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## Asp159 is a critical core amino acid of an IgE-binding and cross-reactive epitope of a dust mite allergen Der f 7

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### ABSTRACT

Der f 7 and Der p 7 are important house dust mite allergens with known structure and suggested biological function recently. However, their IgE-binding determinants remain unknown. The purpose of this study is to identify the IgE-reactive epitopes of Der f 7 and the determinants of IgE-mediated cross-reactivity between Der f 7 and Der p 7. IgE-reactive determinants were identified by immunodot blot inhibition using synthetic overlapping peptides, allergen mutants, and a Der f 7 structural model. Our results showed that synthetic peptides with sequence <sup>156</sup>SILDP<sup>160</sup> on Der f 7 bind IgE in two of the 30 asthmatic serum samples tested. Recombinant Der f 7 I157A, L158A, or D159A mutants have reduced IgE-binding activity. Inhibition experiments confirmed Asp159 as a critical core residue for IgE-binding. Among Der p 7, Der f 7 and Der f 7 mutants with single substitution between residues 156 and 160, only the D159A mutant cannot inhibit significantly IgE-binding against Der p 7. Therefore, Asp159 contributes to IgE-mediated cross-reactivity between Der f 7 and Der p 7. The structural model constructed for Der f 7 suggests that the IgE-binding epitope forms a loop-like structure on the surface of the molecule. In conclusion, Asp 159 is a critical core residue of an IgE-binding and IgE-mediated cross-reactive epitope <sup>156</sup>SILDP<sup>160</sup> of Der f 7. Results obtained from this study provide more information on molecular and structural features related to allergenicity, underlying basis of IgE cross-reactivity between allergens, and in designing safer immunotherapy.

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### 1. Introduction

The prevalence of human atopic disorders including allergic rhinitis, asthma and atopic dermatitis is increasing during the past two to three decades (Sporik and Platts-Mills, 1992; Centers for Disease Control and Prevention (CDC), 2011). House dust mites have been identified as a major risk for allergic sensitization and disease development (Tovey et al., 1981; Sporik et al., 1992). Characterization of mite allergens will be beneficial in the diagnosis and treatment of mite-induced atopic illnesses.

We have previously identified and characterized the major group 7 mite allergens including Der f 7 and Der p 7 through cDNA cloning and expression, monoclonal antibodies preparation and immunological responses analysis (Shen et al., 1993, 1995a,b, 1996, 1997; Hales et al., 2000). The group 7 mite allergens induce IgE

responses in about 50% of mite atopic asthmatic patients (Shen et al., 1993, 1995b). Der f 7 and Der p 7 share 86% amino acid sequence identity and most asthmatic patients with IgE antibodies against Der p 7 have IgE reactivity against Der f 7 (Shen et al., 1995b). We have proposed that Der f 7 is structurally homologous to a human bactericidal permeability increasing protein (BPI)/lipopolysaccharide-binding protein (LBP) which may interact with Toll-like receptors (TLRs) after binding lipopolysaccharide and other bacterially derived lipid ligands (Shen et al., 2011). Mueller et al. reported similar findings by solving the crystal structure of a Der p 7–maltose binding fusion protein (Mueller et al., 2010). It implies that the biological reactivity of the group 7 mite allergens may contribute to their allergenicity.

Cross-linking of allergens to high-affinity Fcε receptors bound human IgE antibodies on the surface of mast cells will induce degranulation and release of inflammatory mediators resulting in immediate-type allergic reactions. Mapping of IgE-reacting antigenic determinants of allergens can further the understanding of allergenicity at molecular and structural levels and provide basis for designing hypoallergenic allergens for better therapeutic strate-

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gies (Takai et al., 2001; Pomés, 2010). However, the IgE-binding determinants on the group 7 mite allergens remain unknown. Here, we report the identification of a linear and cross-reactive IgE-binding epitope on Der f 7. By homology modeling, the IgE-reacting determinant <sup>156</sup>SILDP<sup>160</sup> assumes a loop structure on the surface of Der f 7. Furthermore, Asp159 within the determinant is a critical core amino acid residue and responsible for IgE cross-reactivity between Der f 7 and Der p 7 allergens. Results obtained will aid in the design of hypo-allergenic proteins for therapeutic purposes.

## 2. Methods and materials

### 2.1. Patients' sera

Sera used in the present study were collected from 30 patients with a clinical history of bronchial asthma and previously shown to contain IgE antibodies against the group 7 dust mite allergens (Shen et al., 1993, 1995b). They were stored in aliquots at  $-70^{\circ}\text{C}$ . Serum from healthy laboratory personnel was included as negative control. This study has been approved by the Institutional Review Board of Taipei Veterans General Hospital.

### 2.2. Peptide synthesis and peptide mapping

Twenty overlapping peptides covering the complete sequence of the mature Der f 7 were custom synthesized (Shen et al., 2011) and used in the present study. These 15-residue synthetic peptides are offset by five amino acids. Immunoreactivity of the peptides with human IgE antibodies was determined by immunodot blot inhibition assay. Briefly, 1  $\mu\text{g}$  of rDer f 7 was dotted onto nitrocellulose membranes using a microfiltration apparatus (0.2  $\mu\text{m}$ , Bio-Rad Lab., Hercules, CA, USA). The membrane was blocked with 2% skimmed milk. For inhibition assays, sera from asthmatic patients were preincubated individually, at room temperature for 2.5 h, with individual or various combinations of synthetic peptides (50  $\mu\text{g}$  of each peptide) before immunodot blot analysis. These pre-treated sera were then incubated individually, at  $4^{\circ}\text{C}$  for 16 h, with nitrocellulose membranes dotted with rDer f 7. IgE reactivity against rDer f 7 was detected with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (BD Biosciences Pharmingen, San Diego, CA, USA) and substrate solution as described (Cheng et al., 2008). As a control, individual serum sample was preincubated with 50  $\mu\text{g}$  of a synthetic peptide with sequence encompassing the N-terminus of the mature Asp f 13 allergen (<sup>N</sup>CGLTTQKSAPWGLGSIHKG<sup>C</sup>) before dot-blot immunoassay. Experiments were performed in triplicate and conditions with positive inhibition were repeated at least thrice.

### 2.3. Site-directed mutagenesis

A plasmid encoding Der f 7 is available from previous studies (Shen et al., 1995b). It was used as template in polymerase chain reactions (PCRs) to generate mutants carrying single alanine substitute at S156, I157, L158, D159 or P160 according to published method (Cheng et al., 2008). Primers used in the mutagenesis experiments are listed in Table 1. The newly synthesized DNA carrying the point mutation was purified and inserted into the pQE80 expression vector (Qiagen Inc., Valencia, CA, USA) and transformed into *Escherichia coli* JM109 for expression of Der f 7 mutants. All mutant constructs were confirmed by DNA sequencing and were affinity-purified with Ni-NTA resin columns (Qiagen) according to the manufacturer's instructions. For IgE cross-reactivity studies, purified wild type Der p 7 (GenBank accession no. U37044) with an N-terminal His<sub>6</sub>-tag was also prepared.

**Table 1**  
Primers used in PCR.

| Name                        | Nucleotide sequence in 5'–3'–end orientation                          |
|-----------------------------|-----------------------------------------------------------------------|
| Df7S156A <sub>forward</sub> | 5'– <sup>544</sup> catatttggtggtcttgcaatcttgatcc <sup>572</sup> –3'   |
| Df7S156A <sub>reverse</sub> | 5'– <sup>572</sup> ggatccaagattgcaagaccaccaatg <sup>544</sup> –3'     |
| Df7I157A <sub>forward</sub> | 5'– <sup>550</sup> ggtggtcttccagccttgatccaattttgg <sup>581</sup> –3'  |
| Df7I157A <sub>reverse</sub> | 5'– <sup>581</sup> ccaaaaattggatccaaggtgaaagaccacc <sup>550</sup> –3' |
| Df7L158A <sub>forward</sub> | 5'– <sup>553</sup> ggtcttcaatcgcgatccaattttggcg <sup>583</sup> –3'    |
| Df7L158A <sub>reverse</sub> | 5'– <sup>583</sup> cgccaaaaattggatccgcatgaaagacc <sup>550</sup> –3'   |
| Df7D159A <sub>forward</sub> | 5'– <sup>553</sup> ggtcttcaatcttgcccccaattttggc <sup>582</sup> –3'    |
| Df7D159A <sub>reverse</sub> | 5'– <sup>582</sup> gccaaaaattggggccaagattgaaag <sup>553</sup> –3'     |
| Df7P160A <sub>forward</sub> | 5'– <sup>560</sup> caatcttgatgcaatttttggcggtttatc <sup>590</sup> –3'  |
| Df7P160A <sub>reverse</sub> | 5'– <sup>590</sup> gataaaacgccaaaaattgcatccaagattg <sup>560</sup> –3' |
| pQE-f                       | 5'–cccgaagagtgccacctg–3'                                              |
| pQE-r                       | 5'–gttctgaggtcattactgg–3'                                             |

### 2.4. Dot-blot and dot-blot inhibition immunoassays

One microgram of purified wild type Der f 7, Der p 7, or Der f 7 mutants was dotted onto nitrocellulose membranes as described above. The membranes were blocked with 2% skimmed milk and incubated for 16 h at  $4^{\circ}\text{C}$  with serum samples with and without preincubation with 10  $\mu\text{g}$  of purified recombinant Der f 7, Der p 7 or Der f 7 mutants at room temperature for 2.5 h. The membranes were washed and human IgE antibodies on the membranes were visualized as outlined above. As a control, the same positive serum sample was pre-incubated with 10  $\mu\text{g}$  of bovine serum albumin (BSA, Pierce, Rockford, IL, USA) before immunodot blot against Der f 7 or Der p 7.

### 2.5. Homology-based modeling of antigenic epitopes recognized by IgE antibodies

Molecular modeling of Der f 7 was performed using Swiss-Model programs in the first approach mode (Swiss-Model, Guex and Peitsch, 1997). The coordinates (PDB code 3H4Z) of the Der p 7–maltose-binding protein fusion construct (Mueller et al., 2010) were used in the computation. The antigenic epitopes and the critical core amino acid residues recognized by IgE antibodies were visualized and displayed with the PyMOL 1.3 (<http://www.pymol.org>) or the VMD molecular visualization program (VMD1.8.6) (<http://www.ks.uiuc.edu/Research/vmd/>) (Humphrey et al., 1996).

## 3. Results

### 3.1. Immunodot blot inhibition by synthetic peptides

Twenty synthetic peptides with overlapping sequences covering the entire length of Der f 7 (Shen et al., 2011) were used to identify the IgE-binding determinant(s) on Der f 7. These twenty peptides were divided into four groups in an immunodot blot inhibition assay. Only the group containing peptides Df7-16–Df7-20 inhibited significantly the IgE binding to Der f 7 in two (serum no. 990 and 1045) of the 30 sera tested. A representative result using serum 990 is shown in Fig. 1A. Further analyses demonstrated that any peptide mixture within this group containing peptide Df7-16 (Fig. 1B) or Df7-16 by itself (Fig. 1C) inhibited IgE binding to Der f 7. Furthermore, the corresponding Der p 7 peptide (Dp7-16, <sup>151</sup>HIGGLSILDP<sup>165</sup>) also inhibited IgE-binding against Der f 7 (Fig. 1C). This inhibition was not observed when a control peptide encoding the N-terminus of Asp f 13 was used in the assay (Fig. 1C). Results of Table 2 show data on the average percent inhibition of IgE-binding against rDer f 7 by using peptide(s) shown in Fig. 1 determined with the AlphaEaseFCTM software (version 4.0.0, Alpha Innotech Corporation, San Lean-

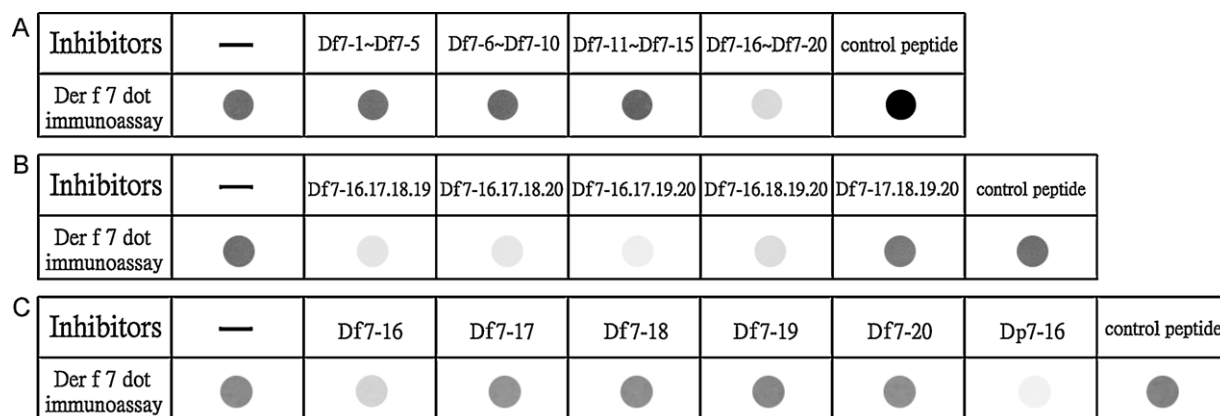


**Fig. 1.** Immunodot blot inhibition of IgE-binding against rDer f 7 by 20 pentadecyl peptides with overlapping sequences offset by five amino acids and cover the complete sequence of the mature Der f 7 [10]. The amino acid sequence of the peptide Dp7-16 is <sup>151</sup>HIGGLSILDPPIFAVL<sup>165</sup>.

**Table 2**

Percent inhibition of IgE-binding in immunodot blotting against rDer f 7.

| Peptide inhibitors | % inhibition <sup>a</sup> |
|--------------------|---------------------------|
| Df7-16–Df7-20      | 62                        |
| Df7-16.17.18.19    | 57                        |
| Df7-16.17.18.20    | 58                        |
| Df7-16.17.19.20    | 67                        |
| Df7-16.18.19.20    | 54                        |
| Df7-16             | 62                        |
| Dp7-16             | 89                        |

<sup>a</sup> Only results of >10% inhibition of IgE-binding, by synthetic overlapping peptides shown in Fig. 1, against rDer f 7 are shown in this table.

dro, CA, USA). Results similar to those described above were also obtained when serum 1045 was used in the dot-blot inhibition assay (data not shown). For control, serum from a healthy person showed negative IgE immunodot blot activity against rDer f 7 (data not shown).

Peptides Df7-15 (<sup>141</sup>EVQRQFANVVNHIGGL<sup>155</sup>) and Df7-17 (<sup>161</sup>IFGVLSVDLTAIFQD<sup>175</sup>) cannot inhibit IgE from binding to rDer f 7 while peptide Df7-16 (<sup>151</sup>HIGGLSILDPPIFGVL<sup>165</sup>) contains an antigenic determinant recognized by IgE antibodies. Hence, the non-overlapping amino acid residues <sup>156</sup>SILDP<sup>160</sup> in peptide Df7-16 are responsible for IgE reactivity against Der f 7.

### 3.2. Immunodot blot reactivity of Der f 7 mutants

In order to determine which amino acid(s) among <sup>156</sup>SILDP<sup>160</sup> is the critical core residue(s) responsible for IgE-binding, site-directed Der f 7 mutants (S156A, I157A, L158A, D159A and P160A) were prepared. The wild-type and Der f 7 mutants have similar far-UV circular dichroism spectra (data not shown) suggesting that the mutations have not changed detectably the overall secondary structure of the proteins. IgE antibodies in serum sample 990 showed reduced immunodot blot reactivity against Der f 7 I157A, L158A and D159A mutants (Fig. 2). Serum 440 with IgE-binding against Der f 7 and does not inhibited by the set of overlapping synthetic peptides was used as control. It bound Der f 7 and its five mutants. In addition, IgE antibodies of both serum samples showed negative dot-blot reactivity against BSA (Fig. 2).

### 3.3. Immunodot blot inhibition by Der f 7 mutants

To affirm the critical core amino acids in IgE-binding against Der f 7, immunodot blot inhibition by Der f 7 mutants was performed. Results in Fig. 3 show that wild-type and Der f 7 mutants can inhibit the IgE-binding of the control serum sample 440 against Der f 7. However, Der f 7 with a D159 to alanine substitution is the only mutant that cannot inhibit significantly the IgE-binding of serum

| serum no. | Immunodot assay |                 |       |       |       |       |     |
|-----------|-----------------|-----------------|-------|-------|-------|-------|-----|
|           | Der f 7         | Der f 7 mutants |       |       |       |       | BSA |
|           |                 | S156A           | I157A | L158A | D159A | P160A |     |
| 440       | ●               | ●               | ●     | ●     | ●     | ●     | ●   |
| 990       | ●               | ●               | ●     | ●     | ●     | ●     | ●   |

**Fig. 2.** Immunodot blot reactivity of IgE antibodies against the wild type and Der f 7 mutants.

| serum no. | Inhibitors |         |                 |       |       |       |       |
|-----------|------------|---------|-----------------|-------|-------|-------|-------|
|           | —          | Der f 7 | Der f 7 mutants |       |       |       |       |
|           |            |         | S156A           | I157A | L158A | D159A | P160A |
| 440       | ●          | ●       | ●               | ●     | ●     | ●     | ●     |
| 990       | ●          | ●       | ●               | ●     | ●     | ●     | ●     |

**Fig. 3.** Immunodot blot inhibition of IgE-binding against rDer f 7 by the wild type and Der f 7 mutants.

| serum<br>no. | Inhibitors |         |         |                 |       |       |       |       |     |
|--------------|------------|---------|---------|-----------------|-------|-------|-------|-------|-----|
|              | —          | Der p 7 | Der f 7 | Der f 7 mutants |       |       |       |       | BSA |
|              |            |         |         | S156A           | I157A | L158A | D159A | P160A |     |
| 440          | ●          | ●       | ●       | ●               | ●     | ●     | ●     | ●     | ●   |
| 990          | ●          | ●       | ●       | ●               | ●     | ●     | ●     | ●     | ●   |

Fig. 4. Immunodot blot inhibition of IgE-binding against rDer p 7 by the wild type Der p 7, Der f 7 and Der f 7 mutants.

sample 990 against Der f 7 (Fig. 3). For control, BSA showed negative inhibition of immunodot blot reactivity of IgE-binding against Der f 7 (Fig. 3). Results similar to those shown in Figs. 2 and 3 were also observed with serum 1045 (data not shown).

Der p 7 and Der f 7 have identical amino acid sequence among residues 156–160 (<sup>156</sup>SILDP<sup>160</sup>) (Shen et al., 1993, 1995b). This region on Der p 7 may also be a determinant responsible for IgE-binding and attributes to IgE cross-reactivity between Der f 7 and Der p 7 allergens. Fig. 1C shows that peptide Dp7-16 with sequence derived from this region of Der p 7 inhibited IgE-binding against Der f 7 and implies that the sequence <sup>156</sup>SILDP<sup>160</sup> may indeed contain a determinant responsible for IgE-mediated cross-reactivity between these two group 7 dust mite allergens.

Our results in Fig. 4 show that inhibitions of IgE binding against Der p 7 in serum sample 440 were detected with Der p 7, Der f 7 or the Der f 7 mutants as inhibitors. However, the Der f 7 D159A mutant was unable to inhibit significantly the immunodot blot reactivity of IgE antibodies against Der p 7 in serum sample 990. As control, BSA showed negative inhibition of immunodot blot reactivity of IgE-binding against Der p 7 (Fig. 4).

### 3.4. Homology protein modeling

The antigenic epitope on Der f 7 and the critical core amino acid residue that are involved in IgE-binding are illustrated on a 3D

model of Der f 7 (Fig. 5). The model was constructed by homology-based protein modeling with the Der p 7–maltose-binding fusion protein (PDB code 3H4Z) as template. The IgE-reacting fragment <sup>156</sup>SILDP<sup>160</sup> locates on the surface and assumes a loop-like structure (Fig. 5).

## 4. Discussion

Der f 7 and Der p 7 are important dust mite allergens. However, the IgE-binding and the IgE cross-reactive epitopes of the group 7 mite allergens have not been characterized. We focused in this study the IgE-reacting epitope(s) of Der f 7 and determinant(s) involve in IgE cross-reactivity with Der p 7. Our results showed a clear inhibition of IgE-binding in two asthmatic serum samples preincubated with the peptide Df7-16 <sup>151</sup>HIGGLSILDP<sup>165</sup>. We further narrowed the responsible sequence to five residues (<sup>156</sup>SILDP<sup>160</sup>) and generated five mutants with single alanine substitution within this region. The I157A, L158A and D159A mutants have decreased IgE-binding but maintain the overall secondary structures of Der f 7 (data not shown). Furthermore, the D159A mutant showed decreased ability in inhibition of IgE-binding to Der f 7. Therefore, the IgE antibodies recognize residues Ile157, Leu158, and Asp 159 on Der f 7, and Asp159 is a major contributor to this interaction. The IgE-binding determinant <sup>156</sup>SILDP<sup>160</sup> of Der f 7 assumes a loop-like structure on the surface of a 3D model of the protein. The decreased IgE-binding of mutant D159A may be attributed to a charge and/or size difference between amino acids aspartate and alanine.

It was found that IgE sequential epitopes of Len c 1 are exposed on the surface of the protein (Vereda et al., 2010). In addition, IgE interactions against the hyaluronidase allergen may also involve a surface-exposed loop-like IgE determinant (Padavattan et al., 2007). Furthermore, the sequential IgE-binding epitopes of Ara h 3 (Rougé et al., 2009) and the 13S globulin allergen of buckwheat (Sordet et al., 2009) are confined to electropositively and/or electronegatively charged residues exposed on the surface of the proteins. Results from Barre et al. also showed that IgE-binding epitopes of the major latex allergen Hev b 2 (1,3-beta-glucanase) were restricted to charged regions on the molecular surface of the protein (Barre et al., 2009). These findings are similar to our observation for Der f 7.

The D159A mutant showed significantly reduced IgE-binding ability compared with that of the wild-type Der f 7. Single amino acid substitution leads to dramatic effects on IgE-binding properties of allergens have been reported (Pomés, 2010; Ferreira et al., 1998; Satoh et al., 2010). For instance, <sup>99</sup>EGVRDLKE<sup>106</sup> is an IgE-binding epitope of a major buckwheat allergen, BWp16, and Asp103 is a critical amino acid in this IgE epitope (Satoh et al., 2010).

IgE antibodies in sera of asthmatic patients are considered to be polyclonal (Vereda et al., 2010) and may recognize several different determinants on an allergen. In this study, only sequential IgE epitopes of Der f 7 were characterized with a series of overlapping synthetic peptides, although linear epitopes may also be part of discontinuous epitopes (Pomés, 2010). In addition, we have

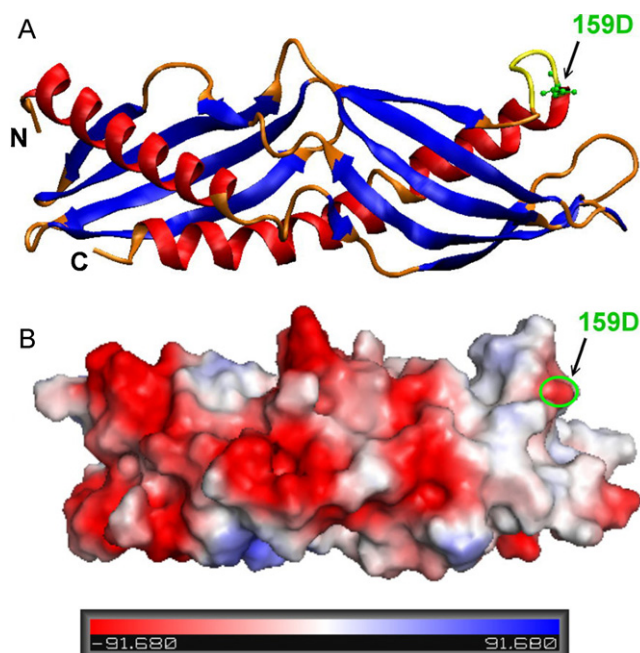


Fig. 5. (A) A ribbon diagram showing the overall fold of Der f 7 allergen. The linear epitope <sup>156</sup>SILDP<sup>160</sup> and the critical core amino acid residue Asp159 recognized by IgE antibodies are colored in yellow and green, respectively. (B) An electrostatically charged surface representation of a Der f 7 allergen 3D model. The electronegatively and electropositively charged regions are colored in red and blue, respectively. The neutral regions are colored white.

determined a single IgE-binding determinant of Der f 7 recognized by IgE antibodies in only two of 30 sera tested. Other linear and/or conformational epitopes recognized by IgE antibodies in sera from remaining patients may not be detected with our present experimental approach. More efforts are needed to identify the whole spectrum of IgE repertoire of Der f 7.

Der f 7 and Der p 7 have 86% protein sequence identity. rDer f 7 is able to inhibit IgE-binding against rDer p 7 (Fig. 4) and vice versa (data not shown). Hence, these two allergens share common IgE-binding epitopes and it is not surprising that a sequence identical to that of the Der f 7 IgE-binding determinant (<sup>156</sup>SILDP<sup>160</sup>) can be found on Der p 7. The peptide Dp7-16 can inhibit significantly IgE-binding against Der f 7. Furthermore, the Der f 7 D169A mutant has reduced ability in inhibiting IgE-binding against Der p 7. Thus, our results suggest that Asp159 is also a key residue contributing to IgE cross-reactivity between Der f 7 and Der p 7. Similarly, Ser112 is an essential amino acid for preserving the structure of a cross-reactive IgE epitope between Bet v 1a and Pru a 1 (Scheurer et al., 1999). In addition, conserved residues of Mala s 11 contribute to its IgE cross-reactivity with human manganese superoxide dismutase, and they are located on the surface of the protein and exposed to the solvent (Vilhelmsson et al., 2008). The determinant on the allergen can thus be reached readily by IgE antibodies.

In conclusion, we have identified a loop-like IgE-binding determinant <sup>156</sup>SILDP<sup>160</sup> on Der f 7 and demonstrated that Asp159 is critical for IgE-binding and for IgE cross-reactivity with Der p 7 allergen. It provides more information on IgE-binding against allergens and cross-reactivity between allergens at the molecular and structural levels. It aids also in designing hypo-allergens for immunotherapy.

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