

Lysine as a Critical Amino Acid for IgE Binding in Phl p 5b C Terminus

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Key Words

Immunoglobulin-E-binding region • Epitopes • Pollen allergen • Phl p 5b • Site-directed mutagenesis

Abstract

Background: Allergens induce the formation of specific immunoglobulin (Ig)E and harbor at least two IgE-binding regions (epitopes) to facilitate crosslinking of basophilic or mast-cell-bound specific IgE antibodies. Studies mapping linear epitopes have shown that these regions often contain charged or hydrophobic amino acids. Nevertheless, these studies are hampered by limited significance due to the often conformational nature of IgE epitopes. This prompted us to study the role of lysines in the context of an intact 3-dimensional model. **Methods:** Major allergen Phl p 5b from timothy grass bears 12 lysines in its C-terminal half. Using site-directed mutagenesis, we substituted all 10 surface-exposed lysines by alanines. **Results:** Although structural integrity of the lysine-deficient mutant was not altered, IgE-binding capacity measured by ELISA inhibition tests and crosslinking activity in ex vivo basophil stimulation and in vivo skin prick tests were significantly diminished. Interestingly, binding of specific IgG antibodies was considerably less reduced by loss of lysines. **Conclusion:** Lysine is an important amino acid for IgE binding in more than one epitope

of major grass pollen allergen Phl p 5b C terminus. Allergenicity, but not IgG binding of the molecule, is substantially diminished by single amino acid substitutions without structural integrity being hampered.

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Introduction

Up to 30% of the people in westernized societies suffer from allergic diseases such as bronchial asthma, allergic rhinitis, urticaria or atopic dermatitis. Allergies are triggered by usually harmless environmental antigens which are thereby named allergens. The type I allergic immune response induced by allergens is mediated by immunoglobulin (Ig)E antibodies. These IgE antibodies bind to effector cells such as mast cells and basophils via the high-affinity IgE receptor. During encounter with the allergen, which leads to crosslinking of at least two IgE antibodies on the sensitized effector cells, mediators are released which in turn induce an allergic inflammatory reaction of the skin or affected mucosa. Though the mechanism by which allergens activate an inflammatory response in already sensitized people is widely understood, the question why allergens may act as harmful molecules at all is still unsolved.

Crucial for the induction of the allergic response is the interaction of an allergen with its specific IgE antibody. With its variable site, the antibody binds to a defined region of the allergen, the so-called IgE epitope or IgE-binding region. It has been demonstrated that IgE epitopes are located on the surface of allergenic molecules and are often conformational [1–8], a fact also supported by observations that allergens show a tremendously reduced activity after impairment of 3-dimensional integrity [9, 10] or loss of large parts of the molecules. Furthermore, IgE antibodies bind to their epitopes with higher affinities than IgG antibodies, the latter often directed against linear epitopes [11, 12]. From what we have learned from epitope mappings with linear peptides, it is important to note that IgE epitopes are primarily composed of charged and hydrophobic amino acids, and many of them contain at least one lysine [2, 13–16]. Interestingly, the two dominant IgE epitopes on a group V grass pollen allergen completely lost their IgE-binding ability when the lysines were exchanged by nonpolar amino acids [2, 17]. Nevertheless, these results are hampered by the fact that they are based on analyses using linear epitopes which do not represent the 3-dimensional structure in which these epitopes are naturally integrated and on which their activity, namely allergenicity, depends.

For our experiments, we chose Phl p 5b C terminus as a model allergen, as the 3-dimensional structure has been solved. Furthermore, this C-terminal part of the molecule exhibits allergenic and biologic activity comparable with the holoprotein [18]. When analyzing the surface of the 3-dimensional structure of Phl 5b C terminus, our model allergen [19], it was striking that 10 out of the 12 lysines were located on the surface of the allergen and only 2 were found in the inner core of the molecule and were involved in the formation of stabilizing salt bridges.

These data led to the hypothesis that lysine might be an important amino acid for the interaction of the major allergen Phl p 5 with IgE antibodies.

Thus, the aim of this study was to determine whether a complete loss of IgE-accessible lysines of the C-terminal part of Phl p 5b would lead to a loss of IgE binding and allergenic activity, as it was shown for the linear epitopes. Therefore, all 10 lysines of Phl p 5b C terminus projecting to the surface of the molecule were exchanged, and helix stability, IgE-binding capacity and the allergenic activity of the mutant were compared with the wildtype allergen. Furthermore, we wanted to know whether this loss would be specific to IgE or also to IgG binding.

Material and Methods

Construction of a Lysine-Deficient Mutant of Phl p 5c C Terminus

The plasmid, used as a template for cloning, contained the Phl p 5b C terminus, which comprises 136 amino acids and contains 12 lysines and one recognition site for *Xho* I at position 61/62. The sequence was flanked by an *Eco*R I recognition site at the N terminus and a *Hind* III site at the C terminus, cloned into the multiple cloning site of pBluescript (pBS SK–, Stratagene, La Jolla, Calif., USA). Two of the lysines, lys³² and lys⁸⁰, are located in the inner core of the molecule and involved in the formation of salt bridges. Therefore, only the remaining 10 lysines (lys¹, lys¹², lys¹⁸, lys⁴², lys⁴⁵, lys⁵⁶, lys⁶⁶, lys⁹⁰, lys¹⁰⁰ and lys¹³⁵) were changed to alanines by site-directed mutagenesis using mutation primers and Phl p 5b C terminus as template [15].

Lysines were exchanged in three steps: first, 5' of the internal *Xho* I restriction site lysine codons were exchanged to alanine codons, resulting in a plasmid named pMut 1. Second, codons for lys⁶⁶ located 3' of the *Xho* I restriction site and the hindmost lysine codon were exchanged to alanine codons (pMut 2), and third, the resulting fragment was cloned into the *Xho* I, *Hind* III restricted pMut 1, resulting in a plasmid called pMut 3. lys⁹⁰ and lys¹⁰⁰ were exchanged to alanines using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction, with pMut 3 as template.

Sequences of all clones were controlled by DNA sequencing (GENTERprise, Mainz, Germany).

Primers used for cloning are given in table 1. Restriction sites are given in italic letters, mutation sites (lys → ala) in bold letters.

Subcloning of Plasmids, Overexpression and Purification of Recombinant Proteins

The mutant lacking all 10 lysines in question (Mut c) and the wildtype protein were subcloned into the hexahistidine-encoding prokaryotic expression vector pProEX HT (Life Technologies), a kind gift from Dr. G. Schramm, Forschungszentrum Borstel, Germany, and were transformed into *Escherichia coli* DH5-α cells (Life Technologies).

Primers used for subcloning were: wildtype forward: *atat gga tcc atc gag gga agg aag atc ccc gcc ggc gag ctg cag atc*; wildtype reverse: *atat aag ctt tca gac ttt ata gcc acc ggc agc gac gg*; mutant forward: *atat gaa ttc atc gag gga agg gcg atc ccc gcc ggc gag ctg cag*; mutant reverse: *atat aag ctt tca gac cgc ata gcc acc ggc agc*.

Restriction sequences are given in italic letters, stop codons are marked in bold. Underlined sequences represent a recognition site for the specific protease factor Xa. This restriction site was inserted in case the hexahistidine sequence would prove to be interfering with experiments.

Wildtype protein was expressed in pProEX HT A and lysine-deficient mutant in pProEX HT B.

Transformed cells were grown in LB medium containing 200 mg/ml ampicillin at 37°C to an optical density of OD₆₀₀ = 0.6. Protein expression was induced by addition of IPTG (isopropyl-D-thiogalactoside) to a final concentration of 0.3 mM. Cells were grown for an additional 4 h and harvested by centrifugation at 4,000 g for 20 min at 4°C.

Proteins were purified under native conditions. Cell pellets were resuspended in 50 ml lysis buffer per liter of culture (50 mM

Table 1. Primers used for site-directed mutagenesis

Primer	Primer sequence	Plasmid serving as template	Resulting plasmid
lys ^{1,12,18} (forward primer)	atat <i>gaa ttc</i> gcg atc ccc gcc gcc gag ctg cag atc atc gac gcg atc gac gcc gcc	pPhl p 5c, C terminus	pMut 1
lys ^{42,45,56} (reverse primer)	atat <i>ctc gag</i> gga tgg gat gca cg c gta ggt gtc gta gcc gcc gcc ggt gct ctc cg c gat gcc cg c gtt gaa g		
lys ⁶⁶ (forward primer)	atat <i>ctc gag</i> gcc gcc gtc g cg cag gcc tac gcc	pPhl p 5c, C terminus	pMut 2
lys ¹³⁵ (reverse primer)	atat <i>aag ctt</i> tca gac cg c ata gcc acc gcc agc		
lys ^{90,100} (forward primer)	c gcg ctg acc g cg gcc atc acc gcc atg tcc gag gta cag g cg gtc agc cag ccc	pMut 3	pMut c
lys ^{90,100} (reverse primer)	ggg ctg gct gac cg c ctg tac ctc gga cat ggc ggt gat gcc cg c ggt cag cg c g		

NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and frozen. After thawing, 1.5 ml lysozyme (10 mg/ml) per liter of culture was added and the samples incubated on ice for 30 min. Afterwards, samples were subjected to ultrasonic treatment six times for 10 s on ice, with a 10-second cooling period between each run. Protease activity in the samples was inhibited by addition of Complete solution, EDTA free (Roche, Germany).

Cellular debris was pelleted at 10,000 *g* for 20 min at 4°C. The supernatant was filtrated (5 µm) and hexahistidine-tagged fusion proteins purified by Ni column chromatography. Ten milliliters Ni-NTA-Agarose (Qiagen, Hilden, Germany) per liter culture lysate was added to the supernatant and incubated for 60 min at 4°C under frequent shaking. The suspension was filled into an empty column (Biorad, Germany), and flowthrough was collected for SDS-PAGE analysis. Ni-agarose-bound proteins were washed with 50 ml washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with 20 ml of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Elution buffer was exchanged to 10 mM phosphate buffer, pH 7.4, using PD-10 columns (Amersham, Freiburg, Germany) and the eluate concentrated with an ultrafiltration device (10 kDa exclusion size; Millipore, Germany).

Proteins in the eluted fractions were further purified by size exclusion chromatography on a HiLoad 16/60 Superdex 200 prep grade column (Amersham) using 50 mM NaH₂PO₄, 100 mM NaCl, pH 7.4, as running buffer at 0.5 ml/min.

Characterization of Proteins by SDS-PAGE

For SDS-PAGE analysis, 2 µg of purified recombinant proteins/lane was separated on denaturing (87 mM DTE) 15% polyacrylamide gels (BioRad mini-Protean call, BioRad, Sydney, Australia). Gels were stained with Coomassie blue.

Circular Dichroism Spectra of Wildtype and Mutant Protein

Circular dichroism (CD) measurements were performed in 10 mM phosphate buffer, pH 7.5, with concentrations of 0.5 mg/ml. Analysis was carried out on a Jasco spectropolarimeter using a cell length of 0.1 cm at 20°C. Spectra were recorded with a scan speed of 20 nm/min and a 1-nm resolution from averaging six scans. Final spectra were corrected by subtracting the respective

buffer spectra measured under identical conditions. Results were normalized and expressed in arbitrary units.

Isolation of IgG Antibodies

IgG antibodies were isolated by using protein G columns (Amersham) according to the manufacturer's instructions. Bound IgG was eluted by using glycine buffer, pH 2.7. The eluate was collected in 1-ml fractions in tubes each containing 60 µl 1 M Tris/HCl, pH 7.4, to adjust pH immediately. Purity of isolated IgG was confirmed by SDS-PAGE analysis. The protein content was adjusted to 2 mg/ml.

Comparison of Mutant and Wildtype Protein by ELISA IgE and IgG Inhibition Tests

Sera of 7 patients who had previously shown positive IgE binding to wildtype protein in ELISA were subjected to an ELISA inhibition test. Sera or isolated IgG fractions were diluted 1:3 in 2% defatted milk, containing various concentrations of wildtype and mutant allergen and incubated at 8°C overnight. On the next day, sera were added to ELISA plates, coated with wildtype protein (10 µg/ml). Residual free anti-wildtype IgE or IgG was measured by ELISA.

MaxiSorp ELISA plates were coated with 100 µl/well (10 µg protein/ml in sodium carbonate buffer, pH 9.6) at 8°C overnight. The next morning, the coating solution was removed, plates were washed once with TBS/Tween (1 M Tris, 1 M NaCl, 25 mM MgCl₂/0.5% Tween) and blocked with 250 µl/well 5% defatted milk in TBS at room temperature for 1 h. Afterwards, wells were washed three times with TBS/Tween. Hundred microliters of pre-incubated sera/IgG antibodies was added. After incubation at 37°C for 2 h, plates were washed three times with TBS/Tween, and detection antibody (alkaline phosphatase-conjugated mouse antihuman IgE or antihuman IgG, diluted 1:1,000 in 2% defatted milk; BD Biosciences) was added and incubated at 37°C for 2 h. After washing all wells three times with TBS/Tween, 100 µl/well AP substrate (1 mg 4-nitrophenyl phosphate disodium/1 ml diethanolamine buffer, pH 9.6; Roth, Germany) was added and, after incubation for 30 min at 37°C, stopped by adding 50 µl/well 1 M NaOH. Absorption was read on an ELISA reader (Dynatech) at 405 nm.

Fig. 1. Sequences of the N terminus (**a**) and a mutant (Mut c) of the Phl p 5c C terminus (**b**; accession number of Phl p 5b: Z27083). All 10 surface lysines were exchanged for alanines (bold, underlined). Only 2 lysines in the inner core (bold, italic) remained. The numbering refers to the residue number in the whole molecule as well as to the residue number in the mutant protein (numbers given in parentheses).

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ADAGY APATP AAAGA AAGKA TTEEQ KLIED INVGF KAAVA AAASV PAADK 50
FKTFE AAFTS SSKAA AAKAP GLVPK LDAAY SVAYK AAVGA TPEAK FDSFV 100
a ASLTE ALRVI AGALE VHAVK PVTEE PGMA 129

AIPAG ELQII DAIDA AFAVA ATAAA TAPAD DKFTV FEAAF NAAIA ESTGG 179 (50)
AYDTY ACIPS LEAAV AQAYA ATVAA APQVK YAVFE AALTA AITAM SEVQA 229 (100)
b VSQPA TGAAT VAAGA ATTAA GAASG AATVA AGGYA V 265 (136)

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For calculation of results, absorption at 405 nm for sera without inhibitory molecules was defined as 100% binding capacity. Inhibition of all other samples was calculated as percentage in relation to 100% binding.

Measurement of Allergenic Activity ex vivo by Basophil Stimulation (Expression of CD203c)

The degree of basophil activation was measured by upregulation of CD203c [20, 21]. Basophils/blood from 5 grass-pollen-allergic patients were used to determine optimal allergen concentrations. Concentrations ranging from 25 pg/ml to 50 ng/ml turned out to be optimal, with antihuman IgE antibody serving as positive and phosphate-buffered saline (PBS) as negative control. Basophil activation was measured by upregulation of CD203c expression in 5 additional patients. Basophil cells were counted, which upregulated CD203c expression above the level of unstimulated cells, and results are given as percentage of total basophils (fig. 6a).

Heparinized blood samples were taken from grass-pollen-allergic patients. Hundred-microliter blood samples were incubated with several dilutions (100 µl each) of wildtype or mutant Phl p 5c C terminus in PBS. PBS alone served as negative control, and antihuman IgE antibody (BD Pharmingen, San Diego, Calif., USA), diluted 1:500, served as positive control. After incubation for 15 min at 37°C, cells were washed in 1 ml PBS/20 mM EDTA and incubated with 10 µl/sample of phycoerythrin-labeled anti-CD203c antibody (Immunotech, Marseille, France) at room temperature in the dark for 45 min. Afterwards, erythrocytes were lysed with 2 ml FACS™ lyzing solution (BD Pharmingen) according to the manufacturer's instructions. Cells were washed with PBS and resuspended in 1 ml 1.5% paraformaldehyde. Analysis of cells was done on a FACS Calibur (BD Pharmingen), equipped with an argon laser. Basophils were defined by specific anti-CD203c antibody labeling. Positively stained cells were analyzed for upregulation of fluorescence intensity after stimulation compared with negative control. The proportion of CD203c-positive cells, shifted above the fluorescence level of negative controls, was calculated.

Measurement of Allergenic Activity in vivo (Skin Prick Test)

Skin prick tests were performed with 4 volunteers, 2 grass-pollen-allergic patients and 2 controls (1 healthy and 1 grass-pollen-allergic patient who does not react with Phl p 5), who had given informed written consent. Wildtype as well as mutant proteins were sterile filtrated, mixed with equal volumes of sterile glycerol and used in three different final concentrations (100, 10 and

1 µg/ml). NaCl (0.9%) and histamine (1.7 mg/ml) skin prick solutions (Allergopharma, Reinbek, Germany) served as controls. Wheal areas were read after 15 min of allergen application and measured in square millimeters.

Results

Site-Directed Mutagenesis of Potential IgE-Binding Sites

The sequence of the mutant lacking 10 of the 12 lysines of the wildtype Phl p 5c C terminus is given in figure 1. The hexahistidine tag for affinity purification and the adjacent spacer sequence followed by a 4-amino-acid recognition sequence (IEGR) of factor Xa are not displayed. Alanines replacing lysines are given in bold, underlined letters, remaining lysines in the inner core of the protein are marked in bold, italic letters.

Purity of Recombinant Proteins

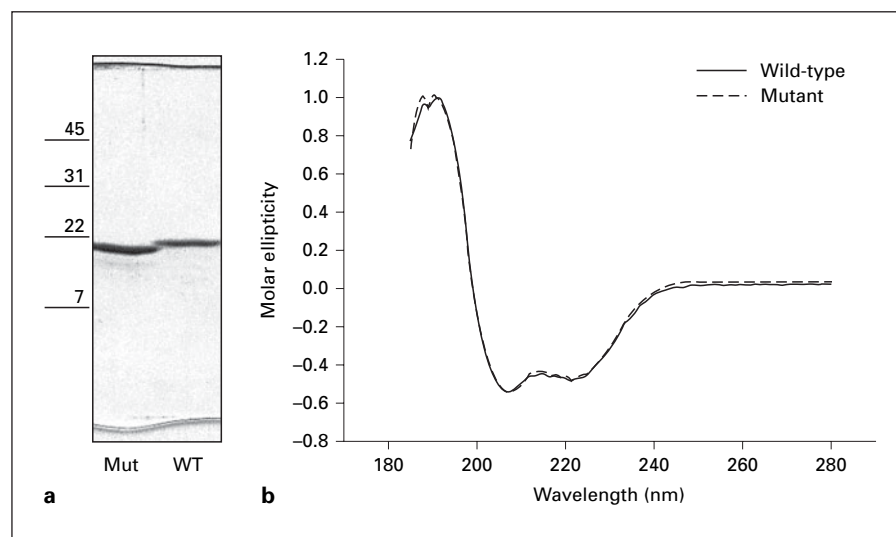
Purity of recombinant proteins was confirmed by SDS-PAGE analysis.

Figure 2a shows a reducing SDS-PAGE of purified wildtype and mutant proteins. The apparent molecular weight of the proteins in SDS-PAGE equals the nominal weights calculated for amino acid composition (16.8 kDa for the mutant and 17.4 kDa for the wildtype allergen, including affinity tags, spacer sequences and protease cleavage sites).

Confirmation of Conserved Secondary Structure

To analyze the potential influence of lysine substitution on the structural integrity of the allergen, allergens were subjected to CD spectroscopy. Figure 2b shows the results from the CD analysis of the wildtype and lysine-deficient Phl p 5b C terminus. Both curves were corrected for the buffer solution and normalized (highest value set at 1.0). There are no significant differences between the curves, indicating that substitution of lysines for ala-

Fig. 2. Recombinant lysine-deficient mutant (Mut) and wildtype (WT) Phl p 5b C termini were separated on a reducing 15% SDS-PAGE (2 μ g/lane each) and stained with Coomassie blue (a). Results are shown as arbitrary units on the y-axis at a given wavelength on the x-axis (b).



nines did not alter the overall secondary structure of the molecule.

Capacity of Mutant and Wildtype Allergen for Inhibition of IgE Binding

In order to examine IgE-binding capacities of mutant and wildtype allergen, both proteins were allowed to compete for binding to antibodies in solution. Despite interindividual differences, the mutant protein showed reduced inhibitory capacity compared with wildtype allergen, which could lead to 100% inhibition. Figure 3 shows inhibition results of 7 individual patients (mean $\pm$ SD). Inhibition induced by mutant allergen in relation to wildtype allergen, measured at the highest concentration tested, ranged from 15 to 57%. About 500 times more mutant allergen was required to inhibit 50% of IgE binding to wildtype allergen as measured by ELISA (mean values: 0.076 μ g/ml of wildtype vs. 37.5 μ g/ml of mutant allergen). Even when increasing the concentration of mutant allergen used for inhibition up to 500 μ g/ml, we could not achieve 100% inhibition of IgE binding to wildtype allergen (data not shown).

Capacity of Mutant and Wildtype Allergen for Inhibition of IgG Binding

To evaluate the significance of lysines for IgE binding, the same 7 sera used for IgE inhibition tests were used for IgG isolation and subsequent IgG inhibition tests.

When analyzing IgG binding, it was most striking that 100% inhibition could not be achieved, even when using 1 mg/ml of inhibiting protein. About 200 times more mu-

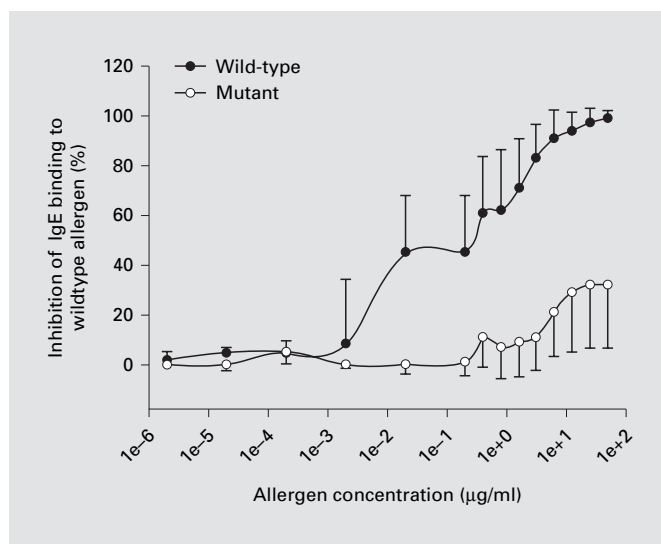


Fig. 3. Results of IgE inhibition studies of 7 individual sera done by ELISA. Mean and standard deviation of inhibition of binding to the wildtype allergen, induced by preincubation of sera with several dilutions of the wildtype and mutant allergen, respectively.

tant allergen was needed for 50% inhibition of maximum IgG binding to wildtype allergen (median: 0.3 μ g/ml of wildtype vs. 59.3 μ g/ml of mutant allergen); this is 2.5 times less than observed for IgE binding.

In comparison with IgE, IgG binding to wildtype allergen is weaker (fig. 4a) but as good as that of IgE to lysine-deficient mutant allergen (fig. 4b).

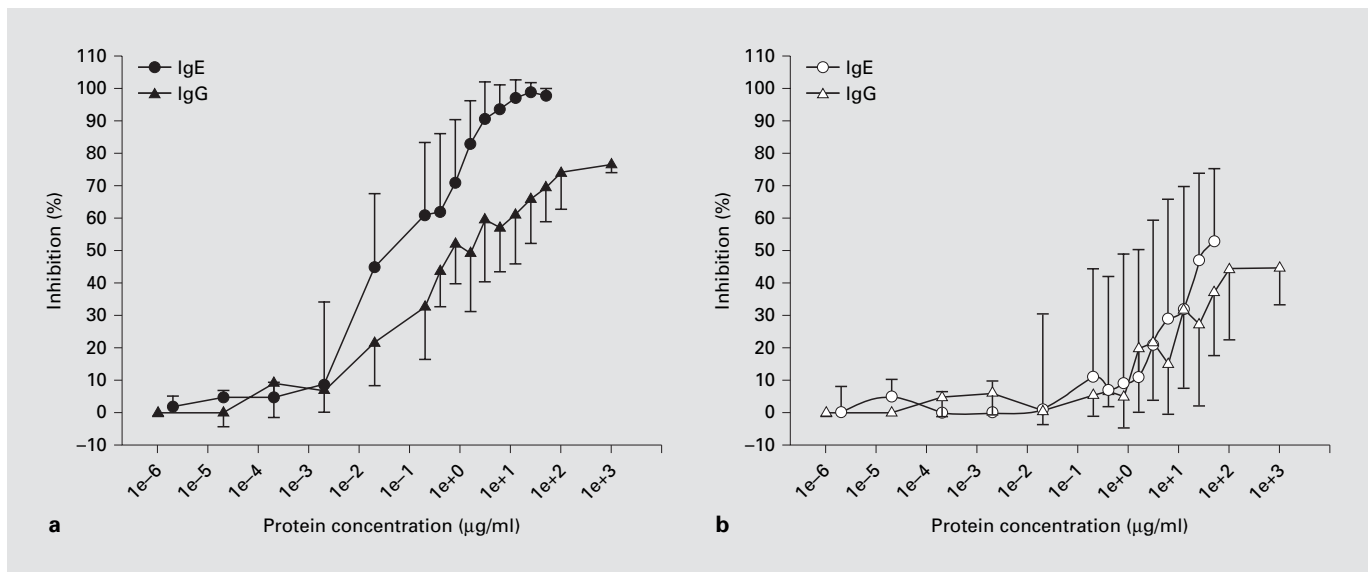


Fig. 4. Comparison of IgE and IgG binding measured by ELISA inhibition for wildtype allergen (**a**) and lysine-deficient mutant (**b**), respectively. Mean and standard deviation of 7 individual sera are given.

Measurement of Allergenic Activity ex vivo by Basophil Stimulation (Expression of CD203c)

After it had been demonstrated that lysine substitution lead to an extensive loss of IgE-binding capacity of the Phl p 5b, it was important to know whether this was also the case for allergenicity (crosslinking capacity). Therefore, various concentrations of the mutant protein and the wildtype allergen were used for basophil stimulation in whole blood.

All patients tested confirmed a higher stimulatory capacity of wildtype allergen compared with the mutant protein. These differences were most prominent when using low protein concentrations (<5 ng/ml) for basophil activation.

For calculation of results, allergen concentration needed for 50% of maximum stimulation (obtained by anti-IgE crosslinking) was compared for both allergens in all patients. Figure 5a shows one representative result out of five comparable experiments and demonstrates the way results were calculated. Only in 1 patient was basophil activation by the mutant tested lower than 50% of maximum in all concentrations. Here, the highest stimulation achieved with the mutant protein was compared with the wildtype protein, resulting in the same CD203c upregulation.

To normalize the highly variable individual results, the concentration measured for the wildtype allergen in

each patient was set to 1, and the concentration of the mutant allergen needed to achieve the same basophil stimulation was calculated as a multiple of the respective wildtype concentration. Figure 5b shows the results for all patients tested, indicating a significantly reduced basophil activation by the mutant compared with the wildtype allergen.

Measurement of Allergenic Activity in vivo (Skin Prick Test)

Since the previous experiments had shown that obviously more than one IgE epitope was affected by lysine deletion, it was necessary to support these results by in vivo testing in a standard human skin prick test model, usually used for diagnosis of allergic sensitization and measuring crosslinking of IgE on skin mast cells.

Neither the healthy nor the allergic control without grass pollen allergy reacted towards dermal provocation with Phl p 5 C terminus with or without lysines. Two Phl p 5b allergic patients reacted positively with both allergen forms. Interestingly, in the titrated skin prick test (fig. 6a, b), the resulting wheal area after 15 min was much lower when using the mutant compared with the wildtype protein.

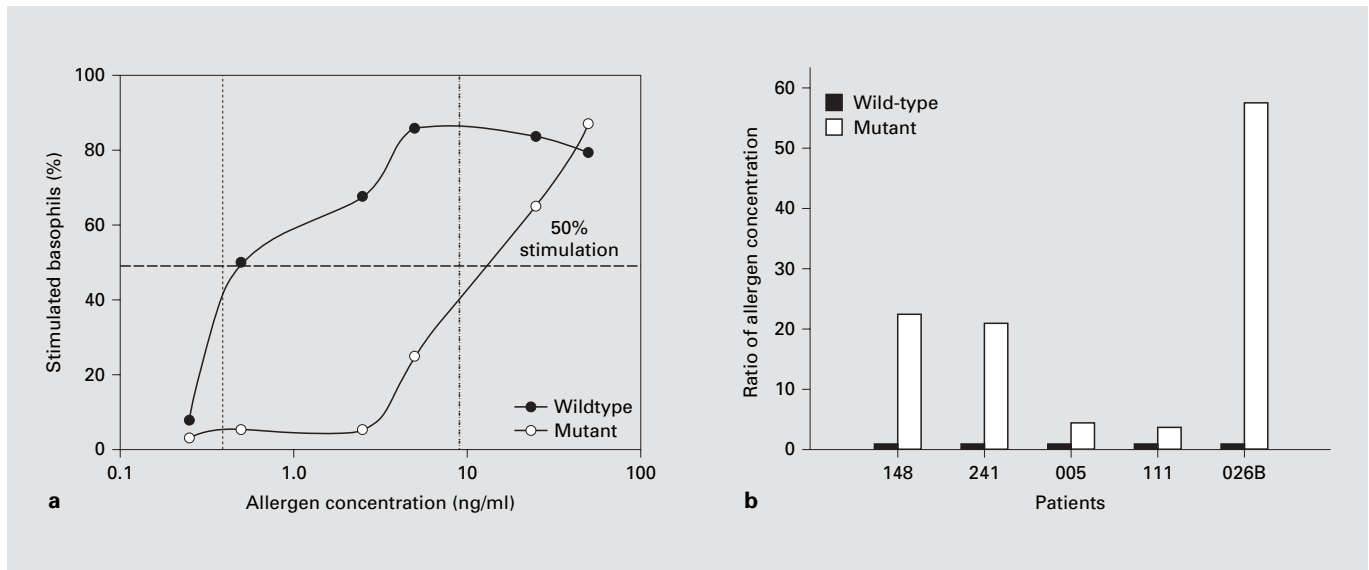


Fig. 5. Comparison of allergenic activity as measured ex vivo by basophil stimulation. **a** The way the 50% stimulatory capacity was read from basophil activation by wildtype and mutant allergen. **b** Results of basophil stimulatory capacity of mutant and wildtype allergen for 5 different patients.

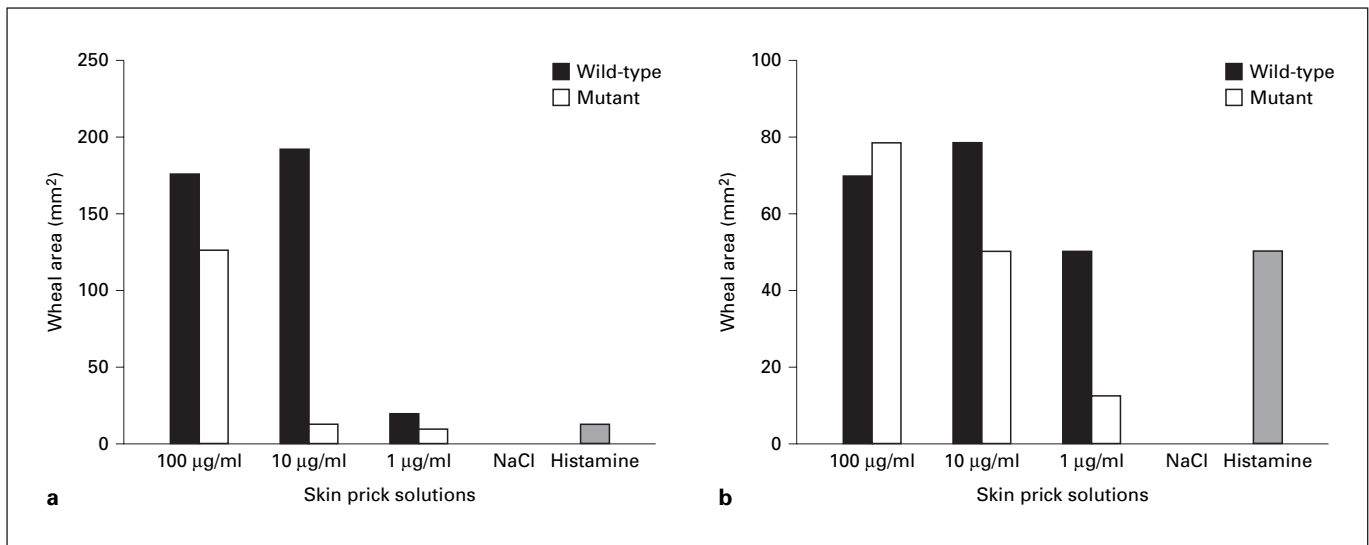


Fig. 6. Results of skin prick tests performed on 2 grass-pollen-allergic patients with three different concentrations of wildtype allergen and lysine-deficient mutant. NaCl induced no skin reaction in either patient, whereas the positive control (histamine) did.

Discussion

Here, we describe for the first time that the substitution of every single lysine located on the surface of a major grass pollen allergen results in a significant reduction

in IgE-binding capacity as well as in allergenic activity, without the integrity of the 3-dimensional structure being lost. Lysines have already been shown to be critical for IgE binding in linear epitope analyses of the related grass pollen allergen Lol p 5 [17] and other allergens [13, 14, 16]

or at least present in IgE-binding regions [1, 12]. But since IgE epitopes are known to be mainly conformational, it was essential to measure the influence of missing lysines using an allergen with intact 3-dimensional structure.

The 3-dimensional structure of Phl p 5b C terminus had revealed that 10 out of the 12 lysines are located on the surface, thereby being available as contact residues for IgE antibodies. These lysines were exchanged for alanines in a mutant allergen. Interpretation of the crystal structure published for the wildtype allergen [19] indicated that 2 out of 12 lysines were relevant for the stability of the molecule by forming intramolecular salt bridges and allowed to predict that substitution of the 10 external lysines by alanines should not affect the conformation of the molecule. This notion was confirmed by the finding that in CD spectroscopy, no differences in the 2-dimensional structures of wildtype and mutant allergen could be detected. Therefore, we feel confident that also the 3-dimensional structure has been retained in the mutant. However, alterations on a microstructural level cannot be excluded by this method and require further crystallographic analysis.

We detected fundamental functional differences for the mutant as compared with the wildtype allergen in vitro and in vivo. Reduced IgE binding in ELISA inhibition and less allergenicity in basophil activation and skin prick test could be demonstrated for the mutant allergen in contrast to the wildtype molecule. These results might address several important features of IgE-binding epitopes. First, lysines are present in several epitopes on the surface of major allergen Phl p 5b, otherwise crosslinking experiments would not have been so drastically affected by the introduced mutations. As demonstrated in earlier studies with Phl p 5b [22], neither single epitope mutants nor naturally occurring isoforms [23] did lead to loss of IgE binding and allergenicity. Second, several IgE-binding epitopes seem to be distributed over the whole molecule, like the lysines themselves, which are protruding from the molecule all over the surface. Furthermore, results of an IgE epitope mapping of a related grass pollen allergen from *Holcus lanatus*, Hol l 5 [24], suggested the presence of several different overlapping IgE-binding regions on the molecule, which might be linear parts of conformational epitopes. To search for conformational epitopes, a mimotope approach conducted with the *Phleum pratense* group 5 allergen Phl p 5a revealed the presence of two conformational epitopes in the C terminus [25]. Interestingly, a variability in recognizing the different epitopes by different allergic patients was observed, in accordance with our finding that the uniform ex-

change of all lysines on the surface did not lead to a similar loss of allergenicity in any of our patients. This indicates that IgE formation during sensitization is affected by individual differences and also demonstrates that lysines, though important for IgE binding, are not the only important amino acids involved in the interaction with the variable site of IgE antibodies. Prospective site-directed mutagenesis of the same single amino acid throughout an allergen with subsequent analysis of antibody binding has never been carried out before. From a study with Lol p 5, an allergen closely related to our model allergen Phl p 5, there are data where other amino acids besides lysine, e.g., phenylalanine, threonine, valine and glycine, within different known linear epitopes, were altered by site-directed mutagenesis [26]. It was demonstrated that single amino acid substitutions could also lead to decreased IgE binding and allergenicity. However, in contrast to our study, the introduced changes resulted in a slight decrease in structural stability, and especially the alterations in the C-terminal part of the molecule showed poor effects. Furthermore, all mutations apart from one were only effective in some of the investigated individuals, and in one example, a couple of changes even enhanced IgE binding.

Comparable studies have been carried out with allergens from latex and peanut where amino acids which had previously proven to be important for IgE binding in linear epitopes were later exchanged and examined in an intact holoprotein [e.g., see ref. 27, 28]. But again, in contrast to our study, often more than one amino acid per linear epitope was exchanged, and in most cases, many different amino acids were mutated in each molecule.

The third feature of IgE epitopes indicated by our study is probably the most interesting one since it demonstrates that the deletion of lysines mainly influences IgE but less IgG binding. Other investigations have already revealed different binding patterns of IgE and IgG, showing that both preferably bind to different amino acid residues [13] and that IgG rather binds to linear epitopes in contrast to IgE [14]. Furthermore, binding affinities of IgG towards allergens, including closely related Phl p 5a, have been shown to be considerably lower than those of IgE [11, 29]. In line with these data, we were never able to completely block the binding of residual IgG to wildtype allergen by incubating it with the allergen overnight, even when using two times more allergen than the amount necessary for inhibiting IgE binding. There are two likely explanations. It may either be due to the lower affinity of IgG compared with IgE [11, 29], or to the fact that not all paratopes were blocked, as the concentration of IgG is

much higher than that of IgE. We cannot completely rule out the latter, but as both inhibition curves of IgG reach a plateau phase at the highest concentrations used, we tend to argue that the different affinities probably account for the observed differences.

Thus, the specific feature of IgE epitopes might be described by certain amino acids, an important one being lysine, and the numerous distribution of IgE epitopes on the surface of allergens. IgE is characterized by higher affinity towards the allergen in contrast to IgG, and multiple recognized epitopes lead to a high avidity of IgE and the ability of crosslinking several IgE antibodies, even when individuals are sensitized towards different IgE epitopes.

However, lysines are not exclusively or predominantly present in allergenic molecules. They can make up to 18% of the amino acid content of allergens, e.g., in Ole e 6, but there are also potent allergens with very few lysines (0.9% in Der p 1), and the same distribution is true for nonallergenic molecules.

Therefore, it may not merely be the content of lysines or other specific amino acids to be found in antibody-binding sites which accounts for allergenic activity but rather, as already mentioned, the location or distribution on the surface of the protein molecule. This question can-

not ultimately be answered until structural information of further allergens will be available. Furthermore, it will be interesting to exchange lysine for a different positively charged amino acid, e.g., arginine, to evaluate the importance of lysine versus the charge of the amino acid.

The results presented here offer a number of perspectives. It will be important to investigate whether lysines are not only responsible for IgE binding but might also play a part in specific IgE induction. This analysis is under investigation. Furthermore, it is also open whether T-cell epitopes are affected in lysine-deficient mutants. Concluding from the position of T-cell epitopes in Phl p 5b, not all of the so far known T-cell epitopes described [22] will be affected. Furthermore, other studies with more pronounced mutations of the allergen describe that T-cell reactivity remains conserved [26]. If lysines or similar amino acids play a critical role in allergenicity in general, this correlation should be considered for the design of hypoallergenic allergens in therapeutic applications.

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