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Localization of antigenic sites on Der p 2 using oligonucleotide-directed mutagenesis targeted to predicted surface residues

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Summary

Background Understanding the molecular nature of allergen–antibody interactions is important to understanding the mechanism of conventional immunotherapy as well as to designing alternative immunotherapeutic strategies. Many important allergens have been cloned and expressed, making it possible to apply recombinant DNA techniques to dissect antigenic determinants.

Objective The aim of this study was to use predictive algorithms and site-directed mutagenesis to investigate monoclonal antibody and IgE antibody epitopes of the major house dust mite allergen Der p 2.

Methods Computer algorithms were used to assess the primary amino acid sequence of Der p 2 and to identify regions of hydrophilic and flexible sequence. Subsequently, site-directed mutagenesis was used to generate amino acid substitutions at hydrophilic residues at positions 44–46 and at position 100. The variants were tested in a competitive inhibition ELISA with four group 2-specific murine monoclonal antibodies and with human IgE antibody from mite allergic patients.

Results Conservative amino acid substitutions at position 44–46 did not distinguish IgE antibody epitopes, but did suggest that these residues are involved in the epitope defined by one monoclonal antibody, 15E11. Non-conservative substitution of proline at this position reduced binding to all four monoclonal antibodies, as well as IgE antibody, by 50–80%. Point mutants at position 100 mapped the epitopes of two monoclonal antibodies, 7A1 and 13A4, previously shown to bind the same region of Der p 2. In addition, the two variants tested at this position showed distinct inhibition curves with these two monoclonal antibodies indicating differences in fine specificity.

Conclusions Using predictive algorithms, in the absence of tertiary structural information, we have been able to localize important B cell determinants on Der p 2. The results suggest that it is possible to modulate antibody recognition of allergens using site-directed mutagenesis and that this approach may provide a new strategy for allergen specific immunotherapy.

Keywords: allergens, antigenic determinants, dust mites, IgE antibody, monoclonal antibody, mutagenesis, secondary structure

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Introduction

During the past decade, many allergens have been isolated, cloned and sequenced, making it possible to dissect the antigenic structure of proteins that cause immediate hypersensitivity. Knowledge of the molecular nature of allergen-antibody (Ab) [1] interactions is important for designing new and more effective immunotherapeutic strategies for the treatment of allergic disease. B-cell epitope mapping studies most commonly involve functional approaches such as chemical modification of the antigen, binding studies with peptides and proteolytic fragments, and analysis of naturally occurring isoforms and point mutants. Structural analyses using X-ray crystallography and nuclear magnetic resonance spectroscopy are technically more difficult. However, the three dimensional structure has been determined for several antigen-antibody complexes using murine monoclonal antibody (MoAb) in complex with protein antigens such as hen egg lysozyme and staphylococcal nuclease. Combinations of these studies have defined the area of the antigen-Ab interface, the binding energies involved, and the role of affinity maturation in stabilizing the antigen-Ab complex [1–5].

Site-directed mutagenesis has been used successfully for epitope mapping of antigens of known three-dimensional structure, where surface residues are targeted [6–9]. The assumptions in this approach are that altering amino acid side chains that are surface exposed has minimal effect on the tertiary structure of the antigen and that changes in Ab binding occurring as a result of an amino acid substitution indicate the importance of that residue to the epitope.

The group 2 allergens are comprised of a family of isoforms that show extensive antigenic crossreactivity. Genomic and cDNA cloning have demonstrated at least three unique Der p 2 sequences and four Der f 2 sequences [10,11]. Crossreactive and species-specific MoAbs have recently been developed; however, epitope mapping using these MoAbs may be confounded by the fact that the isoforms have 2–10 amino acid differences making it difficult to assign a particular region of the allergen to a particular epitope [12–15]. Investigators have addressed the nature of B- cell epitopes on Der p 2 using large polypeptides generated from cDNA fragments as well as short synthetic peptides spanning the entire primary sequence [16,17]. Both studies showed a very low frequency of IgE Ab binding and concluded that the majority of epitopes on Der p 2 are conformational, rather than sequential, in nature. These conformational determinants are stabilized by the three disulfide bonds and in a previous study we used site-directed mutagenesis to investigate the contribution of each of these bonds to the antigenic structure of Der p 2 [18–20].

In this study, we used predictive algorithms to identify surface residues (potential antigenic sites), since the tertiary structure of Der p 2 was not known. These algorithms have been shown to be useful for predicting antigenic determinants [21–23]. Site-directed mutagenesis was used to generate a panel of variants at these positions, and MoAb and IgE Ab binding to the variants and to recombinant (r) Der p 2 was compared using inhibition ELISA. Using this approach, amino acid residues on Der p 2 that form part of epitopes recognized by MoAb and IgE Ab have been identified.

Materials and methods

Oligonucleotide mutagenesis

The cDNA for Der p 2 was obtained from Dr Wayne Thomas, Perth, Australia, and subcloned into the plasmid M13mp19 [24]. Oligonucleotide directed mutagenesis was performed as previously described [20]. Oligonucleotides used to generate amino acid substitution at positions 44–46 and 100 are shown below: (N indicates a mixture of the four deoxyribonucleotides)

Asp44–46: 5′-GCC GTT TTC GAA GCC **NNC/G NNC/G NNC/G** ACA AAA ACG GC-3′;

Lys100: 5′-CCG AAA ATT GCA CCA **NNC/G** TCT GAA AAT GTT GTC G-3′;

Mutants were identified by DNA sequence analysis. The sequences were PCR amplified from the M13 templates using specific 5′ and 3′ terminal primers containing BamH1 and EcoR1 restriction endonuclease sites, respectively. These fragments were subcloned into the BamH1/EcoR1 site of the pGEX2T vector (Pharmacia Biotech) and DNA sequence analysis showed that the sequence in the expression vector was identical to the Der p 2 cDNA except at the targeted codon(s). Each variant was expressed and purified as previously described and protein concentration measured by Bradford Assay [20]. The variants were produced in approximately equal amounts from bacterial expression cultures (1–4 mg/L) and gave a single band of 41 kDa on SDS-PAGE (data not shown).

Production and purification of recombinant (r) Der p 2

The plasmid pGEX2TP2 produces rDer p 2 as a fusion protein with glutathione-S-transferase. The unmodified glutathione-S-transferase (GST) fusion protein used for Ab binding studies was designated rDer p 2; variants were designated by the single letter amino acid abbreviation preceding the position of that residue in the primary sequence, followed by the single letter code for the variant. For example: K100T is the threonine substitution for lysine at position 100. For simplicity, variants at positions 44–46 with multiple substitutions are designated by the substitution at position 44, e.g. N44Q is the substitution of

glutamine, threonine, threonine at positions 44, 45, and 46, respectively.

Secondary structural predictions of Der p 2

Chou Fasman, Neural net, and Garnier-Robson algorithms were used to predict the secondary structure from the primary amino acid sequence deduced from the Der p 2 cDNA [25–27]. The hydrophilicity profile was generated using the Hopp-Woods algorithm, using a window of seven residues, and the flexibility profile was produced with the Karplus and Schultz algorithm [22,23]. All computer aided sequence analysis was performed using a Silcon Graphics work station.

Murine and human Ab to mite allergens

The specificity of the murine MoAb used in this study has been described previously [2–14].The antibodies were used as 50% ammonium sulfate fractions of ascites. Sera from patients with asthma or atopic dermatitis were selected as a source of IgE Ab. Collection of sera used in this study was approved by the Human Investigation Committee of the University of Virginia. Patients were skin-test positive to *D. pteronyssinus* (>4×4 mm weal on skin-prick testing) and had serum IgE Ab to Der p 2 (measured by MoAb modified RAST as previously described) [12].

Competitive inhibition assay

The relative ability of variant antigen to interact with MoAb and IgE Ab was determined by competitive inhibition in a modified ELISA assay as follows: antigen was mixed with Ab to give a final antigen concentration of 0.01–100 μg/mL. The Ab concentration was predetermined to give an end-point OD_{405} of 1 in the ELISA in the absence of any inhibitor. All antigen and Ab solutions were prepared in phosphate buffered saline with 0.05%Tween-20, 1%BSA. The antigen-Ab mixes were allowed to equilibrate overnight at 4 °C and then were pipetted in duplicate into wells of a plastic microtitre plate that had been coated with rDer p 2 at 20 μg/mL. The plates were incubated at room temperature for 2 h and then processed as a standard ELISA using peroxidase conjugated goat anti-mouse IgG or goat anti-human IgE as the secondary antibody. The positive control was rDer p 2 as inhibitor and the negative controls were GST and saline-BSA diluent. Results were expressed as the percentage inhibition of the reaction of Ab in the absence of any inhibitor.

Results and discussion

Structural predictions

Epitope mapping by site-directed mutagenesis is most often applied to proteins of known three dimensional structure, where specific surface residues can be targeted [6–9]. The tertiary structure of Der p 2 has not yet been determined and previous studies in our laboratory suggested that the intact structure, stabilized by three disulfide bonds, was essential for antibody binding [19,20]. In addition, unlike other *Dermatophagoides* allergens, Der p 2 shows no sequence homology with proteins of known three- dimensional structure, so that a structure can not be modelled on other protein structures. This makes the use of predictive algorithms particularly useful for investigating the antigenic structure of Der p 2. Figure 1 shows the secondary structure profiles of the Der p 2 primary amino acid sequence of 129 residues. The three algorithms (Neural net, Chou-Fasman, Garnier-Robson) showed similar profiles. Regions of α-helix were predicted at the amino and carboxy termini and were flanked by regions predicted to be β-sheet, at residues 10–20 and 100–110. The region of sequence between residues 30 and 60 showed overlapping helix and sheet profiles. Turns were predicted by the Garnier-Robson method between residues 20–30 and 70–80, coincident with the C21-C27 and C73-C78 disulfide bonds.

Following the assumption that antigenic determinants are composed of amino acid residues on the protein surface, predictive algorithms were used to target potential surface residues on Der p 2 that scored highest for hydrophilicity and flexibility. These profiles are also shown in Fig. 1. The primary sequence was subjected to the hydrophilicity scoring system of Hopp and Woods, and the scores were averaged over a window of seven residues [22]. Peaks of hydrophilic sequence occurred at the amino-terminus, between residues 20–30, 40–50, 80–90, and additional peaks were centered around the lysine residue at position 100 and aspartic acid residues at positions 113 and 114. Huang *et al.* studied the correlation between hydrophilicity and solvent accessible surface and between chain flexibility and mobility for proteins of known structure. These investigators found that the hydrophilicity profile correlated more consistently with surface area while flexibility had only a limited correlation with mobility [21]. The flexibility profile of Der p 2, generated using the algorithm of Karplus and Schultz, mirrored the hydrophilicity plot; peaks occured between residues 40–50 and at residue 100 (Fig. 1) [23].

Generation of Der p 2 variants

Variants were produced at the two regions that gave the highest hydrophilic and flexible scores: N44, Q45, and N46 were mutagenized as a block and K100 was targeted for point mutagenesis. Oligonucleotides were designed to give all 19 amino acid substitutions at each position with a minimized possibility of introducing stop codons. Variants are shown in Table 1: a total of 22 templates from

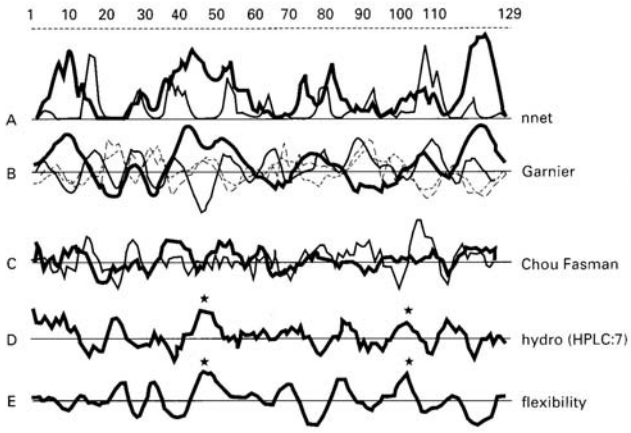

Fig. 1. Analysis of the secondary structure, hydrophilicity, and flexibility of Der p 2. The primary amino acid sequence of Der p 2 (amino acids 1–129) is represented by the dashed line. This sequence was subjected to secondary structure predictions using the algorithms of Holley and Karplus, 'Neural net' (A), Garnier-Robson (B), Chou Fasman (C). Alpha-helical regions are shown by the heavy line and beta sheet regions are shown by the light line. The Garnier-Robson algorithm (B) also predicts turns (long dashes) and random structure (short dashes). The hydrophilicity profile using the Hopp and Woods algorithm is shown in (D) and the flexibility profile using the algorithm of Karplus and Schultz is shown in (E). The residues targeted for mutagenesis are designated (★).

the N44-Q45-N46 targeted mutagenesis reaction were sequenced. Four wild-type sequences were recovered, 10 variants with changes at all three positions, and six variants with one, two, or three codon changes. One variant was sequenced that contained a stop codon at amino acid 46. From the mutagenesis reaction targeted to lysine 100, 10 templates were sequenced: one wild-type sequence was recovered, six point mutants, one stop codon, and two insertions of a single amino acid between lysine 100 and serine 101. The efficiency of mutagenesis was greater than 80%.

Table 1. rDer p 2 variants generated by site-directed mutagenesis

Asn44-Gln45-Asn46 variants:	
Thr Pro Pro	His Pro Leu
Tyr Pro Phe	Ser Leu Leu
His His Leu	Asn Pro His
Tyr His Ile	Asn Leu Pro
Ile His His	Asn His Phe
Pro Thr Tyr	Tyr Gln Pro
Thr Pro Stop	Thr Ser Asn
Gln Thr Thr*	Ser Lys Asn
Pro Pro His*	

Lys100 Variants:
Asp
Pro
Stop
Arg*
Thr*

* Variants selected for Ab binding studies.

Localization of antigenic determinants

Two variants at N44-Q45-N46 were evaluated and the results of the competitive inhibition assays are summarized

Table 2. ELISA inhibition profiles of rDer p 2 and variants[a]

	% Inhibition of antibody binding						
	Monoclonal antibodies				IgE antibodies		
Inhibitor	αDpX	15E11	7A1	13A4	pool[b]	L.W.	F.D.
rDer p 2	80	80	85	85	90	85	80
Surface variants:							
N44Q	75	40	85	85	90	82	88
N44P	9	< 2[c]	50	8	40	30	33
K100T	80	55	30	5	90	82	82
K100R	80	60	75	2	90	83	87
Cysteine variants:[d]							
C21S	80	35	80	75	80	72	93
C73R	14	< 2	65	13	17	53	23
C119Y	80	45	85	80	85	90	92

[a]Maximal inhibition of Ab binding in ELISA using 100 μg/mL rDer p 2 or variant.
[b]Serum pool from seven house dust mite allergic patients.
[c]Values less than the negative control are recorded as < 2; GST and PBS-1%BSA were used as negative controls and showed a maximum of 2% inhibition.
[d]Substitutions at cysteine residues lack the corresponding disulfide bond.

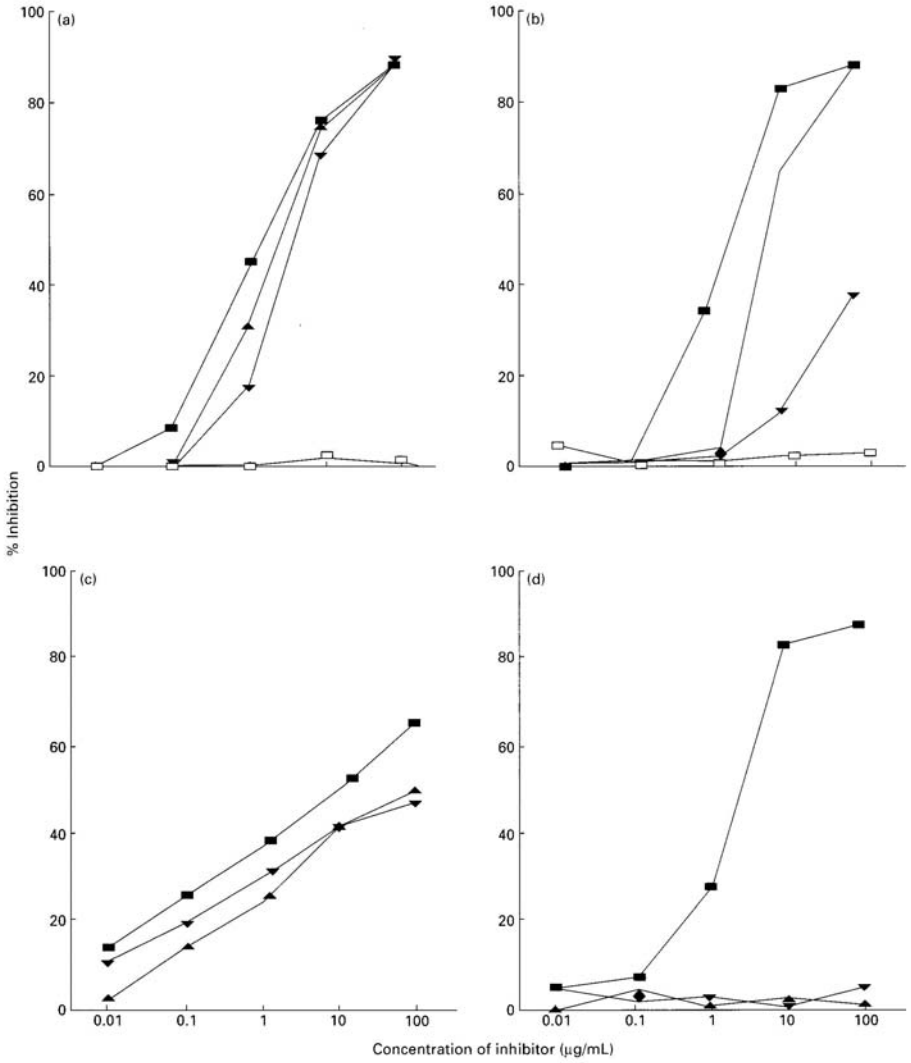

Fig. 2. Monoclonal antibody binding to rDer p 2 variants at K100. Increasing concentrations of rDer p 2 or rDer p 2 variants were used to block binding of MoAb αDpX (a), MoAb 7A1 (b), MoAb 15Ell (c) and MoAb 13A4 (d) to rDer p 2 in a modified ELISA. Inhibitor antigens were rDer p 2 (■), K100R (▲), K100T (▼), and GST (□), the negative control. The results are presented as the percentage inhibition of MoAb binding in the absence of inhibitor.

in Table 2. For this analysis, we considered maximum inhibition of Ab binding of ≤ 50% as a significant reduction in antigenicity. Conservative substitution N44Q gave comparable inhibition curves to rDer p 2 for MoAb αDpX, 7A1, 13A4, and for IgE Ab from four individual patients and a serum pool from seven additional patients. However, this variant had reduced antigenicity for MoAb 15E11, inhibiting binding up to 40% at the highest concentration tested. This result suggests that the 44–46 region is important to the epitope defined by MoAb 15E11. By contrast, the N44P substitution, predicted to introduce a 'helix breaker' motif, failed to inhibit binding of the four MoAb as well as IgE Ab, suggesting that this substitution has effects on several epitopes. Less than 20% inhibition of binding was detected for MoAb αDpX, 15E11, and 13A4 and MoAb 7A1 was inhibited by 50%. IgE Ab binding to the N44P variant was significantly reduced; N44P showed only 30–40% inhibition of binding at 100 μg/mL. Table 2 shows data for two patients, L.W. (atopic dermatitis) and F.D. (asthma). All four individuals and the serum pool gave unique inhibition curves, however, the pattern was consistent; that is, the N44P variant showed markedly decreased antigenicity. This dramatic reduction in reactivity with the polyclonal IgE Ab was also seen in a panel of Der p 2 variants lacking one of the three disulfide bonds (Table 2). We have previously shown that these variants are 10–100-fold less reactive in skin testing and propose that such modified allergens would provide alternative reagents for immunotherapy, with reduced risk of side-effects [20]. The N44P variant provides an additional example of a strategy for modification of IgE binding determinants.

Table 2 also summarized the binding assays using variants at K100. The binding inhibition dose response curves using these substitutions are shown in Fig. 2. The K100R substitution is conservative, maintaining the positively charged side chain, while the K100T substitution removes this charge and introduces a less hydrophilic side chain. These variants gave overlapping inhibition curves with rDer p 2 for MoAb αDpX and IgE Ab. The other MoAb showed distinct binding patterns. The MoAb 13A4 was inhibited by < 10% using either substitution, suggesting that K100 is critical to the epitope structure. Inhibition of binding of MoAb 15E11 reached 55% and 60% with K100T and K100R, respectively, at the maximum concentration tested, indicating that this amino acid may contribute to the 15E11 epitope. However, these values did not meet the criteria set for significant loss of antigenicity. Inhibition of binding of MoAb 7A1 was slightly reduced for K100R but reached only 30% for K100T, thus the positive charge of the side chain at position 100 is critical for MoAb 7A1 binding (Table 2). These results strongly suggest that K100 forms an important part of the epitope defined by MoAb 13A4 and 7A1, two MoAb previously shown to bind to the same region of Der p 2 by cross-inhibition studies [13]. The differential effects of the two substitutions on binding of each MoAb suggest differences in the fine specificity of these antibodies, and underscores the importance of testing multiple substitutions at a given position.

The MoAb 15E11 was sensitive to substitutions at both N44 and K100, although the level of inhibition by the K100 variants did not reach the cut off of ≤ 50% inhibition of binding. It may be that residues 44–46 and 100 are juxtaposed on the surface of Der p 2 and fall within the 15E11 epitope. Recently, we have been able to obtain preliminary NMR spectra of Der p 2 using ^{15}N and ^{13}C labelled recombinant allergen (G. Mueller *et al.* manuscript in preparation). Once the structure is known, we will be able to refine and expand this analysis to residues surrounding N44 and K100 to provide a more detailed map of the antigenic surface of Der p 2.

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