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Generation of a low Immunoglobulin E-binding mutant of the timothy grass pollen major allergen Phl p 5a

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Clinical and Experimental Allergy

Summary

Background Immunotherapy of grass pollen allergy is currently based on the administration of pollen extracts containing natural allergens. Specifically designed recombinant allergens with reduced IgE reactivity could be used in safer and more efficacious future therapy concepts.

Objectives This study aimed to generate hypoallergenic variants of the timothy grass major allergen Phl p 5a as candidates for allergen-specific immunotherapy.

Methods Three deletion mutants were produced in *Escherichia coli* and subsequently purified. The overall IgE-binding capacity of the mutants was compared with the recombinant wild-type allergen by membrane blot and IgE-inhibition assays. The capacity for effector cell activation was determined in basophil activation assays. T cell proliferation assays with allergen-specific T cell lines were performed to confirm the retention of T cell reactivity. Structural properties were characterized by circular dichroism analysis and homogeneity by native isoelectric focusing. The deletion sites were mapped on homology models comprising the N- and C-terminal halves of Phl p 5a, respectively.

Results The double-deletion mutant rPhl p 5a $\Delta(94-113, 175-198)$ showed strongly diminished IgE binding in membrane blot and IgE-inhibition assays. Both deletions affect predominantly α -helical regions located in the N- and C-terminal halves of Phl p 5a, respectively. Whereas deletion of $\Delta 175-198$ alone was sufficient to cause a large reduction of the IgE reactivity in a subgroup of allergic sera, only the combination of both deletions was highly effective for all the sera tested. rPhl p 5a $\Delta(94-113, 175-198)$ consistently showed at least an 11.5-fold reduced capacity to activate basophils compared with the recombinant wild-type molecule, and the T cell proliferation assays demonstrated retention of T cell reactivity.

Conclusion The mutant rPhl p 5a $\Delta(94-113, 175-198)$ fulfils the basic requirements for a hypoallergenic molecule suitable for a future immunotherapy of grass pollen allergy; it offers substantially reduced IgE binding and maintained T cell reactivity.

Keywords allergen-specific immunotherapy, grass pollen group 5, hypoallergen, recombinant allergen

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Introduction

The grass sub-family *Pooideae* includes species that occur world-wide and that produce pollen that can trigger symptoms of allergic disease. Timothy grass (*Phleum pratense*) is one of the most important of these grasses [1], and it produces a number of allergens including the major allergen Phl p 5. This definition as a major allergen is based on the finding that IgE antibodies directed to Phl

p 5 have been detected in the sera of up to 85% of grass pollen-allergic individuals [1, 2].

Phl p 5 is a member of the group 5 allergen family, which includes highly homologous allergens of different grass species. It shows IgE-cross-reactivity to Phl p 6, another major allergen of timothy grass [3]. Natural Phl p 5 consists of two main isoforms, Phl p 5a and Phl p 5b [4], which are products of related, but separate genes [5, 6]. A number of naturally occurring variants of Phl p 5a and Phl

p 5b, consisting of 276–287 and 262–265 amino acids, respectively, were identified by cDNA cloning [5, 7].

Immunotherapy for individuals having grass pollen allergy is performed routinely by administration of pollen extracts containing natural allergens or chemically modified extracts with reduced IgE-binding capacity (allergoids) [8–10]. An important development involves the use of recombinant allergens, which can be produced in high purity and are much easier to standardize [11, 12]. Methods of genetic engineering provide an opportunity to optimize the recombinant proteins for therapeutic use, and the generation of low IgE-binding (hypoallergenic) recombinant allergens especially is one focus of research for a future immunotherapy with a reduced risk of adverse reactions [13, 14].

This study aimed to generate recombinant hypoallergenic variants of Phl p 5a by the introduction of deletions. The decision for a deletion strategy was based primarily on the successful modification of Phl p 5b [15]. We focused on two sequence regions of Phl p 5a, amino acids 94–113 and 175–198 (with reference to NCBI protein database entry CAD87529), which were defined outside previously mapped T cell epitopes [15, 16]. These considerations were based on the fact that allergen-specific immunotherapy is known to act at the T cell level [17, 18].

The single-deletion mutants rPhl p 5a Δ 94–113 and rPhl p 5a Δ 175–98, as well as the corresponding double-mutant rPhl p 5a Δ (94–113, 175–198) were expressed in *Escherichia coli*, subsequently purified and tested for their IgE-binding capacity compared with the recombinant wild-type Phl p 5a. The most promising molecule, the double-deletion mutant rPhl p 5a Δ (94–113, 175–198), was further characterized concerning its capacity for basophil activation, T cell reactivity, isoelectric point and structure formation.

Materials and methods

DNA constructs

PCR was conducted with *Pwo* polymerase (Peqlab, Erlangen, Germany) using a 'T-Gradient' cycler (Biometra, Göttingen, Germany). PCR primers were purchased from Metabion (Martinsried, Germany). The vector pProExHt (Invitrogen, Carlsbad, CA, USA) was used to obtain proteins with an N-terminal hexahistidine tag and a TEV-protease cleavage site.

Plasmid pProExHt rPhl p 5a Δ 175–198. Phl p 5a wild-type encoding cDNA (GenBank entry AJ555152), cloned into an *EheI*/*HindIII* restricted pProExHt vector (plasmid pProExHt Phl p 5a wt) as previously described for a Phl p 5b isoform [11], served as a PCR template for the generation of DNA encoding rPhl p 5a Δ 175–198. At first, two DNA fragments were generated separately. Fragment 1

was generated using primer 1 (sense, gcc gat cta ggc tac ggc ccg gcc) and primer 2 (antisense, aac ata act agt ggc agc gac ctt gaa ggc ggc gtc), and fragment 2 using primer 3 (sense, atc taa cta gta cgg gcg gcg ccta cga g) and primer 4 (antisense, aac ata *aag ctt* tca gac ttt gta gcc acc agt). The PCR products were ligated at their joined *SpeI* site (primers 2 and 3, underlined). The product was amplified with primers 1 and 4 restricted at the *HindIII* site (primer 4, italics), and finally cloned into the *EheI*/*HindIII*-restricted pProExHt vector.

Plasmid pProExHt rPhl p 5a Δ 94–113. Deletion 94–113 was introduced by connecting two cDNA fragments by a PCR technique. In a first round of PCR, fragment 1 was generated using primers 1 and 5 (antisense, gga gct gga ttc ggc ggc gcc ctt ggg, template pProExHt Phl p 5a wt) and fragment 2 using primers 4 and 6 (sense, gcc gcc gaa tcc agc tcc ggc gcg acg cct gag gcc aag tac gac, template pProExHt Phl p 5a wt). The amplified fragments shared a common sequence bridging the deletion site. They were connected in a second round of PCR using primers 1 and 4. The resulting cDNA was cloned as described above.

Plasmid pProExHt rPhl p 5a Δ (94–113, 175–198). The cDNA was prepared in a similar way as described for rPhl p 5a Δ 94–113. Fragment 1 was generated with primers 1 and 5 (template pProExHt Phl p 5a wt) and 2 with primers 4 and 6 (template pProExHt rPhl p 5a Δ 175–198), respectively. They were connected by PCR amplification with primers 1 and 4 and the resulting cDNA was cloned into pProExHt as described above.

Expression and purification of proteins

The recombinant proteins were expressed as fusion proteins with an N-terminal histidine tag in the cytosol of *E. coli* strain JM109 (Promega, Madison, WI, USA). Protein synthesis was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (Merck, Darmstadt, Germany) at 37 °C for 3 h. The obtained cell pellet was sonicated for lysis in 25 mM sodium phosphate buffer, pH 7.5, 50 mM NaCl, 20 mM imidazole (Merck), before centrifugation. The clarified supernatant was applied to a 12.5 mL Ni²⁺-charged HiTrap Chelating column (GE Healthcare, Chalfont St Giles, UK) to capture tagged proteins specifically. The column was eluted with 50 mM sodium phosphate buffer, pH 7.5, 50 mM NaCl, 0.5 M imidazole. Finally, fusion proteins were polished by size exclusion chromatography (SEC) using a Superdex 75 column (GE Healthcare) with 25 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl. Effector cell activation, T cell proliferation and pI determination were investigated with tag-free proteins obtained after cleavage of the histidine tag using histidine-tagged TEV protease (Invitrogen) in accordance with the recommendations of the manufacturer. The recombinant

proteins were separated from the protease and the histidine tag by collecting the flow-through of a second affinity chromatography step, which was performed as before. The proteins were further purified by SEC as described above.

Natural Phl p 5a was purified from a *P. pratense* extract by affinity chromatography using the Phl p 5a-specific monoclonal antibody BG 6 [19]. The extract was passed through a column with the antibody covalently coupled to Protein G Sepharose (GE Healthcare). After washing, nPhl p 5a was eluted with 5 mM Tris/glycine, pH 2.5. Eluted fractions were neutralized immediately.

Analytical size exclusion chromatography, sodium dodecyl sulphate polyacrylamide gel electrophoresis and native isoelectric focusing

For analytical SEC, 30–80 µg of recombinant protein was loaded onto a Superdex 75 HR 10/30 column (GE Healthcare) and eluted with 20 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl.

The column was calibrated with albumin, ovalbumin, chymotrypsinogen A and ribonuclease A (GE Healthcare) using the same buffer. For SDS-PAGE analysis, purified recombinant allergens (1.5 µg/lane, without reduction agent) were resolved on 10% NuPAGE Bis-Tris gels using 1 × MES-buffer (Invitrogen). Native isoelectric focusing (IEF) was performed by resolving the non-fusion proteins (5 µg/lane) on non-denaturing Novex pH 3–10 gels (Invitrogen) according to the manufacturer's instructions. Marker proteins were purchased from GE Healthcare.

Sera and whole-blood samples

Sera and whole-blood samples were obtained from 43 grass pollen-allergic subjects (30 males, 13 females; P1–43). All allergic donors had a clinical history of grass pollen allergy and EAST classes of ≥ 3 for grass pollen-specific IgE (Specific IgE ELISA-RV; Allergopharma, Reinbek, Germany). The age of the patients was in the range of 21–64 years (mean = 37 ± 10.6 years). The donors did not receive corticosteroids during the 4 weeks before the blood donation and were not under the effect of antihistamines.

Immunoblotting

Molecule-specific IgE reactivity was tested with blot strips containing an array of non-denatured recombinant allergens. Briefly, a nitrocellulose membrane (Protran BA-79, Schleicher and Schuell, Dassel, Germany) was pre-incubated in 25 mM Tris, pH 8.5, containing 20% methanol (v/v) and clamped in a multiblot-apparatus MN45 (Bio-metra). The distinct parallel conduits of the apparatus were filled with the proteins dissolved in phosphate-buffered saline (PBS) (200 µg/mL). Following incubation

for 2 h, the conduits were washed with Tris-buffered saline containing 0.05% Tween 80 (TBST). The membrane was cut into strips, which were subsequently blocked by incubation in TBST for 1 h. These strips were incubated overnight in parallel with different individual sera from grass pollen-allergic blood donors (1:3 in TBST). Alkaline-phosphatase-conjugated anti-human IgE (Allergopharma) was used as a secondary antibody. Protein/antibody complexes were visualized with a mixture of NBT and BCIP substrates (Invitrogen).

IgE-inhibition assays

Microtitre plates were coated with 1 µg/mL purified natural Phl p 5a in NaHCO₃ buffer, pH 9.6, overnight. After washing with PBS containing 0.05% Tween 20 (PBST), the plates were blocked with PBST containing 1% BSA. Sera were diluted with PBST containing 0.1% BSA and were pre-incubated in duplicate with serial dilutions of inhibitors (maximum concentration: 20 µg/mL) for 1 h. The samples were applied to the washed plates and incubated for 2 h at room temperature. After further washing steps, an anti-human IgE alkaline phosphatase conjugate (Allergopharma) was allowed to bind for 1 h at room temperature. Detection was performed by incubation with *p*-nitrophenylphosphate (Sigma, Deisenhofen, Germany). Optical density was measured with an ELISA plate reader (ICN, Meckenheim, Germany) at 405 nm. Inhibition was calculated for each dilution using the formula: % inhibition = $(A_1 - \text{NSB}) - (A_2 - \text{NSB}) / (A_1 - \text{NSB})$, where A_1 is the average OD of the positive control wells without inhibitor and A_2 is the average OD of the duplicate wells with an inhibitor. NSB represents the average OD of the negative control wells for unspecific binding (sample without serum). The IgE binding of the rPhl p 5a mutants was too low to perform calculations of allergenic potency by using either a parallel line calculation or the ratio of 50% inhibition [20]. Therefore, the inhibition values determined at 1.25 and 5 µg/mL inhibitor concentration of each single experiment are presented in Table 1.

Basophil activation assays

Basophil activation was detected by measurement of the level of expression of the marker CD203c using flow cytometry as described in detail previously [21]. Briefly, heparinized whole-blood samples were stimulated with serial dilutions of allergens and derivatives in Ultraculture medium (Cambrex, Verviers, Belgium) for 15 min at 37 °C. Activation was stopped by adding PBS with 20 mM EDTA. Labelling of the basophils was performed with CD203c-PE (Beckman Coulter, Krefeld, Germany) for 45 min at room temperature. The lysis of red blood cells was performed with the whole-blood lysing kit (Beckman Coulter) according to the instructions. After washing, cells were analysed

Table 1. IgE-inhibition assay results

Inhibitor	% inhibition*							
	P 4	P 8	P 9	P 10	P 13	P 15	P 20	P 26
nPhl p 5a	84 (73)	84 (78)	95 (89)	86 (74)	96 (84)	92 (83)	95 (84)	94 (84)
rPhl p 5a wt	74 (70)	75 (71)	91 (86)	66 (60)	89 (84)	86 (82)	92 (82)	93 (88)
rPhl p 5a Δ 94–113	50 (44)	59 (56)	83 (77)	61 (54)	73 (66)	62 (54)	92 (84)	47 (45)
rPhl p 5a Δ 175–198	37 (33)	32 (33)	25 (22)	11 (5)	25 (22)	33 (33)	13 (12)	42 (35)
rPhl p 5a Δ (94–113, 175–198)	16 (14)	23 (20)	16 (12)	11 (7)	19 (8)	15 (15)	10 (8)	2 (5)

*Percentage inhibition values at 5 and 1.25 μ g/mL (in parentheses) inhibitor concentration obtained with sera from eight grass pollen-allergic subjects (P). wt, wild-type.

in a flow cytometer (Coulter EPICS XL-MCL ADC, Beckman Coulter). Counting time was adjusted to analyse at least 500 basophils. Specifically activated basophils were calculated as the maximum number of activated basophils obtained by allergen stimulation minus the number of activated basophils in the non-stimulated control sample. Dose–response curves were calculated from single values using 'GraphPad Prism 4.00' (GraphPad, San Diego, CA, USA). The allergen concentration needed to activate 50% of the basophils [C_{50} (pM)] was calculated from the dose response curves. The relative potency (P_{rel}) was defined as the quotient of the C_{50} values of the reference rPhl p 5a wt and rPhl p 5a Δ (94–113, 175–198).

T cell lines and proliferation assays

T cell lines (TCL) were raised from peripheral blood mononuclear cells (PBMC) of grass pollen-allergic individuals with a clinical history of hayfever and EAST classes ≥ 3 (EAST-RV, Allergopharma) with timothy grass pollen extract. The isolation of PBMC was performed with lymphocyte separation media (PAA, Coelbe, Germany) in Leucosep tubes (Greiner, Frickenhausen, Germany) according to the instructions of the manufacturers. All T cell cultures and stimulation experiments were performed in serum-free Ultraculture medium (Cambrex) supplemented with 2 mM Ultraglutamine I (Cambrex), antibiotic–antimycotic solution (Sigma) and 20 μ M mercaptoethanol (Invitrogen). TCL were raised by seeding 1×10^5 PBMC and 10 μ g of nPhl p 5/mL in a 96-well round-bottom culture plate in a total volume of 100 μ L/well. After 5–7 days, the cultures were fed by adding 100 μ L medium with 25 U/mL IL-2 (Strathmann Biotec, Hamburg, Germany). Three to 4 days later, half of the medium was exchanged against fresh medium containing 25 U IL-2/mL and incubated for another 4 days. Fourteen days after set-up of these cultures, a proliferation assay was performed in order to detect allergen-specific T cells. Cultures with stimulation indices of at least two represent a TCL and were expanded. For the proliferation assays, T cells were seeded at 2×10^4 cells/well in a 96-well culture plate in triplicate under stimulation with the allergen (0.3 μ M) and

5×10^4 mitomycin C-treated autologous PBMC/well. After a 48-h incubation at 5% CO_2 , 37 °C and a humidified atmosphere, 1 μ Ci ^3H -thymidine (GE Healthcare) was added to each well and further incubated for 16 h. Cells were harvested on microbeta filter mats with a 96-well cell harvester (Perkin Elmer, Colonia, Germany). The stimulation index (SI) was calculated as the quotient of c.p.m. of the unstimulated control (T cells and mitomycin C-treated autologous PBMC) and allergen stimulation.

Homology modelling and circular dichroism analysis

The 3D model for the N-terminal half of Phl p 5a (amino acids 31–144; NCBI protein database entry CAD87529) was calculated on the basis of a homology alignment with Phl p 6, a highly homologous molecule with published 3D structure (Fedorov et al., 2003; RCSB Protein Data Bank (PDB) entry 1NLX). The amino acid sequence identity in the compared region was 56.7%. The model of the C-terminal half of Phl p 5a (amino acids 154–255) was calculated following a 3D structure of a truncated Phl p 5b molecule [22] (PDB: 1L3P), using a sequence alignment with 79.4% identity. Modelling was performed by using the software 'DS Modeler', a module of 'Discovery Studio Modelling' (Accelrys, San Diego, CA, USA). Circular dichroism (CD) measurements were carried out on a Jasco J-715 spectropolarimeter using 0.02 and 0.05 cm path-length cells equilibrated at 20 °C.

Spectra were recorded with a 0.2 nm resolution at a 50 nm/min scan speed and resulted from averaging three scans. Results were expressed as the molar mean residue ellipticity at a given wavelength. The data were fitted with the secondary structure estimation program according to Yang et al. [23].

Results

rPhl p 5a Δ (94–113, 175–198) shows markedly reduced overall immunoglobulin E binding

The deletion mutants and recombinant wild-type Phl p 5a (rPhl p 5a wt) were purified as soluble histidine-tagged

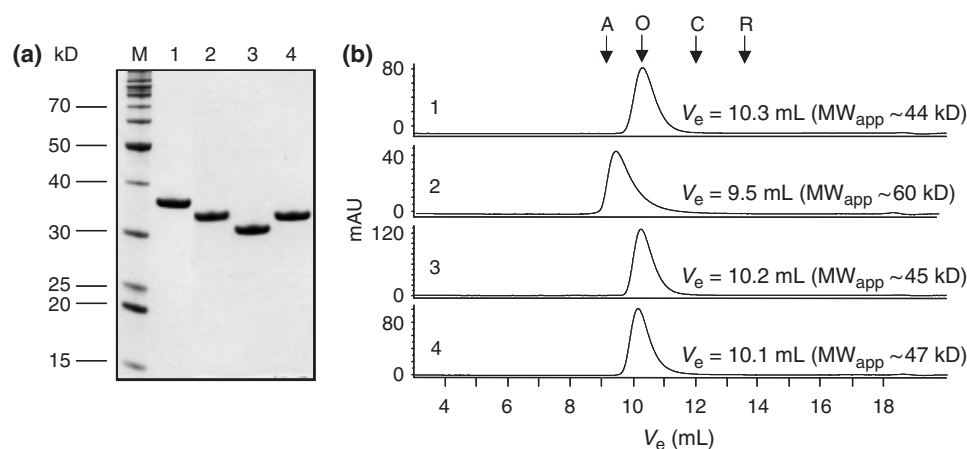


Fig. 1. Analysis of histidine-tagged rPhl p 5a wt (1), mutants $\Delta 94-113$ (2), $\Delta(94-113, 175-198)$ (3) and $\Delta 175-198$ (4). (a) Coomassie-stained 10% Bis-Tris SDS-PAGE (M: molecular weight marker). (b) Analytical SEC. Arrows indicate the elution volume (V_e) of the calibration proteins albumin (A; ~67 kD), ovalbumin (O; ~43 kD), chymotrypsinogen A (C; ~25 kD) and ribonuclease A (R; ~13.7 kD), respectively. MW_{app} , apparent molecular weight; wt, wild-type.

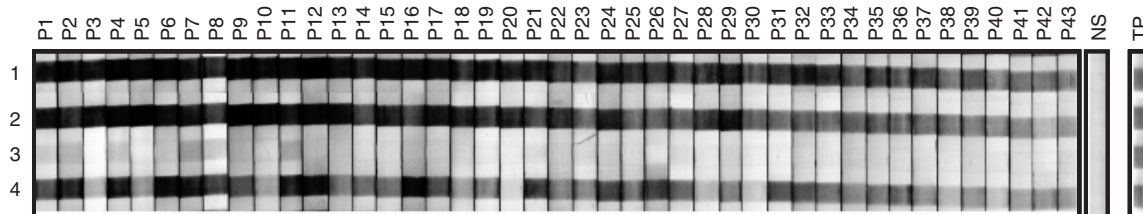


Fig. 2. Blot assay for IgE binding. Membrane strips loaded with non-denatured histidine-tagged rPhl p 5a wt (1), mutants $\Delta 94-113$ (2), $\Delta(94-113, 175-198)$ (3) and $\Delta 175-198$ (4) were incubated in parallel with sera of allergic subjects (P). NS, Sera of a non-allergic subject; TP, total protein staining. wt, wild-type.

proteins. The proteins migrated as single bands in SDS-PAGE analysis and eluted as single peaks in analytical SEC. Neither degradation products nor aggregates were observed (Fig. 1). An immunoblotting assay was performed initially to obtain a rough estimate of the molecule-specific IgE reactivity. Membrane strips containing an array of non-denatured recombinant allergens were incubated in parallel with 43 individual sera from grass pollen-allergic subjects. All the sera contained IgE directed to rPhl p 5a wt (Fig. 2). The IgE binding to the mutant rPhl p 5a $\Delta 175-198$ was strongly reduced in many cases compared with the wild-type molecule (e.g. P3, P10, P20). The deletion of site 94–113 seemed to be less effective in terms of reducing IgE reactivity, but a minor effect was observed with several of the sera examined (e.g. P31–33). IgE binding to the double-deletion mutant was consistently the lowest of all molecules tested. Most of the sera gave no signal, but a few produced a weak signal (e.g. P7, P8, P11).

The immunoblotting technique has at least a theoretical risk of inducing misfolding of the proteins due to the membrane contact. Therefore, we also conducted IgE-inhibition assays in solution with the sera of eight grass

pollen-allergic individuals (Fig. 3; Table 1). The proteins were used at different concentrations to inhibit the binding of serum IgE to natural Phl p 5a (nPhl p 5a) immobilized on microtitre plates. nPhl p 5a used as an inhibitor was able to inhibit the IgE binding to its immobilized counterpart completely (e.g. P20, Fig. 3), but the concentration needed was dependent on the degree of sensitization of the allergic subject (e.g. 84–96% inhibition at 5 μ g/mL nPhl p 5a; Table 1).

Two groups of sera could be distinguished by their IgE-binding characteristics. The first group, represented by P20 in Fig. 3, showed no major differences between the overall IgE binding of rPhl p 5a $\Delta 94-113$ and the recombinant wild-type allergen while the IgE binding to rPhl p 5a $\Delta 175-198$ and to rPhl p 5a $\Delta(94-113, 175-198)$ was considerably lower (Table 1; P10 and 20).

The second group, represented by P4 in Fig. 3, differed from the former in a clearly diminished IgE binding to rPhl p 5a $\Delta 94-113$ compared with the recombinant wild-type allergen. Furthermore, rPhl p 5a $\Delta 175-198$ bound consistently more IgE than the double-deletion mutant, but less than rPhl p 5a $\Delta 94-113$ (Table 1: P4, P8, P9, P13, P15 and P26).

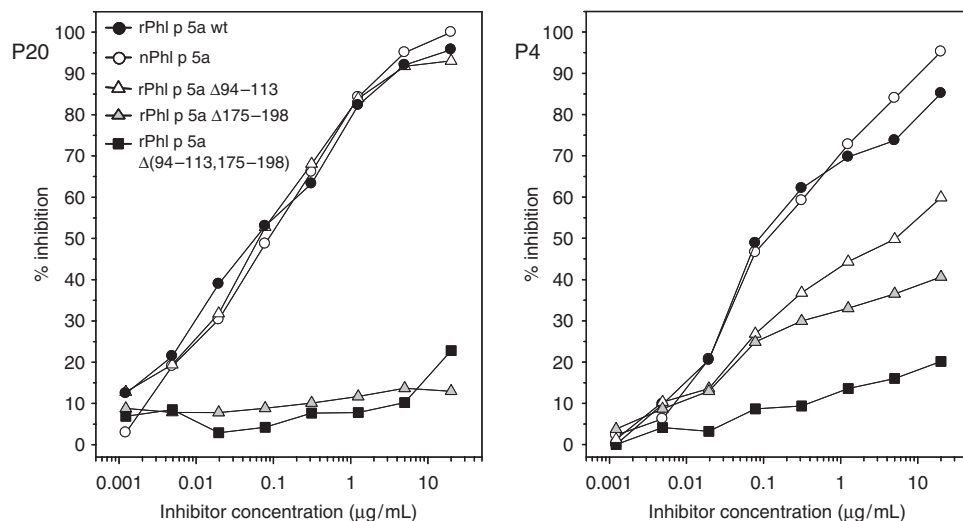


Fig. 3. Results of IgE-inhibition tests with sera of allergic subjects P20 (left) and P4 (right). Symbols represent the mean values of duplicates obtained at eight concentrations of histidine-tagged proteins or nPhl p 5a, wt, wild-type.

The results of the IgE-inhibition assays are in good accordance with the immunoblot experiments. Both single-deletion mutants express less IgE-binding activity than the recombinant wild-type protein, and furthermore $\Delta 175-198$ has a greater effect than $\Delta 94-113$. The double-deletion mutant rPhl p 5a $\Delta(94-113, 175-198)$ has the lowest IgE-binding capacity.

rPhl p 5a $\Delta(94-113, 175-198)$ induces lower effector cell activation

The basophil-activating capacity of the double-deletion mutant compared with wild-type allergens was tested with whole blood from 12 allergic subjects. nPhl p 5a and rPhl p 5a wt induced activation of basophils to a similar extent (Fig. 4; Table 2). The calculated C_{50} values were 0.26–10.15 and 1.26–14.66 pM, respectively (Table 2). In the case of the double-deletion mutant, markedly higher concentrations ($C_{50} = 17.3-8433$ pM) were needed to achieve the same degree of basophil activation. The calculated potency (P_{rel}) of rPhl p 5a $\Delta(94-113, 175-198)$ relative to rPhl p 5a wt ranged between 0.0002 and 0.0873 (Table 2).

rPhl p 5a $\Delta(94-113, 175-198)$ stimulates T cell lines

Table 3 shows the results from T cell proliferation assays with Phl p 5-specific T cell lines originating from 10 allergic individuals. In general, rPhl p 5a $\Delta(94-113, 175-198)$ stimulated T cell proliferation as well as the natural and recombinant wild-type allergens as indicated by similar stimulation indices. Despite certain T cell line-specific variations in the degree of the response, the results indicate an almost complete retention of T cell reactivity in the hypoallergenic molecule.

Structural properties of rPhl p 5a $\Delta(94-113, 175-198)$

Amino acid sequence analysis of Phl p 5a/b has revealed a remarkable sequence similarity of two internal stretches, comprising most of the N- and C-terminal halves, respectively [22]. The N-terminal sequence stretch also shows high homology to Phl p 6 [3]. X-ray diffraction analysis of a truncated Phl p 5b comprising the C-terminal half (PDB: 1L3P) and of Phl p 6 (PDB: 1NLX) has shown that both molecules are folded to a four-helix bundle [22, 24]. However, a 3D structure for the whole Phl p 5a or Phl p 5b or any other group 5 allergen is not available.

We generated two separate homology models of the N- and the C-terminal halves of Phl p 5a using the structures of Phl p 6 and the truncated Phl p 5b as templates to predict the locations of the deletion sites $\Delta 94-113$ and $\Delta 175-198$ on Phl p 5a (Fig. 5). Both deletion sites span predominantly α -helical regions, located in the N-terminal ($\Delta 94-113$; helix 3) and C-terminal four-helix bundle ($\Delta 175-198$; helix 6, and a minor part of helix 5), respectively.

The mutant rPhl p 5a $\Delta(94-113, 175-198)$ and rPhl p 5a wt were analysed by CD spectroscopy. The 20 °C spectra are characterized by two minima at 208 and 222 nm and a characteristic maximum at about 195 nm (Fig. 6a). The CD spectra of both proteins are therefore typical for molecules with a high α -helical structure content. The secondary structure estimations resulting from the fitting procedure yielded a 46.9% α -helical content for the wild-type protein in contrast to 30.5% for the rPhl p 5a $\Delta(94-113, 175-198)$. The mutant migrated as a single band in native isoelectric focusing, indicating a high homogeneity concerning its total surface charge (Fig. 6b). The apparent pI value for rPhl p 5a wt is ~ 8.4 compared with only ~ 6.4 for rPhl p 5a $\Delta(94-113, 175-198)$. This observation is in

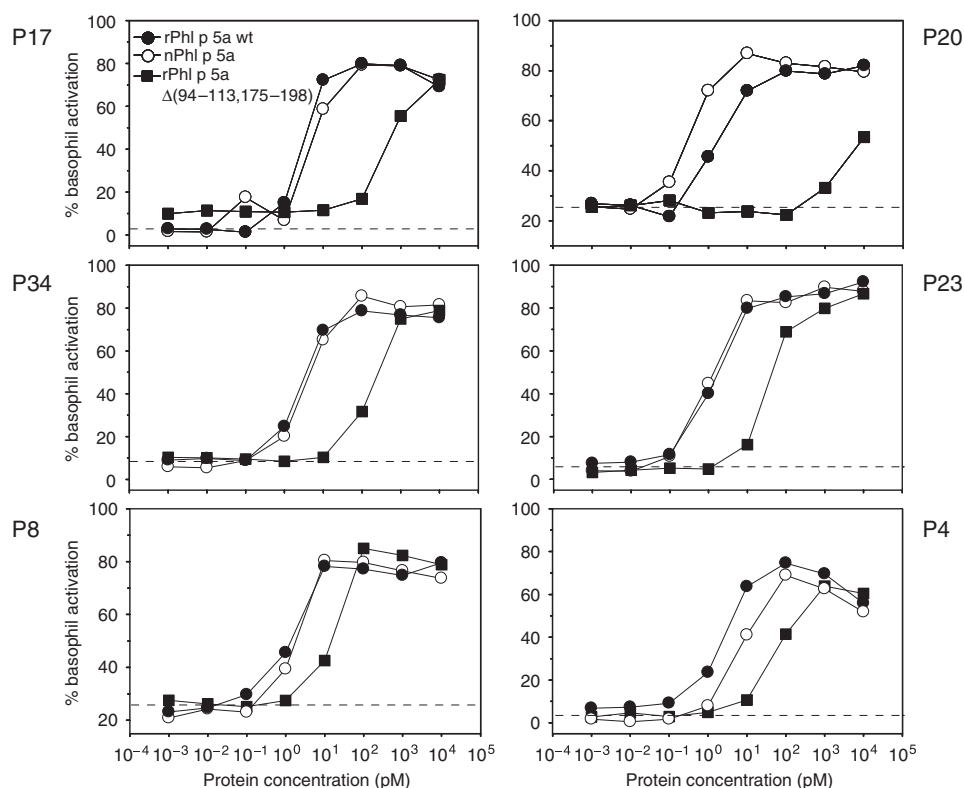


Fig. 4. Basophil activation test results obtained with tag-free rPhl p 5a $\Delta(94-113, 175-198)$, rPhl p 5a wt and nPhl p 5a, respectively. Results from experiments with peripheral blood mononuclear cells of six allergic individuals (P). wt, wild-type.

Table 2. Individual basophil activation test results of 12 grass pollen-allergic subjects (P)

	C_{50} (pM)*			rPhl p 5a $\Delta(94-113, 175-198)$ to rPhl p 5a wt	
	nPhl p 5a	rPhl p 5a wt	rPhl p 5a $\Delta(94-113, 175-198)$	$P_{rel}^{\dagger}$	x fold reduction ‡
P 4	9.30	2.20	95.7	0.0230	43.5
P 8	1.55	1.51	<u>17.3</u>	<u>0.0873</u>	<u>11.5</u>
P 13	4.08	5.34	477.2	0.0112	89.4
P 17	6.44	2.68	466.6	0.0057	174.1
P 20	<u>0.26</u>	1.68	<u>8433.0</u>	<u>0.0002</u>	<u>5019.6</u>
P 23	1.02	<u>1.26</u>	39.2	0.0321	31.1
P 24	1.22	2.57	58.1	0.0442	22.6
P 28	9.43	11.35	198.2	0.0573	17.5
P 29	1.77	2.34	33.7	0.0694	14.4
P 31	<u>10.15</u>	<u>14.66</u>	3967.0	0.0037	270.6
P 34	3.48	2.54	165.1	0.0154	65.0
P 40	1.08	1.45	17.5	0.0829	12.1

*Defined as the concentration needed to activate 50% of specifically activated basophils.

† Relative potency, calculated by C_{50} of rPhl p 5a wt/ C_{50} rPhl p 5a $\Delta(94-113, 175-198)$.

‡ Reduction factor, calculated by C_{50} of rPhl p 5a $\Delta(94-113, 175-198)$ / C_{50} rPhl p 5a wt.

Underlined: minimum and maximum values. wt, wild-type.

line with the decline of the calculated pI values from ~ 8.1 to ~ 6.1 for rPhl p 5a wt and rPhl p 5a $\Delta(94-113, 175-198)$, respectively.

Discussion

In this study, we have demonstrated the successful generation of a low IgE-binding Phl p 5a variant by the introduction of deletions, a technique that was also applied successfully to the shorter isoform Phl p 5b [15]. Apart from the high homology displayed by the two isoforms, there are distinct differences in their IgE and T cell reactivity, and therefore both forms have to be considered for inclusion in a therapeutic formulation [12, 25, 26].

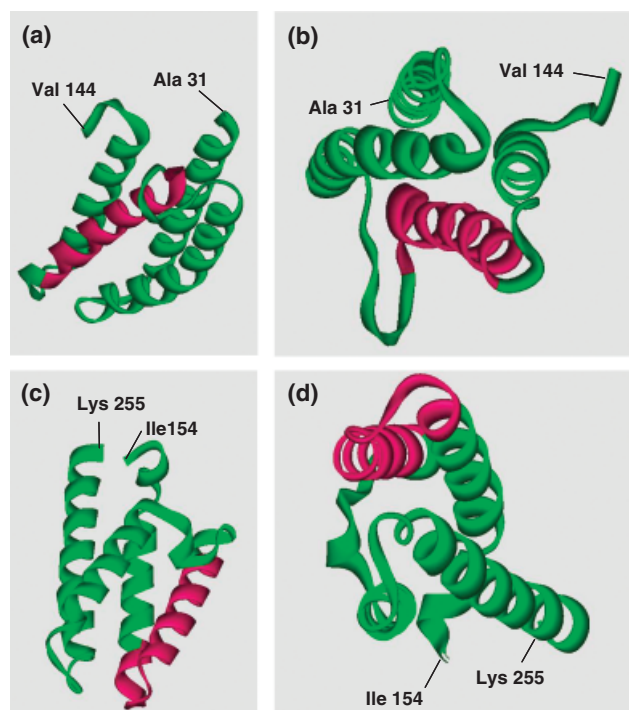
The double-deletion mutant rPhl p 5a $\Delta(94-113, 175-198)$ bound consistently much less IgE than the wild-type allergen as demonstrated by an immunoblot assay and IgE-inhibition tests. Furthermore, the capability of the mutant to activate effector cells was strongly reduced as shown by basophil activation assays. The calculated potency of rPhl p 5a $\Delta(94-113, 175-198)$ was consistently at least 11.5-fold lower than the recombinant wild-type allergen. This confirms the hypoallergenic property of the mutant in a cell-based assay that is closer

Table 3. Proliferative response of Phl p 5-specific T cell lines from 10 grass pollen-allergic individuals (A–J)

T cell line	No allergen		nPhl p 5a		rPhl p 5a wt		rPhl p 5a Δ (94–113, 175–198)	
	c.p.m.*		c.p.m.	SI†	c.p.m.	SI	c.p.m.	SI
A (3.2)	663		6520	9.8	3235	4.9	2908	4.4
B (8.2)	360		7548	21.0	5561	15.5	4798	13.3
C (11.2)	812		4211	5.2	3800	4.7	5815	7.2
C (11.3)	1897		6220	3.3	5471	2.9	6656	3.5
D (19.1)	791		5141	6.5	3698	4.7	5948	7.5
D (19.2)	373		3566	9.6	1247	3.3	969	2.6
E (23.22)	371		8091	21.8	10740	29.0	7732	20.8
E (23.50)	358		2698	7.5	2997	8.4	2373	6.6
F (59.57)	184		1981	10.8	2184	11.9	2096	11.4
G (65.15)	2239		6333	2.8	8873	4.0	9663	4.3
H (70.68)	75		2013	26.8	2174	29.0	2395	31.9
I (89.23)	194		352	1.8	679	3.5	339	1.8
J (116.34)	87		1290	14.8	1260	14.5	1039	11.9

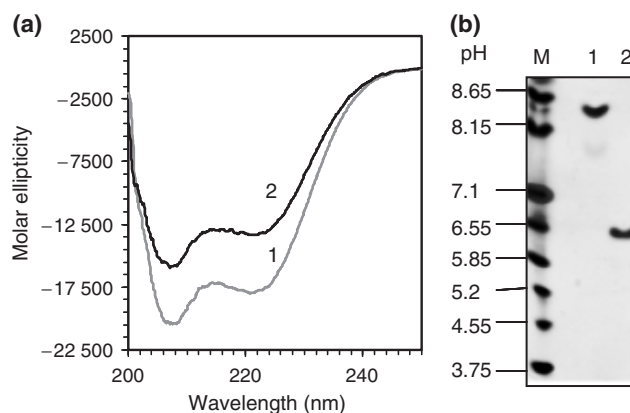
*Mean c.p.m. values from triplicates.

†Stimulation index, calculated by c.p.m. obtained with allergen/c.p.m. of unstimulated control. wt, wild-type.

**Fig. 5.** Homology models of Phl p 5a. (a) and (b) N-terminal model following Phl p 6 (PDB: 1NLX). (c) and (d) C-terminal model following a Phl p 5b fragment (PDB: 1L3P). Deletion sites are coloured in red.

to the *in vivo* situation than the immunoblot and IgE-inhibition assays.

The introduction of mutations carries the risk of generating molecules with undesirable biochemical proper-

**Fig. 6.** (a) CD spectra obtained with tag-free rPhl p 5a wt (1) and rPhl p 5a Δ (94–113, 175–198) (2). (b) Coomassie-stained native isoelectric focusing gel (pH 3–10) with these proteins (M, marker proteins). wt, wild-type.

ties like poor stability, insolubility or aggregation. Phl p 5a Δ (94–113, 175–198) could be obtained in a soluble form and neither degradation products nor aggregates were observed by analytical size exclusion chromatography, SDS-PAGE or native isoelectric focusing. Therefore, we can conclude that the low IgE-binding capacity of the mutant is not attributable simply to degradation or aggregation processes but to loss of IgE epitopes as a result of the deletions.

As there is no 3D model available that comprises the whole sequence of any group 5 allergen, we can only work on the hypothesis that Phl p 5a forms a two-domain structure with two connected four-helix bundles rather than one multi-helix structure [22]. The two separate homology models of the putative N- and the C-terminal domains of Phl p 5a that we generated on the basis of available structural data for Phl p 6 and a C-terminal fragment of Phl p 5b show that the two deletion sites Δ 94–113 and Δ 175–198 span predominantly α -helices, which are participating in the formation of four-helix bundles. Deletion Δ 94–113 (corresponding to helix 3) is located in the N-terminal and deletion site 175–198 (correspond to helix 6 and a small part of helix 5) in the C-terminal four-helix bundle. It is remarkable that the double-deletion mutant is folded to a stable and soluble structure despite the deletion of these large α -helical sequence stretches. The mutant may form at least in part α -helical secondary structures similar to the wild-type as indicated by the CD measurements, but these are most probably rearranged in a different tertiary structure.

The IgE-inhibition and immunoblot data demonstrated that only the combination of both deletions was highly effective in terms of reducing the IgE reactivity. Deletion Δ 175–198 alone clearly reduced IgE binding, but we observed a further improvement by the additional deletion of amino acids 94–113 (Figs 1 and 2; Table 1). This

result underlines previous findings obtained with large fragments of Phl p 5a, that dominant IgE epitopes are located in both the N-terminal and C-terminal halves [27, 28]. IgE-epitope mapping studies with Hol l 5, the related group 5 allergen of velvet grass, suggested that the identified short IgE-binding stretches are parts of large conformational epitopes [29]. An approach for mapping of IgE epitopes on Phl p 5a by using mimotopes from a phage display library also demonstrated the presence of several conformational IgE epitopes in the N- and C-terminal half [30]. The single-deletion mutant rPhl p 5a Δ 94–113 was highly IgE-reactive in a subgroup of sera, which were also characterized by a poor IgE binding to rPhl p 5a Δ 175–198 (Fig. 2, P20). This could be explained by a prevalence of IgE to the C-terminal half within these sera and may be indicative of an unchanged structure with retained conformational IgE epitopes in the C-terminal half of rPhl p 5a Δ 94–113. The presence of separate important conformational IgE epitopes in both halves of Phl p 5a may favour the assumption that the allergen has two IgE-binding four-helix bundles rather than a multi-helix structure, but it remains a goal of future research to clarify this question by structural analysis of a full-length group 5 allergen.

Retained T cell reactivity is an important feature for allergens or allergen preparations that are used for immunotherapy [17, 18, 31]. Our data indicate that both deletion sites do not affect major T cell epitopes, as the double-deletion mutant induced lymphoproliferative responses to a degree comparable with the unaltered allergens. Therefore, it is expected that rPhl p 5a Δ (94–113, 175–198) may effectively target T cells during immunotherapy. In conclusion, rPhl p 5a Δ (94–113, 175–198) combines substantially lowered overall IgE binding with retained T cell reactivity. It is therefore a promising candidate molecule for inclusion in a cocktail of hypoallergenic allergens for a future immunotherapy of grass pollen allergy.

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