

Molecular and immunological characterization of group V allergen isoforms from velvet grass pollen (*Holcus lanatus*)

Gabriele SCHRAMM, Albrecht BUFE, Arnd PETERSEN, Max SCHLAAK and Wolf-Meinhard BECKER

Forschungszentrum Borstel, Biochemische und molekulare Allergologie, Borstel, Germany

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Group V grass pollen allergens belong to the major grass pollen allergens causing reactions of type I allergy. cDNAs coding for two isoforms of the group V allergen of velvet grass pollen (*Holcus lanatus*), a widespread grass species, were isolated from a cDNA library by hybridization with a 5'-terminal reverse-transcribed PCR-derived cDNA probe. Amino acid sequences of the two isoforms, designated rHol I 5.01 and rHol I 5.02, revealed high similarity between them (78% identity) and to group V allergens and their isoforms found in other grass species. Recombinant Hol I 5 isoforms were expressed in *Escherichia coli* and purified as fusion proteins. To compare their immunological reactivities with group-V-specific monoclonal antibodies and patients' IgE, immunoblotting, ELISA and histamine release assay were performed. Interestingly, monoclonal antibody Bo9, specific for group Vb isoforms of timothy grass, bound only to isoform rHol I 5.01, not to rHol I 5.02. On the other side, IgE reactivities of patients' sera revealed no differences between the two isoforms when investigated by immunoblotting and only slight differences when investigated by ELISA. In histamine release assay both isoforms released comparable amounts of histamine from basophils of four individual patients. Thus, the two group V isoforms of velvet grass pollen exhibit differential binding when tested with monoclonal antibodies, i.e. different structure of single epitopes, but negligible differences concerning overall IgE-binding capacity and histamine-releasing capacity.

Keywords: grass pollen allergen; isoform; cDNA cloning; IgE-binding capacity; histamine-releasing capacity.

Up to 20% of the population in Europe and North America suffers from type I allergy caused by grass pollen allergens [1]. Group V grass pollen allergens are known as major allergens and were found in several related grass species [2, 3]. Recently, some of these important allergens were cloned, sequenced and expressed as recombinant proteins [4–7] leading to important information on the structure of these allergenic proteins. Recombinant allergens made possible further investigations on the reaction of the immune system: (a) determination of IgE-binding epitopes and T cell epitopes, e.g. special structures of the molecule, which interact with components of the immune system, and (b) determination of the biological function of the allergen in pollen and its possible contribution to the induction of the type I allergy. The function of some allergens is known: Der p 1, a major house dust mite allergen, displays proteinase activity [8] and was shown to increase the permeability of bronchial epithelium [9].

Knowledge of the interaction of the immune system with an allergen is a prerequisite to develop new successful concepts for specific immunotherapy. The usual therapy for type I allergy, originally introduced by Noon in 1911 [10], is by subcutaneous

injection of natural extracts leading to an immune deviation towards the specific allergens. Unfortunately, natural allergen extracts do not only contain the relevant allergen, but further components to which patients are not sensitized [11, 12]. To improve immunotherapy, the application of pure recombinant allergens was suggested [13–15]. Whether it is sufficient to use one or several major allergens or the whole spectrum of allergens to which one individual patient is sensitized (tailor-made mixture) is currently under discussion [12, 13]. The situation is complicated by the fact that cloning of allergens of different sources revealed the presence of a variety of isoforms characterized by single amino acid exchanges or deletions [4, 7, 16]. Recently, for the birch pollen allergen Bet v 1, isoforms with no or reduced IgE-binding capacity but retained T cell reactivity were described [17]. The authors suggested the application of this hypoallergenic isoform for a more successful immunotherapy without negative side effects such as anaphylactic reactions or the maintenance of the allergic reaction by release of mediators and cytokines from basophils and mast cells.

Velvet grass (*Holcus lanatus*) is a widespread grass and important source of pollen allergens, but until now the group V allergen Hol I 5 was not available as a recombinant protein. Our aim in this study was to clone and express the group V allergen of velvet grass pollen in *Escherichia coli*. We purified two different isoforms of this allergen and characterized their immunological reactivities with monoclonal antibodies and patient's IgE. We observed differences between these two isoforms in their reactivity to a monoclonal antibody, but no substantial differences in IgE-binding capacity or histamine-releasing capacity.

Correspondence to G. Schramm, Forschungszentrum Borstel, Biochemische und Molekulare Allergologie, Parkallee 22, D-23845 Borstel, Germany

Abbreviations. NaCl/Tris, Tris-buffered saline; 0.1 M Tris, 0.1 M NaCl, 2.5 mM MgCl₂ pH 7.4; MBP, maltose-binding protein; 5'-RACE, 5'-rapid amplification of cDNA ends.

Note. The nucleotide sequences of rHol I 5.01 and rHol I 5.02 have been submitted to the EMBL nucleotide sequence data library and are available under accession numbers Z97874 and Z97875, respectively.

Table 1. Sequences of oligonucleotides used for cloning, subcloning and sequencing of rHol I 5.01 and rHol I 5.02. Underlined sequences indicate restriction sites, broken underlined sequences the cleavage sites for the endonuclease factor Xa. The position of the oligonucleotides is given in bp of the nucleotide sequence of rHol I 5.01 and rHol I 5.02, respectively. gsp, gene-specific primer.

Purpose	Primer	Nucleotide sequence in 5'→3' orientation	Position
Cloning	gsp	ATATAAGCTTAAAGACGGTGTACTTGACCT	648–629
	NT-2	ATATAAGCTTGGCGGTCTCGAAGGTCTTGTA	168–148
	degNT-1	ATATGGATCCGC(T, C, A, G)GA(T, C)GC(T, C, A, G)GG(T, C, A, G)TA(T, C,)	1– 32
		AC(T, C, A, G)CC(T, C, A, G)GC(T, C, A, G)GC(T, C, A, G)CC(T, C, A, G)GC	
Sequencing	HLV1	CTACGAGGCATACAAG	549–564
	HLV2	GCGGTGGCAGCGGCAAC	726–709
	HLV3	TGACGGCGTGGACCTCGA	367–350
	HLV4	GGCTCATCCCTCAGCTCA	221–238
	HLV1.1	ACTGAACAGTCCAAGGCC	125–142
	HLV1.2	GATCAGACTTTGTAGCCA	726–709
Subcloning	PV1s	ATATGTCGACATCGAGGGTAGGGCAGACGCCGGATATACAC	1– 19
	PV1a	ATATAAGCTTATCAGCAAAATCACTCAAC	884–866
	PV2s	ATATGGATCCATCGAGGGTAGGCAGAACTACTCGAAGACG	1– 21
	PV2a	ATATAAGCTTATCAACAAAATCGCTCGCC	825–807
	PV1Cts	ATATGGATCCATCGAGGGTAGGCAGAACTGCTCGAAGACG	70– 88

MATERIALS AND METHODS

Patients' sera and monoclonal antibodies. Sera from grass pollen allergic patients were collected in the outpatients department of the Medical Clinic Borstel. Atopic phenotype was confirmed by clinical history, diagnosis and CAP score $\geq$ IV to velvet grass pollen (Pharmacia CAP system, scores according to manufacturer's manual). Control sera were collected from non-atopic donors. The monoclonal antibodies were raised against crude timothy grass pollen extract. Bo1 is directed against group V allergens Phl p 5a and b [18], BG6 against Phl p 5a and Bo9 against Phl p 5b [19].

Immunoscreening of a cDNA library of velvet grass inflorescences. Construction and immunoscreening of a cDNA library of velvet grass inflorescences in the λ ZAP II vector (Stratagene) was described elsewhere [20]. Immunoscreening of the cDNA library was performed with the grass pollen allergen group-V-specific monoclonal antibody Bo1.

Amplification of a 5'-terminal cDNA sequence by reverse-transcription PCR with a degenerated primer. For cDNA synthesis, 1 μ g mRNA from velvet grass inflorescences was reverse transcribed using an internal gene-specific primer (gsp) and 200 U SuperScript reverse transcriptase (Gibco BRL). mRNA template was degraded by 2 U RNase H and the resulting first strand of cDNA was purified over GlassMax spin cartridges (Gibco BRL) according to the manufacturer's instructions. cDNA was subjected to PCR with a nested gene-specific primer (NT-2) and a degenerated N-terminal primer (degNT-1). Primers were synthesized on an automated DNA synthesizer (Cyclon Plus Synthesizer, Milligen/Bioscience) by the phosphor amidite method (for sequences see Table 1). PCR was performed on the Thermo-cycler (Perkin-Elmer) using 1 ng DNA template, 1 μ M primers, 200 μ M dNTPs and 1 U Taq Polymerase (Pharmacia) under the following conditions: 45 s at 94°C, 30 s at 57°C, 2 min at 72°C, 30 cycles.

Screening of the cDNA library by hybridization with a 5'-terminal probe. The 171-bp 5'-terminal cDNA fragment generated by reverse-transcription PCR was used as a hybridization probe for screening of the cDNA library. The probe was labelled with digoxigenin according to the manufacturer's instructions (DIG DNA labeling kit, Boehringer).

Bacteriophage λ plaques were immobilized on nitrocellulose filters following the method of Benton and Davis, [21]. Hybridization was performed under stringent conditions at 68°C in

0.75 M NaCl, 0.075 M sodium citrate, pH 6.8 [21]. For immunological detection of positive phage clones nitrocellulose filters were then incubated in 0.1 M Tris-buffered saline (NaCl/Tris: 0.1 M Tris, 0.1 M NaCl, 2.5 mM MgCl₂, pH 7.4) containing 1% nonfat milk for blocking of free binding sites. The digoxigenin-labelled DNA probe was detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer), diluted 1:5000 in NaCl/Tris plus 1% MMP. Positive phage clones were isolated and the pBluescript SK plasmids containing the inserts were excised *in vivo* with the helper phage Exassist (Stratagene). Automated sequencing of double-stranded plasmid DNA was performed by cycle sequencing using the ABI Prism dye terminator cycle sequencing ready reaction kit on the ABI 377DNA Sequencer (Perkin-Elmer). Sequencing was done with reverse and universal primer (Pharmacia) and gene-specific primers: HLV1, HLV2, HLV3, HLV4 for rHol I 5.01 and HLV1.1 and HLV1.2 for rHol I 5.02 (Table 1).

Expression of the two recombinant Hol I 5 isoforms, rHol I 5.01 and rHol I 5.02. For characterization, the recombinant isoforms were expressed as fusion proteins with maltose-binding protein (MBP) in *E. coli* (MBP expression system, New England Biolabs). cDNA coding for the two isoforms rHol I 5.01 and rHol I 5.02 and a 23 amino-acid N-terminal truncated form of rHol I 5.01 (rHol I 5.01ct), equivalent to rHol I 5.02, were subcloned by PCR into the pMalc expression vector. Specific primers were designed with an AT-clamp, restriction sites (*Bam*HI or *Sal*I for the coding strand, *Hind*III for the complementary strand) and a sequence coding for the cleavage sites of the specific protease factor Xa to allow cleavage of the MBP from the fusion protein (Table 1). Conditions for PCR reactions were as follows: 1 min at 94°C, 1 min at 54°C, 2 min at 72°C, 30 cycles. Cloning followed standard procedures [21] and sequences were confirmed by sequencing using universal and *malE* primers (New England Biolabs).

The fusion protein was induced and purified according to the manufacturer's manual (New England Biolabs). Briefly, *E. coli* JM109 cells, harboring the appropriate plasmids pM5-01, pM5-01ct or pM5-02 were grown as 1-l cultures to $A_{600} = 0.8$. Expression of MBP fusion proteins was induced with 0.3 mM isopropyl thiogalactoside. After incubation for additional 4 h at 37°C cells were harvested by centrifugation. The cell pellet was resuspended in 30 ml column buffer (20 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA). After breaking the cells by passage twice

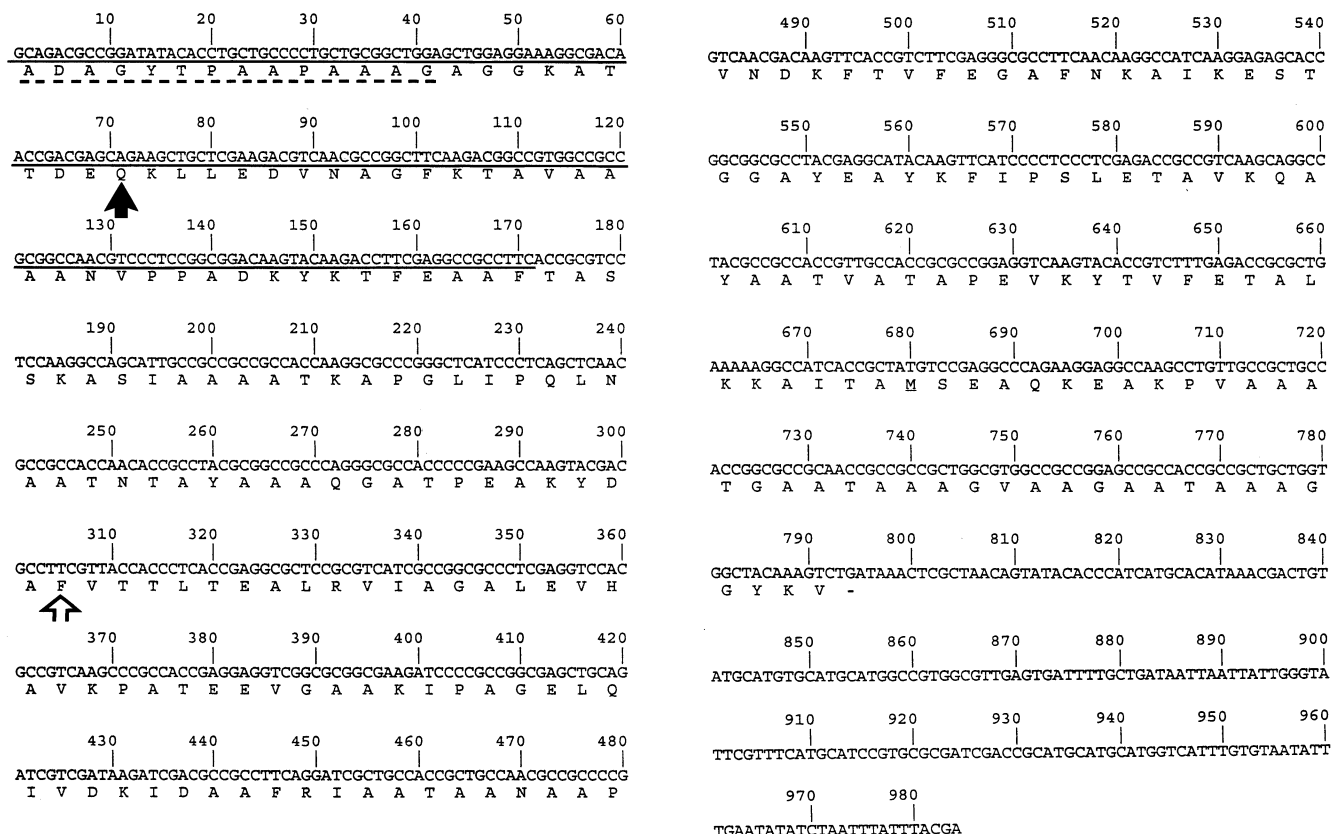


Fig. 1. Nucleotide sequence and deduced amino acid sequence of the rHol I 5.01 isoform. The sequence is shown from left to right in 5'→3' orientation. The underlined 5'-terminal nucleotide sequence indicates the hybridization probe used for screening of the cDNA library by hybridization. The broken underlined amino acid sequences mark the N-terminal sequence of Hol I 5 known from protein sequencing [24]. The solid arrow indicates the N-terminus of the truncated sequence of rHol I 5.01ct, the open arrow indicates the N-terminus of the C-terminal Hol I 5 fragment identified by immunoscreening.

through a French press and subsequent centrifugation at 17 000 *g*, MBP fusion proteins were purified from the supernatant by affinity chromatography using an amylose resin column with a yield of 30 mg fusion protein/l culture.

Antibody binding analysis. For immunoblot analysis, 0.2 µg/cm purified MBP fusion protein was separated by SDS/PAGE (12%) according to Laemmli [22] and transferred onto nitrocellulose membrane (Schleicher & Schüll) by semi-dry blotting for 30 min at 0.8 mA/cm² [23]. Immunological detection was performed with sera of nonatopic donors and atopic patients and supernatants of hybridomas as described elsewhere [20].

For ELISA, 0.1 µg/well purified fusion proteins were coated onto plates (MaxiSorb, Nunc). Plates were blocked with 5% non-fat milk in NaCl/Tris pH 7.4, for 2 h, followed by incubation with monoclonal antibody (dilution 1:40) and patients' sera (*n* = 42) (dilution 1:5) in 2% non-fat milk in NaCl/Tris, pH 7.4. After a 2-h incubation at 37°C, plates were washed with 0.05% Tween in NaCl/Tris pH 7.4, and incubated for a further 2 h at 37°C with alkaline-phosphatase-conjugated anti-(mouse IgG/M) (dilution 1:10000; Dianova) or alkaline-phosphatase-conjugated anti-(human IgE) (dilution 1:2000; Allergopharma). After washing, bound conjugate was detected using 4-nitrophenyl phosphate substrate (Merck). Reaction was detected at 405 nm (ELISA reader MR7000, Dynatech).

Histamine release assay. Granulocytes from grass-pollen-allergic patients were isolated from EDTA blood samples by dextran sedimentation. Cells were incubated with serial dilutions of purified recombinant fusion proteins. The fusion partner MBP was used as negative control. Histamine release was measured

in the cell-free supernatant by radioimmunoassay (Pharmacia). Stimulation with phorbol myristate acetate/ionomycin or anti-IgE was used as positive control. Total histamine was determined after repeated freeze-thawing of the cells.

Computer analysis and nucleotide sequence accession number. Sequence data were analysed using PCGene software (Intelligenetics).

Statistical analyses. Data obtained by ELISA were analysed for statistical significance by two-tailed, paired Wilcoxon test.

RESULTS

Cloning and sequence analysis of the two isoforms of the group V allergen of velvet grass, rHol I 5.01 and rHol I 5.02.

By immunoscreening of the cDNA library from mRNA of velvet grass inflorescences in λ ZAP II vector with the monoclonal antibody Bo1, recognizing specifically group V allergens, we isolated four identical phage clones. Restriction analysis and sequencing revealed that the inserts of all four clones were too short to contain a complete open reading frame (ORF). Sequence comparison with amino acid sequences of the group V allergens of timothy grass pollen, Phl p 5a and Phl p 5b, identified the ORF as a C-terminal fragment of the group V allergens (Fig. 1). By methods like screening of the cDNA library by hybridization with this C-terminal fragment as a probe or 5'-RACE, no cDNA coding for the complete group V allergen of velvet grass pollen could be obtained. However, northern blot analysis of the velvet grass inflorescence mRNA with a closely related cDNA probe of timothy grass pollen revealed the presence of mRNA of com-

PHLP5-0103	ADLGYPATPAAPAAGYTPATPAAPAGAEPAKGATTEEQKLEIKINAGFK	50
PHLP5-0201	..A..A.....AG.A-----D..V...	36
HOL-L-5-01	..A..T..a...AG..-----d...l.Dv....	35
HOL-L-5-02	-----l.Dv...S...	12
PHLP5-0103	AALAAAAGVPPADKYRTFVATFGAASNKAFAEGLSGEPKGAEESSKAAL	100
PHLP5-0201	..v....S..A...fk..E..a..Tss.KA---AAaKA.-----G.	72
HOL-L-5-01	t.v....N.....k..E..a..T.s.KAsI.AAAtKA.-----G.	75
HOL-L-5-02	..v....K.....k..lRa.TVLDRGsTEQSKaE..T.-----MPE.	55
PHLP5-0103	TSKLDAAYKLAYKTAEGATPEAKYDAYVATVSEALRIIAGTLEVHAVKPA	150
PHLP5-0201	VP.....Sv...a.V.....f.sf..slt...v...a.....V	122
HOL-L-5-01	IPQ.N..TNT..Aa.Q.....f.t.lt...v...a.....	125
HOL-L-5-02	s...VD..MA.f.asT.G.Q.....f.t.lt...v...a.....	105
PHLP5-0103	AEEVKV--IPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDA	198
PHLP5-0201	t..PGmAK.....i.d.i.....At...D.....K.	172
HOL-L-5-01	t...GAAK.....ivd.i.....ri.....V.....G...K.	175
HOL-L-5-02	t...PAAK....d...vd.i..s..i.....t...K.	155
PHLP5-0103	IKASTGGAYESYKFIPALEAAVKQAYAAATVATAPEVKYTVFETALKKAIT	248
PHLP5-0201	..E.....dt..C..s.....a.A...a...a..T...	222
HOL-L-5-01	..E.....a.....s..t.....a.....a...a..T...	225
HOL-L-5-02	l.E.....s.....s.....a.....a...a..T...	205
PHLP5-0103	AMSEAQKAAPAAAATA---TATAAVGAATGAATAATGGYKV	287
PHLP5-0201	...V..VsQ...tG.atVAAGa..t.A...s...V.a....	265
HOL-L-5-01	E...V.....Ga...A.V.a...a....	264
HOL-L-5-02	...Q...V.Q.....-----G.ATV.a...t.a....	240

Fig. 2. Alignment of amino acid sequences of the group V allergens rPhl p 5a (PHLP5 0103) and rPhl p 5b (PHLP5 0201) from timothy grass pollen with the sequences of the two Hol l 5 isoforms, rHol l 5.01 and rHol l 5.02. Identical amino acids are indicated by points and gaps by dashes. Capital letters indicate non-conserved amino acid exchanges and small letters well-conserved (similar biochemical properties) amino acid exchanges. Regions of high similarity are marked by grey hatched boxes. Numbers on the right side indicate the position number of the last amino acid residue in the line.

Table 2. Similarity between the amino acid sequences of the rHol l 5 isoforms rHol l 5.01 and rHol l 5.02 and other known group V allergens.

Isoform	Amino acid sequence identity with group V allergens							
	Phl p 5a	Phl p 5b	Lol p 5a	Lol p 5b	Poa p 5 (clone 31)	Poa p 5 (clone 41)	Poa p 5 (clone 60)	Pha a 5 (clone 29)
	%							
Hol l 5.01	73.5	77.7	83.0	72.4	74.2	76.9	73.5	66.7
Hol l 5.02	69.2	73.8	78.8	64.6	70.8	72.1	69.6	64.9

plete size (data not shown). To selectively isolate the 5'-terminus of the cDNA of the group V allergen, mRNA was reversely transcribed and amplified by PCR using a gene-specific internal antisense primer and a degenerated 5'-terminal sense primer, which was deduced from N-terminal protein sequencing of the native Hol l 5 protein [24]. The resulting PCR product encoded the N-terminus of the group V allergen (see Fig. 1). This 171-bp 5'-cDNA sequence was used as a hybridization probe for screening of the cDNA library for a complete Hol l 5 clone. After screening of approximately 2×10^6 plaques, two phage clones were isolated and plasmids were obtained by *in vivo* excision. Both plasmids, pHL5-01 and pHL5-02, were analysed by double-stranded sequencing with vector- and gene-specific primers. The 984-bp cDNA insert of pHL5-01 contained an ORF of 795 bp coding for a protein of 264 amino acids (Fig. 1) while pHL5-02 contained an ORF of 723 bp coding for a protein of 240 amino acids (nucleotide sequence not shown). Alignment of the two cDNA sequences revealed 108 nucleotide exchanges and two gaps of 9 and 12 nucleotides in the coding regions, and the

lack of 69 bp at the 5'-terminus of pHL5-02. Comparing the overlapping nucleotide sequences, both cDNA inserts show a sequence identity of 79.6%. The overlapping amino acid sequences display a similarity of 85.1% and an identity of 78%. Fig. 2 shows an alignment of the amino acid sequences of the recombinant timothy grass pollen allergens rPhl p 5a and rPhl p 5b [16] with the deduced amino acid sequences of clones pHL5-01 and pHL5-02. The protein encoded by pHL5-02 lacks 23 amino acids at the N-terminus, thus representing an incomplete molecule. All four aligned allergen isoforms show a similarity of 75.5% with an identity of 47.2%. Table 2 shows the high similarity of the deduced amino acid sequences of pHL5-01 and pHL5-02 to other known group V grass pollen allergens. Thus, the cDNA inserts of plasmids pHL5-01 and pHL5-02 represent two isoforms of the group 5 allergen of velvet grass pollen, designated Hol l 5.01 and Hol l 5.02.

Expression and immunological characterization of the isoforms rHol l 5.01 and rHol l 5.02. For expression of the recom-

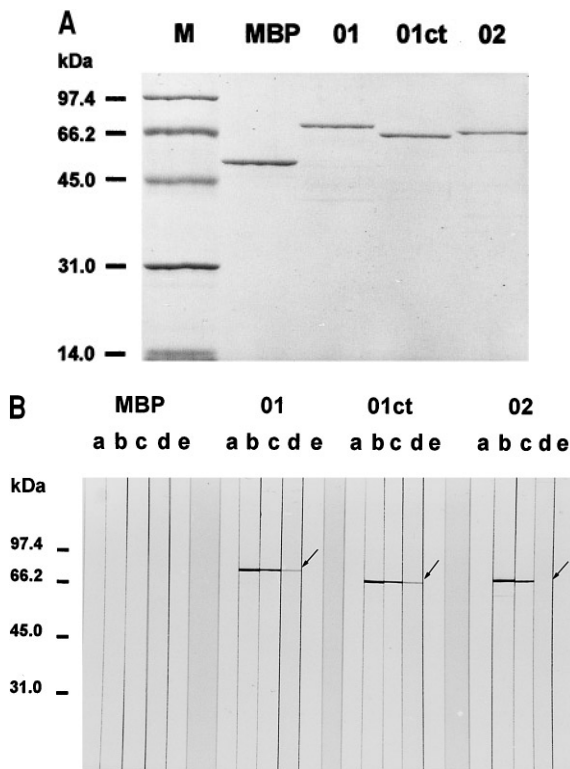


Fig. 3. Expression and immunological characterization of the rHol I 5 isoforms. (A) 12% SDS/PAGE and Coomassie staining of 0.4 µg/cm purified fusion proteins. (M) Low-molecular-mass markers (BioRad), (MBP) maltose-binding protein, (01) MBP-rHol I 5.01, (01ct) MBP-rHol I 5.01ct, (02) MBP-rHol I 5.02. (B) Immunoblotting of the recombinant isoforms of rHol I 5. (MBP) Maltose-binding protein, (01) MBP-rHol I 5.01, (01ct) MBP-rHol I 5.01ct, (02) MBP-rHol I 5.02. 0.2 µg/cm of the purified fusion proteins were analysed by SDS/PAGE and immunoblotted for IgE reactivity of a normal control serum (a) and patient's serum (b) and the reactivity of the monoclonal antibodies Bo1 (c), Bo9 (d) and BG6 (e). Arrows indicate the different reactivity of monoclonal antibody Bo9 to the rHol I 5.01 and rHol I 5.02 isoforms.

binant Hol I 5 isoforms, the cDNA inserts of pHL5-01 and pHL5-02 were subcloned by PCR into the pMalc expression system. To achieve a better comparison of the immunological reactivities of the two isoforms we also cloned a truncated form of rHol I 5.01 (rHol I 5.01ct) missing 23 amino acids at the N-terminus equal to rHol I 5.02. After expression and purification of the MBP fusion proteins by affinity chromatography with amylose resin, they were analysed by SDS/PAGE (Fig. 3A) and western blotting (Fig. 3B). As immune reactants, sera of normal and atopic donors and the monoclonal antibodies Bo1 (group V), BG6 (group Va) and Bo9 (group Vb) were applied. Both isoforms of rHol I 5 were recognized by sera of patients and the group-V-specific monoclonal antibody Bo1. In contrast, group-Va-specific monoclonal antibody BG6 did not react with either of the isoforms. Interestingly, monoclonal antibody Bo9 recognized isoform rHol I 5.01 and the truncated version rHol I 5.01ct, but not rHol I 5.02, suggesting that the sequence differences between the two isoforms lead to different epitopes.

To investigate whether patients' sera reacted differently towards the two isoforms of rHol I 5, we compared the IgE reactivity of 42 sera with the N-terminal truncated form rHol I 5.01ct and rHol I 5.02 by immunoblot analysis and ELISA. By immunoblotting, two out of 42 sera revealed a slightly reduced IgE binding to the isoform rHol I 5.02 compared to rHol I 5.01ct. In all other sera no significant differences concerning the IgE reactivity were observed. Fig. 4 shows the IgE reactivity of ten

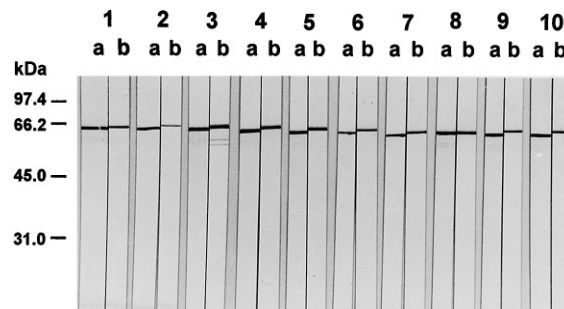


Fig. 4. Comparison of the IgE reactivity of ten different patients' sera (1–10) to the two N-terminal truncated isoforms of rHol I 5 [rHol I 5.01ct (a) and rHol I 5.02 (b)] by immunoblotting.

of the investigated sera with serum 2 as an example for reduced IgE binding. Comparison of the IgE reactivity of both rHol I 5 isoforms by ELISA revealed for rHol I 5.02 a significantly reduced IgE-binding capacity by nearly all 42 investigated sera ($P < 0.01$). But with the exception of serum 2, which shows 2.5-fold reduced IgE reactivity to rHol I 5.02 (as also seen by immunoblotting), the differences of the IgE-binding capacity between rHol I 5.01ct and rHol I 5.02 were very small with medians of absorbances of 0.342 and 0.297, respectively. Results of immunoblot analysis and ELISA suggest comparable IgE-binding capacity of both rHol I 5 isoforms.

To investigate the effect of the slightly reduced IgE binding to rHol I 5.02, we compared the functional activity of both isoforms by histamine release from basophils of four individual patients, including patient 2, who showed clearly reduced IgE binding to the isoform rHol I 5.02 (Fig. 5). Basophils of all four patients released comparable amounts of histamine when stimulated with either rHol I 5 isoform. No histamine release was observed with the fusion partner MBP (data not shown). Thus, the slightly reduced IgE binding had no effect on the histamine-releasing activity of these proteins.

DISCUSSION

In this study two variable (identity of 78%) isoforms of the group V grass pollen allergens of velvet grass (*Holcus lanatus*) were cloned. In contrast to the group I allergen of velvet grass [20], obtaining a complete cDNA clone of the group V allergen proved to be difficult. Although mRNA of complete size was detectable, reversely transcribed cDNA lacked the 5'-terminal part of the sequence. The mRNA of the group V allergen of velvet grass pollen exhibits a G+C content of 68% and seems to display a strong secondary structure, leading to premature dissociation of the reverse transcriptase from template.

Hybridization of the cDNA library with an N-terminal 171-bp probe led to the isolation of two cDNA clones, displaying a high similarity to other known group V allergens. They could be identified as two isoforms of the rHol I 5 allergen and were designated rHol I 5.01 and rHol I 5.02. Unfortunately, the isoform rHol I 5.02 was not a complete clone, lacking 23 amino acids at the N-terminus. In order to compare the antibody binding capacity of these two isoforms, we cloned an equally truncated form of rHol I 5.01 (rHol I 5.01ct). For analysis of the antigenic reactivities three monoclonal antibodies were used, with two of them being able to distinguish between the group V timothy grass pollen isoforms Phl p 5a and Phl p 5b, BG6 and Bo9, respectively. Interestingly, both velvet grass isoforms rHol I 5.01ct and rHol I 5.02 were recognized by the monoclonal antibody Bo1, specific for both group V isoforms of timothy

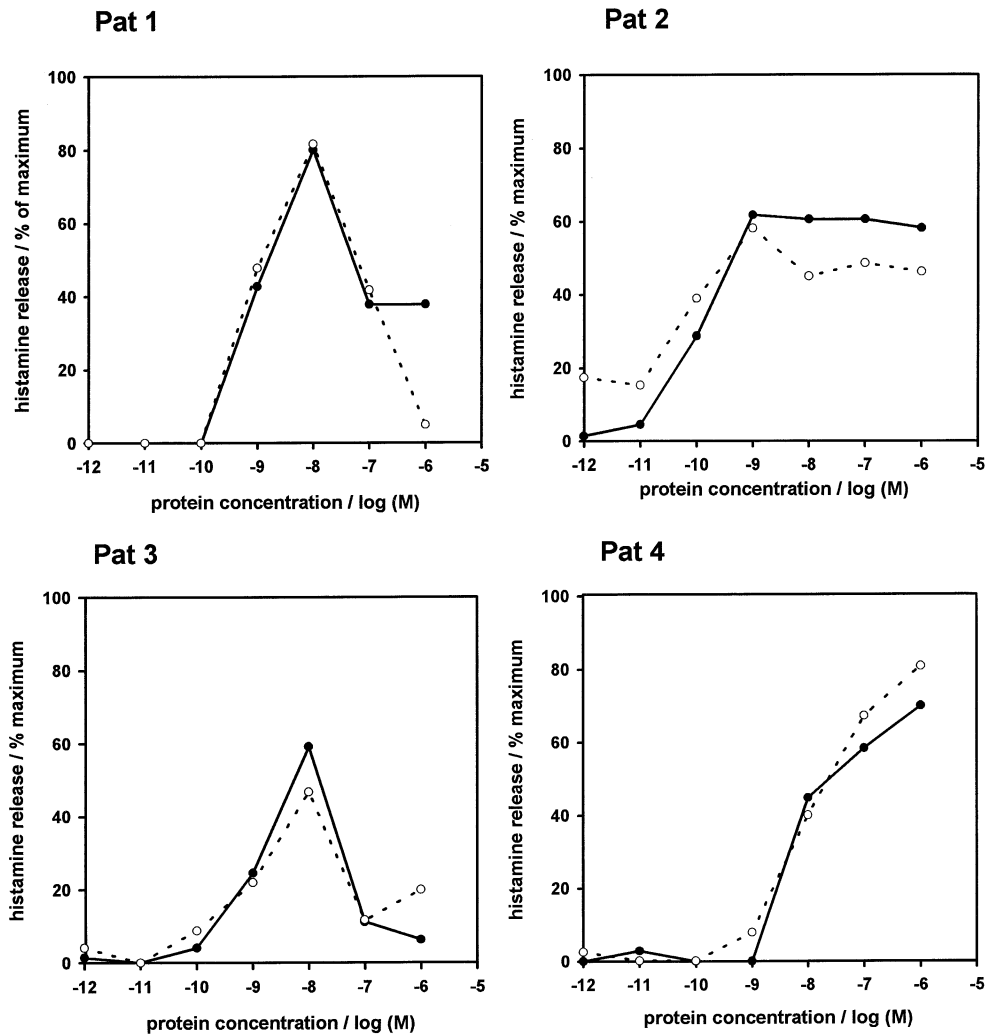


Fig. 5. Comparison of histamine release activity of the two N-terminally truncated isoforms of rHol I 5, rHol I 5.01ct (—) and rHol I 5.02 (---) performed with basophils of four individual patients (Pat 1–4).

grass, none of them were recognized by BG6, specific for 5a isoforms of timothy grass, and only rHol I 5.01ct was recognized by Bo9, specific for 5b isoforms of timothy grass. These results indicate that none of the two velvet grass group V allergens display the epitope structure characteristic for the 5a isoforms. However, rHol I 5.01 displays the epitope characteristic for the 5b isoform. This variable recognition pattern prompted us to compare the IgE-binding capacity of the two isoforms. Immunoblot analysis of 42 allergic patients' sera revealed no significant differences in the IgE binding to the two rHol I 5 isoforms.

To obtain more precise data on the IgE-binding capacity, we investigated the sera by a more sensitive ELISA. We observed slightly reduced IgE binding to the isoform rHol I 5.02 compared to rHol I 5.01ct in nearly all investigated sera ($P < 0.01$) but the differences were negligible. Obviously these small differences could not be visualized by immunoblotting. In contrast to the IgE binding, comparison of the functional properties of these isoforms by histamine release assay revealed that both isoforms released comparable amounts of histamine in a specific and dose-dependent manner. So the slight differences in IgE-binding capacity, detected by ELISA, had no effect on the functional activity, e.g. histamine-releasing capacity of the isoforms. Thus, the two group V allergen isoforms of velvet grass pollen display differential binding to a monoclonal antibody reflecting the presence of different antigenic epitopes on these isoforms, but no

substantial differences in IgE-binding capacity or histamine-releasing capacity. This is in line with a report showing differential monoclonal antibody-binding capacity but comparable IgE-binding capacity of ten isolated isoforms of the timothy grass pollen allergens, Phl p 5a and b [16]. These observations made for grass pollen allergens are in contrast to the situation found for tree pollen allergens, such as Cor a 1 and Bet v 1 [17, 25]. Cloned isoforms of tree pollen allergens showed different IgE-binding capacity. For the birch pollen allergen Bet v 1, authors described a hypoallergenic isoform, which revealed no IgE reactivity but retained T cell reactivity, thus being a good candidate for improved immunotherapy [17]. Two possible explanations for these differences between the group V grass pollen allergens and the birch pollen allergen Bet v 1 are the following. (a) The existence of a higher number of allergen isoforms in tree pollen than in grass pollen: analysis of allergens by two-dimensional electrophoresis revealed over 20 spots of Bet v 1 [26], but only 4–8 spots of different group V grass pollen allergens [19, 24, 27]. (b) The presence of discontinuous IgE-binding epitopes on Bet v 1 resulting in the loss of IgE reactivity by few amino acid exchanges [28, 29]; in contrast, the group V grass pollen allergen rHol I 5.01 of velvet grass pollen bears at least four IgE-binding epitopes, with two of them being continuous epitopes (Schramm, G., unpublished results). Thus, few amino acid exchanges affect only a part, but not all, IgE-binding epitopes and

this effect was compensated by the polyclonal IgE response. Further epitope mapping studies of group V grass pollen should lead to more information on structure and number of IgE-binding epitopes on grass pollen allergens and the development of new suitable concepts for specific immunotherapy of grass pollen allergy.

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