

Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure

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Abstract We assessed the effect of multiple-mutations within one IgE-binding area on allergenicity of Der f 2. The triple-mutant of Der f 2, P34/95/99A, exhibited the most significant reduction of allergenicity and circular dichroism analysis showed that the global structure of Der f 2 was maintained in P34/95/99A. These results indicate that such a strategy is effective when designing allergen-vaccines, which achieve less allergenicity for a broad population of patients without disrupting the global structure. Structurally, Der f 2 is a member of the MD-2 related lipid-recognition proteins. The sites for the triple-mutation located on the characteristically charged entrance of a cavity and corresponded to the regions critical to ligand-binding in the Niemann-Pick type 2 disease protein and MD-2.

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1. Introduction

House dust mites of two species of *Dermatophagoides* (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) associated with various allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis are the most important source of in-door allergens [1]. Group 1 (Der f 1 and Der p 1) and group 2 (Der f 2 and Der p 2) allergens derived from house dust mites are considered major allergens based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract [2].

Der f 2 from *D. farinae* [3] consists of 129 amino acid residues. Its IgE epitopes are dependent on the integrity of the

conformational structure rather than the contiguous sequence of amino acids [4–7]. The amino acid sequence of Der f 2 is homologous to other group 2 allergens from different mite species and the Niemann-Pick type C2 disease protein (NPC2/HE1) with cholesterol-binding activity [8–10]. Recently, a structural relationship between the Der f 2 homologues and MD-2, which associates with Toll-like receptor 4 on the cell surface and is part of the mammalian innate immune system involved in the recognition of lipopolysaccharide (LPS) from gram-negative bacteria [11], was indicated by a PSI-BLAST search [12]. Although NPC2 and MD-2 bind cholesterol [13] and LPS [14], respectively, the ligand(s) for the mite group 2 allergens has not been identified.

Mutant allergens with reduced binding to IgE would be useful for a safer and more effective allergen-specific immunotherapy [15]. The mutation of residues contributing to a key intramolecular interaction of allergen molecules would be an effective and widely applicable way to reduce allergenicity [6,7]. Another strategy is to introduce mutations at the molecular surface without disrupting the global structure. To effectively induce the production of blocking antibodies, a mutant allergen with a nearly intact global structure would have an advantage over one with disruption of global structure [16,17].

In this report, we assess the effect of multiple-mutations in one IgE-binding area, which do not cause a disruption of the global structure, on IgE-binding and basophil histamine-releasing activity using recombinant Der f 2 as a model allergen. We chose Pro95, Pro99, and Pro34, which are close together at one end of the tertiary structure and contribute to IgE-binding [18], as target residues for the double- and triple-mutations. We suggest that such a strategy is also effective when designing allergen-vaccines less allergenic for a wide population of patients without disruption of the global structure. Furthermore, we indicate colocalization of this IgE-binding area and the potential ligand-binding region of Der f 2 on the basis of the 3D structure.

2. Materials and methods

2.1. Cloning of cDNA coding for Der f 2

Total RNA was prepared from cultured mites, and the cDNA fragments of Der f 2 were synthesized, amplified by reverse transcription-polymerase chain reaction, subcloned into vectors and sequenced as described previously [19].

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Abbreviations: CD, circular dichroism; LPS, lipopolysaccharide; NPC2, Niemann-Pick type 2 disease; PVDF, polyvinylidene difluoride; RAST-EIA, radioallergosorbent test-enzyme immunoassay; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay

2.2. Expression and purification of recombinant Der f 2 and its mutants

The cDNA encoding the amino acid sequence of an isoform of wild-type Der f 2, which was designated as clone 11/Der f 2.0103 [3,20] and identical to the clone used in our previous mutational analyses [4–7,18], was subcloned into an expression vector, a modified pGEMEX-1 (Promega, Madison, WI). Expression vectors for mutants of Der f 2 were constructed by site-directed mutagenesis. The wild-type Der f 2 and its mutants were expressed in *Escherichia coli*, refolded, and purified as described previously [18]. The protein concentration was calculated based on the absorbance at 280 nm and the number of tyrosine and tryptophan residues in Der f 2.

2.3. Gel-filtration analysis

Gel-filtration analysis was performed as previously described [18] with some modifications. Briefly, purified recombinant allergens (0.1 ml, approximately 0.1 mg/ml) were subjected to gel-filtration chromatography on a Superdex 75 HR 10/30 (1 cm × 30 cm) (Amersham Biosciences, Piscataway, NJ, USA) at a flow rate of 0.5 ml/min with 50 mM Tris–Cl (pH 7.5) containing 150 mM NaCl and 0.01% sodium azide. Apparent molecular sizes of the recombinant allergens were estimated using standard proteins.

2.4. Circular dichroism

Circular dichroism (CD) spectra were measured on a JASCO J-820 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using a 0.1-cm cell at 25 °C over 190–260 nm. Samples at 0.1 mg/ml were dialyzed against 10 mM potassium phosphate buffer (pH 7.5). The experimental parameters were: bandwidth 1.0 nm, sensitivity 100 millidegrees, step resolution 0.5 nm/datum, scan speed 50 nm/min, and scans 10.

2.5. Dot blot analysis

The sera were obtained from mite sensitive atopic patients with bronchial asthma, allergic rhinitis, and/or atopic dermatitis who proved positive to a skin prick test. Purified recombinant proteins (500 ng of protein/0.1 ml of Tris-buffered saline [TBS]/dot) were immobilized onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA) using a blotting apparatus (Bio-Dot; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The membrane was blocked with BlockAce (Snow Brand, Sapporo, Japan) (diluted 1/4 with TBS and 0.05% Tween 20) at room temperature for 1 h, and then, incubated with mite-allergic patient sera (1/100) for 3 h. After being washed three times with TBS and 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated anti-human IgE antibody (Biosource, Camarillo, CA, USA) (1/1000) for 1.5 h. After being washed again, the membrane was developed with an ECL+ kit (Amersham Biosciences). The chemiluminescence was detected with an LAS-1000 (Fuji Film, Tokyo, Japan). The density of the dots was calculated using the computer software ImageGauge V4.0 (Fuji Film). Group data were statistically analyzed with Prism version 4.0 (GraphPad, San Diego, CA, USA).

2.6. Radioallergosorbent test-enzyme immunoassay

IgE reactivities were measured by radioallergosorbent test-enzyme immunoassay (RAST-EIA) as previously described [5]. Briefly, the diluted serum of each allergic patient was incubated with paper discs coupled with the allergens. Then, the IgE that bound to the disc was detected with β -galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence.

2.7. Inhibition assay

The method used was described previously [7]. Briefly, the diluted serum of each mite-allergic patient was incubated with various concentrations of each inhibitor recombinant protein and then the solution was added to a paper disc coupled covalently with wild-type Der f 2. The IgE that bound to the discs was detected with β -galactosidase-conjugated anti-human IgE antibodies. The percentage of inhibition was expressed as the relative reduction of the fluorescence intensity in each sample to that when no inhibitors were added.

2.8. Histamine release

The method employed was as described [21] using HRT-Shionogi (Shionogi and Co., Ltd., Osaka, Japan) with some modifications. Briefly, basophil-containing leukocytes were directly purified from a

small amount of peripheral blood using magnetic beads coated with a monoclonal antibody. The cell-bead complexes on the magnetic device were then transferred to another microplate, which was coated with streptavidin for enzyme-linked immunosorbent assay (ELISA) and contained allergen solution. After the incubation, the cell-bead complexes on the magnetic device were removed and the histamine content was measured by ELISA. The amount of histamine released after incubation with 0.2 mg/ml of digitonin was used as the total histamine content for the calculation. The percentage of histamine released was calculated as the relative amount of histamine in the supernatant to the total amount of histamine.

2.9. Molecular modeling

Homology models of wild-type Der f 2 clone 11/Der f 2.0103 and its triple-mutant P34/95/99A were both generated using the program MODELLER [22] release 6v2, starting from the refined structure of Der f 2 clone 1/Der f 2.0101 [23], without large steric hindrance. The graphic was produced using the programs Molscript [24] and Raster3D [25].

3. Results

3.1. Expression and purification of single- and multiple-proline mutants of Der f 2

Der f 2 has no *N*-glycosylation site in the deduced amino acid sequence, and the recombinant Der f 2 with an additional methionine at the first N-terminal residue showed the mobility on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and secondary structure identical to the natural Der f 2 purified from mite extract (data not shown). The wild-type, three single-mutants (P34A, P95A, and P99A), three double-mutants (P34/95A, P34/99A, and P95/99A), and a triple-mutant (P34/95/99A) of Der f 2 were overexpressed as insoluble inclusion bodies in *E. coli* cells. The proline was replaced by an alanine residue in these mutants. Each of the refolded and purified proteins was detected as a single band on SDS–PAGE (Fig. 1A). Mobilities of the wild-type and all mutants of Der f 2 were almost equivalent to each other.

3.2. Secondary structures of single- and multiple-proline mutants of Der f 2

Each additional proline mutation caused a small change in the CD spectrum (Fig. 1B). The single-mutants showed small differences from the wild-type Der f 2. The double-mutants showed small differences from the progenitor single-mutants. The triple-mutant P34/95/99A showed a small difference from the double-mutants. However, as the differences were small, it was suggested that the structural changes were local and that the global β -sheet structure of Der f 2 was maintained. Mutation of Pro95 to alanine increased the ellipticity at 220–235 nm (Fig. 1B, the upper panel) in P95A, P34/95A, P95/99A, and P34/95/99A.

3.3. Estimation of molecular sizes by gel-filtration analysis

The purified wild-type Der f 2 and the triple-mutant P34/95/99A were detected as a single peak in gel-filtration analysis, and their apparent molecular weights were 14 and 15 kDa, respectively (Fig. 2). This also suggested that the global structure of Der f 2 was maintained.

3.4. Direct IgE-binding and inhibitory activity of single- and multiple-proline mutants of Der f 2

The reactivity of IgE to the purified Der f 2 mutants immobilized on a PVDF membrane was examined by dot blot

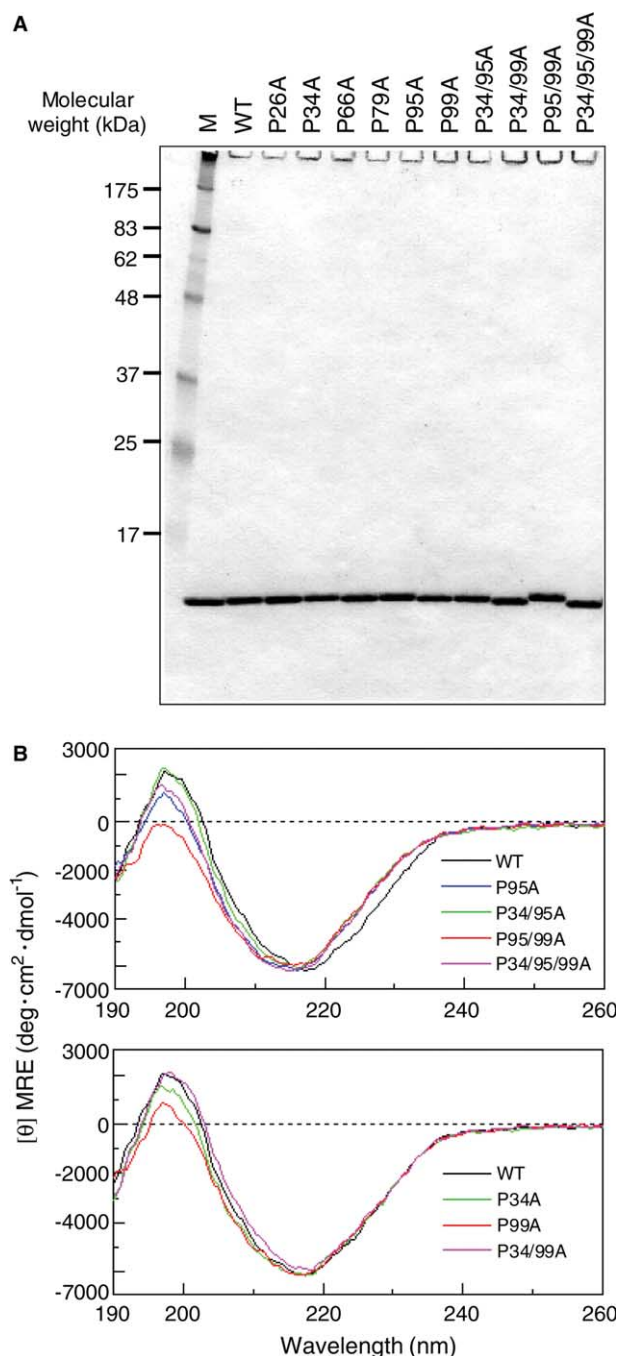


Fig. 1. Purification of single- and multiple-proline mutants of Der f 2. (A) SDS-PAGE (10–20% acrylamide) under reducing conditions. The gel was stained with Coomassie brilliant blue. WT, recombinant wild-type Der f 2. M, molecular weight marker. (B) Circular dichroism. Upper panel: spectra of mutants with P95A mutation. Lower panel: spectra of mutants without P95A mutation.

analysis (Fig. 3). In all sera tested, the single-mutants showed reduced IgE-binding compared to the wild-type Der f 2. The double-mutants showed reduced or equivalent IgE-binding compared to the progenitor single-mutants. By the Tukey post hoc test following with the repeated measures ANOVA, the triple-mutant P34/95/99A showed significantly lower binding than the other mutants except P95/99A (Fig. 3C). By the paired *t* test (two-tailed), P34/95/99A showed significantly

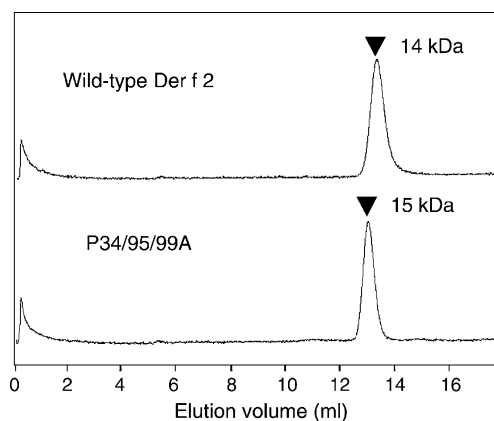


Fig. 2. Elution profiles in gel-filtration analysis of purified wild-type Der f 2 and the triple-mutant P34/95/99A. The apparent molecular weights of the peaks are shown with an arrow.

lower binding than P95/99A ($P = 0.0027$). These results suggested that additional mutations decreased the total avidity of the mutants to the polyclonal IgE antibodies. The pattern of IgE reactivity to the mutants was not always similar among patients (Fig. 3B), suggesting that the contribution of the three proline residues to IgE-binding was not the same for all patients.

Using paper discs covalently coupled with the wild-type or mutants of Der f 2, the reactivity of IgE to the Der f 2 mutants (Fig. 4A) and the capacity of the mutants to inhibit IgE from binding to the immobilized wild-type Der f 2 (Fig. 4B) were examined. The triple-mutant P34/95/99A showed the lowest levels of binding and inhibitory activity.

3.5. Activity of single- and triple-mutants of Der f 2 in stimulating histamine release by basophils

Peripheral blood basophils from mite-allergic volunteers were stimulated with the wild-type, single-mutants, and triple-mutant of Der f 2, and histamine release was measured (Fig. 5). The pattern of histamine-releasing activity of the single-mutants differed among the volunteers. The triple-mutant P34/95/99A showed the lowest activity in all volunteers tested. Threshold concentrations of the triple-mutant necessary for histamine release were approximately 10-fold higher in Volunteers #12 and #13, and 4–6-fold higher in #14 than those of the wild-type Der f 2.

3.6. Characteristic features of the region surrounding Pro 95, Pro99, and Pro34 on the modeled structure

To obtain information on the structural background of the significant reduction of allergenicity of the triple-mutant P34/95/99A, we investigated the features of the region surrounding the sites for the triple-mutation on the basis of a 3D model based on the nuclear magnetic resonance structure of Der f 2 [23,26]. Interestingly, the three proline residues located on the characteristically charged molecular surface, where seven charged residue and the charged C-terminal carboxyl group existed (Arg31, Lys33, Lys96, Lys100, Lys126, Arg128, and Asp129), at one edge of the entrance of a cavity (Fig. 6A, top). As the cavity of Der f 2 was expected to be involved in interaction with the unknown ligand, locations of amino acid residues critical to cholesterol-binding in NPC2 [27] and

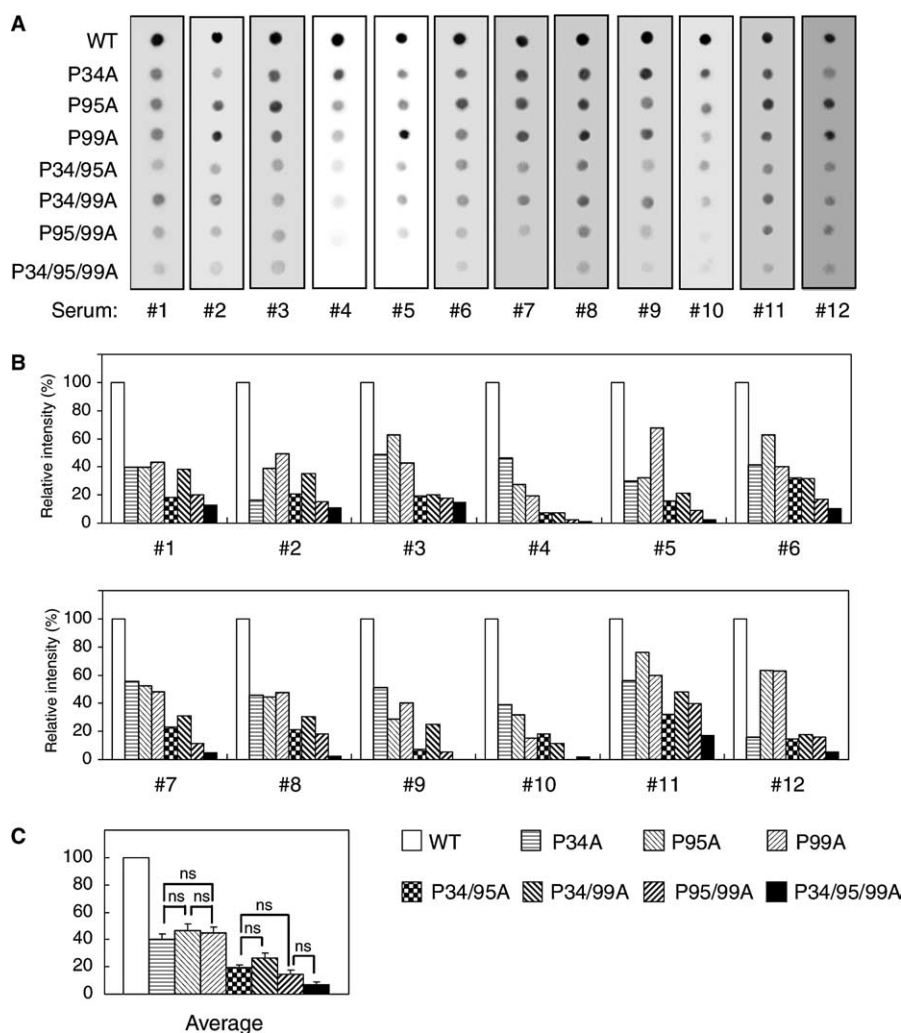


Fig. 3. Dot blot analysis of IgE-binding of single- and multiple-proline mutants of Der f 2. Density of the dots shown in A was measured and the calculated values are shown in B. (C) Data shown represent the means $\pm$ S.E.M. of the 12 sera. Multiple comparison by the Tukey post hoc test following with the repeated measures ANOVA showed all combinations except those indicated are significantly different each other ($P < 0.05$). ns: not significantly different ($P > 0.05$). WT, wild-type Der f 2.

cellular LPS-recognition in MD-2 [28–31] were compared with the location of the triple-mutation of P34/95/99A in Der f 2 (Fig. 6B and C). To our surprise, the loop containing Pro95 and Pro99 of Der f 2 corresponded to the regions critical to the ligand-binding in NPC2 and MD-2 (Fig. 6B). The residue Gly59 of mouse MD-2 corresponding to the residue next to Pro34 of Der f 2 is also involved in LPS-recognition (Fig. 6C).

4. Discussion

The target residues, Pro95, Pro99, and Pro34, are closely located in loops at one end of the tertiary structure (Fig. 6A), which we previously suggested that these residues compose a single IgE-binding area [18]. The patterns of IgE-binding capacity (Figs. 3B and 4) and histamine-releasing activity (Fig. 5) of the mutants were not always similar among patients suggesting that the contribution of each of the three proline residues to allergenicity was not the same for all patients. We have demonstrated that allergenicity of the triple-mutant of Der f 2, P34/95/99A, most significantly decreased for all pa-

tients tested while that of the single- or double-mutants did not decrease for all (Figs. 3–5). The triple-mutant showed a decrease in anaphylactic potential, because histamine release from human basophils was reduced (Fig. 5). The secondary structure (Fig. 1B) and molecular compactness (Fig. 2) are almost identical between wild-type Der f 2 and P34/95/99A. Therefore, the reduction of allergenicity of P34/95/99A was not accompanied by a disruption of global structure. These results suggest that multiple-mutation within one IgE-binding area is effective when designing allergen-vaccines, which achieve hypoallergenicity for a broad population without disrupting global structure, for allergen-specific immunotherapy.

The binding and inhibitory activity, which was assayed in individual tubes for the triple-mutant P34/95/99A, was at relatively significant levels for some sera (Fig. 4) compared with much less direct IgE-binding of the triple-mutant than the wild-type Der f 2, which was assayed in a same plastic bag for all the recombinant proteins dotted onto a PVDF membrane (Fig. 3). Threshold concentrations of the triple-mutant necessary for histamine release were 4–10-fold higher than those of the wild-type Der f 2 (Fig. 5). These results suggest

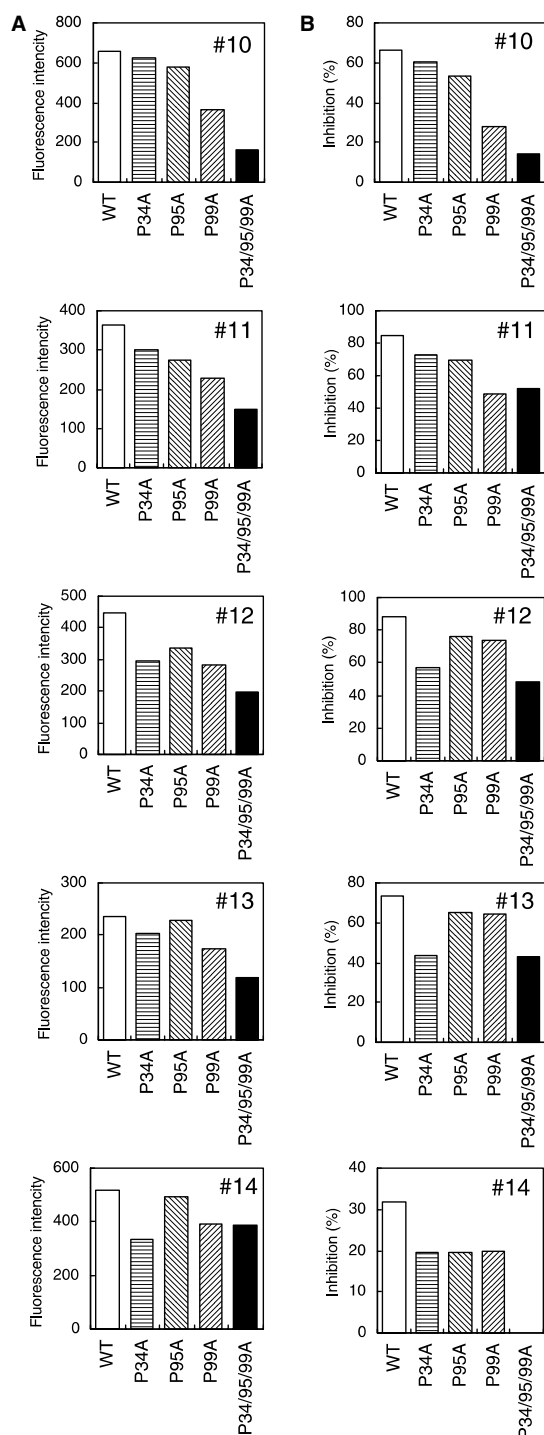


Fig. 4. Direct IgE-binding (A) and inhibitory activity (B) of single- and triple- mutants of Der f 2. The serum dilution was 1/8 for #10–12, 1/4 for #13, and 1/100 for #14. The allergen concentration in the immobilization step was 25 nM for #11 and #14, 50 nM for #10 and #13, and 90 nM for #12 in A, and 100 nM except for #12 (20 nM) in B. WT, wild-type Der f 2.

that the triple-mutant maintains significant numbers of IgE epitopes for some sera although its affinity is lower than the wild-type Der f 2. Disparities between the direct IgE-binding and inhibition experiments for Serum #14 (Fig. 4, #14) might be attributed to that the inhibition assay was performed in the experimental conditions where only 30% inhibition by the

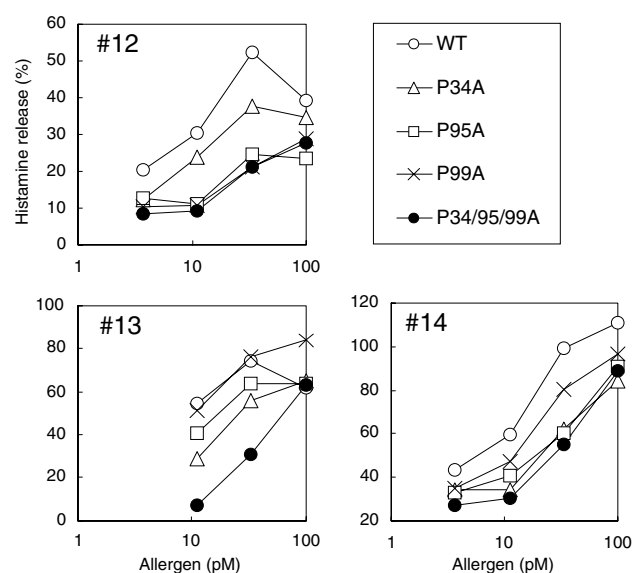


Fig. 5. Activities of single- and triple-mutants of Der f 2 to induce histamine release from basophils. The percentage of histamine released was calculated as the relative amount of histamine in the supernatant to the total amount of histamine. Spontaneous histamine release without stimulation by allergens in Volunteers #12, #13, and #14 was 7.5%, 2.0%, and 20.1%, respectively. The results shown are representative of two or three independent experiments. WT, wild-type Der f 2.

wild-type Der f 2 was observed in Serum #14 (Fig. 4B, #14) while 65–90% inhibition was observed in the other sera (Fig. 4, #10–13). The Der f 2-specific IgE titer of Serum #14 was very high and more extensive dilution (1/100 dilution) than other sera (1/3–1/8 dilution) was needed to detect even this level of inhibition. Further dilution of Serum #14 and/or increase of the inhibitor concentration might be necessary for detection of more significant inhibition by the triple-mutant.

Mutant allergens with reduced binding to IgE would be useful for a safer and more effective allergen-specific immunotherapy [15]. Previously, we proposed that the mutation of residues contributing to key intramolecular interactions to maintain the global structure of an allergen would be an effective strategy to reduce allergenicity [7]. A mutant Der f 2, C8/119S, which lacks a disulfide bond showed markedly reduced allergenic activities, maintained T-cell epitopes, induced Th1 rather than Th2 cytokine production and had hyposensitizing activities when administered to Der f 2-sensitized mice [6,7,32,33]. The compactness of the global structure was disrupted in this mutant [7]. On the other hand, it has been suggested that blocking antibodies belonging to a subclasses other than IgE contribute to the efficacy of allergen-specific immunotherapy [16,34]. The beneficial effect of the blocking antibodies is considered to arise from an impairment of allergic symptoms caused by blocking IgE from binding the allergen, modulating antigen-presentation through the uptake of allergen to antigen-presenting cells through Fc receptors, and/or downregulation of signal transduction through an Fcγ receptor with an inhibition motif. P34/95/99A with a nearly intact global structure might have an advantage in inducing the blocking antibodies. Fig. 6A shows that P34/95/99A modified only a small area of the molecular surface, and the surrounding unchanged areas would induce an IgG fraction that sterically hinders even the binding of P34/95/99 to IgE. Very recently, two mutants of birch pollen

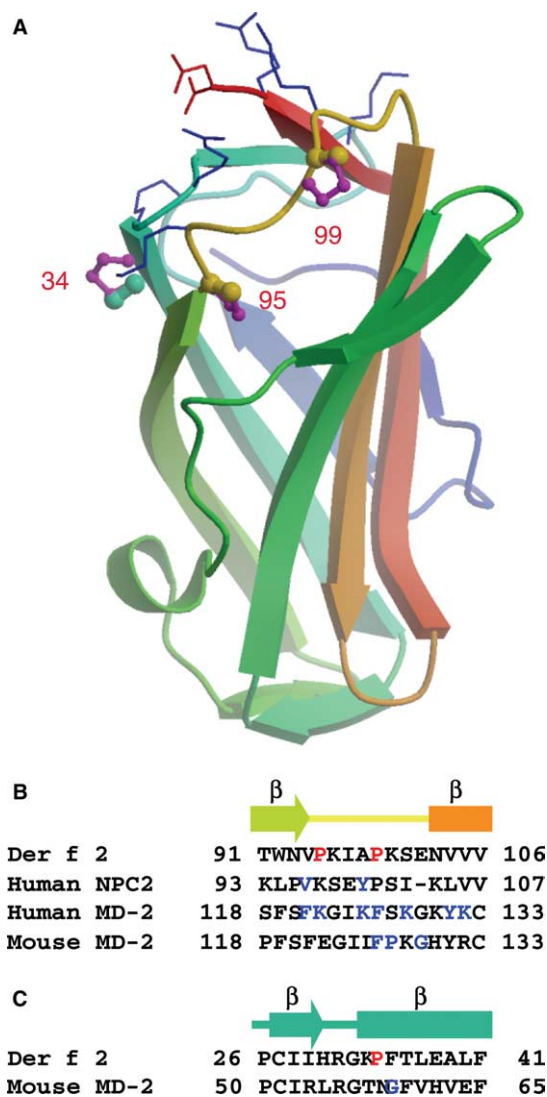


Fig. 6. Characteristic features of the region surrounding the triple-mutation P34/95/99A of Der f 2. (A) The color changes from blue (N-terminus) to red (C-terminus). The charged residues close to the mutations are shown as blue (positive) and red (negative) wires. (B and C) Sequence alignment of Der f 2, NPC2, and MD-2 including the triple-mutation. Blue residues in B and C have been reported to be involved in ligand-recognition.

major allergen Bet v 1 designed on the basis of the similar concept of reduction of IgE-binding without disruption of global structure has been reported, although four or nine amino acid substitutions were introduced into three or five different areas on the molecular surface in the two Bet v 1 mutants, respectively [17], differing from the Der f 2 mutant, P34/95/99A, in which three substitutions were introduced into one area.

Although prolines undergo *cis/trans* isomerization, and depending on the local topology, can induce drastic changes in the secondary and tertiary structure of a protein, the multiple-mutants of Der f 2 retained the global structure. This might be attributable to that the three prolines are positioned on a loop on the top of the molecule and not within structurally essential regions to form α helices or β sheets (Fig. 6A). We consider that the small differences between wild-type Der f 2 and P34/95/99A in secondary structure (Fig. 1B) and apparent

molecular compactness (Fig. 2) are due to local structural change. The spatial relationship of Pro95 and Try92 is appropriate for non-conventional C–H... π interaction [35], so the small change in CD spectra at 220–235 nm on P95A mutation might be due to the loss of this interaction. The change in the apparent molecular compactness suggests a fast equilibrium with a partially unfolded state. The first candidate for the unfolding site would be the loop containing Pro95 and Pro99, whose substitutions with alanine residues would have further increased the flexibility of the loop.

The target residues for the triple-mutation to achieve the significant reduction in allergenicity were revealed to be located on the characteristically charged molecular surface at the edge of the entrance of a potential ligand-binding cavity (Fig. 6A) and to correspond to the region essential to ligand-recognition in NPC2 and MD-2 (Fig. 6B and C). Such a cavity is also reported in the crystal structures of another mite group 2 allergen Der p 2 [36] and a mammalian cholesterol binding protein NPC2 [37], which are homologous to Der f 2. These experimental results and structural information are interesting when considering the relation between the ligand-binding structure and IgE-binding structure. The experimental results suggest this region is, at least, one of the major IgE-binding areas on the molecular surface of Der f 2. Such a characteristic combination of charge distribution, flexibility and proximity to the hydrophobic cleft is generally preferably recognized both by low-molecular ligands and by proteins. But whether other specific mechanisms are involved during sensitization processes in the background of this colocalization of a major IgE-binding area and the region potentially essential to ligand-recognition is still unknown.

In summary, we assessed the effect of multiple-mutations in one IgE-binding region, which do not cause a disruption of the global structure, on allergenicity using recombinant Der f 2 as a model allergen and suggested that such strategy is effective. Furthermore, we showed colocalization of a major IgE-binding area and the potential ligand-binding region of Der f 2 experimentally for the first time.

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