

Note

Reactivities of Mutants of a Major House Dust Mite Allergen Der f 2 to Mouse Anti-Der f 2 Monoclonal Antibodies Analyzed by Immunoblotting

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Received September 22, 2000; Accepted November 6, 2000

A total of sixteen recombinant variants of a major house dust mite allergen Der f 2, the wild-type Der f 2, six cysteine mutants, six proline mutants, and three lysine mutants, were expressed in *Escherichia coli*. The cells were solubilized and run on SDS-PAGE under reducing conditions. Epitopes for five mouse anti-Der f 2 monoclonal antibodies, 1B2, 7C10, 13A4, 15E11, and 18G8, to the recombinant Der f 2 variants were characterized by immunoblot analysis.

Key words: major house dust mite allergen; Der f 2; site-directed mutagenesis; epitope; immunoblotting

Immediate hypersensitivity is caused by the release of mediators such as histamine from mast cells and basophils activated by aggregation of a complex of the high-affinity IgE receptor (FcεRI) on the cell surface and allergen-specific IgE, crosslinked by multivalent allergens. House dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) allergens are major causative factors of various allergic diseases such as asthma, rhinitis, and atopic dermatitis. Der f 2 is a major group 2 allergen from *D. farinae*,^{1–3} and consists of 129 amino acid residues with marked similarity to Der p 2,⁴ the group 2 allergen from *D. pteronyssinus*. IgE epitopes of Der f 2 and Der p 2 are dependent on the integrity of the conformational structure rather than the contiguous sequence of amino acids.^{5–10} We identified the location of three intramolecular disulfide bonds of natural Der f 2 (Cys8–Cys119, Cys21–Cys27, and Cys73–Cys78).¹¹ Recently, the tertiary structures of recombinant Der f 2 and Der p 2 were identified by NMR.^{12,13} Non-fused forms of the recombinant Der f 2 and Der p 2 have an IgE-binding capacity identical to the natural forms.^{14,15} Site-directed mutagenesis has found residues that are important to the IgE-binding capacity.^{7,10,12,16–19}

Analysis of epitopes for monoclonal antibodies

(mAbs) also provides information on the antigenic structure of the allergen molecule. Previously, we established hybridomas secreting mAbs, which recognized both Der f 2 and Der p 2, by immunizing mice with natural Der f 2 purified from mite extract.^{20–22} Epitopes of Der f 2 and Der p 2 for some of the mAbs have been analyzed by using mutants into which a deletion or point mutation was introduced.^{7,8,17,18,23} In this study, we characterized epitopes of the five mouse anti-Der f 2 mAbs by analyzing reactivities of the wild-type and fifteen mutants of Der f 2 to the mAbs by immunoblotting.

The wild-type Der f 2 and its fifteen mutants (Fig. 1) were overexpressed in *E. coli* BL21 (DE3) transformed with the expression vectors. The cells were solubilized in a buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol, and electrophoresed on SDS polyacrylamide gel (SDS-PAGE) (Fig. 2, Coomassie Brilliant Blue). The recombinant Der f 2 molecules except two mutants, K15A and K15/77A, were detected as major bands corresponding to a molecular mass of approximately 15 kDa on SDS-PAGE. Under these reducing conditions, mobilities of K15A and K15/77A were smaller than those of the other recombinant Der f 2 molecules.

The reactivities of five mAbs to the Der f 2 mutants were examined by immunoblot analysis (Fig. 2). Three anti-Der f 2 monoclonal antibodies (mAbs), 13A4, 15E11, and 18G8 (Asahi Beer Pharmaceutical Co, Ltd., Tokyo, Japan), were established from BALB/c mice; and two mAbs, 1B2 and 7C10, from A/J mice. mAb 1B2 showed significantly reduced reactivity only in ΔC. mAb 7C10 showed the most reduced reactivity in K77A, K15/77A, and ΔC followed by 8C, C73/78S, and 8C–119C. mAb 13A4 showed the most reduced reactivity in ΔC and 8C, followed by C8/119S and 8C–119C. Concerning mAb 13A4, reduction of the development of the bands for P34A, P79A, P95A, and P99A was also

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Abbreviations: mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; RAST-EIA, radioallergosorbent test-enzyme immunoassay

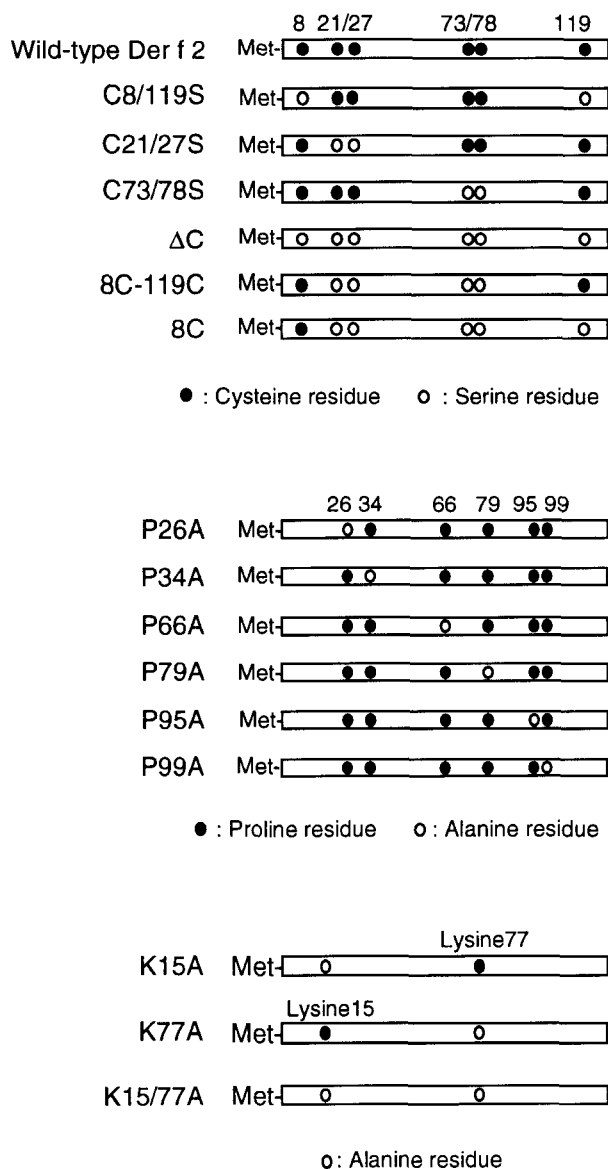


Fig. 1. Schematic Representation of Recombinant Variants of Der f 2.

Wild-type and fifteen mutants of Der f 2 expressed in *Escherichia coli* with a methionine at the first N-terminal residue are shown. Expression vectors for the wild-type Der f 2¹⁴⁾ and thirteen mutants, C21/27S, C73/78S, C8/119S,⁷⁾ C8/21/27/73/78/119S (ΔC), C21/27/73/78S (8C-119C),¹⁰⁾ P26A, P34A, P66A, P95A, P99A,¹⁹⁾ K15A, K77A, and P79A,¹⁶⁾ were constructed as previously described. Those for two mutants, C21/27/73/78/119S (8C) and K15/77A, were constructed by the use of restriction sites and/or site-directed mutagenesis.

observed. mAb 15E11 showed the most reduced reactivity in P79A, ΔC, 8C, 8C-119C, and C73/78S, and significantly reduced reactivity in C8/119S, K77A, and K15/77A. mAb 18G8 showed the most reduced reactivity in ΔC, 8C, and C73/78S, followed by 8C-119C and P79A.

Epitopes detectable in immunoblot analysis after SDS-PAGE under reducing conditions are limited to those that can be refolded on the membrane from polypeptides reduced and denatured by 2-mercap-

toethanol and SDS. Although some conformational epitopes fail to be correctly refolded, immunoblotting is a convenient method for rapid analysis of many unpurified samples. mAb 1B2 strongly bound all the mutants except ΔC. Recently, we confirmed that global disruption of the tertiary structure occurred in purified samples of ΔC, in which all the three intramolecular disulfide bonds were disrupted, C8/119S, in which Cys8-Cys119 bridging N- and C-terminal regions was disrupted, and 8C-119C, in which two bonds, Cys21-Cys27 and Cys73-Cys78, were disrupted.¹⁰⁾ Therefore, mAb 1B2 might recognize an epitope that has the character of a sequential rather than conformational epitope although the location of the epitope is not identified. The reduction of reactivity of mAb 1B2 to ΔC might have resulted from exhaustive disruption of the secondary structure caused by alteration of all six cysteine residues to serine residues.

Reactivities of three mAbs, 7C10, 15E11, and 18G8, to four mutants, C73/78S, ΔC, 8C-119C, and 8C, in all of which Cys73-Cys78 was disrupted, were greatly reduced compared with those to the wild-type Der f 2. mAb 7C10 showed significantly reduced binding to K77A, mAb 18G8 to P79A, and mAb 15E11 to both K77A and P79A. Therefore, we concluded that the disulfide bond, Cys73-Cys78, and the surrounding residue(s) maintain the epitopes recognized by the three mAbs, but the recognized residues are not completely identical to each other. This conclusion is consistent with our previous report that the epitopes for mAb 15E11 and mAb 18G8 were located in the sequence of 67-90.⁸⁾ However, there is a discrepancy between the results of immunoblotting in this study and our previous finding by enzyme-linked immunosorbent assay (ELISA) using purified recombinant Der f 2 molecules that the reactivity of P79A to mAb 15E11 was equivalent to that of the wild-type.²³⁾ We consider this to result from a failure of the protein of P79A to refold on the membrane after SDS-PAGE under the reducing conditions because a similar discrepancy was observed between immunoblotting and radioallergosorbent test-enzyme immunoassay (RAST-EIA) to analyze IgE-reactivity of P79A.¹⁹⁾ Pro79, which is next to Cys78 forming the disulfide bond Cys73-Cys78 and adopts the *cis* conformation, is suggested to play roles in the refolding of Der f 2.¹⁹⁾ The epitope for mAb 15E11 is considered to be dependent on the conformation of the allergen from results in previous studies⁸⁾ and those in the present study that mAb 15E11 showed significantly reduced binding to C8/119S in spite of conservation of Cys73-Cys78.

Nishiyama *et al.* suggested that the epitope for mAb 13A4 includes three residues, Arg128, Asp129, and Lys100, on the basis of their analysis on Der f 2 and that by Smith and Chapman on Der p 2.^{18,23)} Interestingly, these residues²³⁾ and Pro99, Pro95, and

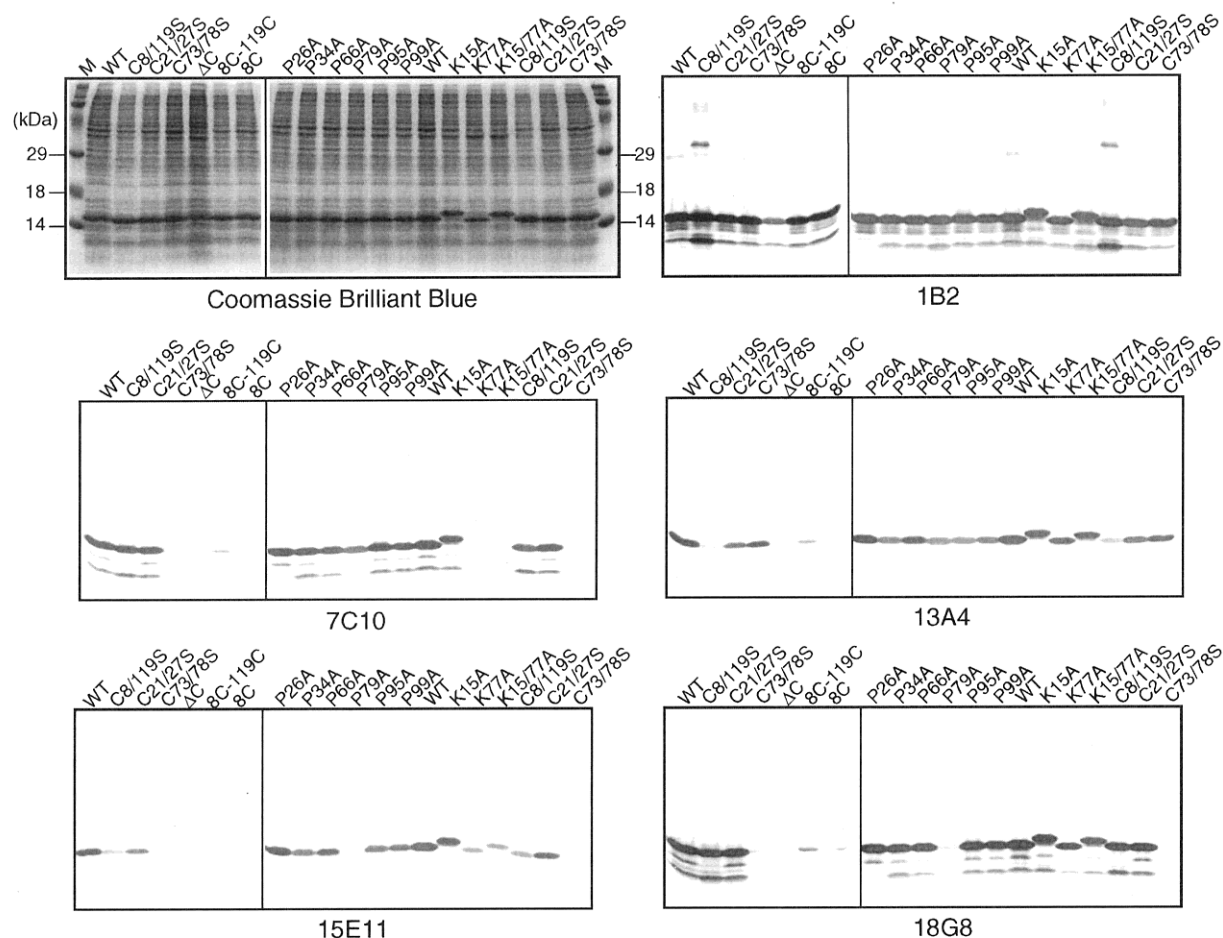


Fig. 2. Expression and Immunoblot Analysis of the Wild-Type and Fifteen Mutants of Der f 2.

Total cellular proteins of *E. coli* BL21 (DE3) transformed with the expression vectors of the wild-type (WT) and fifteen mutants of Der f 2 were run on SDS-PAGE under reducing conditions and electroblotted. The membranes were stained with Coomassie Brilliant Blue, or probed with a combination of a mouse mAb and a horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio Rad, Richmond, CA) with diaminobenzidine (Wako, Osaka, Japan) and H_2O_2 . The reactivities to five mouse mAbs, 1B2, 7C10, 13A4, 15E11 and 18G8, were analyzed. M: molecular weight marker.

Pro34¹⁹⁾ are located in loops at one end of the tertiary structure of Der f 2 and included in an IgE-binding site. Reduction of the development of the bands for three proline mutants, P99A, P95A, and P34A, was observed (Fig. 2). The mutations, P34A, P95A, and/or P99A, affected the reactivity to mAb 13A4 although further analysis using purified proteins should be done to conclude whether the residues are indeed involved in the recognition of mAb 13A4 or not. If they are involved directly, one of the conclusions in our previous study, that the epitope for mAb 13A4 was located in the sequence of 67–90, should be revised because it was based on weak bands of the two deletion mutants, 67–129 and 1–90, in immunoblotting.⁸⁾ A mutation, P79A, also affected the binding to mAb 13A4. This might result from a failure of protein refolding of P79A as mentioned in the last paragraph. Furthermore, mAb 13A4 showed significantly reduced binding to C8/119S. These results are consistent with our previous reports that the epitope for mAb 13A4 is considered dependent on the

conformation of the allergen.⁸⁾

In this study, the reactivities of five mAbs to sixteen recombinant variants of Der f 2 were analyzed by immunoblotting. The epitopes for two mAbs, 1B2 and 7C10, were analyzed for the first time. Information on the epitopes for three mAbs, 13A4, 15E11, and 18G8, previously unreported, was obtained and discussed.

Acknowledgments

We would like to thank Yachiko Hayashi-Shibusawa (Asahi Breweries, Ltd.) for preparation of the mAbs, Dr. Fuyuhiko Inagaki (Hokkaido University), Dr. Hideki Hatanaka (Tokyo Metropolitan Institute of Medical Science), and Saori Ichikawa (Japan Women's University) for helpful comments on the tertiary structure of Der f 2, and Dr. Hirokazu Okudaira (University of Tokyo) for encouragement.

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