

A Nonspecific, Single-Stranded Nuclease Activity with Characteristics of a Topoisomerase Found in a Major Grass Pollen Allergen: Possible Biological Significance

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The major allergen from timothy grass pollen, Phlp5b (*Phleum pratense*), was shown to exhibit ribonuclease activity. It turned out that the C-terminal portion of this molecule was the biologically active domain. Here evidence is presented that the allergen is a single-stranded, sugar-nonspecific nuclease with topoisomerase activity. An isomerase-specific active site was identified, and a non-active mutant was constructed by site directed mutagenesis, and showed no nucleolytic activity. In contrast to the wild type (WT), the mutant did not dimerize. Although the binding capacity of IgE antibodies toward the mutant was reduced as compared to the WT, the allergenic activity was retained. We conclude that the allergen Phlp5b is a single-stranded nuclease with an unusual topoisomerase-like activity. This biological activity is not by itself connected to the allergenicity of the molecule. Whether the enzymatic activity is responsible for the induction of the allergic sensitization and inflammation remains an open question.

Key words: Allergen / Allergenic activity / Nuclease / Phlp5b / Site-directed mutagenesis / Timothy grass pollen / Topoisomerase.

Introduction

By definition, allergens are molecules inducing an allergic sensitization and response in humans predisposed to atopic diseases (King *et al.*, 1994). The result of sensitization is the production of allergen-specific immunoglobulin E (IgE) antibodies which in turn mediate the allergic response leading to symptoms as observed in allergic rhini-

tis, conjunctivitis and bronchial asthma. A major immunological characteristic of allergens is their recognition by IgE-antibodies. Furthermore, they induce the release of mediators such as histamine and leukotrienes from basophils and mast cells by crosslinking at least two IgE-antibodies bound to these cells via the high affinity IgE-receptor (Sutton and Gould, 1993). This biological activity is called allergenicity and is observed in a great number of different molecules isolated from tree, grass and weed pollen, animal dander, mites, mushrooms, foods, bee venom and many other plant and animal species. It remains an open question why these molecules in particular can induce an allergic response while others do not. Other than certain biochemical properties of allergens, such as molecular masses between 8 to 45 kDa, their accessibility to the human mucosa and their stability in the air as aerosols and on the mucosa as solvents, no other reliable features have been identified to explain why they become allergenic (Stewart *et al.*, 1993). It has been shown that allergens must bear at least two IgE-binding epitopes for crosslinking, and usually these epitopes are structurally different (Bufe *et al.*, 1994). As far as has been shown, the sequences of these epitopes do not show a typical pattern for allergens (Schramm *et al.*, 1997). On the other hand the distribution of IgE-binding regions on the allergenic molecule seems to be important for an effective induction of mediator release (Greenspan and Cooper, 1995).

Ever since allergens were first characterized, it has been obvious that they have certain biological functions in physiological systems. A considerable number of allergens are enzymes, others are protease inhibitors, structural, storage and transport proteins (Bufe, 1998). Long ago it was demonstrated that the major mite allergen (Der p 1) is a cysteine proteinase. Interestingly, Der p 1 can augment its own transport through the mucosa by its protease activity and then possibly better participate in the process of sensitization (Herbert *et al.*, 1995). It can also non-specifically trigger allergic inflammation in asthmatic patients (Tomee *et al.*, 1998). The biological function of allergens may thus play an important role in the induction of allergic diseases.

Recently we have determined that the major group V allergen of grass pollen, *Phleum pratense* 5b (Phlp5b) from timothy grass, shows ribonuclease activity (Bufe *et al.*, 1995). This was a surprising finding, since the biological significance of RNases released from pollen is not fully understood, nor can the meaning of RNase activity in the context of allergic sensitization be explained. Pollen RNases may function as defense proteins against viral infection, as components of a degradation complex partici-

pating in the apoptosis of tapetal cell layer, and for nucleoside re-utilization by developing pollen (Matousek *et al.*, 1996). On the other hand, the neurotoxic eosinophilic cationic protein released from eosinophils during an allergic inflammation in the mucosa was shown to be a ribonuclease (Domachowski *et al.*, 1998), so that RNase activity might well be involved in the induction of an allergic reaction.

The RNase specific activity of Phlp5b first identified was relatively low. But it turned out that the allergen gained biological activity by cleavage of the N-terminal portion of the molecule (Bufe *et al.*, 1996). The C-terminal 13 kDa component of Phlp5b (Phlp5b-C-t) showed significantly higher RNase activity than the full-length holoallergen. Since pollen RNases exhibit different nucleolytic activities, like specific and nonspecific restriction of double-strand or single-strand ribonucleic acids, it was in our interest to more closely characterize the properties of the allergenic ribonuclease.

Here we present data that Phlp5b-C-t is a nonspecific, single-strand nuclease with the ability to function as an isomerase. By identification of a putative active nucleolytic site, an inactive recombinant mutant of Phlp5b-C-t was constructed, and was analyzed for its IgE-binding capacity and its allergenic activity.

Results

Nuclease Activity of Phlp5b-C-t

In earlier experiments we demonstrated that the purified natural and recombinant Phlp5b shows ribonuclease activity (Bufe *et al.*, 1995) and that the C-terminal 13 kDa component of this allergen, isolated by crystallization, is the activated form of the parent protein (Bufe *et al.*, 1996). As Figure 1 shows, it is now confirmed that the former 13 kDa component, the purified recombinant Phlp5b-C-t.

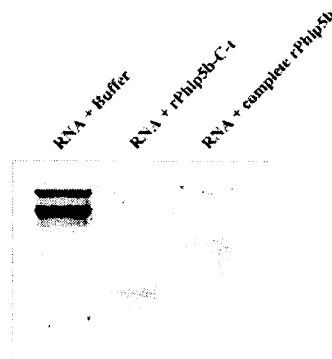


Fig. 1 Cleavage of Total RNA (1 μ g) from Timothy Grass Pollen by Holoallergen Phlp5b and the Active C-Terminal Domain. Lane 1 represents the buffer control (10 mM Tris-HCl), lane 2 rPhlp5b-C-t (1 μ l = 35 pmol) and lane 3 the complete rPhlp5b (2 μ l = 35 pmol). Separation was done on a 1.2% agarose gel after incubation at 56 °C for 45 min. The gel was stained with ethidium bromide

also digested total RNA (lane 2). Lane 3 demonstrates that the holoallergen exhibits lower activity than the C-terminal. Since a number of pollen nucleases exhibit double-strand RNase (dsRNase) activity, we wanted to analyze whether this is also true for the active C-terminal domain. It turned out that Phlp5b-C-t did not degrade dsRNA in the nuclease assay (data not shown). This result indicated that the nuclease activity of Phlp5b-C-t was confined to ssRNA.

To determine the specificity of this nuclease activity we looked at the capacity of Phlp5b-C-t to cleave ssDNA. Figure 2 shows the results of this experiment. In contrast to the buffer-control (lane 3) and BSA (lane 4), Phlp5b-C-t (lane 7) degraded M13 phagemid DNA to a mixture of nu-

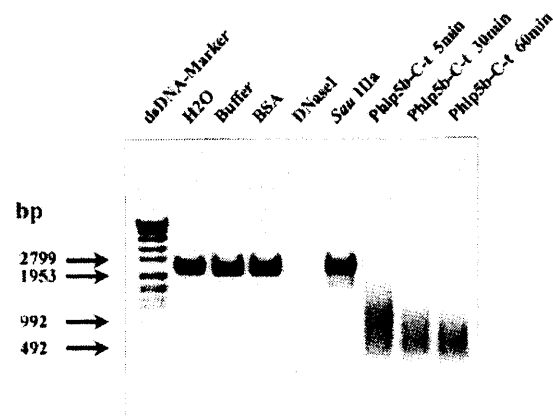


Fig. 2 Cleavage of ssDNA (1 μ g of M13 Phagemid) by Different Nucleases and rPhlp5b-C-t.

M13 was incubated at 37 °C for either 45 min or at the given times with (lane 2): H₂O, (lane 3): buffer, (lane 4): 1 μ l BSA (2 mg/ml), (lane 5): 1 μ l DNase I (2500 units/ml), (lane 6): 1 μ l Sau3A (15000 units/ml) and (lane 7–9): 1 μ l rPhlp5b-C-t (35 pmol). A 1.2% agarose gel was performed as described earlier.

cleotides with sizes between 50 and 1800 bp 5 min after the onset of the reaction. After 60 minutes (lane 9) the degradation was not yet complete. This indicated that the activity of Phlp5b-C-t is a sugar-unspecific nuclease capable of cleaving both ssRNA and ssDNA substrates. It also showed that the activity of the allergen with M13 phagemid DNA was weaker than that of DNase I, which degraded the phagemid nearly completely in about 5 min. Like DNase I the allergen Phlp5b-C-t seemed to exhibit non-specific nuclease activity because the degradation pattern of both were different to that of the specific nuclease Sau3A (lane 6), which led to an incomplete restriction of the M13 phagemid into a fragment of about 1800 bp.

Further evidence for the fact that the nuclease activity of Phlp5b-C-t is independent of nucleotide sequence comes from PAGE experiments performed with M13 phagemid degradation products after cleavage with the different nucleases. While no degradation products were visible on the flow sheet (Figure 3) in the buffer- and BSA-sample (lane A and B), in the DNase I sample (lane C) many peaks

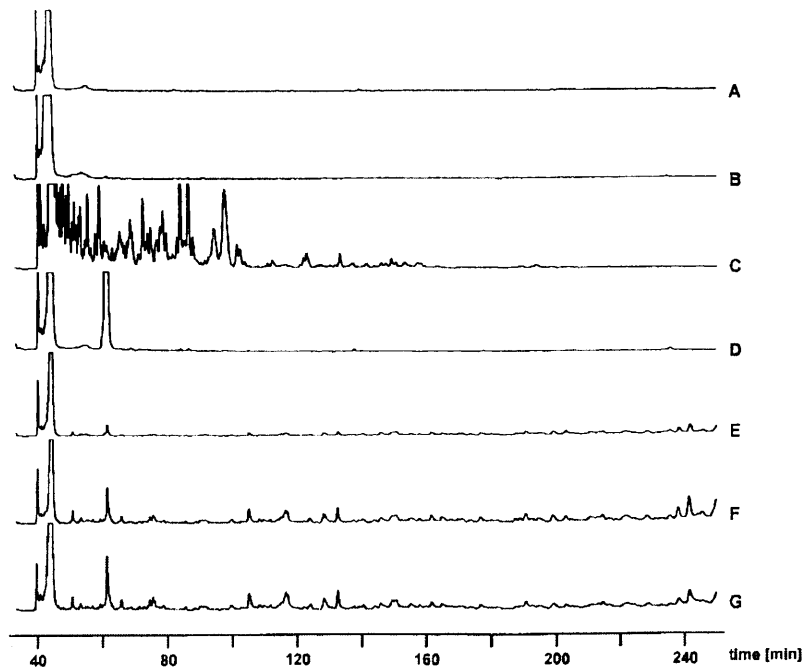


Fig. 3 ssDNA Sequencing of Degradation Products Following Nucleolytic Digestion by Different Nucleases and rPhlp5b-C-t. The flow sheet from the ALF automatic sequencer shows digestion pattern by (lane A) = M13 + buffer, (lane B) = M13 + BSA, (lane C) = M13 + DNase I, (lane D) = M13 + *Sau3A*, (lane E) = M13 + rPhlp5b-C-t stopped after 5 min, (lane F) = M13 + rPhlp5b-C-t stopped after 30 min and (lane G) = M13 + rPhlp5b-C-t stopped after 60 min. Concentrations were the same as shown in Figure 2. Time indicates the minutes of specific fragment detection after loading of samples on the gel.

appeared already between 40 to 100 min after the products entered the gel, indicating the presence of numerous small DNA fragments degraded nonspecifically. In the *Sau3A*-sample (lane D) only one fragment was identified at 63 min. By linkage analysis of this restriction site with the respective part in the sequencing lanes of M13 (data not shown), we showed that the peak region represents the only specific site of *Sau3A* [GATC] present in the plasmid. This confirmed the specific nucleolytic activity of this enzyme even in ssDNA. In all three Phlp5b-C-t samples (lane E to G) numerous peaks with increasing intensity from E to G could be seen between 50 to 250 min. None of the peaks revealed a specific restriction pattern when linked to the sequencing lanes of M13 (data not shown). These results clearly demonstrate that Phlp5b-C-t cleaved single-stranded M13 phagemid DNA in a nonspecific manner.

Isomerase Activity of Phlp5b-C-t

We then wanted to determine whether Phlp5b-C-t could also degrade double-stranded DNA. When incubating Phlp5b-C-t with the cloning vector pQE9, we surprisingly found no restriction of the plasmid, but instead observed the disappearance of the supercoiled portion of pQE9. Figure 4 shows a time dependent gradual fading of the supercoiled status accompanied by an increase of the religated circular plasmid form without any further indication for a restriction of the double-stranded DNA. Taken to-

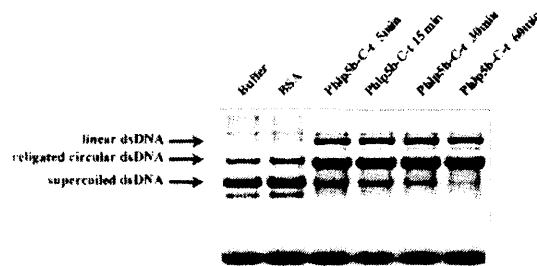


Fig. 4 Cleavage of dsDNA by rPhlp5b-C-t. pQE was incubated at 37 °C for either 45 min or at the given times with (lane 1): buffer, (lane 2): 1 μ l BSA (2mg/ml), and lane 3–6: 1 μ l rPhlp5b-C-t (35 pmol). 1.2% agarose gel electrophoresis was performed as described earlier.

gether these experiments indicate that Phlp5b-C-t is an RNase with nonspecific single-stranded nuclease and isomerase-like activity.

Putative Active Site of a Topoisomerase Found in Phlp5b-C-t

In order to more closely characterize the identified isomerase activity of Phlp5b-C-t, a data bank screening was conducted. Amino acid sequences with greater than 15% identity to known isomerases were not found (data not shown). But interestingly, the active site of topoisomer-

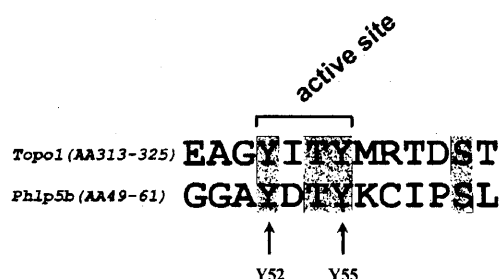


Fig. 5 Active Site of Topoisomerase I from *E. coli* (Topo I) Aligned to a Homologous Site in Allergen Phlp5b. Hatched boxes show identical amino acids.

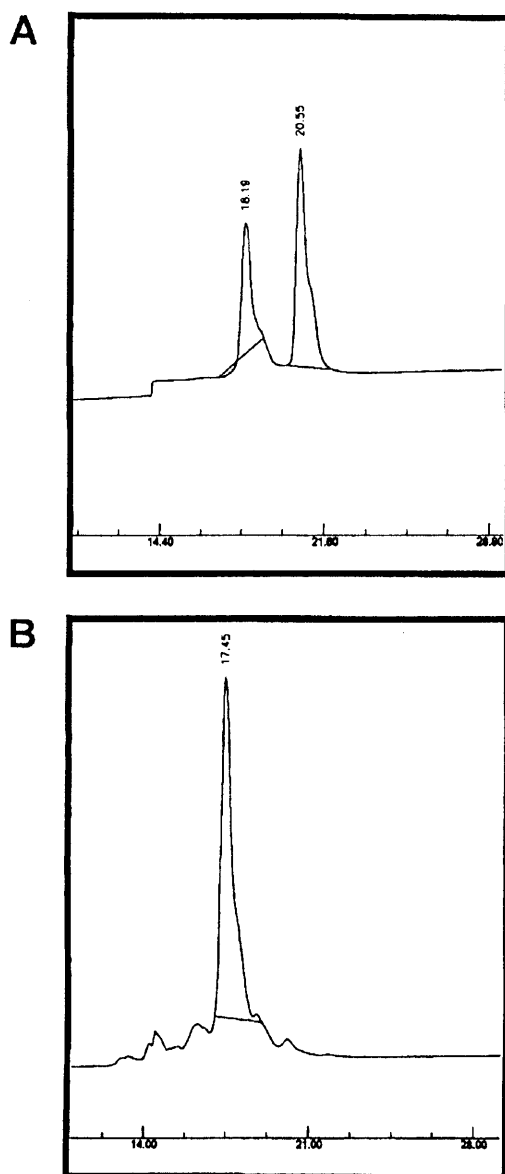


Fig. 6 Analytical HPLC Flow Sheet of Purified rPhlp5b-C-T-WT (A) and rPhlp5b-C-T-MUT (B). Time in minutes, peaks in mV voltage.

ase I from *Escherichia coli* (Tse-Dinh and Wang, 1986), intensively characterized and found to consist of a four amino acid stretch, shows high identity to a region in the Phlp5b-C-t sequence (Figure 5). The presence of this putative active site sequence in our nuclease further confirms the specificity of the demonstrated isomerase activity of Phlp5b-C-t.

Mutagenesis of the Putative Active Site of Phlp5b-C-t

Tyrosine residues have been shown to be actively involved in the cleavage of nucleic acids, resulting in a transesterification of an active site tyrosine creating a covalent 3'-phosphotyrosyl (DNA-enzyme) intermediate and liberating a 5' hydroxyl (5'-OH) (Burgin, 1997). To investigate the biological relevance of this putative active site of Phlp5b-C-t, the two tyrosines were changed to phenylalanine and aspartic acid, respectively (Y52F and Y55D), by site-directed mutagenesis. After the mutant had been cloned and checked by sequencing (data not shown), recombinant Phlp5b-C-t-Mut was expressed and purified following the same procedure as used for purification of recombinant Phlp5b-C-t. Figure 5 represents the analytical HPLC flow sheets of both the Phlp5b-C-t-WT (Figure 6A) and the mutant (Figure 6B). The WT showed two peaks, one of which, eluting at 18.19 min, probably represents the dimeric fraction whereas the second one at 20.55 min contains the monomeric protein (see Bufe *et al.*, 1994). The mutant protein was eluted only in one peak at 17.45 min, suggesting that the mutation led to a structural change of the molecule, possibly losing the potential to dimerize. This is confirmed by SDS-gel analysis of the mutant protein under non-reducing conditions showing no dimerization (data not shown). Furthermore, we can show that the mutant form lost its nuclease activity (Figure 7A and B). In contrast to Phlp5b-C-t-WT and the holoallergen the mutant did not degrade single-stranded M13 phage-mid DNA and did not uncoil double-stranded plasmid pQE. Taken together, one of the two, or both, tyrosine residues in the putative active site of Phlp5b-C-t-WT are involved in the cleavage of nucleic acids and also in the structural stabilization of protein dimers.

Reduced IgE-Binding Capacity and Retained Allergenicity of Phlp5b-C-t-Mut

We then wanted to know whether the mutations of Phlp5b-C-t-WT also led to changes of antibody-binding and altered allergenicity. Therefore Phlp5b-C-t-WT and the mutant were coupled to an ELISA-plate under equimolar conditions and binding capacity of IgG and IgE antibodies from 15 grass-pollen allergic patients was measured. No difference in IgG-binding between the WT and the mutant could be observed, in contrast IgE-binding to the mutant compared to the WT is significantly, but not completely, reduced (Figure 8). Allergenic activity was determined in a skin prick test assay, a procedure regarded as a relevant *in vivo* test for a specific allergic response.

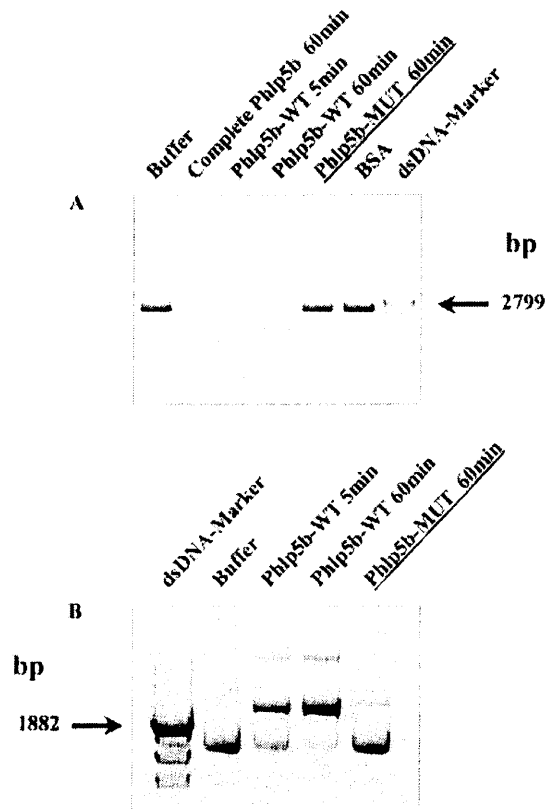


Fig. 7 Analysis of DNA Cutting Activity of rPhlp5b and Derivatives.

(A) Cleavage of ssDNA (1 µg of M13 phagemid) by holoallergen rPhlp5b, rPhlp5b-C-T-WT and rPhlp5b-C-T-MUT.

M13 was incubated at 37 °C for either 45 min or at the given times with (lane 1): buffer, (lane 2): 2 µl rPhlp5b (35 pmol), (lane 3/4): 1 µl rPhlp5b-C-t-WT (35 pmol), (lane 5): 1 µl rPhlp5b-C-t-MUT (35 pmol), (lane 6): 1 µl BSA (2 mg/ml).

(B) Cleavage of dsDNA (1 µg of pQE) by rPhlp5b-C-T-WT and rPhlp5b-C-T-MUT.

pQE was incubated at 37 °C for either 45 min or at the given times with (lane 2): buffer, (lane 3/4): 1 µl rPhlp5b-C-t-WT (35 pmol), (lane 5): 1 µl rPhlp5b-C-t-MUT (35 pmol). 1.2% agarose gel electrophoresis was performed as described earlier.

The results in Figure 9 show that the allergenicity of the mutant is retained because the allergic skin reaction is identical to that of the WT. In summary, the loss of nucleolytic activity of the grass pollen allergen does not lead to a reduction of its allergenic activity, although the capacity of IgE-binding is specifically and substantially diminished.

Discussion

The biological activity of allergens is becoming more and more the focus of interest because of a growing body of evidence that these activities may promote the immunological conditions necessary for specific sensitization toward allergens (Bufe, 1998). Many biological functions of

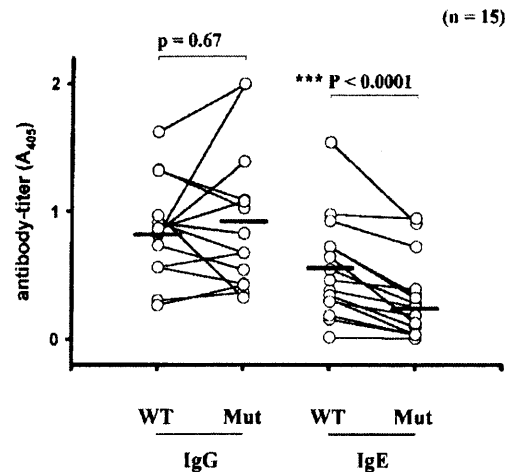


Fig. 8 Comparison of rPhlp5b-C-T-WT (WT) and rPhlp5b-C-T-MUT (Mut) Specific IgG- and IgE-Antibody Binding as Measured by ELISA (n = 15).

Lines represent the mean of the extinctions measured for the respective 15 values of IgG directed against Phlp5b-C-t-WT. IgG directed against Phlp5b-C-t-MUT, IgE directed against Phlp5b-C-t-WT and IgE directed against Phlp5b-C-t-MUT. Data were analyzed by Wilcoxon's two-tailed test. A $p < 0.05$ was defined to be statistically significant, p with *** is highly statistical significant.

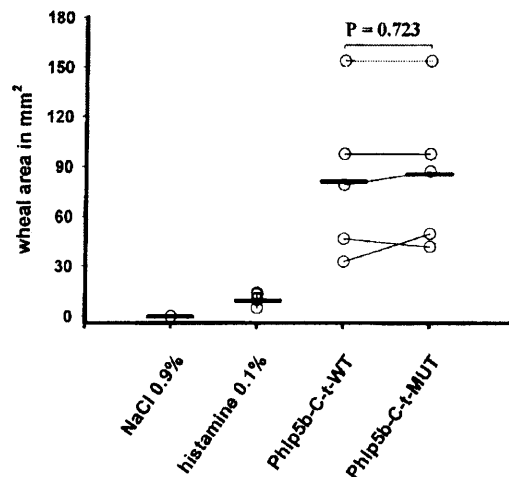


Fig. 9 Allergenic Activity of rPhlp5b-C-T-WT and rPhlp5b-C-T-MUT as Measured in the Skin Prick Test Assay (n = 5).

NaCl 0.9% was the negative control, histamine 0.1% the positive control. Lines represent the mean of the wheal areas induced in the respective 5 patients. NaCl 0.9% (negative control), histamine 0.1% (positive control), 20 µl Phlp5b-C-t-WT (1×10^{-6} M) and 20 µl Phlp5b-C-t-MUT (1×10^{-6} M). Data were analyzed using Wilcoxon's two-tailed test. A $p < 0.05$ was defined to be statistically significant.

allergens have been discovered by homology screening; for some, activity has been determined in biological assays. We have recently identified that two major allergens from grass and tree pollen have ribonuclease activity (Bufe *et al.*, 1994, 1996), a novel finding since nuclease activities

have not been associated with allergenic molecules so far. In this paper we specify the nucleolytic activity of the group V allergen of timothy grass (Phlp5b), one of the most important molecules inducing allergic sensitization and responses in many grass pollen allergic patients (Matthiesen and Löwenstein, 1991). We demonstrate that Phlp5b is a sugar-nonspecific nuclease that degrades ssRNA and ssDNA. Most interestingly the allergen does not cleave dsDNA but shows functional characteristics of a topoisomerase. No enzymes with topoisomerase activity from pollen have yet been described, so that the biological significance of this finding remains unknown. Phlp5b is located in the cytosol (Grote *et al.*, 1997) and is quickly released from the pollen grain once the cell is humidified (Vrtala *et al.*, 1993). As an RNase, it may be involved in the defense against viral infections (Matousek *et al.*, 1996). On the other hand it could as well participate in the process of germination (Knox, 1973) or possibly pollen-stigma recognition processes (Gaget *et al.*, 1984). Other authors have suggested that an additional biological function of topoisomerase may be the identification, through cleavage, of ribonucleotides misincorporated in DNA (Burgin, 1997). This could be one step of a RNA/DNA processing reaction necessary to provide enough nucleotides for an effective growth of pollen tubes. It is an interesting speculation that a nuclease such as our allergen, capable of catalyzing several different nucleolytic reactions, is important for a successful germination.

Active sites, usually found in ribonucleases, were not found in Phlp5b sequence. Therefore it is of note that a region highly homologous to the active site of topoisomerase I in *E. coli* was identified (Lynn and Wang, 1989). Since elimination of the tyrosine residues essentially involved in the cleavage of ssDNA resulted in the loss of activity, it is most probable that this active site is responsible for the nucleolytic properties of Phlp5b. Preliminary data from the crystal structure of Phlp5b-C-t (Rajashankar *et al.*, 1998) showed that Y52 is responsible for dimer formation. Therefore the mutation of both tyrosine residues necessarily led to the loss of dimerization. Further mutation experiments should demonstrate whether the catalytic domain is confined to Y55 or whether dimerization itself is essential for the nucleolytic activity. As the allergen is located mainly in the cytoplasm of pollen cells (Grote *et al.*, 1997) and, when exported, seems to act in the extracellular compartment, Phlp5b is not a classical isomerase as are the other known enzymes of this group. In contrast, it exhibits more features of a multivariable enzyme involved in different biological processes not yet defined.

With the construction of an enzymatically inactive variant of an allergen, we are now able to further analyze the influence of the biological activity of allergenic molecules on allergic sensitization and allergic response. Like other authors reporting on different allergens (Müller *et al.*, 1995), we could show that the inactive mutant of Phlp5b did not lose its allergenic activity in terms of inducing an allergic response. Although the IgE-binding capacity of the mutant is reduced, allergenicity is retained. This result con-

firms earlier observations showing that features other than IgE-binding alone are responsible for the allergenic activity of an allergen. It seems as if spacing of IgE-binding epitopes on the allergenic molecule, a certain molecular weight and globular structures are more important for effective crosslinking of IgE-antibodies on the effector cells (Bufe *et al.*, 1998). These features are obviously not changed when the nucleolytic activity of Phlp5b is knocked out by a defined site-directed mutation.

Thus we present more evidence that biological activity of allergens probably has no influence on the allergic response, but it may well be directly involved in the process of sensitization and the induction of allergic inflammation. There are several hints for this kind of activity as demonstrated for allergens with proteolytic activity (Schulz *et al.*, 1998). Up until now we have no evidence that the nucleolytic activity of Phlp5b would be responsible for the induction of a classical inflammatory process as observed in allergy. But it is interesting to note that one of the major toxic substances released from eosinophils, and inducing inflammatory reactions especially in allergic patients, the eosinophilic cationic protein (ECP), is a ribonuclease (Slifman *et al.*, 1986). Although the nucleolytic activity is not directly responsible for the toxic effect of ECP (Rosenberg, 1995), an allergen with nucleolytic activity that enters the human mucosa in high concentration might still be able to induce an inflammatory reaction in an as yet undescribed manner.

In summary, the biological activity of the major allergen Phlp5b from grass pollen may be involved in germination processes, has probably no influence on the allergic response, but may well induce the allergic inflammation and thus the sensitization toward allergens. Phlp5b-C-t-WT and its mutant will be helpful tools to analyze this activity *in vitro* and in animal model of allergy.

Materials and Methods

RNase Activity Gel and Double Stranded RNA Digestion

RNase activity gel was performed as described elsewhere (Bufe *et al.*, 1995). Analysis of double-stranded RNA cleavage was done according to the method described by Matousek *et al.* (1996).

Single- and Double-Stranded DNA Digestion and Agarose Gel

Single-strand phagemid M13 (Pharmacia LKB Biotechnology, Sweden) and double-strand plasmid pQE9 (Qiagen, Chatsworth, CA, USA) were purchased; DNA markers, nuclease DNase I and restriction enzyme *Sau3A* were from Boehringer (Mannheim, Germany), bovine serum albumin from ICN Pharmaceuticals (Irvine, CA, USA). 2.5 µg M13 phagemid or 1 µg pQE9 plasmid as substrates were incubated in appropriate restriction buffer (buffer H from Boehringer) for 30 min or given time with different substances in given concentrations. The mixture was then separated on a 1.2% agarose gel stained with 0.003% ethidium bromide.

Separation of Cleaved Single Stranded M13 DNA and Parallel Sequencing of M13 Plasmid

After incubation of M13 phagemid DNA for 30 min with different substances the restricted DNA was precipitated and used with a T7Sequencing kit (Pharmacia) in a slightly modified version: after annealing of fluorescence stained universal primer, a usual sequencing reaction was performed with T7-polymerase without adding dideoxy nucleotides. The reaction was stopped after 5 min and the different mixtures were separated and analyzed on an ALF automatic sequencer (Pharmacia, Uppsala, Sweden). Non-restricted M13 phagemid was sequenced in one direction parallel to the gel separation of the restricted fragments according to the dideoxy method of Sanger *et al.* (1977) using the T7-polymerase sequencing kit (Pharmacia).

Sequence data were analyzed on PCGENE Software (Intelligence, Geel, Belgium).

Site-Directed Mutagenesis

Site-directed mutagenesis of T52P (TAC to TTC) and T55D (TAC to AAC) in Phlp5b-C-t was performed by PCR amplification using pPhlp5bCt-WT (wild type) as template and two sequence-specific primers having exchanged the appropriate basepairs for the mutation in the anti-sense primer:

Phlp5bMut1-sens:
ATATGGATCCAAGATCCCCGCCGCGAG;

Phlp5bMut1-anti:
ATATCTCGAGGGATGGGATGCACTTGTGGTGTGAAGGCGC.

PCR conditions were: 95 °C 1 min, 56 °C 1 min, 72 °C 2 min, 30 cycles. The PCR product was purified from a 1.2% agarose gel, restricted with *Bam*HI and *Xho*I, and ligated into pPhlp5bCt-WT restricted with identical enzymes and cloned. The new plasmid was called pPhlp5bCt-mut1, the mutations were controlled by sequencing, and the recombinant proteins were purified by a method described previously (Gehlhar *et al.*, 1997).

Gel Filtration of Recombinant Proteins

Purified wild type (P5Ct-WT) and mutant (P5Ct-Mut1) were analyzed using gel-filtration on a Superdex 7S HR 10/30 column (Pharmacia) at room temperature as described elsewhere (Bufe *et al.*, 1995).

Phlp5b-Specific Antibodies by ELISA

Human IgG and IgE binding of 15 different sera (from patients with proven grass pollen allergy to P5Ct-WT and -Mut1) was tested using an ELISA-technique explained earlier (Gehlhar *et al.*, 1999).

Histamine Release Assay and Skin Prick Tests

Allergenicity of P5Ct-WT and -Mut1 was tested in a histamine release assay using basophils of a grass pollen allergic patient, and in Skin Prick Tests following the guidelines for clinical diagnosis of allergic diseases. Both procedures are described elsewhere (Bufe *et al.*, 1998).

Statistical analysis

Statistical analysis was carried out using the statistical program SPSS (SPSS GmbH Software, Munich, Germany). Paired data were analyzed by Wilcoxon's two-tailed test. A $p < 0.05$ was defined to be statistically significant.

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