



DETERMINATION OF THE N- AND C-TERMINAL SEQUENCES REQUIRED TO BIND HUMAN IgE OF THE MAJOR HOUSE DUST MITE ALLERGEN Der f 2 AND EPIOTOPE MAPPING FOR MONOCLONAL ANTIBODIES

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Abstract—B cell epitopes of the major house dust mite allergen Der f 2 from *Dermatophagoides farinae* were analysed using deletion mutants of Der f 2 expressed as fusion proteins in *Escherichia coli*. The reactivities of these partial Der f 2 molecules to human anti-mite IgE antibodies in atopic patients and to murine anti-Der f 2 monoclonal antibodies (mAbs) were examined by immunoblotting. A C-terminal deletion mutant of Der f 2, 1–123, had almost the same reactivity to human IgE as the whole Der f 2 (1–129) and an N-terminal deletion mutant of Der f 2 (25–129) still had weak reactivity. On the other hand, in two deleted Der f 2 molecules, 1–120 and 30–129, reactivity was lost in spite of long overlapping sequences. These results suggest that the human IgE antibodies to Der f 2 in atopic patient sera recognize the conformational structures dependent on the tertiary structure of Der f 2, including disulfide bond formations, rather than the contiguous sequences of amino acids. The sequences 1–24, 25–29 and 121–123 were revealed as the minimum N- and C-terminal amino acid sequences required for IgE binding. Contrastingly, all three murine mAbs bound to the smaller deletion mutants, 1–90 and 67–129, suggesting that the cores of the epitopes for these mAbs exist in the 24 amino acid sequence of Der f 2, 67–90 overlapping the sequential human IgE epitope on Der p 2, the equivalent allergen from *Dermatophagoides pteronyssinus*. These findings are important for the understanding of the antigenic structure of Der f 2 and for the manipulation of the allergen for immunotherapy. © 1997 Elsevier Science Ltd.

Key words: Der f 2, epitope, IgE, monoclonal antibody, house dust mite, allergen.

INTRODUCTION

House dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) allergens have been identified as major causative factors associated with various allergic diseases such as bronchial asthma and atopic dermatitis (Voochorst *et al.*, 1967; Miyamoto *et al.*, 1968). Immediate hypersensitivity is caused by the release of mediators from mast cells and basophils activated with aggregation of the complexes of the high-affinity IgE receptor (FcεRI) on the cell surface and allergen-specific IgE, crosslinked by multivalent allergens (Ishizaka and Ishizaka, 1984; Metzger *et al.*, 1986). IgE antibodies in the majority of sera of patients allergic to house dust mites have binding activities to both of the groups of house dust mite allergens, group 1 (Der f 1 and Der p 1, *M_r* 25 000) and group 2 (Der f 2 and Der p 2, *M_r* 14 000) (Platts-Mills and Chapman, 1987; Haida *et al.*, 1985; Yamashita *et al.*, 1989; Yasueda *et al.*, 1986). Therefore, studies of the B

(Lind *et al.*, 1988; Lombardero *et al.*, 1990; Greene and Thomas, 1992; Shoji *et al.*, 1996; Chua *et al.*, 1991; van't Hof *et al.*, 1991; Nishiyama *et al.*, 1995; Smith and Chapman, 1996) and T cell epitopes (Yssel *et al.*, 1992; van Neerven *et al.*, 1993; O'Hehir *et al.*, 1993; O'Brien *et al.*, 1995; Tsitoura *et al.*, 1996) of the major allergens have been carried out to clarify their relationships to immunogenicity and to apply such epitope moieties to immunotherapy (Valenta and Kraft, 1995; Hoyne *et al.*, 1995).

Previously we isolated cDNA clones encoding Der f 2, the group 2 allergen from *Dermatophagoides farinae* (Yuuki *et al.*, 1990), all of which encode proteins consisting of 129 amino acid residues with remarkable sequence homology to Der p 2, the group 2 allergen from *Dermatophagoides pteronyssinus* (Chua *et al.*, 1990). Der f 2 and Der p 2 proteins contain six cysteine residues, that form three intramolecular disulfide bonds (Nishiyama *et al.*, 1993), and no N-glycosylation sites. Ourselves and others have examined the B cell epitopes of Der f 2 and Der p 2 by the use of recombinant DNA techniques (Nishiyama *et al.*, 1995, 1994; Iwamoto *et al.*, 1996; Chua *et al.*, 1991; Smith and Chapman, 1996) and synthetic

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peptides (van't Hof *et al.*, 1991). Chua *et al.* (1991) reported that partially deleted Der p 2 molecules expressed from random fragments of cDNA encoding Der p 2 did not effectively react with IgE antibodies in the sera of adult patients allergic to house dust mite. Moreover they generated and analysed only five deleted Der p 2 molecules to which deletions were introduced at relatively long intervals along the sequence. As we describe here, to obtain more information on the B cell epitopes of Der f 2, another group 2 house dust mite allergen, we generated numbers of more precisely deleted molecules. Various deleted mutants of Der f 2 cDNA were prepared and expressed in *Escherichia coli* as fusion proteins. The resultant modified Der f 2 proteins of various lengths were used for comparison of the reactivities to IgE antibodies in atopic patient sera and to three murine anti-Der f 2 monoclonal antibodies (mAbs). Finally, the sequences 1–24, 25–29 and 121–123 were revealed as the N- and C-terminal amino acid sequences required for IgE binding. On the other hand, the cores of the epitopes for murine anti-Der f 2 mAbs were revealed to exist in the peptide composed of 24 amino acid residues, 67–90.

MATERIALS AND METHODS

Sera and mAbs

Sera were obtained from 10 mite sensitive atopic patients with allergic rhinitis, bronchial asthma and/or atopic dermatitis who proved positive to a prick skin test. The murine anti-Der f 2 mAbs (13A4, 15E11 and 18G8) used in this study have been described previously (Akagawa *et al.*, 1992).

Expression of Der f 2 cDNA

The expression vector for the fusion with maltose binding protein designated pMFL11-c was constructed by insertion of the Der f 2 cDNA fragment generated by digestion of pFL11 (Yuuki *et al.*, 1991) with restriction enzymes to the *Stu* I site of pMAL-c (Mania *et al.*, 1988). The expression vector of the fusion with β -galactosidase named pFL502 was constructed from pFL11 by deletion of the 3' terminal region of the β -galactosidase gene to approximately half size. The control plasmid named pFL500 encoding only the half-sized β -galactosidase was also constructed. *E. coli* transformed with these vectors were cultured and induced to express the fusion proteins (Mania *et al.*, 1988; Yuuki *et al.*, 1991).

Immunoblotting

Samples from the cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8% acrylamide) (Laemmli, 1970) and the gels were used for immunoblotting (Towbin *et al.*, 1979). Total proteins transferred onto each nitrocellulose membrane (Bio Rad, Richmond, CA, U.S.A.) and those bound to IgE antibody in each patient serum were detected, as previously described (Tovey and Baldo, 1984), with a minor modification of the use of peroxidase-

labeled goat anti-human IgE polyclonal antibody (Cappel, Malvern, PA, U.S.A.). Total proteins transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon: Millipore Co., Bedford, MA, U.S.A.) and those bound to anti-Der f 2 polyclonal antibody in murine antiserum and mAbs were detected as previously described (Yuuki *et al.*, 1991).

Construction and screening of the expression vectors of deleted Der f 2s

Restriction enzymes and exonuclease III were used for the modification and deletion of the cDNA (Henikoff, 1984). Deletion of the 3' terminal of the cDNAs was performed as follows. pMFL11-c and pFL502 were double-digested with *Sal* I and *Pst* I. After the reaction of deletion and the end repair, SURF linker (Pharmacia, Uppsala, Sweden) containing stop codons was ligated with each of them. The levels of deletion were controlled by changing the time of exonuclease III treatment. Deletion of the 5' terminal of the cDNAs was performed as follows. pMFL11-c was digested with *Kpn* I and blunt ended. After the deletion and the end repair, the fragments were digested with *Hind*III and fractionated by polyacrylamide gel electrophoresis. DNA fragments ranging from 300 to 400 base pairs were recovered and ligated with the *Stu* I/*Hind* III fragment of pMAL-c. *E. coli* TBI was transformed with the ligated DNA and clones expressing the deleted cDNA were screened by colony immunoassay using murine anti-Der f 2 antiserum (Yuuki *et al.*, 1991). The fragments encoding two deleted Der f 2, 67–129 and 85–129, were generated by the use of restriction sites. Sequences of deleted cDNA inserts were determined using a 373A DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

Expression and IgE binding of the Der f 2 fusion proteins

Der f 2 was expressed as fusion proteins with maltose binding protein or with the half molecule of β -galactosidase. As shown in Fig. 1, both fusion proteins reacted clearly with murine anti-Der f 2 IgG polyclonal antibody and human anti-Der f 2 IgE antibody in a patient serum. This result suggests that the recombinant Der f 2 molecules are applicable to the epitope study. Therefore, we prepared deleted Der f 2 cDNAs of various sizes, and examined the reactivities of expressed proteins to human IgE in mite-sensitive atopic patient sera and to three murine anti-Der f 2 mAbs, as described.

Reactivities of deleted Der f 2 molecules of various lengths to IgE in mite-sensitive atopic patient sera

Der f 2 cDNAs serially lacking the 5' terminal or 3' terminal region were expressed as fusion proteins and assayed for IgE reactivities. The screening system, composed of exonuclease III treatment, colony immunoassay and immunoblotting, facilitated the screening of numbers of serially deleted Der f 2 mutants and the fine analysis

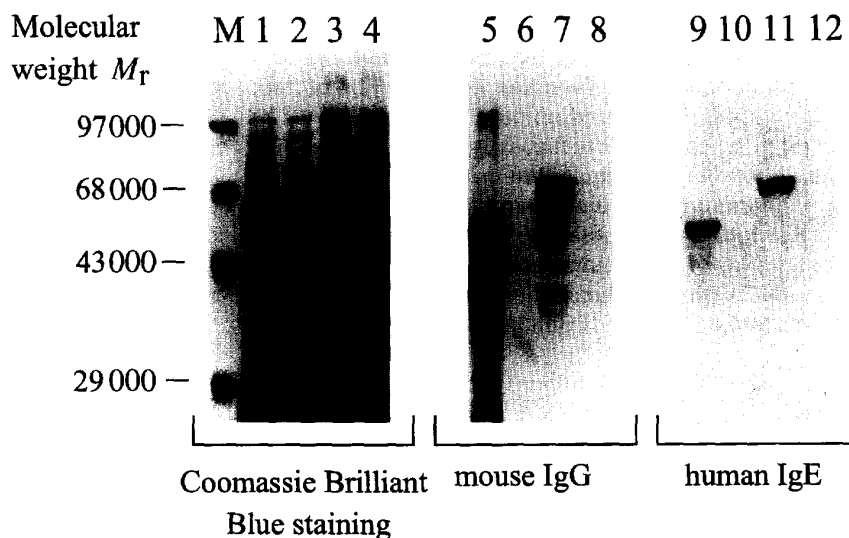


Fig. 1. Reactivity of human IgE antibody to Der f 2 expressed as fusion proteins. Total cellular proteins of *E. coli* TBI transformed with the expression vector of the Der f 2 fusion protein with maltose binding protein: pMFL11-c (lanes 1, 5, 9), or the control vector: pMAL-c (lanes 2, 6, 10), or those of *E. coli* MC1061 transformed with the expression vector of the Der f 2 fusion protein with β -galactosidase: pFL502 (lanes 3, 7, 11), or the control vector: pFL500 (lanes 4, 8, 12) were subjected to SDS-PAGE (8% acrylamide) and electroblotted. The membranes were stained with Coomassie Brilliant Blue (lanes M, 1, 2, 3, 4) or probed with murine anti-Der f 2 antiserum (lanes 5, 6, 7, 8) or a mite sensitive human atopic serum (lanes 9, 10, 11, 12).

of the required sequences to bind human IgE. Those clones which expressed the smallest fusion proteins reactive to IgE in terms of molecular weight or the largest fusion proteins without reactivities to IgE, were selected and their deleted cDNA inserts sequenced. The results of immunoblotting using a mite sensitive atopic patient serum are shown in Fig. 2. The same result was obtained with the tested serum of all 10 atopic patients (data not shown). Among the proteins lacking C-terminal residues of Der f 2, the polypeptide 1–123 reacted with IgE antibody to the same extent as the whole Der f 2 protein (1–

129) but the polypeptide 1–120 had no detectable reactivity. Among the proteins partially lacking N-terminal residues of Der f 2, the polypeptide 25–129 had remarkably low reactivity compared to the whole Der f 2 molecule. Another polypeptide 30–129 had no detectable reactivity. Polypeptides from the more extensively deleted Der f 2 cDNA fragments were not reactive to IgE antibody (data not shown).

Since neither of the overlapping polypeptides, 1–120 or 30–129, had detectable reactivity with human IgE, IgE epitopes of Der f 2 did not appear to consist of simply

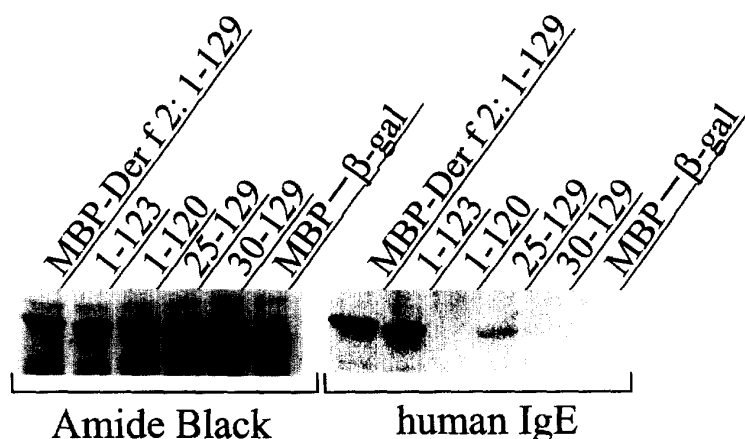


Fig. 2. Reactivity of human IgE antibody to proteins expressed from deleted Der f 2 cDNA. Total cellular proteins of *E. coli* TBI transformed with the expression vector of the Der f 2 fusion protein with maltose binding protein: pMFL11-c (MBP-Der f 2:1–129), its deletion mutants (1–123, 1–120, 25–129, 30–129) or the control vector: pMAL-c (MBP- β -gal) were subjected to SDS-PAGE (8% acrylamide) and electroblotted. The membranes were stained with Amide Black or probed with human atopic serum.

sequential polypeptides. Contrastingly, the polypeptide 1–123 was fully reactive with human IgE while the polypeptide 25–129 was only weakly reactive. Therefore, the sequences 1–24, 25–29 and 121–123 contained amino acid residues directly involved in the interaction to human IgE or maintaining the structure of Der f 2 required to react with human IgE (Fig. 4). Interestingly, within and around these sequences there are four cysteine residues, Cys8, Cys21, Cys27 and Cys119 (Fig. 5). The binding activities to the human IgE of group 2 allergens of house dust mite are not affected by treatment at 100°C for 10 min but are

sensitive to the reduction and the alkylation (Lombardero *et al.*, 1990). We previously determined the location of three disulfide bonds of native Der f 2 (Cys8–Cys119, Cys21–Cys27 and Cys73–Cys78) (Nishiyama *et al.*, 1993). Therefore, the three disulfide bonds appeared essential to the group 2 allergens for retaining the rigid structure needed to be reactive to human IgE. Disruption of the disulfide bonds might occur in those polypeptides which had no or low reactivities to human IgE. The polypeptide 25–129 still retained detectable reactivity with human IgE but it was much lower than that of the

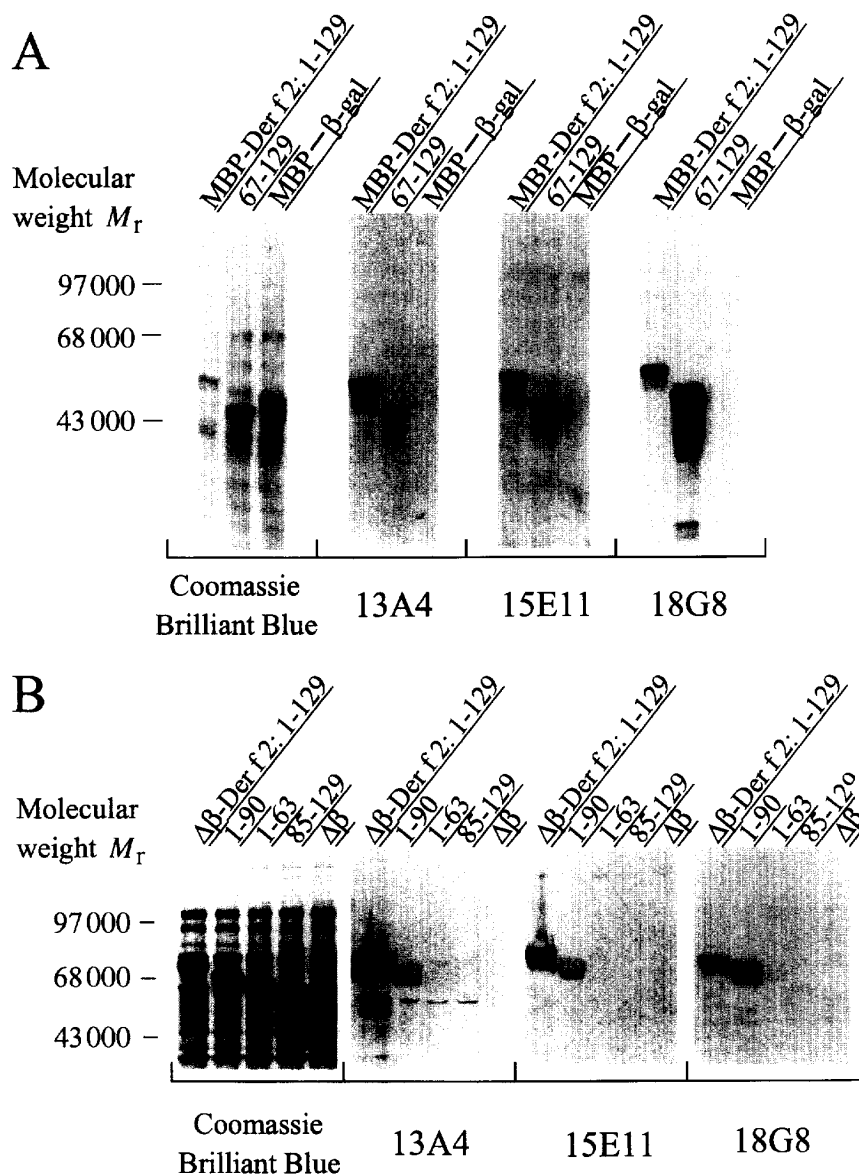


Fig. 3. Epitope mapping of Der f 2 for three murine mAbs. (A) Total cellular proteins of *E. coli* TB1 transformed with pMFL11-c: the expression vector of the Der f 2 fusion protein with maltose binding protein (MBP-Der f 2:1–129), its deletion mutant (67–129) or pMAL-c: the control vector (MBP-β-gal); or (B) those of *E. coli* MC1061 transformed with pFL502: the expression vector of the Der f 2 fusion protein with β-galactosidase (Δβ-Der f 2: 1–129), its deletion mutants (1–90, 1–63, 85–129) or pFL500: the control vector (Δβ) were subjected to SDS-PAGE (8% acrylamide) and electroblotted. The membranes were stained with Coomassie Brilliant Blue or probed with three murine mAbs, 13A4, 15E11 and 18G8.

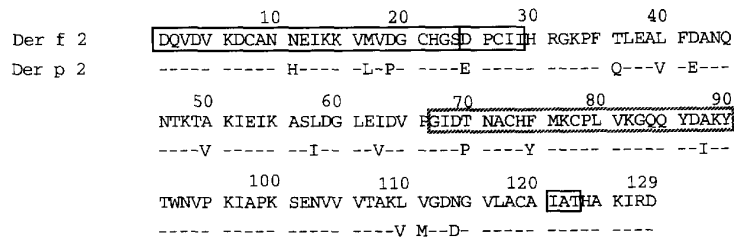


Fig. 4. Comparison of the deduced primary structures of mature Der f 2 (clone 11) (Yuuki *et al.*, 1990) and Der p 2 (Chua *et al.*, 1990). Identical amino acids at the same position are indicated by (-). N- and C-terminal sequences required to bind human IgE (1-24, 25-29 and 121-123) are boxed with a solid line. The sequence containing the epitopes for the three mAbs, 13A4, 15E11 and 18G8 (67-90) is boxed with a dashed line.

whole Der f 2 molecule. Some of the IgE epitopes were lost by the deletion of amino acids 1-24 because the peptide 25-129 could not completely inhibit the IgE binding to the recombinant whole Der f 2 molecule as described previously (Nishiyama *et al.*, 1995). Reduction of the binding affinities of the rest epitopes might also occur by deletion. On the basis of the results described here, we generated more N-terminal deletion mutants (4-129, 6-129, 11-129, 16-129 and 21-129), analysed their IgE reactivities (Nishiyama *et al.*, 1995) and showed that the reactivity of the peptide 11-129 was markedly reduced compared to that of the peptide 6-129. Furthermore, the incorrect disulfide bond of Cys8-Cys73, instead of the correct bonds of Cys8-Cys119 and Cys73-Cys78, was observed in a portion of the directly expressed peptide 1-120 (Nishiyama *et al.*, 1995), equivalent to the peptide expressed as a fusion protein that was not reactive to IgE. These results suggest the importance of the disulfide bond of Cys8-Cys119. Recently Smith and Chapman reported that two mutant Der p 2 molecules, C8G and C119Y, in both of which a single mutation had been introduced at each of the two cysteine residues involved in the disulfide

bond of Cys8-Cys119, maintain the same reactivity to IgE as the wild type Der p 2. The discrepancy between our findings and theirs might result from differences between either Der f 2 and Der p 2, the patients, the amino acids substituted for cysteine, or the preparation of recombinant products.

Epitope analysis for three murine mAbs by the use of deleted Der f 2 molecules

Epitope mapping of Der f 2 for murine mAbs is also important for understanding the antigenic structure of Der f 2 recognized by polyclonal human IgE. The reactivities of four fusion proteins with three different murine mAbs were examined by immunoblotting. The results are shown in Fig. 3. All of the three murine mAbs reacted to the polypeptides 1-90 and 67-129 although the reactivities of 13A4 and 15E11 were reduced compared with those to Der f 2 (summarized in Fig. 5). Based on this observation, we concluded that the core of the epitopes for the three murine mAbs are located in the amino acid sequence, 67-90, but some of the N- and C-terminal

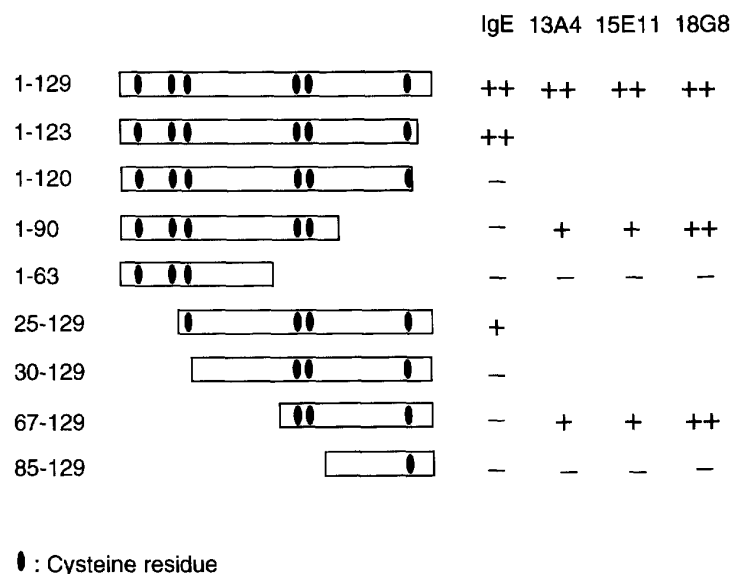


Fig. 5. Reactivities of partial Der f 2 polypeptides with human IgE antibody and three murine mAbs. The results shown in Figs 2 and 3 are summarized here. (++) denotes markedly positive reaction, (-) denotes no reaction and (+) denotes positive but not strong reaction.

residues contribute to the high affinities of Der f 2 to 13A4 and 15E11.

Previously we reported that the mAb 18G8 partially competed with 13A4 and 15E11 in inhibition studies (Akagawa *et al.*, 1992). The results suggest that the epitope for 18G8 overlaps with or lies near those for 13A4 and 15E11 within the 24 amino acid sequence of 67–90 or that conformational change of the epitopes for 13A4 and 15E11 is caused by the binding of 18G8 to Der f 2.

As shown in Fig. 4, three amino acid residues (Thr70, Phe75 and Ala88) differ between the clone 11 of Der f 2 (Yuuki *et al.*, 1990), used in the present study, and Der p 2 (Chua *et al.*, 1990). The epitope for the mAb 15E11 is expected to be dependent on either of the three residues since it was strongly reactive with Der f 2 but much less reactive to Der p 2 (Akagawa *et al.*, 1992; Ovsyannikova *et al.*, 1994).

Interestingly, it is reported that the sera of some atopic patients showed IgE reactivities to the synthetic Der p 2 peptide 65–78 (van't Hof *et al.*, 1991), which overlapped with the Der f 2 peptide 67–90 shown to contain the core of the epitopes for the three murine mAbs in the present study. This suggests that the residues in this region are antigenic in both mouse and human.

Recently, the mechanisms and new strategies of allergen-specific immunotherapy have been vigorously analysed (Creticos *et al.*, 1996; Secrist *et al.*, 1995; Suko *et al.*, 1995; Stampfli *et al.*, 1995; Durham, 1995) and studies that encourage the use of recombinant allergens for immunotherapy have been reported (Valenta and Kraft, 1995; Hoyne *et al.*, 1995). In this study we have performed the B cell epitope analysis of the major house dust mite allergen Der f 2 using numbers of deletion mutants expressed in *Escherichia coli*. Consequently, we have shown that IgE epitopes of Der f 2 were highly dependent on the integrity of conformational structure, revealed the sequences 1–24, 25–29 and 121–123 as the minimum N- and C-terminal sequences required for IgE binding and located the cores of the epitopes for murine anti-Der f 2 mAbs in the peptide composed of 24 amino acid residues, 67–90. These findings are important for the understanding of the antigenic structures of the house dust mite group 2 allergens and for their manipulation for immunotherapy.

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