

Nuclear Magnetic Resonance Structure-Based Epitope Mapping and Modulation of Dust Mite Group 13 Allergen as a Hypoallergen¹

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IgE-mediated allergic response involves cross-linking of IgE bound on mast cells by specific surface epitopes of allergens. Structural studies on IgE epitopes of allergens are essential in understanding the characteristics of an allergen and for development of specific allergen immunotherapy. We have determined the structure of a group 13 dust mite allergen from *Dermatophagoides farinae*, Der f 13, using nuclear magnetic resonance. Sequence comparison of Der f 13 with homologous human fatty acid-binding proteins revealed unique surface charged residues on Der f 13 that may be involved in IgE binding and allergenicity. Site-directed mutagenesis and IgE binding assays have confirmed four surface charged residues on opposite sides of the protein that are involved in IgE binding. A triple mutant of Der f 13 (E41A_K63A_K91A) has been generated and found to have significantly reduced IgE binding and histamine release in skin prick tests on patients allergic to group 13 dust mite allergens. The triple mutant is also able to induce PBMC proliferation in allergic patients with indices similar to those of wild-type Der f 13 and shift the secretion of cytokines from a Th2 to a Th1 pattern. Mouse IgG serum raised using the triple mutant is capable to block the binding of IgE from allergic patients to wild-type Der f 13, indicating potential for the triple mutant as a hypoallergen for specific immunotherapy. Findings in this study imply the importance of surface charged residues on IgE binding and allergenicity of an allergen, as was also demonstrated in other major allergens studied. *The Journal of Immunology*, 2006, 176: 4852–4860.

Dust mites have been one of the major causes of allergy and asthma in the world. To date, >20 groups of allergenic proteins have been identified and characterized from dust mites. The group 2 allergen from dust mites is one of the most well studied allergens, and its three-dimensional structure has been well studied by using x-ray crystallography and nuclear magnetic resonance (NMR)⁴ spectroscopy (1, 2). The group 13 allergen belongs to the fatty acid binding protein family and has been cloned and characterized from a number of dust mites, namely *Blomia tropicalis* (3), *Acarus siro* (4), *Lepidoglyphus destructor* (5), and *Tyrophagus putrescentiae* (6). Prevalence studies have revealed that ~11% of the patients' sera tested have shown IgE binding to Blo t 13, the first characterized group 13 allergen from dust mites (3). We have cloned the group 13 allergen from *Dermatophagoides farinae*, Der f 13, by using expressed sequence tag approach (our unpublished data; GenBank accession no. AY283293) and used it as the target allergen for this study.

The primary response of the human body to an allergen is sensitization, which involves the differentiation of CD4⁺ T cells into Th2 cells. Th2 cells are responsible for producing Th2 cytokines that are necessary for the Ig class switching to IgE in B cells (7). Immediate symptoms of allergy require cross-linking of the high affinity receptor, FcεRI, by allergen-bound IgE to induce the release of inflammatory mediators (7, 8). Recent research in the treatment of allergies has mainly focused on immunotherapy by modulating allergen-specific T cells to shift the cytokine response from the Th2 to the Th1 pattern. Immunotherapy with bee venom has resulted in a reduction of Th2 cytokines like IL-4 and IL-5 but an increase in the secretion of a Th1 cytokine such as IFN-γ (9). Cytokines such as IL-4 and IL-5 play a critical role in the development of Th2 cells, whereas Th1-type cytokines (IFN-γ, TNF-α, and IL-12) play negative regulatory roles in the development of Th2 cells. IL-4 and IL-13 cytokines are able to promote differentiation of CD4⁺ T cells to Th2 cells and stimulate the Ig class switching of B cells to IgE (7). By reversing the response to the Th1-type cytokines, the production of blocking IgG Abs will be induced while giving rise to non-IgE-mediated allergy immune response. Studies have shown that blocking IgG Abs induced in immunotherapy were able to block IgE from binding to its epitope on the allergen in both the mouse model and clinical studies (10–12). Therefore, by blocking the cross-linking of the IgE receptor on mast cells, the release of biological mediators such as histamine and leukotrienes can be impeded.

Previous immunotherapy attempts were conducted by administering increasing dosages of allergens in the form of allergen extracts, peptides, or recombinant allergen proteins. However, direct usage of allergen extracts or recombinant allergens in immunotherapy might cause local and systemic effects such as anaphylactic shock and asthma attacks (7). Immunotherapy with birch pollen extract has been found to sensitize patients to develop IgE against new allergenic components (13), casting doubt on the use of the allergenic extract as a safe vaccine. These side effects can be

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⁴ Abbreviations used in this paper: NMR, nuclear magnetic resonance; CD, circular dichroism; FABP, fatty acid-binding protein; NOESY, nuclear Overhauser effect spectroscopy; 3A, E41A_K63A_K91A; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium salt.

avoided by other strategies such as the use of a T cell epitope-containing peptide or mutated recombinant allergens that lack epitopes necessary for binding to IgE (12). Similar studies (10) conducted recently on a major pollen allergen, Bet v 1, have shown that mutation of surface residues involved in IgE binding can generate hypoallergens with reduced IgE binding that retain the ability to induce Th1 rather than Th2 cell-type responses.

Fatty acid-binding proteins (FABPs) are small cytosolic proteins that facilitate the transport and solubility of fatty acids. In humans they are highly tissue specific and have been characterized from at least eight different tissues (14). Three-dimensional structures of these different FABPs have been extensively studied and found to be highly conserved, although their sequence similarities could vary between 20 and 70%. These proteins adopt a mixed α - β structure, with a typical β barrel made up of 10 antiparallel β strands and covered with a pair of α helices at one end (15–17).

As human FABP are nonallergenic and can be found abundantly in humans, we have conducted a structural analysis of Der f 13 and compared its structure and sequence with those of human FABP to identify putative IgE binding epitopes. Cross-comparison of human FABP and several group 13 allergens has identified several key surface residues of Der f 13 that are mostly conserved among group 13 allergens but are vastly different from their corresponding positions in human FABP. Further mutagenesis studies have confirmed that four of these residues are essential for IgE binding and cross-linking. In the present study, we report the structural study and IgE epitope mapping of Der f 13 as well as the generation of a Der f 13 triple mutant as a hypoallergen that has reduced IgE binding but retained T cell reactivity. By comparing the sequence and structure of allergen with nonallergenic homologous proteins in humans, we can identify key residues that are involved in IgE binding and generate vaccine candidates that can be used for allergen-specific immunotherapy.

Materials and Methods

Subcloning and site-directed mutagenesis

A DNA insert of Der f 13 was generated by PCR using an upstream oligonucleotide primer containing a *Bam*H I restriction site and a downstream oligonucleotide primer containing a stop codon and a *Xho*I restriction site. The DNA insert was ligated into a modified pET-32a plasmid (Novagen) and transformed into DH5- α competent cells. Colonies were screened by PCR, and the sequence of the insert was verified by DNA sequencing (Big Dye v3.1; Applied Biosystems). Mutant constructs were prepared by PCR-based overlap extension using designed oligonucleotide primers (1st BASE) with mismatches (18). A mutated DNA insert was subcloned into the same expression vector as described above. For GST-tagged proteins used in ELISA experiments, constructs were subcloned using a similar method into pGEX-4T1 plasmid (Amersham Biosciences).

Expression and purification of recombinant Der f 13

Plasmid containing the DNA insert of Der f 13 or its mutants was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression. Overnight cultures of the transformed bacterial cells were grown in 1 liter of Luria broth containing 100 μ g/ml ampicillin, and protein expression was induced with a 0.3 mM final concentration of isopropyl β -D-thiogalactoside at 37°C. The protein was expressed as a His-tagged soluble protein and purified using a Ni-NTA resin (Qiagen). The His tag was cleaved by thrombin (5 U/mg protein; Sigma-Aldrich), and the protein was further purified using a HiLoad 16/60 Superdex 75 prep grade (Amersham Biosciences) size exclusion chromatography column on the AKTA fast protein liquid chromatography system (Amersham Biosciences) in Tris-Cl buffer (50 mM Tris-Cl (pH 7.9) and 500 mM NaCl). Protein concentration was determined by UV absorption at 280 nm in a Hitachi spectrophotometer. GST-tagged proteins were expressed as described above and purified using glutathione-Sepharose 4B column (Amersham Biosciences).

Circular dichroism (CD) spectropolarimetry

CD experiments were conducted with 10 μ M protein in 50 mM acetate buffer (pH 4.5) at room temperature. The spectra were acquired with a

J-810 spectropolarimeter (Jasco) using a quartz cuvette with 1-mm path-length (Hellma). The spectra were recorded at a wavelength region from 190 to 260 nm with 0.1-nm resolutions using a scan speed of 50 nm/min and averaged for 10 scans.

NMR experiments

Purified Der f 13 protein was concentrated to ~1 mM using Centrprep YM-3 (Millipore) and exchanged into buffer containing 50 mM acetate buffer (pH 4.5) with 10% deuterium oxide. NMR experiments were performed at 298 K on a Bruker Avance 500 MHz NMR spectrometer equipped with a cryoprobe. Backbone assignments were conducted by HNCACB (19) and CBCA(CO)NH (20) experiments using uniformly labeled ¹⁵N and ¹³C protein sample. Side chain proton assignments were obtained from H(CCO)NH, (H)CC(CO)NH (21), and HCCH-total correlation spectroscopy (22) experiments. Stereo-specific assignments of methyl groups were obtained by ¹H-¹³C heteronuclear single quantum coherence of a 10% ¹³C-labeled protein sample (23). Proton distance constraints were obtained from three-dimensional ¹⁵N-edited nuclear Overhauser effect spectroscopy (NOESY) and ¹³C-edited NOESY with 100-ms mixing time. Hydrogen bond restraints were determined by recording ¹H-¹⁵N heteronuclear single quantum coherence of uniformly ¹⁵N labeled protein exchanged into 100% deuterium oxide buffer for 4 h. NMR data was processed using NMRPipe (24) and analyzed by NMRView (25).

Structures were solved by using the software CYANA (26) with nuclear Overhauser effect restraints from ¹⁵N-NOESY and ¹³C-NOESY experiments, hydrogen bond restraints, and dihedral angle restraints predicted by the program TALOS (27). Structures were calculated by DYANA using standard torsion angle dynamics protocol, and 10 conformers with the lowest target function values were selected for further energy minimization in AMBER 7.0 (28). The final 10 structures were checked with Procheck-NMR (29) and deposited in the Protein Data Bank (accession code 2A0A).

Specific IgE binding ELISA experiment

For ELISA experiments, all proteins used were GST-tagged fusion proteins for maximum binding on the ELISA plate. The volumes of all samples and reagents used in ELISA experiments were 100 μ l, whereas the washing steps were conducted three times with 200 μ l of PBS plus 0.05% Tween 20 (PBS-T) each. Sera from patients were diluted 1/3 with 1% BSA in PBS and preabsorbed with a 0.5 mg/ml final concentration of GST protein overnight at 4°C in a 1000- μ l reaction. Wild-type Der f 13 or mutants were coated overnight at 4°C onto Maxisorp ELISA plate (Nunc) at 1 μ g of protein per well. Plates were washed with PBS-T and blocked with PBS plus 1% BSA for 30 min at room temperature. The plates were washed again with PBS-T and incubated with preabsorbed sera for 2.5 h at room temperature. After washing with PBS-T, biotin-conjugated anti-human IgE mAb (mouse IgG isotype, lot no. M075594; BD Pharmingen) diluted 1/250 in PBS plus 1% BSA was added and incubated for 2 h. Following washing with PBS-T, avidin-conjugated HRP (BD Pharmingen) diluted 1/1000 in PBS with 1% BSA was added and incubated for another 30 min. The plates were then thoroughly washed with PBS-T, and 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich) was added. The color reaction was observed through absorbance measurements at 655 nm using an ELISA plate reader. An assay on each sample was conducted in duplicate, and result was reported as the mean value.

Inhibition ELISA experiment

Maxisorp ELISA plate (Nunc) was coated with a GST-Der f 13 protein (1 μ g in 100 μ l) overnight at 4°C. Sera (final dilution of 1/3) were preabsorbed with a 0.5 mg/ml final concentration of GST protein and various amounts (0.01, 0.1, 1, 10, and 50 μ g) of Der f 13 or the E41A_K63A_K91A triple mutant of Der f 13 (henceforth designated the 3A mutant) in a 100- μ l reaction. After overnight incubation, plates were washed three times with 200 μ l of PBS-T and blocked with 100 μ l of PBS plus 1% BSA for 30 min at room temperature. The plates were washed again with PBS-T and incubated with the preabsorbed sera for 2.5 h at room temperature. Subsequent Abs incubation and colorimetric development were done as described above. The result was reported as mean value from two separate assays.

Skin prick test

Written consents were obtained from allergic patients before the skin prick tests. The tests were conducted with 2 μ l of recombinant protein (0.02 mg/ml) in PBS buffer containing 50% glycerol. All proteins and buffer used in these experiments were filter sterilized with a 0.22- μ m filter membrane. Skin prick was conducted with sterile lancet, and measurement of wheal and erythema was conducted after a 20-min interval. Histamine (10 mg/ml)

Table I. Structural statistics for the NMR solution structure of Der f 13

Data Collection	Statistics
Restraints Used for Calculation	
Total NOE ^a restraints	2299
Intraresidue	832
Sequential	604
Medium range	303
Long range	560
Hydrogen bond distance restraints	92
Dihedral angle constraints	132
CYANA target function	1.55 ± 0.48
Maximum violation for upper limits (Å)	0.32 ± 0.09
Maximum violation for torsion angles (degree)	2.75 ± 0.84
AMBER energies (kcal/mol)	
Total energy	-4817 ± 40
Ramachandran statistics (%)	
Most favored	71.8
Additionally allowed	22.6
Generously allowed	4.3
Disallowed	1.3
Root mean square deviation from mean structure (Å)	
Backbone atoms (residues 6–131)	0.59 ± 0.08
All heavy atoms (residues 6–131)	1.13 ± 0.09

^a Nuclear Overhauser effect.

was included as positive control, and PBS buffer was included as a negative control.

PBMC proliferation and cytokine expression

PBMCs were isolated from fresh blood by using a general Ficoll-Hypaque gradient centrifugation technique. PBMCs were cultured in 100 µl of RPMI 1640 and 10% FBS medium in 96-well plates at 1×10^5 cells per well. One hundred microliters of wild-type Der f 13 or 3A mutant was added to the cells at a final protein concentration of 100 µg/ml. PBMCs were incubated for 6 days in a 5% CO₂ incubator at 37°C. Cells were tested for proliferation by using the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium salt (WST-1) colorimetric method according to standard manufacturer's protocol (BioVision). Absorbance was measured at a 450-nm wavelength with a reference wavelength of 650 nm. Proliferation was reported as the stimulation index using the formula $(S - C)/C \times 100$, with S as the absorbance of stimulated cells and C as the absorbance of control nonstimulated cells. For the measurement of cytokines, supernatants of the cell cultures at day 6 were harvested. Cytokine concentrations were determined by using human Th1/Th2 BioPlex cytokine assay (Bio-Rad) according to the standard protocols from manufacturer for Th1 and Th2 cytokines. Assay on each sample was conducted in duplicate, and the result was reported as mean value.

Mouse immunization

Mouse immunization experiments were conducted to compare the abilities of the wild-type and the 3A mutant of Der f 13 to induce IgG Abs specific for Der f 13. Eight-week-old female BALB/c mice were used in our studies. Groups of 5 mice were each injected i.p. with 200 µl of Ag containing

15 µg of purified Der f 13 or the 3A mutant conjugated with 1.25 mg/ml aluminum hydroxide gel (Sigma-Aldrich) every 2 wk. Buffer containing adjuvant aluminum hydroxide was similarly injected into another mouse as buffer control. Preimmune and postimmune blood samples after the sixth injection were drawn by orbital bleeding, and sera were kept at -20°C until analysis. Animals were maintained in the Animal Holding Unit of the Faculty of Medicine, National University of Singapore, according to local guidelines.

Inhibition of human IgE binding to Der f 13 by specific mouse IgG antibodies

The volumes of all samples and reagents used in ELISA experiments were 100 µl, whereas the washing steps were conducted three times with 200 µl of PBS-T each time. GST-tagged Der f 13 was coated overnight at 4°C onto a Maxisorp ELISA plate (Nunc) at 1 µg of protein per well. Plates were washed with PBS-T and blocked with PBS plus 1% BSA for 30 min at room temperature. The plates were washed again with PBS-T and pre-incubated with 1/10 diluted mouse serum from control mouse or from mice immunized with either Der f 13 or 3A mutant. The wells for control were incubated with PBS plus 1% BSA alone. Plates were incubated for 2.5 h at room temperature. After incubation, plates were washed with PBS-T and incubated with 1/5 diluted pooled sera (from patients D2 and H3) in PBS plus 1% BSA for 2.5 h. After washing with PBS-T, biotin-conjugated anti-human IgE mAb (mouse IgG isotype, lot no. M075594; BD-Pharmingen) diluted 1/250 in PBS plus 1% BSA was added and incubated for 2 h. Following washing with PBS-T, avidin-conjugated HRP (BD Pharmingen) diluted 1/1000 in PBS plus 1% BSA was added and incubated for another 30 min. The plates were then thoroughly washed with PBS-T, and 100 µl of TMB substrate (Sigma-Aldrich) was added. The color reactions were observed through absorbance measurements at 655 nm. Assay on each sample was conducted in duplicate, and the result was reported as mean value. Percentages of inhibition of human IgE binding to Der f 13 after preincubation with mouse sera were determined with the following formula: percentage of inhibition of IgE binding = $100 - (OD_i/OD_c) \times 100$. OD_i and OD_c represent absorbance values after preincubation with mouse sera and BSA control, respectively.

Approval

The human studies involved in this report have been reviewed and approved by the Institutional Review Board of the National University Hospital and the Hospital Ethics Committee of the Kangar Kerbau Women's and Children's Hospital.

Results

Three-dimensional structure of Der f 13 and its site-directed mutagenesis

Der f 13 protein was expressed as a His-tagged fusion protein and purified using Ni-NTA affinity chromatography. The His tag was cleaved away using thrombin, and the protein was subjected to further purification by size exclusion chromatography. Typically, ~30 mg of protein can be obtained from 1 liter of bacterial culture. The purified wild-type and mutant Der f 13 proteins appeared as a single band with an approximate size of 15 kDa when viewed by SDS-PAGE. Der f 13 is a highly soluble protein, and the structure

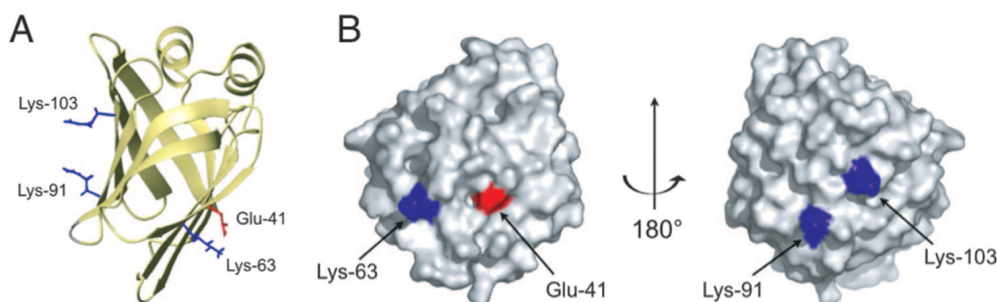


FIGURE 1. Solution structure and IgE binding epitope residues of Der f 13. **A**, Ribbon diagram showing locations of epitope residues found on Der f 13. The four charged surface residues that are involved in IgE binding are shown in wire frame models. **B**, Surface diagrams of Der f 13 show that the four charged residues, Glu⁴¹, Lys⁶³, Lys⁹¹, and Lys¹⁰³, are located on two opposite sides of the protein and form two separate regions of the IgE epitope on Der f 13. Negatively charged residues are colored red, and positively charged residues are colored blue.

Table II. Analysis of charged amino acids compositions of major allergens and their human homologs^a

	Lys	Arg	Basic (%)	Asp	Glu	Acidic (%)	Epitope Residues
<i>Der f 13</i>	18	6	18.3	9	15	18.3	<i>E41,K63,K91,K103</i>
FABP1	15	2	13.3	4	13	13.3	
FABP2	14	7	15.9	8	13	15.9	
FABP3	14	4	13.5	11	8	14.2	
FABP4	14	6	15.1	11	9	15.1	
FABP5	14	6	14.8	8	12	14.8	
FABP6	12	2	10.9	5	11	12.5	
FABP7	12	5	12.8	10	10	15.1	
FABP8	18	7	18.9	5	9	10.6	
<i>Der p 2</i> (Ref. 32)	14	2	12.4	9	7	12.4	<i>H30,R31,K33,S57,K96,I97,E102</i>
NPC2	12	1	8.6	6	6	8.0	
<i>Equ c 1</i> (Ref. 31)	11	9	12.5	13	18	19.4	<i>R26,E82,K129,E148</i>
Lipocalin 9	7	10	9.6	8	13	11.9	
<i>Bet v 1</i> (Refs. 10, 33)	15	3	11.3	9	14	14.4	<i>E42,N43,I44,E45,G46,N47,G48,G49,P50,G51</i>
<i>Hev b6.02</i> (Ref. 30)	2	1	6.9	3	2	11.6	<i>R5,K10,E29,Y30,Q38</i>

^a The contents of positively and negatively charged amino acid residues in selected major allergens are compared with their human homologs. The allergenic proteins are italic and boldfaced. Der f 13 is compared with human FABPs (see Fig. 2 for abbreviations), whereas Der p 2 and Equ c 1 are compared with the homologous human NPC2 and lipocalin 9, respectively. All of the allergens listed are found to have a higher overall percentage of charged residues compared to their homologous human proteins. The residues identified as IgE binding epitopes in these allergens are listed and found to be mostly charged and polar residues.

was solved by using NMR spectroscopy to a high resolution (Table I). The NMR chemical shift and structure coordinates were deposited in the BioMagResBank (accession number 6724) and the Protein Data Bank (accession code 2A0A), respectively. The solution structure of Der f 13 closely resembles the typical fold of a FABP family protein. A β strand at the N-terminal region of the protein is followed by a helix-turn-helix motif and then another nine antiparallel β strands (Fig. 1A). The 10 β strands form a β barrel, with the two α helices situated at one end of the barrel. When compared with other human FABPs, Der f 13 has a much higher percentage composition of both positively and negatively charged residues (Table II). In total, Der f 13 consists of 18.3% positively charged residues and 18.3% negatively charged residues.

Selection of amino acid residues for site-directed mutagenesis was based on the amino acid sequence alignment of Der f 13 and its isoforms with eight known human FABPs (Fig. 2). Residues that have distinctly different properties (e.g., charged vs hydrophobic residues) in Der f 13 and nonallergenic human homologues were selected for mutation. Of the 18 residues selected in Der f 13, 17 of them were either positively or negatively charged residues,

whereas most of their corresponding residues in human FABP were either hydrophobic or polar or have opposite charges. The other residue, Asn⁴⁶, is a polar residue, whereas its corresponding residue is a glycine residue in human FABP. All of these residues were surface-exposed as confirmed by the three-dimensional structure of Der f 13. Each of the 18 residues was individually mutated to alanine residues to determine the effect of mutation on IgE binding to Der f 13 using sera from patients allergic to group 13 dust mite allergens.

Reduced binding to serum IgE

All of the wild-type and mutant Der f 13 proteins used for the ELISA experiments were GST tagged, because the untagged Der f 13 proteins did not bind well on microtiter plate surface. The patient sera used in all ELISA experiments were blocked with GST protein, and all experiments were conducted with the GST protein being the negative control. Sera from four Singaporean and two South African patients who were allergic to group 13 allergens were used for the IgE binding assays. The 18 single mutants of Der

FIGURE 2. Sequence alignment of Der f 13 with other group 13 dust mite allergens and human FABPs. Residues shaded gray are surface residues found to have different properties between Der f 13 and human FABPs and have been selected for mutagenesis studies. Boxed residues are found to have significantly lower IgE binding when mutated to alanine residues. FABP1, liver FABP; FABP2, intestinal FABP; FABP3, muscle and heart FABP; FABP4, adipocyte human FABP; FABP5, psoriasis-related FABP; FABP6, ileal FABP; FABP7, brain FABP; and FABP8, myelin FABP. Lepd13, Blot13, Tyrp13, and Derf13 are group 13 dust mite allergens from *L. destructor*, *B. tropicalis*, *T. putrescentiae* and *D. farinae*, respectively.

FABP1	----MSFSGKYQLQSQENFEAFMKAIGLPEELIQKGDIKGVSEIVQNGKHFKFTIT-AG	55
FABP2	----MAFDSTWKVDRSENVDKFMKMGVNIIVKRKLAHDNLKLTITQEGNKFTVKES-SA	55
FABP3	----MVDALGTLKWLVDKSNFDDYMKSLGVGFATRQVASMTPKPTTIEKNGDILTLKTH-ST	57
FABP4	----MCDAFVGTWKLVSSENFDDYMKGVGFATRKVAGMAKPNMTISVNGDVITIKSE-ST	57
FABP5	MATVQQLEGRWRVLVDKSGFDEYMKELGVGIALRKMAMAKPDCIITCDGKNLTIKTE-ST	59
FABP6	----MAFTGKFEMSEKNIYDEFMKLLGISSDVIEKAHNFKIIVTEVQQDQDFTWSQHYG	56
FABP7	----MVEAFCATWKLTSNQNFDEYMKALGVGFATRQVGNVTKPTVVISQEGDKVIVRTL-ST	57
FABP8	----MSNKFGLTWKLVSSENFDDYMKALGVGLATRKLGSLAKPTVVISKKGDIITIRTE-ST	57
Lepd13	----MANIAGQYKLDKSENFDDYMKALGVGLVFLVKTAAKTVPKPTLEVAVDGDYIFRSL-ST	56
Blot13	----MP-IEGKYKLEKSDNFDFKFLDELGVGFVMTAAKTLKPTLEVVDVQGDYVFRSL-ST	55
Tyrp13	----MVQLNGSYKLEKSDNFDAFLKELGVNFVTRNLAKSASPTVEIVDGDYTIKTS-ST	56
Derf13	----MASIEGKYKLEKSEKDFEFLDKLGVGFVMTAAKTLKPTLEVVAIENDQYIFRSL-ST	56

FABP1	SKVIQNEFTVGECELEMTGKVKTVVQLGEGNKLVT-----FKNIKSVTELNGDIIT	110
FABP2	FRNIEVVFELGVTFNYNLADGTELGRGWSLEGKLIKFKRTDNGNELNTVREIIGDELV	115
FABP3	FKNTEISFKLGVFEDETTADDRKVKSIIVTLDDG-KLVHLQKWDG-QETTLVRELIDGKLI	115
FABP4	FKNTEISFLLGQFEDEVTTADDRKVKSIIVTLDDG-VLVHVQKWDG-KSTTIKRKLVDKLV	115
FABP5	LKTTQFSCTLGKFEETTADGRKTQTVCNFTDG-ALVQHQEWG-KESTITRKLKDGKLV	117
FABP6	GHTMTNKFVTGKESNIQTMGGKTFKATVQMEG-GKLVVN-----FPNYEQTSEIVGDKLV	110
FABP7	FKNTEISFQLGEEFDETTADDRNCKSVVSLDGD-KLVHIQKWDG-KETTFVREIKDGKLV	115
FABP8	FKNTEISFLLGQFEDEVTTADNRKTSIIVTLQRG-SLVNQVQRWDG-KETTIKRKLVDKLV	115
Lepd13	FKNTEIKFKLGEEFEEDRADGKRVKTVIVKDGDKNFVQTYGD--KEVKVVRFEFGDEVE	114
Blot13	FKNTEIKFKLGEEFEEDRADGKRVKTVVNEKGDNKFVQTYGD--KEVKVVRFDQGDV	113
Tyrp13	LKNSIEKFKLGEEFEEDRADGKRVKTVSVTKEGDNKLVQVQKGD--KPVTVVRFSEGLT	114
Derf13	FKNTEIKFKLGEEFEEDRADGKRVKTVIQKEGDNKFVQTYQFGD--KEVKVIRFNGDEV	114

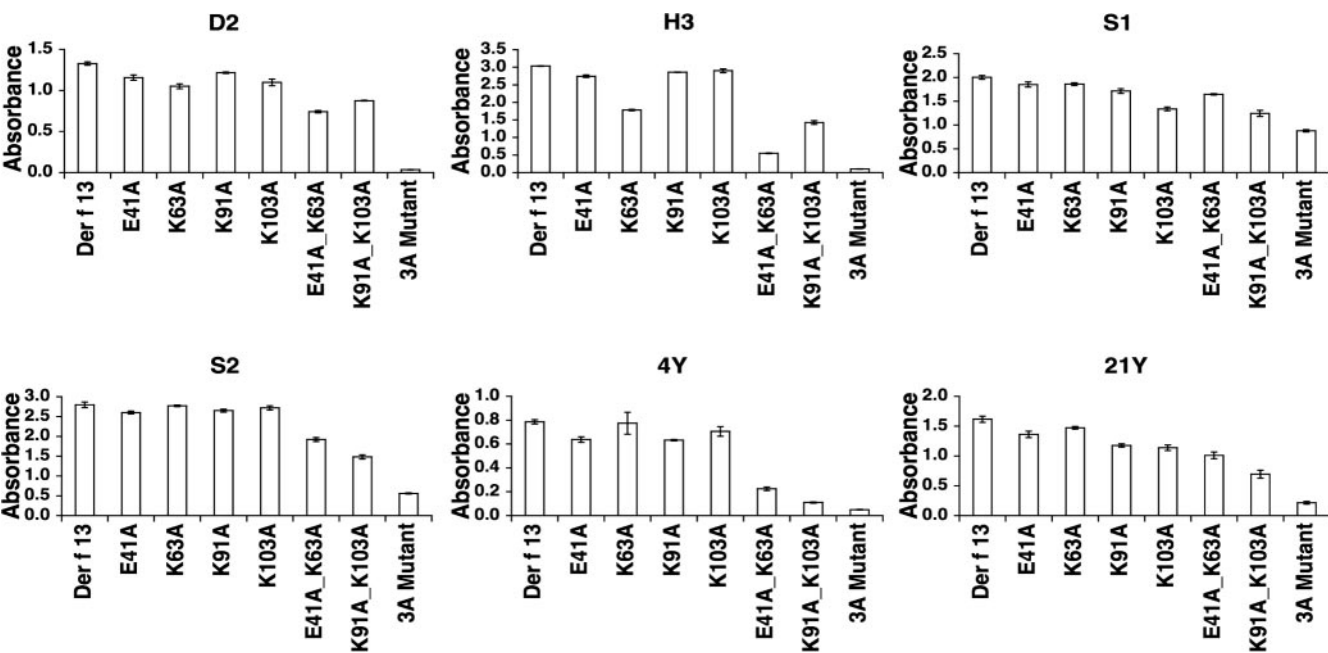


FIGURE 3. IgE binding assay of wild-type and mutant Der f 13. For all patients, except S1, there were significant reductions in IgE binding for the two double mutants, E41A_K63A and K91A_K103A, and the 3A triple mutant, E41A_K63A_K91A, compared with single mutants. Patients D2, H3, S1, and S2 are Singaporeans, whereas patients 4Y and 21Y are South Africans. Note that the y-axes are of different scales and upper limits.

f 13 were tested for IgE binding in our preliminary ELISA screening using serum from patient H3, which showed the highest level of IgE binding, and serum from patient D2, which is among the sera with the lowest IgE binding levels (Fig. 3).

IgE binding ELISA experiments have shown that E41A, K63A, K91A, and K103A are four of the 18 single mutants that have significantly lower IgE binding compared with the wild-type protein (Fig. 3), whereas the other mutations did not affect IgE binding (data not shown). These four residues, coincidentally, are all charged residues and are exposed on the surface of the protein. They are located on the $\beta 2$ strand, the $\beta 4$ strand, the $\beta 7$ strand, and the $\beta 8$ strand, respectively (Fig. 1B). The four residues can be grouped into two patches based on their proximity to each other, with Glu⁴¹ and Lys⁶³ on one side and Lys⁹¹ and Lys¹⁰³ on the opposite side of the β barrel. The inter-residue distance between Glu⁴¹ and Lys⁶³ is ~ 7.8 Å, whereas that of Lys⁹¹ and Lys¹⁰³ is 11.5 Å. The two double mutants, E41A_K63A and K91A_K103A, of Der f 13 showed further reductions in IgE binding (Fig. 3), suggesting that these residues are essential for IgE binding and may form two separate groups of IgE epitope on opposite sides of the protein. A similar trend of reduction in IgE binding was also observed when these mutant Der f 13 proteins were tested against sera from the other two Singaporean (S1 and S2) as well as the two South Africans (4Y and 21Y) (Fig. 3). IgE binding on the 3A mutant of Der f 13 was also determined and found to be the lowest among all the mutants tested. When sera from patients D2, H3, 4Y, and 21Y were used, IgE binding was reduced to $<15\%$ for the 3A mutant as compared with wild-type Der f 13. The IgE binding was reduced to 43 and 21%, respectively, when sera from patients S1 and S2 were used (Fig. 3). These data show that the three mutated residues are common IgE epitopes among most of the patients tested irrespective of their varied degrees of IgE binding for Der f 13, but small discrepancies in the exact IgE epitopes does exist between different patients. Identities of the IgE epitopes are further verified by inhibition ELISA studies showing that preincubation of the sera from patients D2 and H3 with 3A mutant, but not wild-

type Der f 13, failed to sequester IgE that remained available to bind on Der f 13 (Fig. 4).

Skin prick test

To determine the abilities of the wild-type and 3A mutant of Der f 13 to cross-link IgE in vivo to release inflammatory mediators, we conducted skin prick tests on patients D2 and H3, who are allergic to group 13 dust mite allergens. Wild-type Der f 13 and 3A

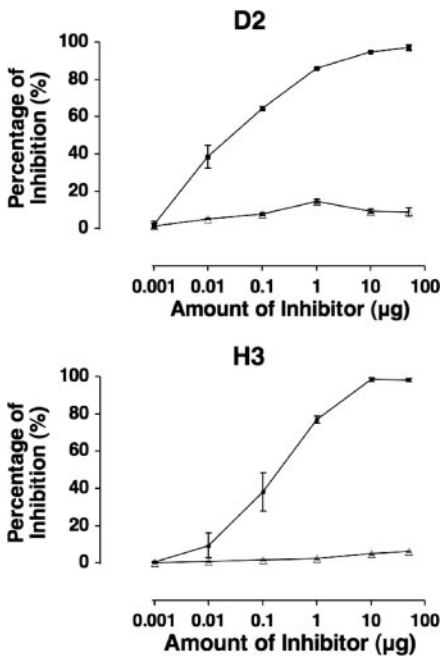


FIGURE 4. Inhibition IgE binding assay of wild-type and mutant Der f 13. IgE binding to Der f 13 was inhibited in both patients with wild-type Der f 13 (■). The 3A triple mutant (△) was unable to inhibit IgE binding to Der f 13 for both patients.

mutant proteins were both tested, and histamine and PBS were used as positive and negative controls, respectively. Both patients showed strong reactions to the wild-type Der f 13 similar to the level produced by the histamine control, although the 3A mutant showed a negative skin prick result with wheal diameter of <2 mm (Table III). To verify that the 3A mutant of Der f 13 has lost its IgE binding and cross-linking abilities due to disruption of the specific IgE binding epitope rather than its overall structure, we compared the CD spectra of wild-type and mutant Der f 13 to monitor their structural changes. The far-UV CD spectrum of Der f 13 showed a typical β -sheet structure with a single minimum peak at ~ 218 nm and another maximum at ~ 195 nm. The overall secondary structure of the 3A mutant remained unchanged and resembled that of the wild-type Der f 13 protein (Fig. 5A). The elution volume of the 3A mutant remained the same as that of the Der f 13 in the size exclusion chromatography, suggesting that the 3A mutant protein did not aggregate or oligomerize under the buffer conditions used (Fig. 5B).

T cell reactivity and shifted cytokine release profile

Immunogenicities of wild-type and 3A mutant of Der f 13 were determined by measuring T cell proliferation in the PBMCs of patients when challenged with either of these proteins. PBMCs were isolated from fresh blood samples from allergic patients using the standard Ficoll-Hypaque gradient centrifugation method. Mixed T cell populations in PBMCs were stimulated with 100 μ g/ml wild-type or the 3A mutant of Der f 13. The degree of T cell proliferation was measured after 6 days of incubation by determining mitochondrial dehydrogenase activity using the WST-1 reagent. Similar degree of proliferation was observed between cultures stimulated with either the wild-type or the 3A mutant of Der f 13 (Fig. 6). Overall, patient H3, who showed a higher degree of IgE binding for Der f 13, also had a higher PBMC stimulation index than patient D2. In the PBMC culture of patient D2, stimulation indexes for the wild-type and the 3A mutant of Der f 13 were 22.5 and 23.6%, respectively, whereas the stimulation indexes for wild-type and 3A mutant of Der f 13 were 53.8 and 52.1% respectively in the PBMC culture of patient H3.

Although the degrees of T cell proliferation stimulated by the wild-type and the 3A mutant of Der f 13 are similar, the proliferation could follow either a Th1 or a Th2 pattern. The pattern of T cell proliferation can be determined by measuring the amount of different cytokines being produced and released into the supernatant of the stimulated PBMC cultures after 6 days of incubation with the respective proteins. The level of IL-13 was used as a marker for the Th2 pattern, whereas that of IFN- γ was used as indicator for the Th1 pattern (Fig. 7). The level of each cytokine was compared between PBMC cultures stimulated with the wild-

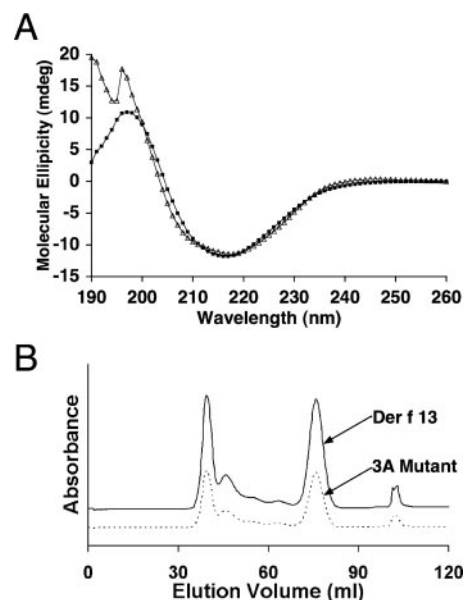


FIGURE 5. CD spectra and size exclusion chromatography elution profiles of the wild-type and the 3A mutant of Der f 13. *A*, Far-UV CD spectra of the wild-type (■) and the 3A mutant (△) of Der f 13. Both proteins showed a spectrum typical of a β -sheet protein with minima at ~ 218 nm. Spectra shown were averages of 10 scans and were recorded at room temperature. *B*, Size exclusion chromatography elution profiles of Der f 13 (solid line) and 3A mutant (broken line). The peaks corresponding to the active fractions are labeled.

type and then 3A mutant of Der f 13 for patients D2 and H3. PBMC cultures from both patients showed a Th2 pattern of T cell proliferation when stimulated with wild-type Der f 13, with an increased level of IL-13 compared with the 3A mutant. Increased levels of IFN- γ were observed in both patients when their PBMC cultures were stimulated with the 3A mutant of Der f 13 compared with the wild-type protein, indicating a Th1 pattern of T cell proliferation. Major differences were observed, especially in patient D2, with the levels of IL-13 cytokine almost completely suppressed in PBMC cultures stimulated with the 3A mutant, while the level of IFN- γ was notably increased. In patient H3, total suppression of IL-13 was not observed when PBMCs were stimulated with the 3A mutant, but a significant decrease in the level was detected compared with that in PBMCs stimulated with wild-type Der f 13. The secretion level of IFN- γ was enhanced when the PBMCs of patient H3 were stimulated with the 3A mutant compared with wild-type Der f 13. These data suggest that patient D2, who had lower IgE binding to Der f 13, can be switched more effectively from a Th2 to a Th1 cytokine profile upon hypoallergen treatment as compared with patient H3.

Blocking of IgE binding by mice IgG induced from 3A mutant

Strong and specific IgG responses were induced in both groups of mice immunized with the wild-type or the 3A mutant of Der f 13. To measure the inhibitory effect of the IgG raised on IgE binding, mouse antisera were assessed with inhibition ELISA using pooled human sera from patients D2 and H3. Mouse sera from both immunization groups were able to inhibit the IgE binding to Der f 13 at a percentage >60%, whereas a couple of the immune sera from each group were able to inhibit IgE binding to >80% (Fig. 8). Serum from the control mouse (immunized with buffer and adjuvant) was unable to block the binding of patient IgE to Der f 13.

Table III. Skin prick tests for wild-type Der f 13 and 3A mutant^a

	Patient			
	D2		H3	
	Wheal	Erythema	Wheal	Erythema
PBS	—	—	—	—
Der f 13	++	++	+	++
3A mutant	—	—	—	—

^a Patients D2 and H3 were skin pricked with 0.02 mg/ml wild-type Der f 13 and 3A mutant. One plus sign (+) represents a marginal positive reaction (wheal and erythema diameter of at least 4 mm and 20 mm, respectively, whereas two plus signs (++) refer to a reaction similar to that observed for histamine control. Negative sign (—) denotes negative reaction with wheal diameter of 2 mm or less and erythema diameter of <15 mm.

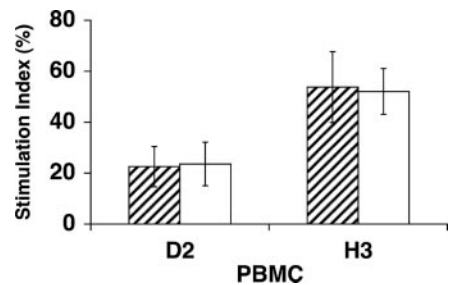


FIGURE 6. PBMC proliferation induced by the wild-type and the 3A mutant of Der f 13. PBMCs from Der f 13-allergic patients D2 and H3 showed similar PBMC proliferation when stimulated with either the wild-type (striped bar) or the 3A mutant (empty bar) of Der f 13. Proliferation was measured with the WST-1 colorimetric method and expressed as stimulation index (%) compared with control PBMCs without stimulation.

Discussion

FABP plays an important role in the trafficking and transport of fatty acids in human tissues. It has been proposed that members of the FABP family are involved in the cellular uptake of lipids and their transport to sites of metabolism. A group of allergens (group 13) isolated from the dust mite that causes allergic response and pathological effect in humans is identified as part of the FABP family. Studies on the allergenic properties of the dust mite FABP are still lacking due to its lower prevalence of allergenicity as compared with that of other major groups of allergens from dust mites, such as group 1 and group 2 allergens. The group 13 dust mite allergen Der f 13 shares a medium high sequence homology with human FABPs, with the closest one being human brain FABP having 39.1% amino acids identity and 58.6% homology. The solution structure obtained in our study revealed that Der f 13 adopts the typical β barrel fold of a FABP very similar to that of other human FABPs. Human FABP, however, could neither bind to serum IgE from patients allergic to Der f 13 nor elicit skin prick reactions in those patients (data not shown). Based on the solution structure of Der f 13, we can identify those exposed amino acid residues that are distinctly different from human FABPs but conserved among group 13 allergen by analyzing their sequences. These residues are likely to be the IgE epitopes and may even be the key residues that gave rise to the allergenicity of FABP in dust mites.

Initial sequence alignment with human FABPs has revealed 18 surface residues on Der f 13 that were subsequently mutated to alanine residues individually via site-directed mutagenesis (Fig. 2). IgE binding studies by ELISA shown that four of these residues, Glu⁴¹, Lys⁶³, Lys⁹¹, and Lys¹⁰³, were essential for IgE binding by Der f 13. These four identified IgE binding epitope residues all happen to be charged amino acids and are situated at two separate sites on opposite sides of the protein (Fig. 1B). These residues are also conserved among other group 13 allergens, including Blo t 13 (3), Lep d 13 (5), and Tyr p 13 (6), except for residue Lys¹⁰³, which is replaced by a threonine in Tyr p 13. Additional experiments on the 3A mutant showed that mutation of all three residues, E41A, K63A, and K91A, can abolish almost entirely the binding of Der f 13 to IgE from both of our patients, D2 and H3. Similar reductions in IgE binding for the Der f 13 mutants were also observed when tested on sera from another four Singaporean and South African patients. Among all six patients, except patient S1, significant reductions in IgE binding were detected when the two double mutants and the 3A mutant of Der f 13 were tested, indicating that the epitope residues identified are common epitopes for the binding of IgE from different patients. The triple mutant 3A has

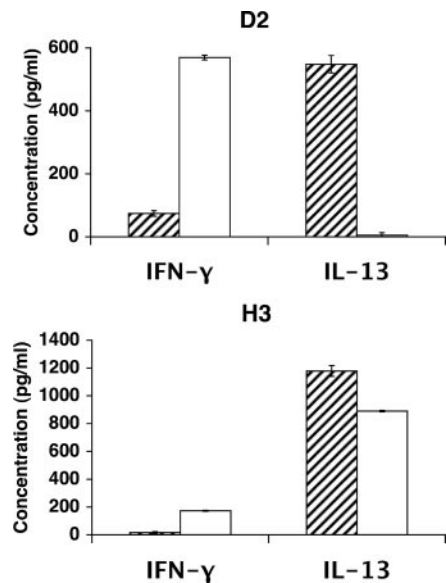


FIGURE 7. Profiles of cytokine secretion induced by the wild-type and the 3A mutant of Der f 13. PBMCs from patients D2 and H3 were stimulated with the wild-type (striped bar) or the 3A mutant (empty bar) of Der f 13. Cytokine IFN- γ is used as a marker for Th1 type proliferation, whereas IL-13 secretion represented a Th2 type proliferation. Both patients showed an increased level of IFN- γ secretion and, at the same time, a reduced secretion of IL-13 when stimulated with the 3A mutant of Der f 13. Stimulation with wild-type Der f 13 showed a typical allergic response with much higher level of IL-13 compared with IFN- γ .

also shown reduced skin prick reactivity for patients D2 and H3 in vivo, demonstrating that the IgE epitopes on Der f 13 have been brought down, reducing IgE cross-linking and, thus, the amount of the inflammatory mediators being released.

To substantiate the feasibility of using the 3A mutant as a hypoallergen in a specific allergen vaccination, PBMC proliferation and cytokine profile assays were conducted. PBMCs from allergic patients showed similar proliferations when stimulated with either Der f 13 or the 3A mutant, indicating that the 3A mutant of Der f 13 still retained its immunogenicity and T cell epitope. As anticipated, PBMCs from group 13 allergic patients released an increased level of IL-13, a Th2 cytokine, upon stimulation with wild-type Der f 13. In contrast, stimulation with the 3A mutant increased the level of Th1 cytokines such as IFN- γ with a simultaneous reduction in the level of IL-13. These observations indicated that the 3A mutant was able to switch T cell proliferation

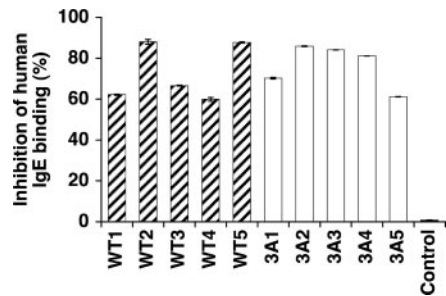


FIGURE 8. Percentage of inhibition of human IgE binding to Der f 13 after preincubation with immunized mouse serum. Sera were used from two separate groups of five mice immunized with either wild-type Der f 13 (WT1–5) or 3A mutant (3A1–5). Serum from mouse immunized with buffer was included as control.

from a Th2 to a Th1 pattern, which is a critical criterion for immunotherapy using hypoallergens. The mutations not only disrupted the IgE binding epitopes but also altered the allergenic property of Der f 13. This kind of switch in cytokines pattern upon hypoallergen treatment, however, seems to be more effective in patients who show a weaker IgE binding to the allergen.

The 3A mutant retains the ability to induce Der f 13-specific IgG even after the removal of the IgE binding epitopes, as demonstrated in the mice immunization experiments. Antisera from mice immunized with the 3A mutant were able to inhibit the binding of human serum IgE to Der f 13, indicating the potential of this hypoallergen to induce blocking IgG. The blocking ability of the induced IgG is critical for competing with IgE for the epitope binding sites on the allergen (7). Despite the fact that the epitope residues have been removed in the 3A mutant, the induced IgG still retains a blocking effect comparable to that of IgG from mice sera immunized with wild-type Der f 13. This result is likely due to IgG raised against the 3A mutant targeted to adjacent regions around the IgE binding epitopes that produces a steric hindrance effect blocking IgE binding to Der f 13. Other than the blocking effect, it has also been shown in other studies (7, 11) that the IgG Abs raised are able to reduce Th2 activation by preventing the IgE-mediated presentations of allergens to T cells.

Combining NMR solution structure and sequence comparison analysis, we have identified potential residues that are essential for IgE binding and allergenicity of Der f 13. By disrupting the specific binding epitopes of IgE while maintaining its overall protein structure and T cell epitopes, we have generated a potential group 13 dust mite hypoallergen that in the future may be used for vaccination against dust mite allergy. In addition to the group 13 dust mite allergen, other studies have also used a similar approach to generating hypoallergens by mutating specific surface residues on allergens, including Bet v 1 (10), Hev b 6.02 (30), and Equ c 1 (31).

IgE epitope mapping studies on major allergens such as Der p 2 (32), Bet v 1 (10, 33), Equ c 1 (31), and Hev b 6.02 (30) have also revealed the importance of charged and polar residues on IgE binding and cross-linking (Table II). In Equ c 1, residues mapped to the IgE binding epitopes all consist of charged residues like lysine, arginine, and glutamic acid. The IgE binding epitopes of Hev b 6.02 have been mapped to the residues Arg⁵, Lys¹⁰, Glu²⁹, Tyr³⁰, and Gln³⁸ by site-directed mutagenesis. Mutation of a single charged residue, Glu⁴⁵, to Ser⁴⁵ has completely abolished binding of Bet v 1 to a mAb that cross-inhibits IgE binding to Bet v 1 (33). The conserved residue Glu⁴⁵ has also been implicated as an important IgE binding residue in other major pollen allergens from trees of the Fagales order, as well as in cross-reactive food allergens of the Bet v 1 family such as Pru av 1, Mal d 1, and Pyr c 1 (34, 35). Epitopes mapped on Der p 2 (32) using the hydrogen exchange technique and mutagenesis studies are comprised of the residues His³⁰, Arg³¹, Lys³³, Ser⁵⁷, Lys⁹⁶, Ile⁹⁷, and Glu¹⁰². In this study, we have mapped charged residues Glu⁴¹, Lys⁶³, Lys⁹¹, and Lys¹⁰³ as IgE binding epitopes of Der f 13. When compared with nonallergenic human FABPs, Der f 13 also showed a considerable higher overall percentage (36.6%) of charged residues compared with the average of 28.3% in human FABPs. A similar trend is also observed in Der p 2, which is comprised of 24.8% charged residues and is significantly higher than the 16.6% of charged residues in the NPC2 protein (36), the most closely related human homologue of Der p 2. Equ c 1, a lipocalin from horse, has a total of 31.9% of charged residues compared with that of 21.5% in human lipocalin 9 (37). The abundance of charged residues found in major allergens, especially when compared with their homologs in human, gives a strong indication that charged residues could play an

important role in IgE binding and allergenicity of these allergens. The abundance of charged residues on allergens may imply that a higher solubility could favor their allergenic properties, or it could simply mean that charged residues are more preferable epitopes for IgE recognition. There are still no known structural features or conserved sequences that allow us to identify an allergen. Our findings, however, strongly suggest that the abundance of surface charged residues is highly related to the allergenicity of a protein.

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Disclosures

The authors have no financial conflict of interest.

References

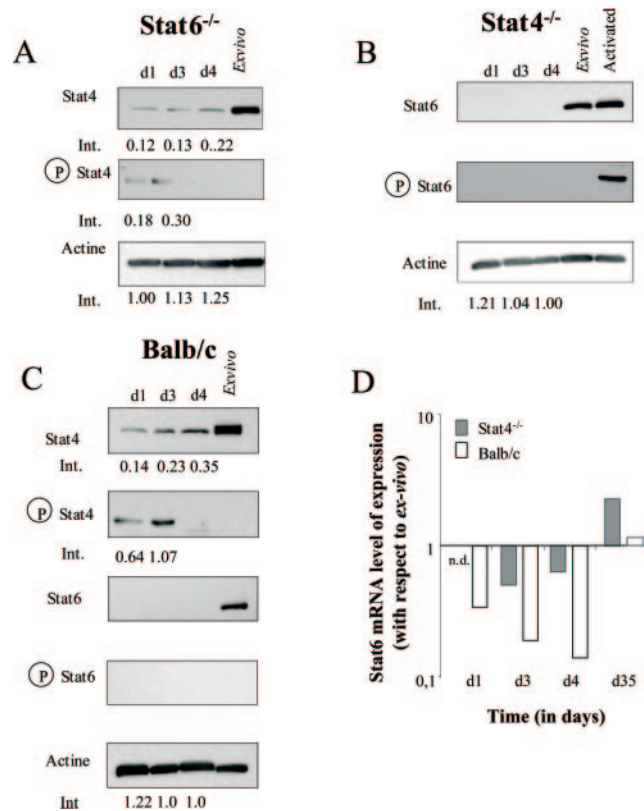
- Derewenda, U., J. Li, Z. Derewenda, Z. Dauter, G. A. Mueller, G. S. Rule, and D. C. Benjamin. 2002. The crystal structure of a major dust mite allergen Der p 2, and its biological implications. *J. Mol. Biol.* 318: 189–197.
- Mueller, G. A., D. C. Benjamin, and G. S. Rule. 1998. Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. *Biochemistry* 37: 12707–12714.
- Caraballo, L., L. Puerta, S. Jimenez, B. Martinez, D. Mercado, A. Avjioglu, and D. Marsh. 1997. Cloning and IgE binding of a recombinant allergen from the mite *Blomia tropicalis*, homologous with fatty acid-binding proteins. *Int. Arch. Allergy Immunol.* 112: 341–347.
- Eriksson, T. L., P. Whitley, E. Johansson, M. van Hage-Hamsten, and G. Gafvelin. 1999. Identification and characterisation of two allergens from the dust mite *Acarus siro*, homologous with fatty acid-binding proteins. *Int. Arch. Allergy Immunol.* 119: 275–281.
- Eriksson, T. L., O. Rasool, S. Huecas, P. Whitley, R. Cramer, U. Appenzeller, G. Gafvelin, and M. van Hage-Hamsten. 2001. Cloning of three new allergens from the dust mite *Lepidoglyphus destructor* using phage surface display technology. *Eur. J. Biochem.* 268: 287–294.
- Jeong, K. Y., W. K. Kim, J. S. Lee, J. Lee, I. Y. Lee, K. E. Kim, J. W. Park, C. S. Hong, H. I. Ree, and T. S. Yong. 2005. Immunoglobulin E reactivity of recombinant allergen Tyr p 13 from *Tyrophagus putrescentiae* homologous to fatty acid binding protein. *Clin. Diagn. Lab. Immunol.* 12: 581–585.
- Valenta, R. 2002. The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* 2: 446–453.
- Turner, H., and J. P. Kinet. 1999. Signalling through the high-affinity IgE receptor Fc ϵ RI. *Nature* 402: B24–B30.
- Jutel, M., W. J. Pichler, D. Skrbic, A. Urwyler, C. Dahinden, and U. R. Muller. 1995. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen-stimulated T cell cultures. *J. Immunol.* 154: 4187–4194.
- Holm, J., M. Gajhede, M. Ferreras, A. Henriksen, H. Ipsen, J. N. Larsen, L. Lund, H. Jacobi, A. Millner, P. A. Wurtzen, and M. D. Spangfort. 2004. Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. *J. Immunol.* 173: 5258–5267.
- van Neerven, R. J., T. Wikborg, G. Lund, B. Jacobsen, A. Brinch-Nielsen, J. Arved, and H. Ipsen. 1999. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4⁺ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J. Immunol.* 163: 2944–2952.
- Vrtala, S., T. Ball, S. Spitzauer, B. Pandjaitan, C. Suphioglu, B. Knox, W. R. Sperr, P. Valent, D. Kraft, and R. Valenta. 1998. Immunization with purified natural and recombinant allergens induces mouse IgG1 antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. *J. Immunol.* 160: 6137–6144.
- Moverare, R., L. Elfman, E. Vesterinen, T. Metso, and T. Haahtela. 2002. Development of new IgE specificities to allergenic components in birch pollen extract during specific immunotherapy studied with immunoblotting and Pharmacia CAP system. *Allergy* 57: 423–430.
- Zimmerman, A. W., H. T. van Moerkerk, and J. H. Veerkamp. 2001. Ligand specificity and conformational stability of human fatty acid-binding proteins. *Int. J. Biochem. Cell Biol.* 33: 865–876.
- Rademacher, M., A. W. Zimmerman, H. Ruterjans, J. H. Veerkamp, and C. Lucke. 2002. Solution structure of fatty acid-binding protein from human brain. *Mol. Cell. Biochem.* 239: 61–68.
- Gutierrez-Gonzalez, L. H., C. Ludwig, C. Hohoff, M. Rademacher, T. Hanhoff, H. Ruterjans, F. Spener, and C. Lucke. 2002. Solution structure and backbone dynamics of human epidermal-type fatty acid-binding protein (E-FABP). *Biochem. J.* 364: 725–737.
- Balendiran, G. K., F. Schnutgen, G. Scapin, T. Borchers, N. Xhong, K. Lim, R. Godbout, F. Spener, and J. C. Sacchettini. 2000. Crystal structure and thermodynamic analysis of human brain fatty acid-binding protein. *J. Biol. Chem.* 275: 27045–27054.

18. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51–59.
19. Wittekind, M., and L. Mueller. 1993. HNCACB, a high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with the α - and β -carbon resonances in proteins. *J. Magn. Reson. B* 101: 201–205.
20. Grzesiek, S., and A. Bax. 1993. Amino acid type determination in the sequential assignment procedure of uniformly $^{13}\text{C}/^{15}\text{N}$ -enriched proteins. *J. Biomol. NMR* 3: 185–204.
21. Grzesiek, S., J. Anglister, and A. Bax. 1992. Correlation of backbone amide and aliphatic side-chain resonances in $^{13}\text{C}/^{15}\text{N}$ enriched proteins by isotropic mixing of ^{13}C magnetization. *J. Magn. Reson. B* 101: 114–119.
22. Kay, L. E., G. Y. Xu, A. U. Singer, D. R. Muhandiram, and J. D. Forman-Kay. 1993. A gradient-enhanced HCCH-TOCSY experiment for recording side-chain ^1H and ^{13}C Correlations in H_2O samples of proteins. *J. Magn. Reson. B* 101: 333–337.
23. Szyperski, T., D. Neri, B. Leiting, G. Otting, and K. Wuthrich. 1992. Support of ^1H NMR assignments in proteins by biosynthetically directed fractional ^{13}C -labeling. *J. Biomol. NMR* 2: 323–334.
24. Delaglio, F., S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, and A. Bax. 1995. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6: 277–293.
25. Johnson, B. A., and R. A. Blevins. 1994. NMRView: a computer program for the visualization and analysis of NMR data. *J. Biomol. NMR* 4: 603–614.
26. Herrmann, T., P. Guntert, and K. Wuthrich. 2002. Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA. *J. Mol. Biol.* 319: 209–227.
27. Cornilescu, G., F. Delaglio, and A. Bax. 1999. Protein backbone angle restraints from searching a database for chemical shift and sequence homology. *J. Biomol. NMR* 13: 289–302.
28. Pearlman, D. A., D. A. Case, J. W. Caldwell, W. S. Ross, T. E. Cheatham, S. DeBolt, D. Ferguson, G. Seibel, and P. Kollman. 1995. AMBER, a package of computer programs for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculations to simulate the structural and energetic properties of molecules. *Comput. Phys. Commun.* 91: 1–41.
29. Laskowski, R. A., J. A. Rullmann, M. W. MacArthur, R. Kaptein, and J. M. Thornton. 1996. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8: 477–486.
30. Karisola, P., J. Mikkola, N. Kalkkinen, K. J. Airenne, O. H. Laitinen, S. Repo, O. T. Pentikainen, T. Reunala, K. Turjanmaa, M. S. Johnson, et al. 2004. Construction of hevein (Hev b 6.02) with reduced allergenicity for immunotherapy of latex allergy by commutation of six amino acid residues on the conformational IgE epitopes. *J. Immunol.* 172: 2621–2628.
31. Lascombe, M. B., C. Gregoire, P. Poncet, G. A. Tavares, I. Rosinski-Chupin, J. Rabillon, H. Goubran-Botros, J. C. Mazie, B. David, and P. M. Alzari. 2000. Crystal structure of the allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. *J. Biol. Chem.* 275: 21572–21577.
32. Mueller, G. A., A. M. Smith, M. D. Chapman, G. S. Rule, and D. C. Benjamin. 2001. Hydrogen exchange nuclear magnetic resonance spectroscopy mapping of antibody epitopes on the house dust mite allergen Der p 2. *J. Biol. Chem.* 276: 9359–9365.
33. Spangfort, M. D., O. Mirza, H. Ipsen, R. J. Van Neerven, M. Gajhede, and J. N. Larsen. 2003. Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. *J. Immunol.* 171: 3084–3090.
34. Mirza, O., A. Henriksen, H. Ipsen, J. N. Larsen, M. Wissenbach, M. D. Spangfort, and M. Gajhede. 2000. Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J. Immunol.* 165: 331–338.
35. Neudecker, P., K. Lehmann, J. Nerkamp, T. Haase, A. Wangorsch, K. Fotisch, S. Hoffmann, P. Rosch, S. Vieths, and S. Scheurer. 2003. Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (*Prunus avium*) and celery (*Apium graveolens*): correlating IgE reactivity with three-dimensional structure. *Biochem. J.* 376: 97–107.
36. Frolov, A., S. E. Zielinski, J. R. Crowley, N. Dudley-Rucker, J. E. Schaffer, and D. S. Ory. 2003. NPC1 and NPC2 regulate cellular cholesterol homeostasis through generation of low density lipoprotein cholesterol-derived oxysterols. *J. Biol. Chem.* 278: 25517–25525.
37. Suzuki, K., J. J. Lareyre, D. Sanchez, G. Gutierrez, Y. Araki, R. J. Matusik, and M. C. Orgebin-Crist. 2004. Molecular evolution of epididymal lipocalin genes localized on mouse chromosome 2. *Gene* 339: 49–59.

CORRECTIONS

Sanchez-Guajardo, V., J. A. M. Borghans, M.-E. Marquez, S. Garcia, and A. A. Freitas. 2005. Different competitive capacities of Stat4^{-/-} and Stat6-deficient CD4⁺ T cells during Lymphopenia-driven proliferation. *J. Immunol.* 174: 1178–1187.

In Figure 3, the blot representing Stat4 phosphorylation was duplicated by mistake in panels A and C. This correction does not affect the validity of the study or its conclusions. The corrected figure is shown below.



Liu, R., A. La Cava, X.-F. Bai, Y. Jee, M. Price, D. I. Campagnolo, P. Christadoss, T. L. Vollmer, L. Van Kaer, and F.-D. Shi. 2005. Cooperation of invariant NKT cells and CD4⁺CD25⁺ T regulatory cells in the prevention of autoimmune myasthenia. *J. Immunol.* 175: 7898–7904.

The sixth author, Shamsher S. Saini, was inadvertently omitted from the author line. The corrected author and affiliation lines are shown below.

Ruolan Liu,* Antonio La Cava,[†] Xue-Feng Bai,[‡] Youngheun Jee,* Mary Price,* Shamsher S. Saini,[§] Denise I. Campagnolo,* Premkumar Christadoss,[§] Timothy L. Vollmer,* Luc Van Kaer,[¶] and Fu-Dong Shi^{2*}

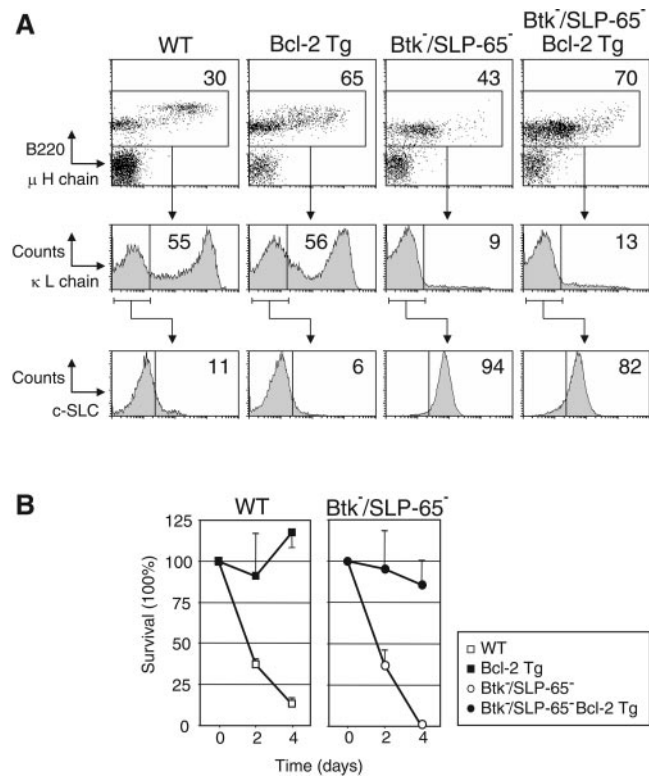
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Cudrici, C., F. Niculescu, T. Jensen, E. Zafranskaia, M. Fosbrink, V. Rus, M. L. Shin, and H. Rus. 2006. C5b-9 terminal complex protects oligodendrocytes from apoptotic cell death by inhibiting caspase-8 processing and up-regulating FLIP. *J. Immunol.* 176: 3173–3180.

The third author’s last name is misspelled. The correct name is Timothy Jansen.

Kerseboom, R., V. B. T. Ta, A. J. E. Zijlstra, S. Middendorp, H. Jumaa, P. F. van Loo, and R. W. Hendriks. 2006. Bruton’s tyrosine kinase and SLP-65 regulate pre-B cell differentiation and the induction of Ig light chain gene rearrangement. *J. Immunol.* 176: 4543–4552.

In Figure 4A, the label for the bottom row of histograms was duplicated by mistake in the label of the row above it. The label for the bottom row should read “Counts versus c-SLC” as stated in the figure legend. The corrected figure is shown below.



Chan, S. L., S. T. Ong, S. Y. Ong, F. T. Chew, and Y. K. Mok. 2006. Nuclear magnetic resonance structure-based epitope mapping and modulation of dust mite group 13 allergen as a hypoallergen. *J. Immunol.* 176: 4852–4860.

In Table II, the epitope residues cited for Equ c 1 are incorrect. The corrected table is shown below.

Table II. Analysis of charged amino acids compositions of major allergens and their human homologs^a

	Lys	Arg	Basic (%)	Asp	Glu	Acidic (%)	Epitope Residues
<i>Der f 13</i>	18	6	18.3	9	15	18.3	<i>E41,K63,K91,K103</i>
FABP1	15	2	13.3	4	13	13.3	
FABP2	14	7	15.9	8	13	15.9	
FABP3	14	4	13.5	11	8	14.2	
FABP4	14	6	15.1	11	9	15.1	
FABP5	14	6	14.8	8	12	14.8	
FABP6	12	2	10.9	5	11	12.5	
FABP7	12	5	12.8	10	10	15.1	
FABP8	18	7	18.9	5	9	10.6	
<i>Der p 2</i> (Ref. 32)	14	2	12.4	9	7	12.4	<i>H30,R31,K33,S57,K96,I97,E102</i>
NPC2	12	1	8.6	6	6	8.0	
<i>Equ c 1</i> (Ref. 31)	11	9	12.5	13	18	19.4	
Lipocalin 9	7	10	9.6	8	13	11.9	
<i>Bet v 1</i> (Refs. 10, 33)	15	3	11.3	9	14	14.4	<i>E42,N43,I44,E45,G46,N47,G48,G49,P50,G51</i>
<i>Hev b6.02</i> (Ref. 30)	2	1	6.9	3	2	11.6	<i>R5,K10,E29,Y30,Q38</i>

^a The contents of positively and negatively charged amino acid residues in selected major allergens are compared with their human homologs. The allergenic proteins are italic and boldfaced. Der f 13 is compared with human FABPs (see Fig. 2 for abbreviations), whereas Der p 2 and Equ c 1 are compared with the homologous human NPC2 and lipocalin 9, respectively. All of the allergens listed are found to have a higher overall percentage of charged residues compared to their homologous human proteins. The residues identified as IgE binding epitopes in these allergens are listed and found to be mostly charged and polar residues.

In **Discussion**, in the last sentence of the fifth paragraph, inclusion of Equ c 1 and the respective citation (Ref. 31) are incorrect. The second sentence of the sixth paragraph has also been revised. The corrected sentences are shown below.

In addition to the group 13 dust mite allergen, other studies have also used a similar approach to generate hypoallergens by mutating specific surface residues on allergens, including Bet v 1 (10) and Hev b 6.02 (30).

In Equ c 1, regions of the protein mapped as IgE binding epitopes consist mostly of charged and polar residues.