG
145	V	K	P	A	A	E	E	V	K	V	I	P	A	A	E	L
481	CAG	GTC	ATC	GAG	AAG	GTC	GAC	GCC	GCC	TTC	AAG	GTC	GCT	GCC	ACC	GCC
161	Q	V	I	E	K	V	D	A	A	F	K	V	A	A	T	A
529	GCC	AAC	GCC	GCC	CCC	GCC	AAC	GAC	AAG	TTC	ACC	GTC	TTC	GAG	GCC	GCC
177	A	N	A	A	P	A	N	D	K	F	T	V	F	E	A	A
577	TTC	AAC	GAC	GAA	ATC	AAG	GCG	AGC	ACG	GGC	GGC	GCC	TAC	GAG	AGC	TAC
193	F	N	D	E	I	K	A	S	T	G	G	A	Y	E	S	Y
625	AAG	TTC	ATC	CCC	GCC	CTG	GAG	GCC	GCC	GTC	AAG	CAG	GCC	TAC	GCC	GCC
209	K	F	I	P	A	L	E	A	A	V	K	Q	A	Y	A	A
673	ACC	GTC	GCC	ACC	GCG	CCG	GAG	GTC	AAG	TAC	ACT	GTC	TTT	GAG	ACC	GCA
225	T	V	A	T	A	P	E	V	K	Y	T	V	F	E	T	A
721	CTG	AAA	AAG	GCC	ATC	ACC	GCC	ATG	TCC	GAG	GCA	CAG	AAG	GCT	GCC	AAG
241	L	K	K	A	I	T	A	M	S	E	A	Q	K	A	A	K
769	CCG	CCG	CCG	CTG	CCA	CCG	CCA	CCG	CAA	CCG	CCG	CCG	TTG	GCC	GCC	ACC
257	P	P	P	L	P	P	P	P	Q	P	P	P	L	A	A	T
817	GGC	GCC	GCC	ACC	GCC	GCT	ACT	GGT	GGC	TAC	AAA	GTC	TGA	TCA	ACT	CGC
273	G	A	A	T	A	A	T	G	G	Y	K	V	*			
865	GAA	TAG	TAT	ACA	TCA	ATC	ACG	CAC	ATA	TAC	GAC	CGT	ACG	TAT	GTG	TGC
913	ATG	CAT	TGC	CGT	CGC	GTC	GAG	CAA	TTT	TGC	TGA	TAA	TTA	ATT	CTT	GAG
961	TTT	TCG	TTG	CAT	GCA	TCC	GCG	CGT	TAT	AGA	GCG	CAT	GCA	CGC	ATT	GCC
1009	ATT	TGT	GTA	TAA	TAA	TC										

Expression of fusion proteins

Expression of fusion proteins was induced with 2 mmol/L isopropyl-B-D-thiogalactopyranoside (IPTG) in 10 ml cultures of *E. coli* K 12 XL1-Blue containing the recombinant pBluescript plasmids. After 0 and 5 hours of induction, lysates were analyzed on Western blot with patients' sera and mAbs.

N-terminal and C-terminal recombinant peptides and PCR peptide

The C-terminal clone pPHLP5A1311 was detected by immunoscreening of the library as incomplete clone in correct reading frame, isolated, and sequenced. By restriction of a 5' end fragment of pPHLP5A1913 at a *Xho* I (CTCGAG) restriction site (at 418 bp in ■Fig. 1) and subcloning, we constructed an N-terminal peptide of 120 amino acids, which overlaps the C-terminal pPHLP5A1311 by 24 amino acids. A recombinant peptide (PCR peptide) of 59 amino acids was constructed from base pair 292 to 470 by PCR. Amplification was performed at 94° C for 30 seconds, 54° C for 30 seconds, and 72° C for 1.5 minutes for 30 cycles with pPHLP5A1913 as template. The sequences of the primers are:

5' end: ATATGGATCCGAGGCGCTCCGCATCAT

3' end: ATATAAGCTTAGACGGTGAACCTTGTCGTTG

The PCR product was purified from 1.5% agarose gel, restricted with *Bam*H I and *Hind* III and ligated into the expression vector pQE9 (Qiagen, Chatsworth, Calif.). The correct reading frame was controlled by sequencing, and the recombinant protein was purified on a Ni²⁺-nitrilotriacetic acid-resin column according to the instructions of the manufacturer (Qiagen).

Purification of IgE antibodies

The method of IgE antibody purification was adopted and modified from a protocol that Donovan and Baldo²⁴ used for analyzing cross-reactivities of IgE antibodies between rye grass pollen and wheat endosperm proteins. We performed affinity purification of epitope binding IgE antibodies in two ways.

1. Recombinant phages were plated out at high plaque density on 55 mm plate and overlaid with IPTG-soaked nitrocellulose filters. Filters were then washed, blocked with Tris-buffered saline (pH 7.4) containing 10% fetal calf serum, and incubated with a well characterized patient's serum. After intensive washing, the IgE antibodies were eluted with a glycine elution buffer (10 mmol/L glycine; 100 mmol/L NaCl; 10% fetal calf serum, pH 2.5) and neutralized with 1 mol/L Tris/HCl, pH 8. The affinity-purified antibodies were used to probe Western blot strips of whole native allergen extract or lysate of fusion proteins.
2. Allergen bands of native pollen extract or fusion proteins of lysates were cut out from Western blots. These strips were used as described above for affinity purification of IgE antibodies.

Control subjects' and patients' sera

Control serum samples were acquired from six different healthy persons who had no clinical history of allergy, IgE antibodies in normal range, and negative RAST results. We collected patients' sera in the Outpatients Allergy Department at the Medical Clinic of Borstel and the Pediatric Allergy Department of AK Heidelberg in Hamburg, Germany. Atopic phenotype was confirmed by clinical history and diagnosis, based on a highly positive skin prick test response to grass pollen allergens and specific IgE antibodies greater than 17.5 kU/L as measured in the CAP System (Pharmacia, Sweden).

Computer analysis

Sequence data were analyzed on GeneWorks and PCGENE Software (Intelligenetics, Geel, Belgium) and on DNASIS- and PROSIS-Software (Pharmacia LKB Biotechnology, Sweden). The correlation coefficient was calculated on KWIKSTAT (Alan C. Elliott, New York, N.Y.).

Nucleotide sequence accession number

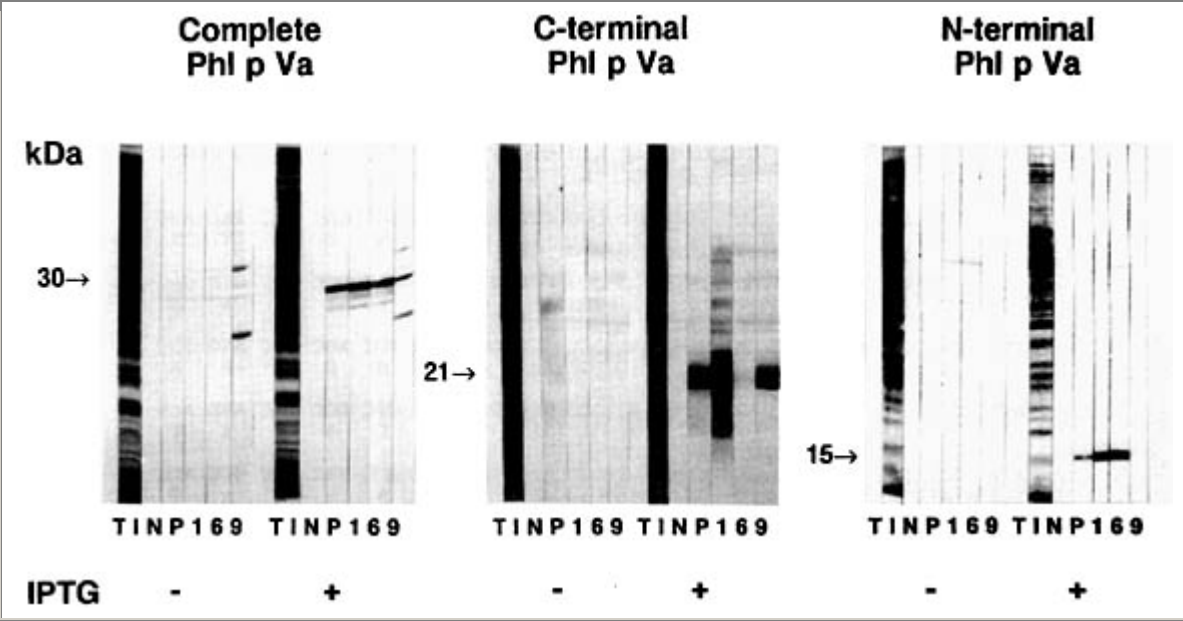
The nucleotide sequence of pPHLP5A1913 has been submitted to the EMBL Nucleotide Sequence Data Library and has been assigned accession number X70942.

Results

cDNA sequences of *Phl p* Va isoallergens

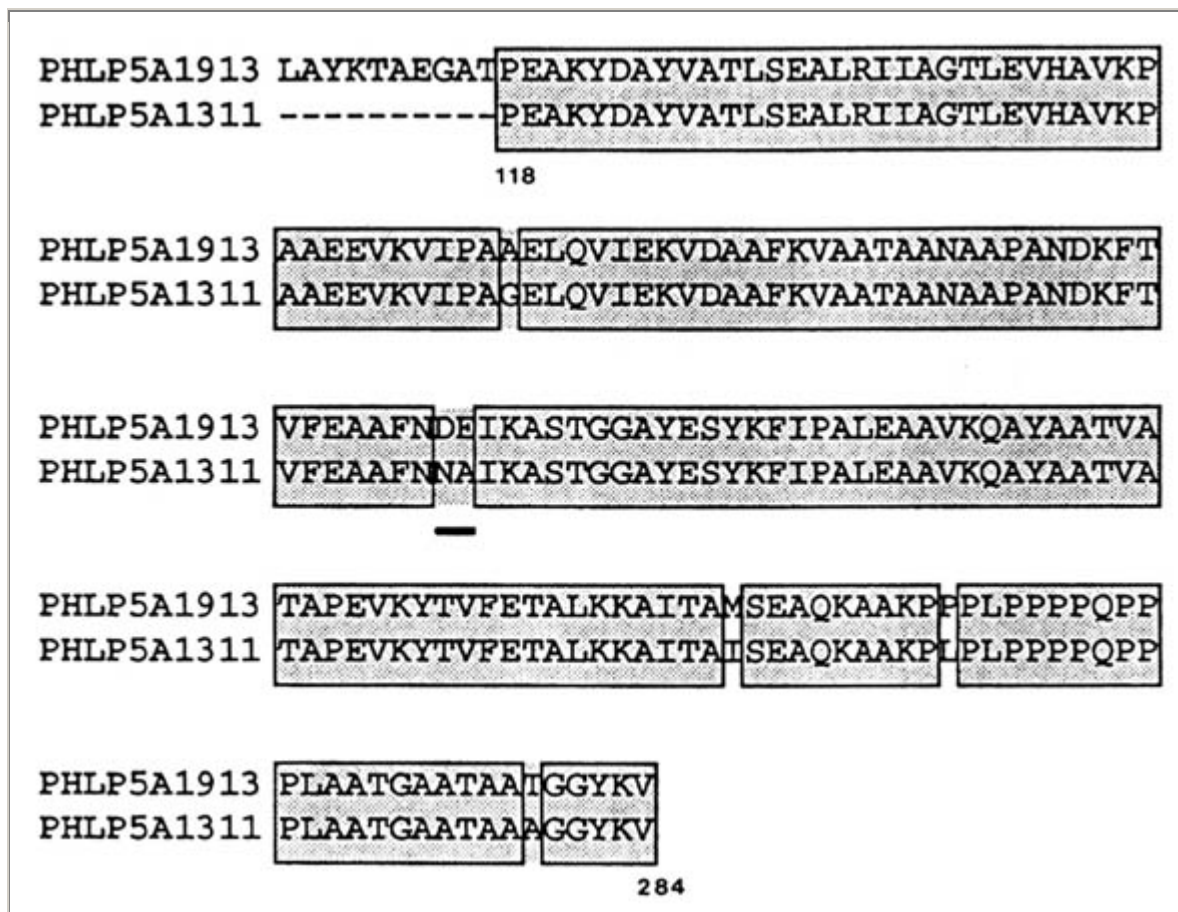
Immunoscreening of the timothy grass pollen cDNA library with mAb Bo1 identified several clones, two of which code for different isoallergens of *Phl p* Va. ■Fig. 2 shows the complete sequence of clone PHLP5A1913 encoding 91% of the complete protein. The sequence encoding the remaining 30 amino acids was cloned by anchored PCR (underlined). The complete nucleotide sequence is made up of a 855 bp open reading frame and a TGA stop codon. The possible polyadenylation site is indicated. The clone was identified by its immunoreactivity with mAb (■Fig. 2) which is known to be specific for *Phl p* Va¹¹ and additionally by an internal amino acid sequence, which was sequenced previously by Edman degradation²¹ of a native *Phl p* Va fragment.

FIG. 2. Western blot of non-IPTG- and IPTG-induced fusion proteins of complete *Phl p* Va, N-terminal and C-terminal recombinant peptides. *T*, Tris buffer saline control of second antibody; *I*, India ink; *N*, healthy control serum; *P*, patient's serum; 1, mAb Bo1; 6, mAb BG6; 9, mAb Bo9.



The comparison of the deduced amino acid sequences of pPHLP5A1311 and pPHLP5A1913 in ■Fig. 3 indicates that pPHLP5A1311 as incomplete C-terminal clone covers 59% of a complete *Phl p* Va isoallergen. Six amino acid differences correspond to seven nucleotide differences, whereas the underlined differences of the two amino acids (aspartic acid vs asparagine and glutamic acid vs alanine) in pPHLP5A1913 and pPHLP5A1311 change negatively charged residues against hydrophobic residues. This can influence the charge of the complete proteins as shown by the calculated isoelectric points of the 166 C-terminal–deduced amino acids of both isoallergens, which differ in their isoelectric points by 2.22 pH units (data not shown).

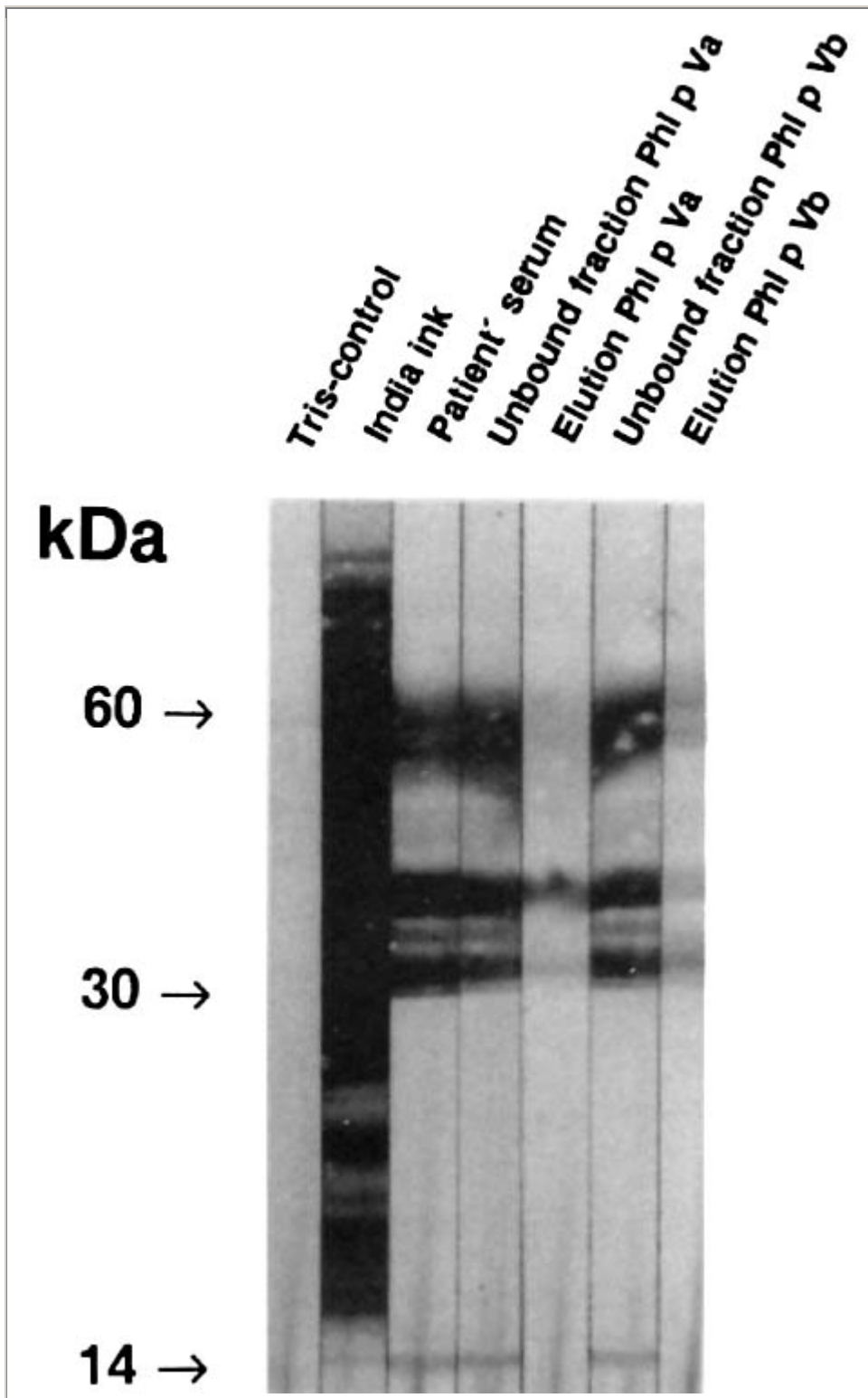
FIG. 3. Comparison of the deduced amino acid sequences of the isoallergenic clones pPHLP5A1913 and pPHLP5A1311 in the C-terminal part. Blocks show the consensus sequence, and shading indicates the homologous amino acid residues.



Identification of group V epitopes

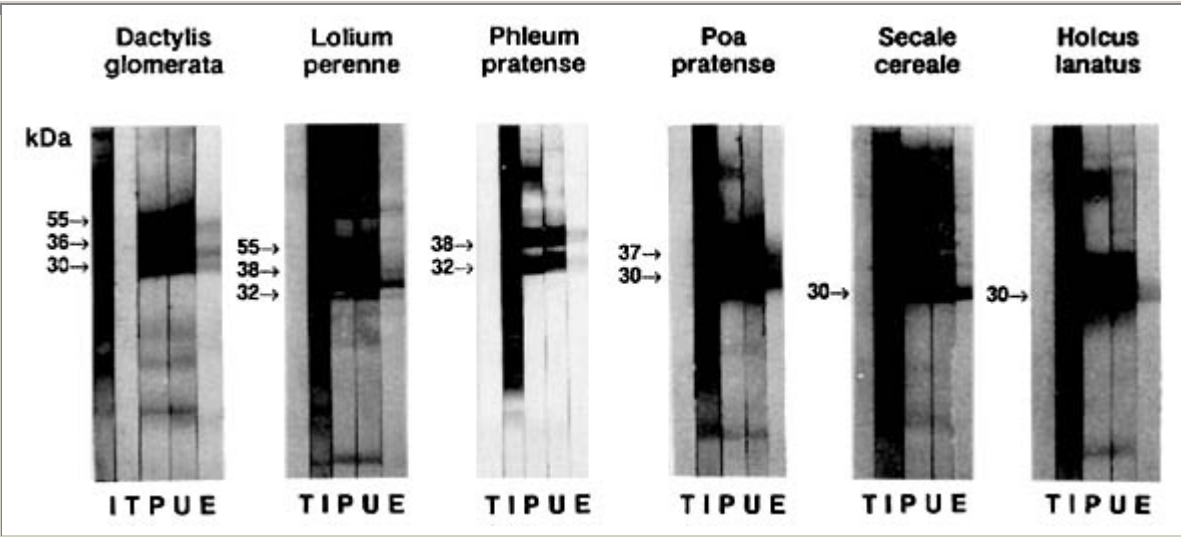
The Western blot of native timothy grass pollen extract in Fig. 4 shows the reversed immunoreactivities of affinity-purified IgE antibodies eluted from native *Phl p* Va and *Phl p* Vb. *Phl p* Va-eluted IgE antibodies recognize *Phl p* Va, and less intensely *Phl p* Vb. *Phl p* Vb IgE antibodies bind to *Phl p* Vb, *Phl p* Va, and additionally to two proteins of about 60 kd. Thus both isoallergenic complexes at 32 and 38 kd, which belong to group V allergens, seem to bear both identical and nonidentical epitopes because the 32 kd epitopes are present also on proteins at 60 kd. *Phl p* Va is highly homologous to major allergens *Lol p* V²⁵ (81.1%), *Poa p* IX⁶ (89.6%), and *Hol I* V²⁶ (85.2%), but there is no homology to any protein with known function (data not shown).

FIG. 4. Western blot of timothy grass pollen extract showing the results of reverse epitope selection by affinity-purified specific IgE antibodies recognizing native *Phl p* Va and *Phl p* Vb. IgE antibodies were detected by an alkaline phosphatase-labeled monoclonal anti-IgE antibody.



Recombinant *Phl p Va* affinity-purified IgE antibodies bind to all group V allergens on six different grasses (■ Fig. 5). According to the grass pollen species, the eluted IgE antibodies in strip E show different binding patterns of group V allergens, all sharing IgE-reactive epitopes with the recombinant fusion protein pPHLP5A1913.

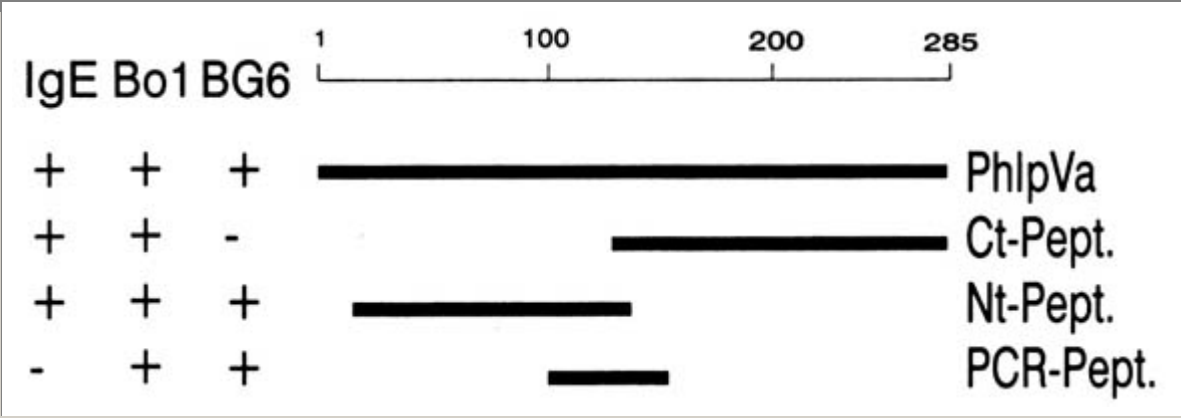
FIG. 5. Immunoreactivities of affinity-purified IgE antibodies, which have been eluted from recombinant *Phl p Va* (pPHLP5A1913) isoallergen, now binding to pollen extracts of six different grasses. *I*, India ink; *T*, Tris buffer saline control of second antibody; *P*, patient's serum; *U*, unbound fraction of antibodies; *E*, elution of recombinant *Phl p Va*-specific IgE antibodies.



Identification of different IgE epitopes on pPHLP5A1913

For further epitope characterization of the complete *Phl p* Va allergen, we compared pPHLP5AXHOI prepared by subcloning and expressing pPHLP5A1913 as *Xho* I restriction fragment representing the N-terminal peptide with the expressed pPHLP5A1311 as the C-terminal peptide. Both fragments overlap by 24 amino acids. When the fusion proteins are compared by their immunoreactivities on Western blots (■Fig. 2), it can be seen that both are recognized by IgE from patient's serum and by mAb Bo1. This means that pPHLP5A1913 bears at least two IgE-reactive epitopes. Monoclonal antibody BG6 binds only to the N-terminal, and mAb Bo9 binds only to the C-terminal peptide. To exclude the presence of a common IgE-reactive epitope in the overlapping region of the two recombinant peptides, it is shown in ■Fig. 6 that a PCR peptide representing the overlapping region is not recognized by IgE antibodies. According to the immunoreactivities of the PCR peptide, mAb Bo1 may bind to an epitope in the center of the allergen.

FIG. 6. Immunoreactivities of the complete *Phl p* Va (*PhlpV*), the C-terminal (*Ct-Pept.*), the N-terminal peptide (*Nt-Pept.*), and a PCR peptide (*PCR-Pept.*) to patients' IgE mAb Bo1 and mAb BG6 shown in accordance with the length of amino acid sequences of peptides. Plus sign indicates positive reactivity in immunoblot; minus sign indicates no immunoreactivity



To determine whether the IgE-reactive epitopes on the C-terminal and N-terminal peptides are different, we carried out a cross-inhibition test in which serum IgE antibodies were preincubated with different concentrations of the C-terminal and N-terminal peptides and then used as a probe on Western blots of the different fragments. The results are shown in ■Fig. 7. IgE antibodies directed against the C-terminus are inhibited completely by the C-terminal but not by the N-terminal peptide and vice versa.

FIG. 7. Cross-inhibition test with patient's serum and recombinant peptides of *Phl p* Va. Serum (DS) was preincubated in an inhibition mix with complete pPHLP5A1913 lysate, the C-terminal

(Ct), and the N-terminal peptide lysate (Nt) in different dilutions of lysates. SK⁺ is lysate from *E. coli* K 12 containing nonrecombinant plasmid. The inhibition mix was then used as probe on Western blots with SK⁺ lysate, complete pPHLP5A1913 (Com), C-terminal and N-terminal peptide. Numbers from 0 to 4 indicate band intensity. As a result, those bands with greatest inhibition are presented. BSA, Bovine serum albumin; Tris, Tris/HCl buffer.

INHIBITION MIX						
B L O T S		PS & BSA	PS & Tris	PS & SK ⁺	PS & Ct	PS & Nt
	SK ⁺	0	0	0	0	0
	Com	4	4	4	1	2
	Ct	4	4	4	0	4
	Nt	4	4	4	4	0

IgE immunoreactivities of patients' sera to native allergens of timothy grass pollen and recombinant peptides of *Phl p Va*

In Fig. 8 the intensities of IgE immunoreactivities of 11 patients with atopic phenotype defined by elevated specific serum IgE antibodies (CAP System > 17.5 kU/ml) and positive skin prick test responses to grass pollen allergens are shown. The healthy control subjects show no IgE reactivity at all (data not shown). All patients react to the C-terminal, and 73% react to the N-terminal peptide of *Phl p Va*. The intensity of IgE binding to both peptides on Western blots correlates positively with the specific grass pollen IgE level measured by Cap test (correlation coefficient: N-terminus = 0.79, $p = 0.003$; C-terminus = 0.50, $p = 0.115$). The binding patterns of patients' IgE to the different groups of allergens and to the two *Phl p Va* peptides varies individually. The serum IgE of one patient (No. 9) does not recognize the N-terminal peptide but shows intense binding to the C-terminus. The binding patterns of the IgE antibodies of a patient only allergic to grass pollen are most equally distributed to the different epitopes.

FIG. 8. Variability of IgE immunoreactivities in Western blots of native timothy grass pollen extract and recombinant C-terminal and N-terminal peptides. *Pat.*, Patient number; *g6-IgE*, results of specific timothy grass pollen Cap test in kilounits per liter; *SPT*, results of skin prick test with timothy grass pollen extract; *Sens.*, sensitization patterns measured by RAST and skin prick test; *poly*, polysensitized patients; *grass*, only grass pollen-sensitized patients; *IV*, native *Phl p IV*; *Va*, native *Phl p Va*; *I*, native *Phl p I*; *Vb*, native *Phl p Vb*; *N-term.*, recombinant N-terminal peptide; *C-term*, recombinant C-terminal peptide.

Pat.	g6-IgE kU/l	SPT	Sens.	IV	Va	I	Vb	N-term.	C-term.
1	247	+++	poly	1	1	1	1	1	1
2	213	++++	poly	2	2	0	1	2	3
3	202	++++	poly	1	1	2	2	2	2
4	39	++++	poly	0.5	0.5	0.5	0.5	0	0.5
5	94	++	poly	0	0	1	1	0	0.5
6	75	++++	poly	1	0.5	0	0.5	1	3
7	558	++++	grass	3	3	3	3	3	4
8	318	++++	poly	0.5	0.5	2	2	1	1
9	163	+++	poly	2	2	0.5	0.5	0	3
10	81	?	poly	0.5	0.5	1	1	0	1
11	75	+++	poly	1	1	1	1	0.5	2

Discussion

In this report we present the cDNA sequence and fusion protein expression of a specific *Phl p Va* isoallergenic clone, which has been identified by its immunoreactivity to well characterized mAbs, by specific IgE from patients' sera, and by sequence comparison to known external and internal N-terminal amino acid sequences.²¹ pPHLP5A1913 represents only 91% of the native protein, but its fusion protein shows immunologic reactivity similar to that of the native allergen. This indicates that the IgE-reactive epitopes are present on the recombinant protein. By mixed oligonucleotide primed amplification²³ we were able to determine the cDNA sequence of the 5' end of *Phl p Va*. We used this anchor as a primer for the isolation of the complete cDNA sequence of the already identified isoallergen. The application of these combined cloning strategies is now widely accepted^{1, 27-29} and was necessary because the mAb Bo1, which was used for immunoscreening of the cDNA library, mainly detected a more C-terminal epitope and therefore preferentially bound incomplete fusion proteins.

Sequence homologies of *Phl p Va* to *Lol p V*, *Poa p IX*, and *Hol I V* clearly indicate that these recombinant allergens seem to belong to one group of allergens, as defined by Marsh et al.³⁰ We demonstrated by specific epitope-selected IgE antibodies that these grass pollen allergens express at least one common epitope. This structural similarity, identified by common IgE-reactive epitopes, may be a marker for their group V characteristics. The great degree of homology indicates similar biologic function of allergenic proteins,⁵ and we assume that like certain major tree pollen allergens, these grass pollen allergens belong to a gene family.³¹ Isoallergens are defined as proteins with identical molecular weight but different isoelectric points.^{10, 32, 33} Several explanations have been suggested for this phenomenon, namely the difference in carbohydrate structures attached to hydroxyproline residues³⁴ and posttranslational modifications of the protein core.³⁵ We can show by sequence comparison of two isoallergenic clones that the different isoelectric points may be induced by nonhomologous amino acid exchanges. This explains the different behavior of isoallergens in electrophoresis and once again stresses the fact that isoallergens constitute a gene family or even a gene cluster in the plant.³⁶

Cross-reactivities between related grass pollen allergens are defined by a high degree of epitope similarity and have been determined by specific mAb³⁷ and polyclonal antisera.³⁸ These common epitopes are present on native and recombinant *Phl p Va*, as data in this article clearly demonstrate. Which of the two newly identified different IgE epitopes is of this common character remains to be determined. Patients' sera recognize IgE epitopes on both the C-terminal and N-terminal recombinant peptide of *Phl p Va*. In a cross-inhibition test with patients' sera we have shown that the C-terminal and N-terminal peptides are not inhibited by each other. Similar results were found by Zhang et al.³⁹ who showed that a recombinant fusion protein of *Poa p IX* rKGB 60 and two overlapping C-terminal and N-terminal peptides of these clones, which are all highly homologous to *Phl p Va*, are recognized by human IgE to different degrees. In contrast to Zhang et al.,³⁹ who used pooled serum, we tested IgE reactivities of 11 allergic patients with N- and C-terminal

peptides of *Phl p* Va. We found that the immunoreactivities are individually different, and one patient even expressed no IgE-reactive binding to the N-terminus of the allergen.

Like other authors,^{14, 15, 40-42} we have found heterogeneity in specific IgE immunoreactivity to major timothy grass pollen allergens. This observation can be extended to the level of one molecule because our data show that recognition of different epitopes on one recombinant isoallergen can be individually different. Thus it is clinically important to define the different relevant IgE-reactive epitopes on grass pollen allergens and to determine the individual reactivity to these structures. This will elucidate the biologic significance and the therapeutic relevance of the heterogeneity of allergens in the future.

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