Original Article

The different modes of binding of the dust mite allergens, Der f 7 and Der p 7, on a monoclonal antibody WH9 contribute to the differential reactivity

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Abstract *Background*: Der f 7 and Der p 7 are important house dust mite allergens. An IgE-binding inhibition monoclonal antibody WH9 reacts ten folds stronger against Der p 7 than to Der f 7. The purpose of this study is to identify the antigenic determinant(s) and the structural basis of Der f 7 recognize by WH9.
Methods: WH9-reactive determinant(s) on Der f 7 was identified by immunoblot and immunoblot inhibition. The 3-D binary complex structures of WH9 and the group 7 allergens were simulated with homology modeling and docking methods.
Results: WH9 reacted with the Der f 7 f9 fragment. Among the five site-directed Der f 7 mutants, WH9 showed reduced immunoblot reactivity against Der f 7 S156A, D159A and P160A mutants. Only the wild-type protein and the Der f 7 I157A and L158A mutants can inhibit significantly the WH9-binding against Der f 7. The structural model of the Der f 7-WH9 complex suggests residues S156 and D159 of Der f 7 can bind to WH9 via potential hydrogen bonds.
Conclusion: The structure models of Der f 7-WH9 and Der p 7-WH9 complexes revealed that the

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differential modes of binding of Der p 7 and Der f 7 allergens on WH9 contribute to the differential reactivity of WH9 against the Der f 7 and the Der p 7 mite allergens.

Introduction

Allergens from house dust mites including *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are worldwide and major risks leading to allergic sensitization and disease manifestations.[1,2] We have previously identified an important group 7 house dust mite allergens (Der f 7 and Der p 7) that can induce IgE responses in about 50% of mite atopic asthmatic patients.[3-8] The group 7 mite allergens are 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.[9-11] Der p 7 and Der f 7 have similar overall folds and bind weakly to polymyxin B via a comparable binding site.[9-11] The results imply the biological reactivity of the group 7 mite allergens may contribute to their allergenicity.

Characterization of antigenic/allergenic determinants of major allergens and their modes in reacting with specific antibodies at molecular and structural levels will be beneficial in better understanding of disease mechanisms and design of better therapeutic strategies. The IgE-binding determinants on the group 7 mite allergens remain largely unknown. We have recently identified a linear (^{156}SILDP^{160}) and cross-reactive IgE-binding epitope on Der f 7.[11] The D159 within the determinant is a critical core amino acid residue and responsible for IgE cross-reactivity between the Der f 7 and the Der p 7 allergens.[11]

We have generated a series of mouse monoclonal antibodies (MoAbs) to characterize group 7 mite allergens.[4-7] MoAbs WH9, WH22 and HD19 react against both Der p 7 and Der f 7 allergens which share an amino acid sequence identity of 86%. All three antibodies are able to inhibit the binding of IgE antibodies against Der p 7.[6,7]

Recently, through in vitro mutagenesis experiments in combination with in silico antibody structure modeling and computational docking, we created a model of the Der p 7-WH9 binary complex to gain insight into the molecular interactions between Der p 7 and WH9.[12] The results reveal residues S156, I157, L158, D159 and P160 of Der p 7 associate with WH9 via potential hydrogen bonds, electrostatic and hydrophobic interactions. L158 and D159 are critical residues on Der p 7 that interact with WH9 which can inhibit IgE-binding to Der p 7.[12]

However, the MoAb WH9-Der p 7 interaction is 10-fold stronger than that of WH9-Der f 7.[5] To elucidate the potential mechanisms and provide a more comprehensive feature of the antigenic/allergenic determinants of the group 7 mite allergens, we analyzed the antigenic determinant of Der f 7 recognized by MoAb WH9. We utilized recombinant overlapping Der f 7 protein fragments,

allergen mutants, and constructed structural models of WH9 with both group 7 mite allergens. The results suggest that the different modes of binding contribute to the differences in reactivity of WH9 against the Der f 7 and the Der p 7 mite allergens.

Methods

Recombinant Der f 7 fragment proteins

Ten recombinant Der f 7 protein fragments (Df7-f1 to Df7-f10) with overlapping sequences offset by ten amino acids were generated to cover the complete sequence of the mature Der f 7. All ten Der f 7 protein fragments were expressed as GST (glutathione-S-transferase)-fusion and His$_6$-tagged proteins. A plasmid encoding Der f 7 (GenBank accession no. S80655) is available in our laboratory[5] and was used as template in polymerase chain reactions (PCRs). Primers employed to generate cDNA inserts encoding the ten Der f 7 fragments are shown in Table 1. The PCR products encoding Df7-f1 to Df7-f7 and Df7-f10 were purified and restricted individually with BamHI and SmaI. The

Table 1 Primers used in PCR to generate ten recombinant Der f 7 protein fragments (Df7-f1 to Df7-f10) with overlapping sequences offset by ten amino acids.

Name	Nucleotide sequence in 5′ to 3′-end orientation
Df7-f1$_{forward}$	5′-cgggatcc^{1}gatccaattcactatgataaa^{21}-3′
Df7-f1$_{reverse}$	5′-tcccccgggtta^{60}aatggcatcatcaatagc^{43}-3′
Df7-f2$_{forward}$	5′-cgggatcc^{31}gaaatcaacaaagctattgat^{51}-3′
Df7-f2$_{reverse}$	5′-tcccccgggtta^{120}tttatcggcatgatcagg^{103}-3′
Df7-f3$_{forward}$	5′-cgggatcc^{91}ccaatgaaagtacctgatcat^{111}-3′
Df7-f3$_{reverse}$	5′-tcccccgggtta^{180}ctcaatgtttcgcatggc^{163}-3′
Df7-f4$_{forward}$	5′-cgggatcc^{151}aaaggtgaattagccatgcga^{171}-3′
Df7-f4$_{reverse}$	5′-tcccccgggtta^{240}accctcttcacctttgac^{223}-3′
Df7-f5$_{forward}$	5′-cgggatcc^{211}ggtgatgctaatgtcaaaggt^{231}-3′
Df7-f5$_{reverse}$	5′-tcccccgggtta^{300}atcatattccatcgagac^{283}-3′
Df7-f6$_{forward}$	5′-cgggatcc^{271}cacgatgatatcgtctcgatg^{291}-3′
Df7-f6$_{reverse}$	5′-tcccccgggtta^{360}atcttgaatatccgaaat^{343}-3′
Df7-f7$_{forward}$	5′-cgggatcc^{331}accactcatgtcatttcggat^{351}-3′
Df7-f7$_{reverse}$	5′-tcccccgggtta^{420}aaaagatgtcattgttatgtt^{403}-3′
Df7-f8$_{forward}$	5′-tcccccggg^{391}ggatgaaggtaacataacaatg^{411}-3′
Df7-f8$_{reverse}$	5′-tcccccgggtta^{480}tggatccaagattgaaag^{463}-3′
Df7-f9$_{forward}$	5′-tcccccggg^{451}gcatattggtggtctttcaatc^{471}-3′
Df7-f9$_{reverse}$	5′-tcccccgggtta^{540}ttccttacgtacggtgtc^{523}-3′
Df7-f10$_{forward}$	5′-cgggatcc^{511}gctattttccaagacaccgta^{531}-3′
Df7-f10$_{reverse}$	5′-tcccccgggtta^{588}attttttttccaattcacg^{571}-3′

PCR products encoding Df7-f8 and Df7-f9 were purified and restricted with SmaI. The restricted PCR products were ligated individually into the pQE30 vector (Qiagen Inc., Valencia, CA, USA) and transformed into *Escherichia coli* JM109. These pQE30 plasmids were purified from JM109 cultures, then restricted and ligated into the pGS-21a vector (GenScript, Piscataway, NJ, USA) for expression of Der f 7 fragments in *E. coli* M15 as GST fusion proteins with His$_6$-tags at both termini. All constructs were confirmed by DNA sequencing and recombinant fusion proteins were affinity-purified with Ni-NTA resin columns (Qiagen) according to the manufacturer's instructions. Wild type Der f 7 (S80655) with an N-terminal His$_6$-tag, as well as the His$_6$-tagged GST protein for control experiments were also isolated with Ni-NTA resin columns.

SDS-PAGE and immunoblotting

The interaction between the purified recombinant proteins and MoAb WH9 against group 7 mite allergens was analyzed by SDS-PAGE-immunoblotting essentially as described.[4,5,7] MoAb FUM20 against serine protease major fungal allergens was used as negative control.[13]

Site-directed mutagenesis

Der f 7 mutants carrying single alanine substitute at S156, I157, L158, D159 or P160[11] were affinity-purified with Ni-NTA resin columns (Qiagen) and used in this study. The mutation on the construct was confirmed by DNA sequencing and the recombinant proteins were affinity-purified with Ni-NTA resin columns (Qiagen) according to the manufacturer's instructions.

Immunoblot inhibition

For immunoblot inhibition studies, culture supernatant from hybridoma WH9 was firstly incubated with 5 μg of purified recombinant wild-type Der f 7 or their mutants at room temperature for 2.5 h before incubating with polyvinylidene difluoride (PVDF) blots containing purified Der f 7 at room temperature for 1 h. As a control, the same culture supernatant from hybridoma WH9 was pre-incubated with 5 μg of bovine serum albumin (BSA, Pierce, Rockford, Illinois, USA) before immunoblot against Der f 7.

Homology modeling of MoAb WH9

The structure of the variable regions of MoAb WH9 was built as described in a previous study.[12] Accordingly, the amino acid sequences of the variable domains of the heavy and light chains of WH9 were compared with entries in the Protein Data Bank and the structures of the variable regions of the heavy and light chains were constructed from the best matches. The crystal structure of the orthorhombic form of the IgG1 Fab fragment (in complex with a tubulin peptide) with PDB codes of 3QNZ[14] was selected as the template. Model building and molecular visualization were carried out using VMD software.[15]

Molecular docking of Der f 7-WH9 complex

Docking of the variable region of WH9 to the crystallographic structure of Der f 7 (PDB ID: 3UV1)[10] was performed using the ZDOCK program.[16] The Der f 7-WH9 complex model was initially subjected to energy minimization using the CHARMM program[17] with a harmonic constrained force of 100 (kcal/mol Å^2) on the backbone atoms, then on the Cα atoms, followed by gradually removing the constraints. A further 5×10^5 steps of minimization were performed to optimize the Der f 7-WH9 complex structure for analysis.

The Der f 7-WH9 and Der p 7-WH9 complexes were superimposed by using the variable domains of the heavy and light chains of the Der f 7-WH9 complex as the template for structural alignment. The Kabsch algorithm[18] was used to move the heavy and light chains of Der p 7-WH9 complex onto those of Der f 7-WH9 complex and the minimum root-mean-square deviations (RMSD) were obtained accordingly.

Results

Immunoblot reactivity of WH9 against overlapping Der f 7 protein fragments

The antigenic determinant of Der f 7 recognized by WH9 was mapped with ten overlapping Der f 7 protein fragments (Df7-f1 — Df7-f10) offset by ten amino acids and together cover the complete sequence of the mature Der f 7. WH9 reacted with the wild type and Df7-f9 which has a sequence of ^{151}HIGGLSILDPIFGVLSDVLTAIFQDTVRKE^{180} (Fig. 1). According to our previous study,[19] the binding of MoAb HD12 against Der f 7 could be blocked by peptides with the linear sequence ^{46}GILDF^{50}. In this study, MoAb HD12 reacted with the wild type and fragment Df7-f3 that has a sequence of ^{31}PMKVPDHADKFERHVGILDFKGELAMRNIE^{60} (Fig. 1). Both MoAbs WH9 and HD12 did not show immunoblot reactivity against the purified His$_6$-tagged GST protein (Fig. 1). For control, MoAb FUM20 against fungal serine protease allergens[13] showed negative reactivity against the wild type Der f 7 or its fragments and the GST protein (Fig. 1).
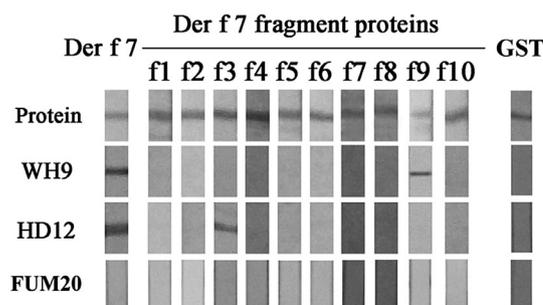

Figure 1. Immunoblot activity of MoAbs WH9, HD12 and FUM20 against rDer f 7, ten recombinant Der f 7 protein fragments with overlapping sequences offset by ten amino acids and cover the complete sequence of the mature Der f 7, and GST. "Protein" indicates recombinant Der f 7, Der f 7 protein fragments fused to GST and GST, blotted onto PVDF membranes and stained by Coomassie blue.

Immunoblot reactivity of WH9 against Der f 7 mutants

WH9 reacts with both Der p 7 and Der f 7 mite allergens.[4,5] Our previous results showed that S156, L158, D159 and P160 on Der p 7 are the WH9-binding determinant.[12] Thus, the antigenic determinant of Der f 7 recognized by WH9 may be related to the sequence of [156]SILDP[160] within the Df7-f9.

In order to determine the amino acid residue(s) that contributes to WH9-binding, site-directed Der f 7 full-length mutants including S156A, I157A, L158A, D159A and P160A[11] were used in the present study. Our results demonstrated that WH9 showed reduced immunoblot reactivity against the Der f 7 S156A, D159A and P160A full-length mutants. However, the I157A and L158A full-length mutants can still be recognized by WH9 (Fig. 2, panel A). This experiment has been repeated at least three different times and a representative result is shown in Fig. 2, panel A. Reduced reactivity against these five Der f 7 mutants was not detected when HD12[7] was used in immunoblotting (Fig. 2).

Immunoblot inhibition by Der f 7 mutants

To affirm the role of amino acids S156, D159 and P160 in WH9-binding against Der f 7, immunoblot inhibition by Der f 7 full-length mutants was performed. Only the wild-type and the Der f 7 I157A and L158A full-length mutants can inhibit significantly WH9-binding against Der f 7 (Fig. 2, panel B). BSA cannot block the WH9-binding against Der f 7. Thus, inhibition experiments confirmed S156, D159 and P160 as critical core residues for WH9-binding against Der f 7. This experiment has also been repeated at least three different times and representative results are shown in Fig. 2, panel B.

Structural overview and intermolecular interactions between Der f 7 and WH9

The binary complex of Der f 7 and WH9 was modeled via computational docking (Fig. 3, left panel). The structure of

Figure 2. (A) Immunoblot reactivity of MoAbs WH9 and HD12 against the wild type and Der f 7 full-length mutants. "Protein" indicates recombinant Der f 7 and five Der f 7 full-length mutant proteins blotted onto PVDF membranes and stained by Coomassie blue. (B) Immunoblot inhibition of WH9-binding against rDer f 7 by the wild type, Der f 7 full-length mutants and bovine serum albumin (BSA).

the variable regions of MoAb WH9 was generated with homology modeling.[12] The X-ray crystal structure of Der f 7 has been reported by Tan et al.[10] Fig. 3 shows the six CDRs (H1, H2, H3, L1, L2 and L3) of the antibody form a cleft for binding to the Der f 7 determinant which assumes a loop like structure containing residues S156, D159 and P160.

The potential interactions between Der f 7 and WH9 are shown in Fig. 3, right panel. The model of the Der f 7-WH9 complex suggests only two potential regions that contribute to the binding of Der f 7 with WH9. The loop-like antigenic determinant of Der f 7 (backbone in red and the residue side chains in yellow) interacts with the residues on CDR-H3 (in blue) and CDR-L2 (in green) of WH9. Two residues (S156 and D159) from Der f 7 and three residues (Y101 of CDR-H3, Y53 and N57 of CDR-L2) from WH9 are in close proximity to each other. The potential interactions are summarized in Table 2.

Of the three CDRs on the heavy chain of WH9, only CDR-H3 is in potential interaction from Der f 7 (Fig. 3, right panel and Table 2). The distance between the OH (Y101) of the CDR-H3 and the amide N of S156 on Der f 7 is 4.78 Å, which could be a potential H-bond. The distance is a little too far for direct interaction in the static model but they can possibly interact via a bridging water molecule.

Among the three CDRs of the WH9 light chain, only CDR-L2 is in contact with Der f 7, where the residues Y53 and N57 on CDR-L2 are in contact with S156 and D159 of Der f 7 (Fig. 3, right panel and Table 2). The $O\delta_2$ atom of D159 is within the H-bonding distance from the OH (Y53) and the $N\delta_2$ (N57) on the CDR-L2. In addition, the $O\eta$ atom of Y53 on CDR-L2 is 3.68 Å from the amide N of S156 on Der f 7.

In this study, the surface and cartoon representations of structural models of Der f 7-WH9 and Der p 7-WH9[12] complexes derived from computer-guided modeling and docking are presented in the left and the right panel of Fig. 4, respectively.

Discussion

Der p 7 and Der f 7 may contribute to allergic sensitization in about 50% of house dust mite atopic patients.[3,5] Therefore, it is important to elucidate their antigenicity and/or allergenicity. In this study, inhibition experiments using allergen mutants suggest that S156, D159 and P160 on a loop-like structure of Der f 7 are critical core amino acids contributing to MoAb WH9-binding. The Der f 7-WH9 structural model suggests that residues S156 and D159 of Der f 7 binds WH9 via potential hydrogen bonds. However, our previous study demonstrated that WH9 showed reduced immunoblot reactivity against Der p 7 with S156A, L158A, D159A and P160A mutations. The Der p 7-WH9 structural model suggests residues S156, I157, L158, D159 and P160 on Der p 7 interacts extensively with WH9 via seven potential hydrogen bonds, two electrostatic and four hydrophobic interactions.[12] Consequently, WH9 reacts approximately ten folds stronger against Der p 7 than to Der f 7.

Inhibition experiments confirmed residues S156, D159 and P160 contribute to WH9-binding against Der f 7 (Fig. 2). However, the structural model of the Der f 7-WH9 complex suggests only residues S156 and D159 of Der f 7 may contribute to WH9 binding. It should be noted that the
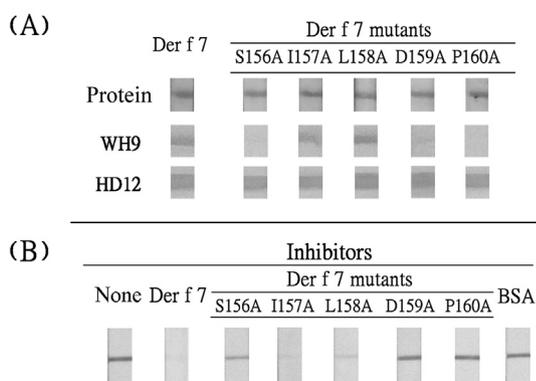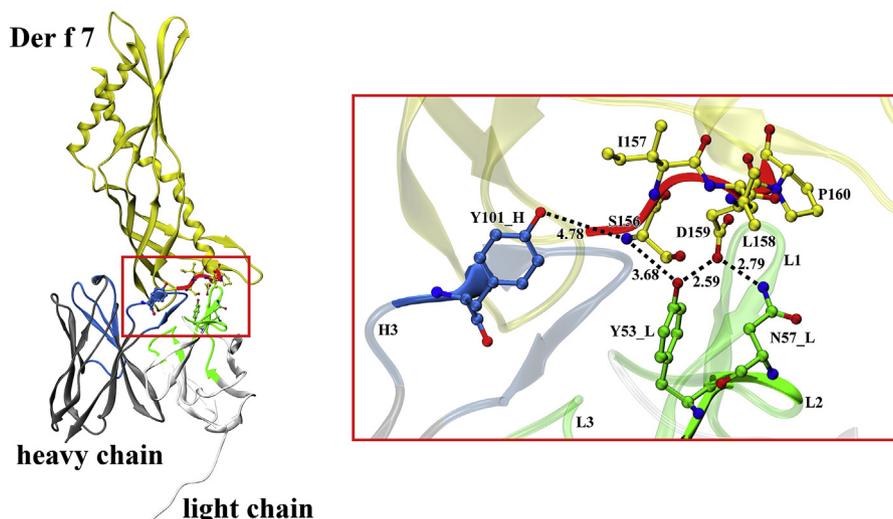
Figure 3. Cartoon representation of Der f 7-WH9 complex derived from computer-guided modeling and docking (left panel) and a close up view around the loop-like determinant of Der f 7 ([156]SILDP[160]) recognized by WH9 (right panel). Der f 7, the loop-like determinant, heavy chain of WH9, light chain of WH9, VH-CDR and VL-CDR are colored in yellow, red, dark gray, light gray, blue and green, respectively. The highly potential hydrogen bonds are indicated in black dash lines. Nitrogen and oxygen atoms are shown in blue and red, respectively. Interatomic distances (see Table 2) are depicted in Å.

model is based on docking experiments utilizing the crystallographic structure of Der f 7 and the homology model of WH9. Implications from docking experiments are only suggestive and should be confirmed with biological assays. For instance, the docking results of Harrington et al.[20] suggested H20 on the alpha subunit of sickle cell hemoglobin interacted with the E22 on the beta subunit of a neighboring tetrameric protein to promote sickle cell hemoglobin polymerization. In reality, these two residues contribute minimally to the formation of sickle cell hemoglobin fibers.[21]

Amino acid residue D159 of Der f 7 and two amino acid residues (Y53 and N57) from the CDR-L2 region of WH9 are in close proximity and within hydrogen bonding distances (Table 2 and Fig. 3). The model suggests strongly that D159 is the major residue among three critical residues (S156, D159 and P160) that interacts with WH9. We have also identified previously D159 as a critical core residue of an IgE-binding and IgE-mediated cross-reactive epitope [156]SILDP[160] of Der f 7.[11]

The distance between Y53 of CDR-L2 and S156 of Der f 7 is 3.68 Å and apparently slightly outside hydrogen bonding distance. However, recent experimental and theoretical studies have emphasized the fact that macromolecules in solution are conformational ensembles that dynamically

converting from one form to the other,[22–24] while crystallographic structures (and homology models derived from crystallographic templates) give only static snapshot views of proteins fluctuating among many conformations.[24] It is conceivable that Der f 7 and/or WH9 can adopt a solution conformation that transiently brings S156 and Y53 into hydrogen bonding distance.

The distance between Y101 of CDR-H3 and Ser156 is 4.78 Å, and probably too far for molecular interaction.
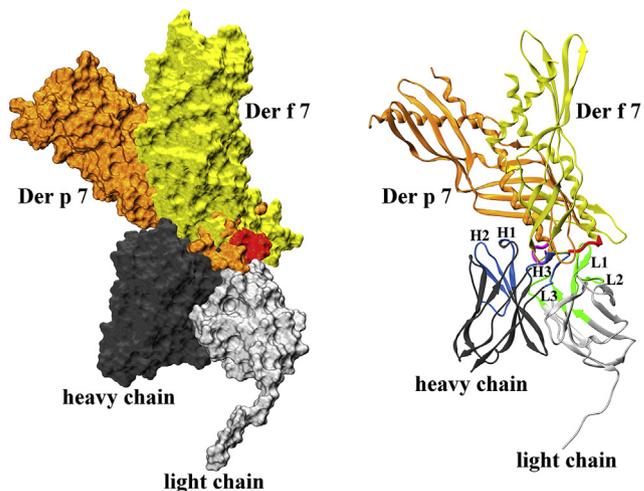

Figure 4. Structural models of Der f 7-WH9 and Der p 7-WH9[12] complexes derived from computer-guided modeling and docking. Surface and cartoon representations of the two complexes are in the left panel and the right panel, respectively. Der f 7, Der p 7, the loop-like determinants of Der f 7 and Der p 7 recognized by WH9, heavy chain of WH9, light chain of WH9, VH-CDR and VL-CDR are colored in yellow, orange, red, purple, dark gray, light gray, blue and green, respectively.

Table 2 Distances between interacting residues in the Der f 7-WH9 complex.

Chain	MoAb WH9	Der f 7	Distance (Å)
VH			
CDR-H3	OH (Y101)	N (S156)	4.78
VL			
CDR-L2	OH (Y53)	N (S156)	3.68
		$O\delta_2$ (D159)	2.59
	$N\delta_2$ (N57)	$O\delta_2$ (D159)	2.79

However, careful scrutiny of the template molecule (3QNZ) used in our homology modeling reveal the presence of a water molecule near the N-terminal of the CDR-H3 region. We cannot exclude the possibility of the presence of such a water molecule in the Der f 7-WH9 complex and acts as a bridge between Ser156 and Y101 of CDR-H3. Further experiments are needed to verify this hypothesis.

Results obtained from this study also showed that unlike that of the Der p 7-WH9 complex, the L158 of Der f 7 does not protrude into the pocket formed by the six hypervariable regions of WH9 (Fig. 4). The structural model of the Der p 7-WH9 complex suggests five residues (S156, I157, L158, D159 and P160) on Der p 7 interact with WH9.[12] However, the structural model of the Der f 7-WH9 complex constructed in this study suggests that only two residues (S156 and D159) on Der f 7 may interact with three residues of WH9 via four potential hydrogen bonds (Table 2).

The overall structure of Der f 7 is homologous to that of Der p 7.[10] An alignment of the structural models shows an RMSD value of 1.22 Å. The most significant structural difference between Der f 7 and Der p 7 is in the $\beta1-\beta2$ loop region, which contains a determinant (^{46}GILDF^{50}) recognizes by the Der f 7-specific MoAb HD12.[9] The corresponding region on Der p 7 has an amino acid sequence of ^{46}GILDL^{50} and it does not bind with MoAb HD12. Interestingly, the distance between the OD2 of D159 and the CD2 of L50 is 14.3 Å in Der p 7.[10] In contrast, the D159 of Der f 7 is located closely to its $\beta1-\beta2$ loop with a distance of 7.7 Å between the OD1 of D159 and the CZ of F50.[10] Thus, the narrow gap between the $\beta1-\beta2$ and the WH9 determinant-containing (^{156}SILDP^{160}) loops on Der f 7 may create a steric hindrance that distances the L158 of Der f 7 from the CRDs of WH9 (Figs. 3 and 4).

For proteins like Der f 7 and Der p 7 that are highly similar, however, based on results obtained from this study, may have different binding modes as presented in this manuscript. The observations are supportive of the fact that the interaction between Der f 7 and WH9 is weaker than that of Der p 7 and WH9. Altogether, these results explain our previous finding that WH9 prepared via immunization of BALB/c mice with recombinant Der p 7 could demonstrate a stronger immunological reactivity against Der p 7 than to Der f 7.[5]

In conclusion, results obtained in this study suggest that S156 and D159 are critical core residues of Der f 7 for MoAb WH9-binding. In addition, results from molecular modeling and docking suggest that different modes of binding contribute to the differential reactivity of WH9 against Der f 7 and Der p 7 mite allergens. Our results provide bases for better design of therapeutic strategies against human atopic disorders.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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