

Hydrogen Exchange Nuclear Magnetic Resonance Spectroscopy Mapping of Antibody Epitopes on the House Dust Mite Allergen Der p 2*

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New strategies for allergen-specific immunotherapy have focused on reducing IgE reactivity of purified recombinant allergens while maintaining T-cell epitopes. Previously, we showed that disrupting the disulfide bonds of the major house dust mite allergen Der p 2 resulted in 10–100-fold less skin test reactivity in mite-allergic subjects but did not change *in vitro* T-cell proliferative responses. To provide a more complete picture of the antigenic surface of Der p 2, we report here the identification of three epitopes using hydrogen protection nuclear magnetic resonance spectroscopy. The epitopes are defined by monoclonal antibodies that are able to inhibit IgE antibody binding to the allergen. Each monoclonal antibody affected the amide exchange rate of 2–3 continuous residues in different regions of Der p 2. Based on these data, a number of other residues were predicted to belong to each epitope, and this prediction was tested for monoclonal antibody 7A1 by generating alanine point mutants. The results indicate that only a small number of residues within the predicted epitope are functionally important for antibody binding. The molecular definition of these three epitopes will enable us to target limited positions for mutagenesis and to expand our studies of hypoallergenic variants for immunotherapy.

Epidemiologic studies suggest that between 10 and 20% of the world population exhibits some form of IgE-mediated hypersensitivity, which is manifested as asthma, atopic dermatitis, or allergic rhinitis (1). A number of studies have shown that sensitivity to house dust mite allergens is the most important risk factor for asthma (1, 2). More than 10 mite allergens have been defined, and the 14-kDa Group 2 allergens (Der p 2¹ and Der f 2) are considered major allergens because of the fact that 80–90% of patients have specific IgE Ab to these allergens (3).

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¹ The abbreviations used are: Der p 2, major group 2 allergen from *D. pteronyssinus*; Der f 2, major group 2 allergen from *D. farinae*; Ab, antibody; mAb, monoclonal antibody, rDer p 2, recombinant Der p 2

Previously we reported that Der p 2 is structurally a member of the immunoglobulin superfamily, although the function of the allergen remains unknown (4).

Therapy for allergic disease includes allergen avoidance, pharmacotherapy, and allergen-specific immunotherapy. Recently, new strategies for immunotherapy have been proposed with the aim of improving efficacy, patient compliance, and associated risks (5). Our studies have focused on the generation of hypoallergenic variants; the underlying hypothesis is that reducing IgE reactivity will reduce IgE-mediated side effects (6). The mapping of epitopes on Der p 2 and Der f 2 is an important step toward the development of hypoallergenic variants. Using murine mAb and sera from mite-allergic subjects, we have shown that the epitopes on the Group 2 allergens are conformational and that the three disulfide bonds stabilize this structure (6, 7). Mutational analysis of surface residues found that substitution of threonine for lysine at position 100 had reduced avidity for mAb 7A1, and mutations of residues 44–46 affected the avidity of a second mAb, α DpX (8). A third mAb, 6D6, belongs to a group of mAb that recognize the different naturally occurring isoforms of Der p 2 at residue 114² (9, 10). Although the above studies have been informative, they provide an incomplete map of the epitopes and do not necessarily identify those residues on Der p 2 that contribute significantly to the binding energy.

Epitopes can also be mapped by measuring changes in amide hydrogen exchange rates of the antigen that occur as a result of the formation of an immune complex (11–13). These rates can be conveniently measured using NMR techniques. This method was first used by Patterson *et al.* (11) to localize the epitope of an anti-cytochrome *c* mAb. We have subsequently measured similar effects in a number of anti-lysozyme antibodies (13). In general, those residues that are in the structural epitope (those that either contact the Ab or are buried by it) show the largest reduction in exchange rates.

Once the location of the epitope has been obtained using amide exchange measurements the residues, which are important for mAb binding, can be systematically identified using scanning alanine mutagenesis (14–16). The mutation to ala-

with first residue altered from Asp to Ser; ELISA, enzyme-linked immunosorbent assay; MOPS, 4-morpholinepropane sulfonic acid, HSQC, heteronuclear single quantum correlation spectroscopy; t1, time of evolution in the indirectly detected time domain (¹⁵N); t2, time of evolution in the directly detected time domain (¹H); NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect.

² Smith, A. M., Benjamin, D. C., Hozic, N., Derewenda, U., Smith, W., Thomas, W. R., Gafvelin, G., van Hage-Hamsten M., and Chapman, M. D. (2001) *J. Allergy Clin. Immunol.*, in press.

nine reduces the side chain to as small as possible without substantially altering the secondary structure. Usually, glycine and proline are not altered, because both of these residues strongly influence the configurational entropy of the peptide chain. This approach has been used to map B-cell epitopes in a number of different model systems. Jin *et al.* (14) studied 43 different mutants of human growth hormone in combination with 21 different monoclonal antibodies. A study by Dall'Acqua *et al.* (15) introduced alanine mutations into both the antibody and antigen (lysozyme) and reported the structure of a complex between the antibody and one of the lysozyme mutants. Benjamin and Perdue (16) characterized the interaction of 70 mutants of staphylococcal nuclease with 10 different mAb. All of these studies found that an average of 3 to 4 residues were energetically important to mAb binding. In this study, a residue was classified as being energetically or functionally important if the free energy of binding of the mutant to mAb binding was 1.0 kcal/mol or greater. The studies of growth hormone and staphylococcal nuclease examined virtually the entire surface of the respective proteins, and the study of lysozyme involved residues known to be in contact with Ab from crystallographic studies. In this study, the hydrogen exchange data were used to provide a starting point for a more focal analysis of the epitopes on Der p 2.

EXPERIMENTAL PROCEDURES

Production of rDer p 2—Recombinant Der p 2 (rDer p 2) was expressed and purified from *Escherichia coli* cultures as previously described (9). Briefly, the protein was recovered from the insoluble fraction of the cell sonicate and resolubilized with 6 M guanidine (one-fifth the original cell culture volume), and after dialysis against buffer (10 mM Trizma (Tris base), 1 mM EDTA, pH 8.5), the protein was purified by mAb affinity chromatography. ^{15}N -Labeled protein was obtained by growing the bacteria on a minimal medium with ^{15}N -ammonium sulfate as the sole nitrogen source.

Monoclonal Antibodies—The murine mAb used in this study were produced at the University of Virginia Lymphocyte Culture Center and have been described in detail elsewhere (17). The mAb αDpX was produced by Dr. Rob Aalberse and colleagues (18). Radioimmunoassay and ELISA have shown that this panel of 8 mAb defines three antigenic regions on Der p 2 (17), and the mAb 7A1, αDpX , and 6D6 were selected as representative of these regions. For this study, mAb were purified from ascites fluid by precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed by affinity chromatography using an rDer p 2 antigen affinity column. Protein concentrations were calculated using extinction coefficients of $E_{0.1\%} = 1.42$ and $E_{0.1\%} = 0.72$ for murine IgG and rDer p 2, respectively. The resins used for the mAb purification and the amide proton exchange rate protection studies (described below) were constructed using Affi-Gel-10 (Bio-Rad Laboratories, Hercules, CA) according to manufacturer protocols. Briefly, 25 mg of protein was coupled per 1 ml of gel bed in a buffer of 0.1 M MOPS, 80 mM NaCl, pH 7.5. Typically greater than 95% of the protein coupled to the matrix. The capacities of the columns were as follows: rDer p 2 column, 27 mg of mAb; mAb 6D6 column, 5 mg of rDer p 2; mAb 7A1 column, 10 mg of rDer p 2; and mAb αDpX column, 13 mg of rDer p 2.

Monoclonal Ab Inhibition of IgE Binding to rDer p 2—Increasing concentrations of mAb were used to inhibit IgE binding to rDer p 2 in a modified enzyme immunoassay. The antigen was bound directly to the microtiter plate or presented by mAb αDpX . Sera were added along with increasing concentrations of mAb so that the final concentration of serum was 1:4 or 1:8, and the concentration of mAb ranged from 0.01 to 100 $\mu\text{g}/\text{ml}$. IgE binding was detected using biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and streptavidin horseradish peroxidase (Sigma). The percent inhibition was calculated from the A_{405} of IgE Ab binding to rDer p 2 in the absence of inhibitor mAb. The positive control experiment used the anti-Der p 2 chimeric mAb 2B12-IgE developed by Schurmann *et al.* (19). Sera from 4 mite-allergic subjects (radioallergosorbent assay positive to *Dermatophagoides pteronyssinus* extract), pooled sera from 7 additional subjects, and 1 radioallergosorbent assay negative control subject were tested.

Measurement of Amide Exchange Rates—The amide exchange kinetics of the mAb-rDer p 2 complex was measured using the method described by Williams *et al.* (13). Briefly ^{15}N -rDer p 2 was loaded on the

mAb column in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 100 mM NaCl) and washed with three column-volumes of phosphate-buffered saline. This was followed with three more column-volumes of NMR sample buffer (10 mM sodium phosphate, 200 mM K_2SO_4 , 10 mM NaCl, 1 M EDTA, pH 6.6) made with 99% D_2O . Time 0 was considered the time at which the second volume of buffer was added. The columns were left at 4 °C for 48 h and then rinsed with 3 volumes of D_2O before elution with 0.2 M acetic acid. The eluted protein was titrated to pH 3.2 using NaOH to quench the amide exchange. The 48-h control sample was made by concentrating and diluting the protein 3 times with the deuterated exchange buffer. The exchange was considered initiated after the first dilution, because the sample was greater than 94% D_2O at this point. After a 48-h incubation at 4 °C the sample was titrated to pH 3.2 with HCl to quench the reaction. The protein was then placed into column elution buffer by 3 rounds of concentration and dilution. The control sample for the 0-h time point and the sample used for the assignment of the spectra were in H_2O containing 0.2 M acetic acid.

Collection of NMR Spectra—All NMR experiments were carried out on a Varian Unity Plus spectrometer operating at a proton frequency of 500 MHz. Amide peak intensities were obtained from two-dimensional HSQC spectra acquired using 256 t_1 points and 1024 t_2 points and with 64 transients (20). The three-dimensional HSQC-NOESY spectra (21) were obtained as previously described (9). All spectra were transformed and analyzed using Felix 95.0 (Biosym/Molecular Simulations, San Diego, CA) with standard protocols.

Prediction of Epitopes and Construction of Alanine Point Mutants—Residues potentially within a given epitope were predicted by taking the midpoint between the two protected residues that were furthest apart and determining which residues had atoms that were less than 12.5 Å from this point. A 12.5-Å radius circle would have an area of 490 Å² whereas a sphere of this radius would have a surface area of 1,960 Å² (22). Considering that the surface of protein antigen epitopes are convoluted, the area predicted should exceed the 600–900-Å² surface area of known protein epitopes (22) and thus over-predict the size of the epitope.

The accessible surface area was determined using a 9.0-Å probe (23). Residues with accessible side chains (see Table II) were changed to alanine using the QuickChange kit from Stratagene (La Jolla, CA). Plasmid DNA was isolated using Wizard Plus minipreps (Promega, Madison, WI) and sequenced using automated methods. Because a polymerase chain reaction-based method was used to create the mutations, the entire coding sequence of each mutant was sequenced to ensure that no unintended changes were introduced.

Competitive Inhibition ELISA—The ability of rDer p 2 and the various alanine mutants to inhibit mAb binding to solid phase rDer p 2 was measured using a goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) conjugated to horseradish peroxidase (9, 24). The results were calculated as the percentage inhibition using the value of maximum inhibition by rDer p 2 as 100% inhibition. The curves were fit to a sigmoidal function using Sigma Plot (SPSS Science, Chicago, IL) to determine the concentration of 50% inhibition (IC_{50}). Relative binding constants were calculated, according to Equation 1, as follows.

$$\text{rel } K_A = \text{IC}_{50\text{native}} / \text{IC}_{50\text{mutant}} \quad (\text{Eq. 1})$$

The change in free energy of the binding reaction as a result of the mutation was calculated from Equation 2 (13),

$$[\Delta\Delta G = -RT \ln (\text{rel } K_A)] \quad (\text{Eq. 2})$$

where the standard error in the determination of the $\Delta\Delta G$ is ~ 0.1 kcal/mol. Consequently, a $\Delta\Delta G$ greater than 0.5 kcal/mol was considered significant.

RESULTS

Effect of mAb on IgE Ab Binding—The mAb used in this study have been characterized by a variety of techniques including inhibition radioimmunoassay and ELISA and have been shown to define three antigenic regions on Der p 2 (17). To demonstrate that these antigenic regions also represent IgE-binding regions, we determined the ability of each mAb to inhibit the binding of IgE from allergic sera. Fig. 1, panel A shows the control experiment using the murine-human chimeric IgE mAb that contains the 2B12 variable region. The data in panel A clearly show that inhibition of binding of the 2B12-IgE hybrid occurs only when the inhibiting mAb bind to

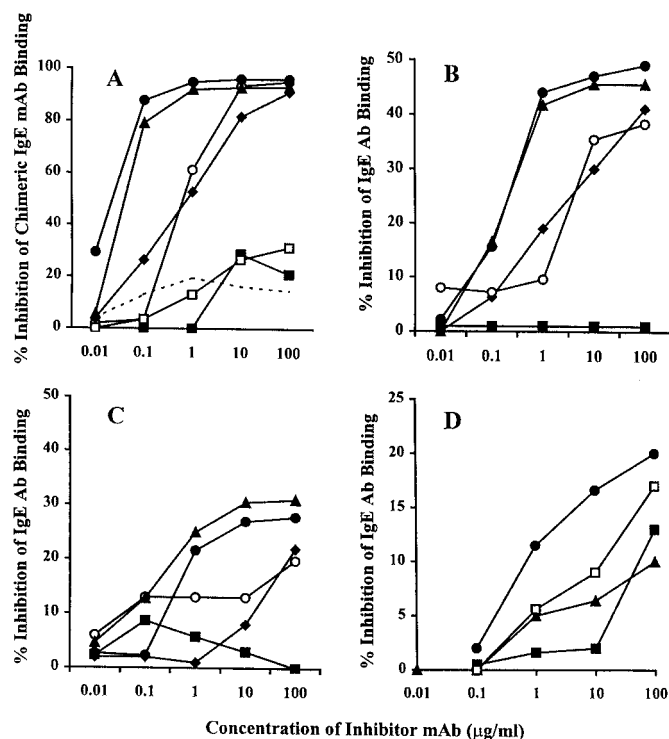


FIG. 1. **mAb inhibition of IgE binding to Der p 2.** Increasing concentrations of mAb were used to inhibit IgE Ab binding to rDer p 2 presented by mAb α DpX in an ELISA. *Panel A* shows the inhibition of the mouse-human chimeric IgE mAb (containing the mAb 2B12 combining site). Inhibitor mAb were 2B12 (open circles), 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), 6D6 (filled diamonds), α DpX (open squares), and an isotype matched control mAb 10A6 (dashed line). *Panels B* and *C* show the inhibition of serum IgE binding from two mite-allergic subjects, B. R., and M. W. The inhibitor mAb were 2B12 (open circles), 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), 6D6 (filled diamonds). *Panel D* shows inhibition of pooled sera IgE Ab by 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), and α DpX (open squares). Values plotted in *panels B, C*, and *D* have been corrected for background binding, determined using the mAb 10A6, which was $\sim 5\%$.

the same antigenic region as does the 2B12 mAb itself. For example, only mAbs 1D8, 6D6, 4G7, and 2B12, which were previously shown by classical competitive inhibition studies to bind to the same antigenic regions on Der p 2 (17), are capable of inhibiting the binding of the 2B12-IgE chimeric mAb in a dose-dependent manner. In contrast, mAb 7A1 and α DpX, each of which binds to one of two other nonoverlapping regions, do not inhibit. These results are in complete agreement with a more detailed study on the competitive inhibition of these and other mAb for binding to Der p 2 (17). *Panels B* and *C* show the ability of mAb to competitively inhibit binding of IgE in sera from two house dust mite-allergic subjects, and *panel D* shows results using pooled sera from 7 additional mite-allergic subjects. Each serum gave unique curves, and the maximum inhibition at 100 μ g/ml ranged from 10 to 50% for any given mAb. Thus, the ability of the mAb to inhibit IgE binding to rDer p 2 suggests that these mAb are appropriate markers for IgE epitopes.

Amide Exchange Experiments—Fig. 2 shows sections of HSQC spectra of rDer p 2 for experiments conducted with mAb 7A1. Peaks for residues 73, 94, 97, and 101 are shown. The *panels* show spectra acquired in H_2O (*left*), after 48 h in D_2O (*center*), and after 48 h in D_2O of a complex of Der p 2 and mAb 7A1 (*right*). These spectra clearly show that residues 94 and 101 were strongly protected from amide proton exchange while complexed with mAb. In contrast residue 97 was weakly protected, and residue 73 was not protected at all. A similar

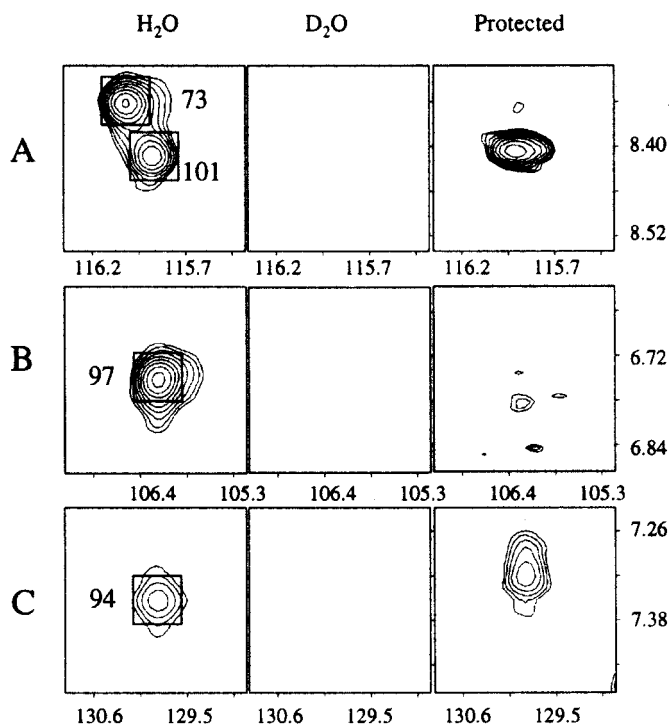


FIG. 2. **Representative exchange data for mAb 7A1.** Portions of the HSQC spectra of Der p 2 containing resonances from residues 73, 101 (*panel A*), 97 (*panel B*), and 94 (*panel C*) are shown. The *left column* of spectra were obtained for a sample in H_2O . The *middle column* of spectra were obtained from a sample of Der p 2 in D_2O . The *right column* (*Protected*) of spectra were obtained from a sample that was bound to mAb 7A1 for 48 h in the presence of D_2O .

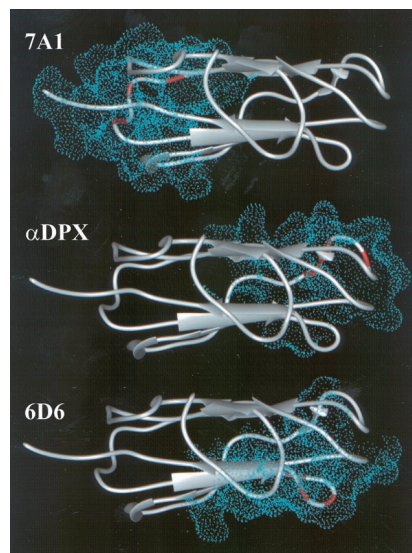


FIG. 3. **Epitopes of mAb 7A1, mAb α DpX, and mAb 6D6.** Residues that were found to have altered exchange rates are mapped on the structure of Der p 2. For each epitope those residues that were found to be protected from amide-proton exchange by mAb are colored red. The molecular surface for the residues predicted to be within each epitope is displayed with cyan-colored dots. The residues that form the α DpX epitope reside primarily on the upper β -sheet whereas those that form the 6D6 epitope reside primarily on the bottom β -sheet.

analysis of spectra obtained for rDer p 2 complexed with mAb α DpX and mAb 6D6 (data not shown) showed that residues 72, 73, and 75 were protected by mAb α DpX, and residues 111 and 116 were protected by mAb 6D6. The protected residues are highlighted on the structure of rDer p 2 in Fig. 3. Also displayed in Fig. 3 are the accessible surfaces of those residues that were predicted to be involved in the full structural epitope.

TABLE I
Amino acid residues of Der p 2 within predicted epitopes

mAb 7A1	mAb α DpX	mAb 6D6
18–20	47–52	7–13
27–37	68–81	41–55
56–63	84–91	70–73
92–106	110–112	76, 80
123–129		107–120

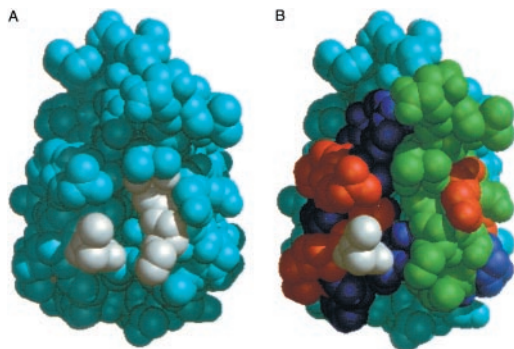


FIG. 4. **Functionally important residues in the 7A1 epitope.** *A*, space-filling model showing the residues (white) protected by the 7A1 mAb during the NMR amide-proton protection assay. *B*, the residues important for mAb 7A1 binding are mapped onto a surface representation of Der p 2. The view is looking down the end of the β -barrel where mAb 7A1 is proposed to interact. The color scheme is as follows: red, residues that when mutated to alanine significantly affect the binding of Der p 2 to mAb 7A1. White, Lys-97, which was the only residue protected in the amide-protection assay, which, when mutated, significantly affected binding. Green, residues that when mutated to alanine did not affect antibody binding. Blue, residues that were not mutated.

The amino acid residues predicted to be in the epitope for each mAb are listed in Table I.

Alanine Scanning Mutagenesis of the mAb 7A1 Epitope—A number of mutants were constructed to test the contribution to binding of different residues in the predicted epitope for mAb 7A1 (Table II). We selected mAb 7A1, because previous studies had shown that Lys-100 was important for binding (8). Only those amino acids with side chain exposure to solvent were analyzed. For example, although Trp-92 and Val-94 are both within the predicted epitope, they do not show any side chain exposure to solvent. Also systematically excluded from mutagenesis were glycine and proline residues. The binding of mAb 7A1 to these mutants was measured using competitive inhibition ELISA, and the calculated IC_{50} values for these mutants are shown in Table II. A total of 8 residues (amino acids 30, 31, 33, 57, 93, 96, 97, and 102) showed a $\Delta\Delta G$ value greater than 0.5 kcal/mol, strongly suggesting that each of these residues contribute significantly to the binding affinity (Fig. 4).

The reduction in binding affinity, which occurred as a result of the mutation, was likely to be caused by changes in the direct interaction of the mAb with the altered residue. Alternatively, the mutation may have affected the secondary or tertiary structure of Der p 2. As a control, the alanine mutants were used to inhibit mAb α DpX binding to rDer p 2. The only mutant that did not interact with mAb α DpX in a manner similar to the native protein was N93A (data not shown). Thus the effect of this mutation cannot be definitively attributed to direct interactions of residue Asn-93 with mAb 7A1.

Although the control experiments with mAb α DpX could identify gross changes in the structure, it was possible that more subtle changes occurred in the mutant proteins. Consequently, a detailed analysis of the K33A mutant was performed to assess the magnitude of the changes in the structure of Der p 2 due to this mutation. The HSQC spectrum of K33A was

TABLE II
Mutants of Der p 2 and the change in $\Delta\Delta G$ of binding mAb 7A1

Residue	IC_{50} Native	IC_{50} Mutant	rel K_A	$\Delta\Delta G$
	ΔG	ΔG	Native/mutant	kcal/mol
His-30	2.36	5.64	0.42	0.52 ^a
Arg-31	2.36	30.00	0.08	1.51 ^a
Lys-33	2.36	10.82	0.22	0.93 ^a
Ser-57	2.39	10.65	0.23	0.88 ^a
Asp-59	2.63	2.17	1.21	-0.11
Leu-61	2.63	2.66	0.99	0.01
Val-63	2.75	3.93	0.70	0.21
Asn-93	1.80	11.04	0.16	1.07 ^b
Lys-96	2.75	13.51	0.20	0.94 ^a
Ile-97	2.61	12.79	0.20	0.94 ^a
Lys-100	2.47	2.71	0.91	0.06
Ser-101	2.47	2.83	0.87	0.08
Glu-102	2.75	6.43	0.43	0.50 ^a
Asn-103	2.47	2.96	0.84	0.11
Val-105	2.61	2.42	1.08	-0.05
Thr-123	2.12	2.10	1.01	-0.07
His-124	1.80	2.39	0.75	0.17
Lys-126	1.80	2.53	0.71	0.20
Ile-127	2.39	2.69	0.89	0.07
Arg-128	2.39	4.29	0.56	0.35
Asp-129	2.61	4.20	0.62	0.28

^a Mutants showing $\Delta\Delta G$ greater than 0.50 kcal/mol.

^b The mutant N93A failed to interact with the α DpX control mAb.

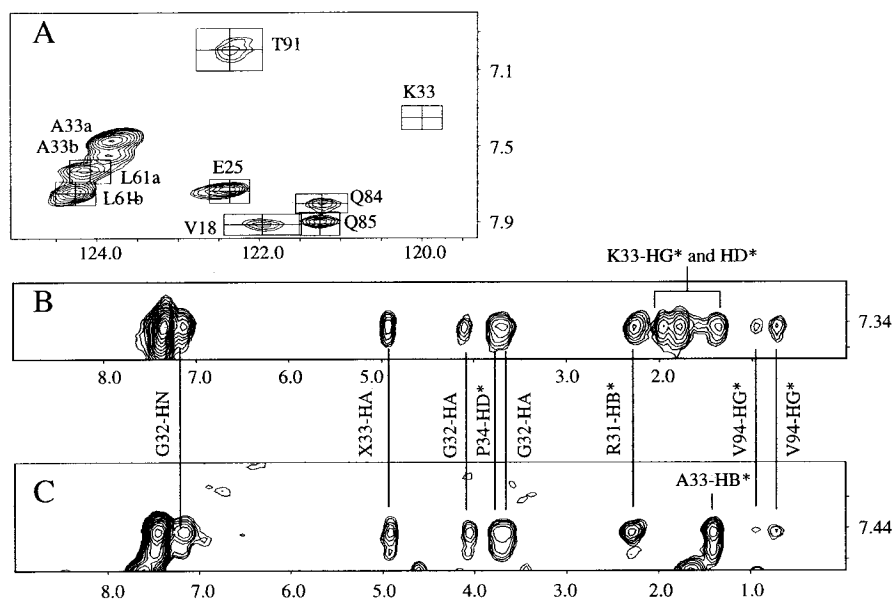
very similar to rDer p 2, and a portion of this spectrum is shown in Fig. 5A. The boxes overlaid on this section of the spectrum indicate the position of peaks in the HSQC spectrum of rDer p 2. One peak disappeared (at 120 ppm nitrogen and 7.32 ppm proton), this peak corresponded to Lys-33. Two new peaks from Ala-33 appeared that were not in the spectrum of rDer p 2. The presence of two peaks indicated that this residue exchanged slowly between two different conformations. However, the structure of these two conformations must be similar based on the similar pattern of NOEs for Ala-33 shown in Fig. 5C. In an analysis of the full HSQC spectra, the only other peaks that shifted in the spectrum of K33A were Gly-32 and Val-94.

The HSQC spectrum shown in Fig. 5A suggests that the replacement of Lys-33 by Ala was not totally benign. To show that the changes in chemical shifts were not indicative of a significant structural rearrangement of the K33A protein, a three-dimensional ^{15}N - ^1H HSQC-NOESY was acquired to detect changes in interproton distances. A section of this spectrum, corresponding to the amide proton of Ala-33, is shown in Fig. 5C. For comparison the region of the same spectrum for residue Lys-33 in rDer p 2 is shown in Fig. 5B. Indicated on these spectra are the NOE assignments from the previous structure determination. The same sequential and long range NOEs were found in the spectra of rDer p 2 and the spectrum of the K33A mutant. The NOEs from residue Ala-33 that are similar to those from Lys-33 are the sequential NOEs to the alpha protons of residues 32 and 34 and the long range peaks to the methyl protons of Val-94. Examining the NOEs from the new amide peak indicated that the differences in the spectra were the lack of intraresidue peaks due to the Lys-33 side chain protons and a new peak that was likely the methyl of Ala-33. These changes would be expected because of replacement of Lys by Ala. An examination of the NOEs from Val-94 and Gly-32 showed similar results; the pattern of NOEs did not change as a result of the K33A mutation (data not shown). These results indicate that there are no large structural changes induced by the K33A mutant; thus, this residue is likely to be in direct contact with mAb 7A1.

DISCUSSION

To date, no data exist describing IgE Ab binding sites for any allergen at the molecular level. The lack of allergen-specific

FIG. 5. NMR spectra of the K33A Der p 2 mutant. A region of the HSQC spectrum of K33A is shown in *panel A*. The boxes indicate the previous positions of resonances for rDer p 2. The amide peak of residue 33 has moved from the right side of the spectrum (empty box) to the left of the spectrum (unboxed peaks). The two new peaks are from Ala-33. *Panels B* and *C* show a slice of the HSQC-NOESY spectrum for the amide of residue Lys-33 in rDer p 2 (*panel B*) whereas *panel C* shows the spectrum for the amide of residue Ala-33 in K33A. Annotated on these spectra are the NOE assignments. The Ala-33 peak in *panel C* contains the same sequential and long range NOEs as Lys-33 but lacks the intraresidue peaks of the Lys-33 side chain. This spectrum also contains a new peak that is likely the methyl group of Ala-33.



monoclonal IgE Ab precludes epitope mapping using crystallographic analysis of allergen-antibody complexes. The NMR methods used here and in other studies (11–13) have clearly shown that it is possible to obtain an approximate mapping of epitopes using this technique. The fidelity of the epitope map depends on several factors, the most significant being the ability to assign resonances and follow the exchange kinetics of these resonances. In the case of Der p 2 89 residues were assigned at pH 3.2. Of these, 31 residues exhibited exchange rates that were either too rapid or too slow to evaluate for protection. Although the NMR experiments were performed under conditions that minimized amide exchange (pH 3.2) the time required to prepare the sample and acquire the NMR spectra was significantly longer than the half-life of the amide proton. This time allowed potentially protected residues with very fast exchange rates in the free protein to exchange with the deuterated buffer. Additional residues were removed from the analysis if their amide hydrogens exchanged too slowly for a significant difference in protection to be seen with the incubation times used here.

Even in the context of these experimental constraints, the residues that were protected are in close proximity to other residues that have been previously implicated to influence the binding of that particular mAb. For mAb 7A1, this study showed that residues 101, 97, and 94 were protected whereas previous studies had shown that mutations of Lys-100 reduce the affinity of Der p 2 for mAb 7A1 (8). For the second mAb, α DpX, the protected residues were 72, 73, and 75. Residue Cys-73 forms a disulfide bond with Cys-78, and mutants that lacked this disulfide, as well as chemically reduced Der p 2, showed reduced affinity for α DpX and IgE Ab (6, 7). The final mAb examined was 6D6, which belongs to a group of mAb that recognizes different naturally occurring Der p 2 isoforms at position 114, either asparagine or aspartic acid (9, 10). The protected residues for mAb 6D6 were 111 and 116. Interestingly, mutation of Lys-100 to alanine had no effect whereas previous studies showed that mutation to threonine did affect 7A1 binding (8), although, in the latter study, mutation to arginine had no effect. Other studies also suggest that different mutations of the same residue can have varying effects on antibody binding (24, 25).

Because the protection data for all three mAbs correlate well with previous mutagenesis data it is likely that the protected residues are within the structural epitope. Finding only 2–3

residues with different exchange rates in Der p 2 is consistent with studies in other systems. For example, in a study of exchange rate differences for three mAb binding to lysozyme, 2–5 residues within the structural epitope for a given mAb were protected (12). Other studies that used this method to localize mAb epitopes generally compared exchange rates for free antigen *versus* antigen protected by mAb (11, 13). Based on those results, we chose to only measure the differences in amide exchange after 48 h to rapidly assess where the mAb might bind. This time was chosen, because it resulted in a large difference between the strongly protected residues that are contained in the epitope *versus* residues with different exchange rates due to long range conformational changes (12, 13). A more extensive examination of exchange rates with more time points may reveal other protected residues. Although the results shown in Fig. 1, *panel A* show no competitive inhibition (*i.e.* no outright blockage) of 2B12 binding by the 7A1 and α DpX mAb, it is still possible that the affinity of one mAb could be affected by the presence of another mAb, although there is no evidence for such in this study or others previously reported (17) in which reciprocal inhibition studies were conducted.

It is possible that binding of mAb to Der p 2 causes a conformational change in a region recognized by another mAb. Indeed, using three mAb specific for the antigen hen egg white lysozyme, we have shown that upon binding, conformational effects are observed at sites distal to the epitope (12, 13). However, these conformational effects are not sufficient to prevent binding of another mAb at the distal site. In that case and in the present study, if there are long range changes in amide protection, *i.e.* protected residues remote from the bound antibody that are within the epitope of another antibody, the affinity must be affected on the basis of thermodynamic arguments. However, it is impossible to predict the size or direction of the change from the current data, but they cannot be larger than the typical remote protection factors of about 1–2 kcal/mol (12, 13). Although we have not conducted experiments with Der p 2 similar to the lysozyme experiments, one would expect to see similar results, *i.e.* long range conformational effects. Nevertheless, also similar to the studies in the lysozyme-anti-lysozyme system, it is clear from the results shown in Fig. 1 (*panel A*) and from studies previously reported on mAb binding to Der p 2 (17) that the failure of the 7A1 and α DpX mAb to inhibit 2B12 binding cannot be accounted for by conformational effects. This strongly supports the conclusion that inhibition by

the other mAbs is due to binding at the same site as the 2B12 mAb. The same arguments apply directly to the data on mAb inhibition of binding of IgE from patient serum, whether it be from one patient or a pooled serum from several patients. Binding of a mAb inhibits the binding of any IgE that binds to the same, or overlapping, epitope(s). It is important to recognize that binding of a single mAb can significantly reduce total patient IgE binding even when the patient IgE can be expected to have maximum heterogeneity as in the case of the pooled sera.

To further characterize the epitopes localized by the exchange data, we utilized the known buried surface area of other protein-antibody complexes to predict which residues may be contained within epitopes on Der p 2. A potential difficulty associated with this method of identifying the structural epitope is illustrated for mAb α DpX. Previous mutagenesis studies implicated residues 44–46 in the binding of α DpX to Der p 2 (8). These residues are 15–19 Å from the protected residues and are thus well outside the predicted epitope. It is possible that the residues protected by mAb α DpX (amino acids 72, 73, and 75) are on the opposite side of the epitope from residues 44–46, and the true center of the epitope lies between these two clusters. A more likely possibility is that alteration of residues 44–46 resulted in significant changes in the conformation of Der p 2. In the original study (8) these residues were changed from NQN to HPP. The 44–46 HPP mutant reacted poorly with both α DpX and 7A1, as well as with the other two mAbs studied. The epitopes for the α DpX and the 7A1 antibodies are shown here to be quite distant. This implies that the triple mutation likely affects the structure of Der p 2 and that residues 44–46 do not directly contact α DpX. An evaluation of the structure of the HPP mutant by another technique, such as NMR, would help resolve this issue.

Although all three of the mAb studied here will bind simultaneously to Der p 2, the predicted epitope for mAb α DpX overlaps considerably with the predicted epitope for mAb 6D6. Because both mAb can bind Der p 2 at the same time (17), the residues that are protected must not be central to the epitopes. This gives the impression that the mAb α DpX epitope is rotated away from the protected residues of mAb 6D6 or *vice versa*. In the case of lysozyme, two mAb were shown to interact with the same residues but with different atoms, and the two mAb could simultaneously bind the antigen (26). These results provide the precedent that epitopes of mAb 6D6 and mAb α DpX could be closely associated and still simultaneously bind Der p 2.

To test the predicted epitope of mAb 7A1, single alanine mutants were generated, and the contribution of their side chain to the free energy of mAb binding was determined. Alanine scanning mutagenesis has been used to examine the energetic contribution of side chains to antibody interactions for human growth hormone, Staphylococcal nuclease, and lysozyme (14–16). These studies found that the $\Delta\Delta G$ values ranged between 1 and 4.5 kcal/mol, but the majority of residues showed $\Delta\Delta G$ between 1.0 and 2.0 kcal/mol. Residues with $\Delta\Delta G$ values in the neighborhood of 1.0 kcal/mol or greater were considered to be functionally important. In each case, only 2 to 5 residues met this criterion for any given antibody. The energetically significant residues were found to be discontinuous with respect to the primary sequence. In our study a total of 7 residues showed changes in $\Delta\Delta G$ greater than 0.5 kcal/mol. Of these, 5 would be considered to be functionally important for mAb binding based on the criteria stated above. In studies of this type it is essential to show that no large changes have occurred in the structure because of the alanine mutation. In this study one mutant, K33A, was chosen for a more detailed structural analysis, because it showed a large difference in

$\Delta\Delta G$, and the chemical shift of its amide is resolved in NMR spectra. Although there are a few minor perturbations in the spectra, the NOE analysis indicated that no significant changes occurred because of the substitution of the alanine at position 33 in the uncomplexed Der p 2. In addition, the mutagenesis data reported here are confirmed by a recent report on the effect of mutagenesis of the Der f 2 allergen on binding of two mAb that see the same antigenic regions as do the 7A1 and α DpX (27).

There are two possible explanations for the observation that changing Asn-93 to an alanine significantly affects binding of both the 7A1 and α DpX mAb. First, Asn-93 may indeed have been recognized by both mAb although competitive inhibition showed that cross-inhibition did not occur. The crystal structures of two lysozyme-anti-lysozyme mAb complexes showed that the two mAb could bind to the same residue, albeit at different atoms, and yet not competitively inhibit (26). Second, we have shown that binding of mAb at one region can cause conformational effects elsewhere in the antigen molecule (12, 13). The side chain of Asn-93 forms two hydrogen bonds with the main chain nitrogen and oxygen of Val-94. These same two Val-94 atoms also form hydrogen bonds with main chain atoms of Lys-33 for which independent mutational evidence suggests it is a binding residue. Thus mutation of Asn-93 to alanine could indirectly affect 7A1 binding through Lys-33.

Considering the number of residues found to be protected from exchange, the number of residues found to be energetically important, and the distribution of these residues, Der p 2 and the mAbs that interact with it fit the general paradigm of protein-antibody interactions. The energetics of the interaction of Der p 2 with mAb 7A1 is similar to that found with other antigen-antibody complexes; only a small number of discontinuous residues contribute significantly, as individual amino acids, to the binding affinity. These results will provide insights into the molecular nature of the epitopes of this clinically important antigen and will serve as a basis for the generation of modified forms of Der p 2 for immunotherapy.

Of particular importance to the use of sequence variants in immunotherapy are our results comparing the reactivity of patient IgE with natural and rDer p 2 (6, 9). These studies showed that the IgE of some patients reacted with natural and not with rDer p 2. Natural Der p 2 is a mixture of isoforms that differ at positions 40, 47, 127, and 114 (28). The rDer p 2 used in these studies corresponds to the rDer p 2.0101 isoform that carries aspartic acid at position 114. Subsequent experiments have clearly shown that native reactivity could be restored by changing position 114 of the rDer p 2 to asparagine, the amino acid found in the other Der p 2 isoforms. Taken together, these results strongly suggest that the patient sera that did not react with rDer p 2.0101 were specific for a single site that included position 114. In addition, the results presented here suggest that the patient IgE Ab recognized a limited number of epitopes on Der p 2, in that 50% of IgE reactivity could be inhibited by a single mAb (Fig. 1). Overall, these results suggest that Der p 2, as seen by at least a subset of patients, could be considered monovalent (a single epitope) or paucivalent (a few epitopes), rather than polyvalent. Thus, selected amino acid changes within each known epitope may produce a null allergen permitting its use in immunotherapy without fear of hyperreactivity because of preexisting IgE Ab. All changes required to produce this null allergen must be made on the same molecule. If a separate variant allergen was produced for each epitope and a mixture of these variants used for immunotherapy, each variant in the mixture would possess all the other native epitopes, and although the overall concentration of each epitope would be reduced, the mixture would react with preex-

isting IgE with the risk of inducing hypersensitivity reactions during immunotherapy.

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