

Lys, Pro and Trp Are Critical Core Amino Acid Residues Recognized by FUM20, a Monoclonal Antibody against Serine Protease Pan-Fungal Allergens

Lin-Hau Lee^a Ming F. Tam^b Hong Chou^a Hsiao-Yun Tai^a Horng-Der Shen^a

^aDepartment of Medical Research and Education, Taipei Veterans General Hospital, and ^bInstitute of Molecular Biology, Academia Sinica, Taipei, Taiwan, ROC

Key Words

Epitope mapping • FUM20 • Serine protease allergens

Abstract

Background: Alkaline/vacuolar serine proteases comprise a major group of pan-fungal allergens from several prevalent airborne fungal species. It is of importance to characterize antigenic determinant(s) recognized by monoclonal antibodies against these major allergens. **Methods:** The antigenic determinant of fungal serine proteases recognized by a monoclonal antibody, FUM20, was analyzed by dot immunoassay of synthetic peptides immobilized on cellulose membrane. Results obtained were confirmed by wild-type recombinant protease and its mutants. The epitopes were mapped to the structure of serine proteases by molecular modeling. **Results:** A linear epitope encompassing 9 amino acids from Pen ch 18 (⁶EKNAPWGLA¹⁴) binds FUM20. The corresponding peptide (⁵AKGAPWGLA¹³) from Rho m 2 also binds FUM20. Substitution of K6, P9 or W10 with alanine in this peptide resulted in drastic loss of FUM20 binding. Rho m 2 mutants with single K6A, P9A, P9G, W10A or W10F substitute showed negative immunoblot reactivity against FUM20. However, the Rho m 2 K6R mutant can bind FUM20. Three-dimensional structural models of the FUM20 antigenic de-

terminants on serine proteases were constructed. The lysine residue critical for FUM20 interaction is on the surface of the proteases and solvent accessible. The critical core residue proline is located at the beginning of an α -helix. **Conclusions:** The lysine, proline and tryptophan residues located on the N-terminal region of fungal serine proteases are critical core amino acid residues recognized by FUM20, a monoclonal antibody against serine protease pan-fungal allergens. These findings advance our understanding of the antigenic structures responsible for the antigenicity of serine protease allergens.

Copyright © 2007 S. Karger AG, Basel

Introduction

Airborne fungi contribute significantly to human respiratory atopic disorders [1]. However, among various causative allergens such as house dust mites, pollens and fungal spores, mold allergens are the least characterized. Hence, fungal extracts for clinical usage have not been successfully standardized [2, 3]. Characterization of major fungal allergens and development of allergen-specific monoclonal antibodies (mAbs) should be useful in clinical diagnosis of mold allergy [4, 5].

We have recently identified alkaline and/or vacuolar serine proteases as a group of highly conserved pan-fungal allergens of prevalent airborne *Penicillium* and *Asper-*

L.H. Lee and M.F. Tam contributed equally to this study.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1018–2438/07/1433–0194\$23.50/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Horng-Der Shen
Department of Medical Research and Education
Taipei Veterans General Hospital
Shih-Pai, Taipei 11217, Taiwan (ROC)
Tel. +886 2 2875 7392, Fax +886 2 2875 1562, E-Mail hdshen@vghtpe.gov.tw

Table 1. Immunoblot reactivity of the mAb FUM20 against different fungal serine protease major allergens

Allergens	Reactivity with FUM20	Amino acid sequences of peptides corresponding to that of the FUM20-reacting Pen ch 18 peptide	References
<i>Vacuolar serine protease</i>			
Pen ch 18	+	⁶ EKNAP-WGLA ¹⁴	10, 11
Rho m 2	+	⁵ AKGAP-WGLA ¹³	9
Pen c 18	+	⁶ EKNAP-WGLA ¹⁴	8, 10
Pen o 18	+	⁶ EKNAP-WGLA ¹⁴	8, 10
Asp f 18	+	⁶ EKSAP-WGLA ¹⁴	7, 10
<i>Alkaline serine protease</i>			
Pen ch 13	–	⁵ QRNAPSWGLS ¹⁴	8, 10
Pen c 13	–	⁵ QSNVPSWGLA ¹⁴	8, 10
Asp f 13	+	⁵ QKGAP-WGLG ¹³	7, 10
Asp fl 13	+	⁵ QKSAP-WGLG ¹³	6, 10

gillus species [6–8] as well as the frequently encountered yeast *Rhodotorula mucilaginosa* [9]. Our immunoblotting data showed that IgE antibodies against components of these fungal species could be detected in 15–30% of serum samples from asthmatic patients [6–9]. Most of the positive serum samples showed IgE binding to the 32-/34-kDa serine protease(s) with a >60% frequency in different fungal species tested [6–9].

For the characterization of these major fungal allergens, mAbs against different epitopes of these serine proteases have been generated [10]. It was found that mAb FUM20 reacted with the vacuolar serine proteases from *R. mucilaginosa* (Rho m 2), *P. chrysogenum* (Pen ch 18), *P. citrinum* (Pen c 18), *P. oxalicum* (Pen o 18), *A. fumigatus* (Asp f 18), and the alkaline serine proteases from *A. fumigatus* (Asp f 13) and *A. flavus* (Asp fl 13) [9, 10]. However, it does not react with the alkaline serine proteases from *P. chrysogenum* (Pen ch 13) and *P. citrinum* (Pen c 13) [10] (table 1). FUM20 may thus be useful in characterizing serine protease pan-fungal allergens in different fungal species and in standardizing various diagnostic fungal extracts. It is also of interest to characterize the epitope(s) of the serine protease allergens recognized by FUM20.

We have previously reported that FUM20 can bind a synthetic peptide with sequence encompassing the N-terminal 23 residues of the mature Pen ch 18 [11]. mAb FUM20 also recognizes a class of pan-fungal allergens. It can be utilized to compare cross species homologues of pan-fungal allergens to pinpoint the epitope region and key features needed for antigen-antibody interaction. In

this study, the antigenic determinant and critical core amino acid residues of serine protease allergens recognized by mAb FUM20 were delineated by immunoassay of synthetic peptides with sequence similar to or slightly varied from those found on serine proteases. We identified three amino acids near the N-terminus of the proteases that are critical for the recognition of these proteins by mAb FUM20. Recombinant proteins with single amino acid substitutions were then used in immunoassays to affirm these results.

Materials and Methods

Peptide Synthesis and Peptide Mapping

The production of FUM20 antibody has been reported previously [10]. Peptides were custom synthesized on derivatized cellulose sheets (Genesis Biotech, Taipei, Taiwan, ROC). Immunoassay of peptides was performed essentially as described [11]. The membranes were blocked with 1% skimmed milk and incubated with hybridoma culture supernatant containing mAb FUM20 at room temperature for 1 h. The membranes were washed and incubated for 1 h with affinity-purified, horseradish peroxidase-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) at room temperature. The blots were washed and developed using an enhanced chemiluminescence kit (Amersham Bioscience, Piscataway, N.J., USA). mAb WH9 against Der p 7 [12] was used as negative control.

Site-Directed Mutagenesis and Immunoblotting

A plasmid carrying the coding sequence of Rho m 2 is available in the laboratory [9] and used as template for polymerase chain reactions (PCR). Rho m 2 mutants carrying a single amino acid substitute at K6, P9 or W10 were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif., USA) according to the manufacturer's instructions. Briefly, two synthetic oligonucleotide primers containing the desired mutation, each complementary to opposite strands of the vector, were extended during PCR by *PfuTurbo* DNA polymerase (Stratagene). Primers employed in the generation of these mutants and the resulting substituted amino acids are listed in table 2. DNA isolated from *Escherichia coli* is usually dam methylated and susceptible to *Dpn I* digestion. Thus, following standard PCR, the parental dsDNA was restricted with *Dpn I* while the newly synthesized DNA carrying the mutation was transformed into *E. coli* Top10F' cells for protein expression. All mutant constructs were confirmed by DNA sequencing.

Recombinant proteins were expressed with an N-terminal His₆-tag and purified in denatured form with Ni-NTA resin as described [9]. Affinity-purified proteins were further separated on denaturing polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membranes (0.45 µm; Millipore, Bedford, Mass., USA). The membranes were blocked with 1% skimmed milk. FUM20-binding abilities to wild-type recombinant Rho m 2 and its mutants were examined essentially as described above. The blots were finally developed with enzyme substrates as described [9, 10].

Table 2. Primers used in PCR

Name	Nucleotide sequence in 5' to 3'-end orientation
Rho m 2-K6A-F	GAGTTTACTGTTGCTGCGGGTGCTCCATGGGGT
Rho m 2-K6A-R	ACCCCATGGAGCACCCGCGAGCAACAGTAAACTC
Rho m 2-K6R-F	GAGTTTACTGTTGCTGCGGGTGCTCCATGGGGT
Rho m 2-K6R-R	ACCCCATGGAGCACCCGCGAGCAACAGTAAACTC
Rho m 2-P9A-F	GTTGCTAAAGGTGCTGCGTGCGGGTTTGGCACGT
Rho m 2-P9A-R	ACGTGCCAAACCCACGCGAGCACCTTTAGCAAC
Rho m 2-P9G-F	GTTGCTAAAGGTGCTGGCTGGGGTTTGGCACGT
Rho m 2-P9G-R	ACGTGCCAAACCCACGCGAGCACCTTTAGCAAC
Rho m 2-W10A-F	GCTAAAGGTGCTCCAGCGGGTTTGGCACGTATC
Rho m 2-W10A-R	GATACGTGCCAAACCCGCTGGAGCACCTTTAGC
Rho m 2-W10F-F	GCTAAAGGTGCTCCATTTGGTTTGGCACGTATC
Rho m 2-W10F-R	GATACGTGCCAAACCAATGAGCACCTTTAGC

Homology-Based Modeling of Antigenic Epitopes Recognized by FUM20

Molecular modeling of fungal serine proteases was performed using Swiss-Model programs in first approach mode [13, 14]. Five proteinase K (*Tritirachium album*) structures (2pkc, 1pj8A, legqA, 1cnmA and 1bjrE) that have 42.3% amino acid sequence identity with Rho m 2 were selected as templates in the computation. The antigenic epitopes and the critical core amino acid residues on the models recognized by FUM20 were visualized and displayed with the WebLab Viewer version 3.1 (Accelrys, San Diego, Calif., USA). Solvent accessibility of surface residues is calculated with the GETAREA program (<http://www.scsb.utmb.edu>) [15].

Results

Epitope Mapping with Synthetic Peptides

mAb FUM20 reacts with Rho m 2, Pen ch 18, Pen c 18, Pen o 18, Asp f 18, Asp f 13 and Asp fl 13 [9, 10] (table 1). In addition, FUM20 reacts with a synthetic peptide with sequence encompassing the N-terminal 23 residues of the mature Pen ch 18 [11]. Hence, four overlapping peptides (fig. 1) of 8–9 residues in length covering the sequence of these 23 amino acids were custom synthesized for immunoassay. The results in figure 1 indicate that FUM20 reacted with peptide No. 2. This peptide covers residues 6–14 of Pen ch 18 (⁶EKNAPWGLA¹⁴).

The sequence of this peptide was then aligned with the corresponding region of other fungal serine protease allergens (table 1). Additional peptides of 9–10 amino acids in length (fig. 2) were synthesized accordingly for immunoassay. Results in figure 2 indicate that FUM20 reacted strongly with the Pen ch 18 (fig. 2, peptide 1) and the Rho m 2 (fig. 2, peptide 7, ⁵AKGAPWGLA¹³) peptides. Substitution of K6 (peptide 8), P9 (peptide 10) or W10 (peptide 11) of the Rho m 2 peptide with an alanine abolished the

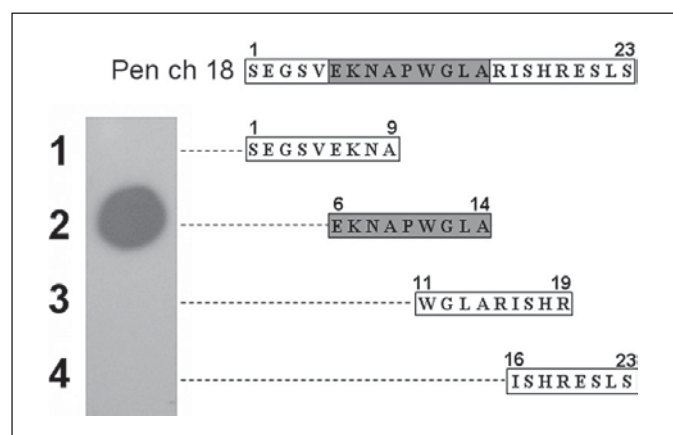


Fig. 1. Immunoreactivity of mAb FUM20 against four different synthetic peptides (peptides 1–4) with overlapping sequences encompassing the N-terminal 23 amino acids of the mature Pen ch 18.

FUM20-binding activity. However, Rho m 2 peptides with an alanine substitution at G7 (peptide 9), G11 (peptide 12) and L12 (peptide 13) retained their immunoreactivity with FUM20.

Results in figure 2 also indicate that insertion of a serine between P10 and W11 of the Pen ch 18 (fig. 2, peptide 2) blocked the reactivity with FUM20. With this insertion, the sequence of the peptide is very similar to that of Pen ch 13 (fig. 2, peptide 4) or Pen c 13 (fig. 2, peptide 6). These two peptides did not react with FUM20. By removing S10 from Pen ch 13 (fig. 2, peptide 3), relatively weak immunoreactivity with FUM20 can be detected. Deletion of the corresponding serine residue from Pen c 13 (fig. 2, peptide 5) did not improve the immunoreactivity of the peptide with FUM20. For control, mAb Der p 7

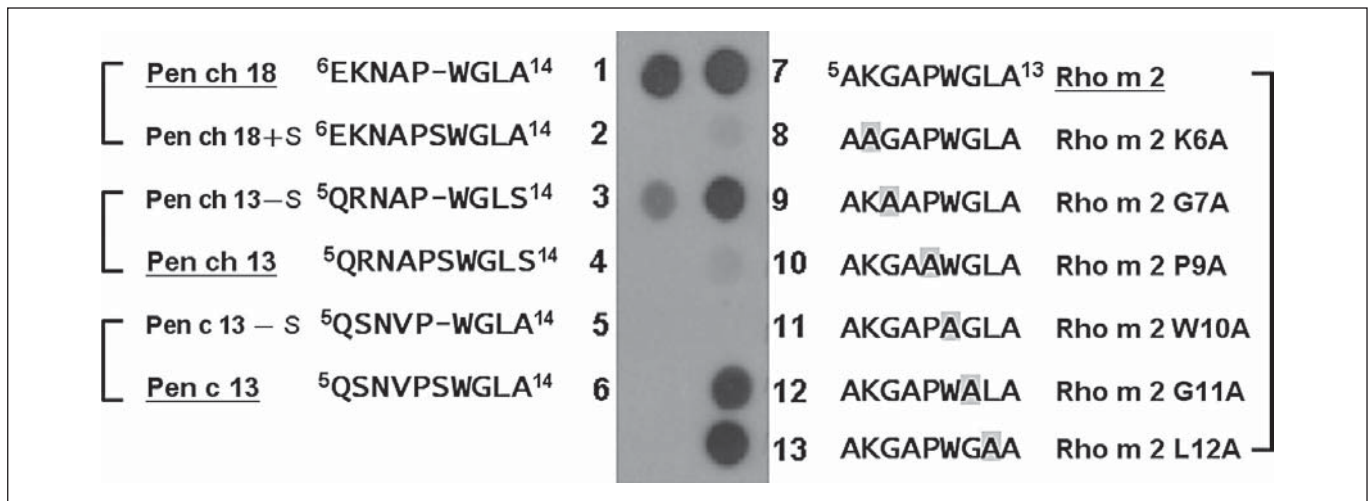


Fig. 2. Immunoreactivity of mAb FUM20 against peptides synthesized according to sequences from Pen ch 18 (Nos. 1 and 2), Pen ch 13 (Nos. 3 and 4), Pen c 13 (Nos. 5 and 6) and Rho m 2 (Nos. 7–13). Some of the peptides contain single amino acid substitution, addition or deletion.

showed negative reactivity against all the synthetic peptides (data not shown).

Epitope Mapping with Recombinant Proteins

Results from immunoassay with Rho m 2 peptides (fig. 2, peptides 8, 10 and 11) indicate that K6, P9 and W10 are important for FUM20 binding. The corresponding residues on Rho m 2 were mutated and the FUM20 binding ability of the wild type and Rho m 2 mutants were determined. The results in figure 3 show clearly that among the six recombinant mutants, only Rho m 2-K6R demonstrated immunoblot reactivity against mAb FUM20. Nonetheless, the intensity of the binding of the Rho m 2-K6R mutant is 22% less than that of the wild-type Rho m 2, as quantified by the AlphaEaseFC™ software (version 4.0.0, Alpha Innotech, San Leandro, Calif., USA).

Homology Modeling

The antigenic epitope on fungal serine proteases and the critical core amino acid residues that are involved in FUM20 binding are illustrated as 3D models (fig. 4). They are constructed by homology modeling with the proteinase K structures as templates. The critical core lysine (yellow) residue locates on the Rho m 2 protein surface (fig. 4a) and is solvent exposed (ratio: 82.7%) as analyzed by the GETAREA program [15]. Solvent accessibility was calculated with the coordinates of the model since the proteinase K templates used do not have a lysine near the N-terminus. The proline residue (red) critical for

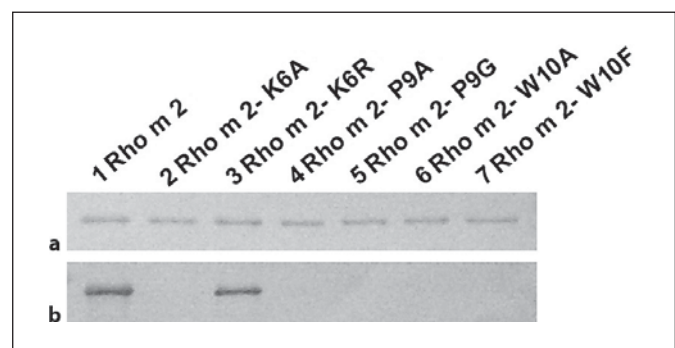


Fig. 3. Coomassie blue-stained protein profiles of the wild type and the Rho m 2 mutants on polyvinylidene difluoride membrane (a) and their immunoblot reactivities against mAb FUM20 (b).

FUM20 interaction is located at the beginning of an α -helix (fig. 4b). The indole ring of the tryptophan (green) on the carboxyl side of the proline residue is inserted into an empty space and partially buried in the protein (fig. 4b). This region of Rho m 2 and Pen ch 18 differs by only two residues (table 1). Hence the model for Pen ch 18 (fig. 4c) adopts a similar structure at this region. However, Pen c 13 and Pen ch 13 have a serine insertion between the proline and tryptophan residues. The additional serine probably disturbed the relative spatial orientation of the key residues required for FUM20 binding (fig. 4d, e). Consequently, Pen ch 13 and Pen c 13 are not reactive with FUM20 [10].

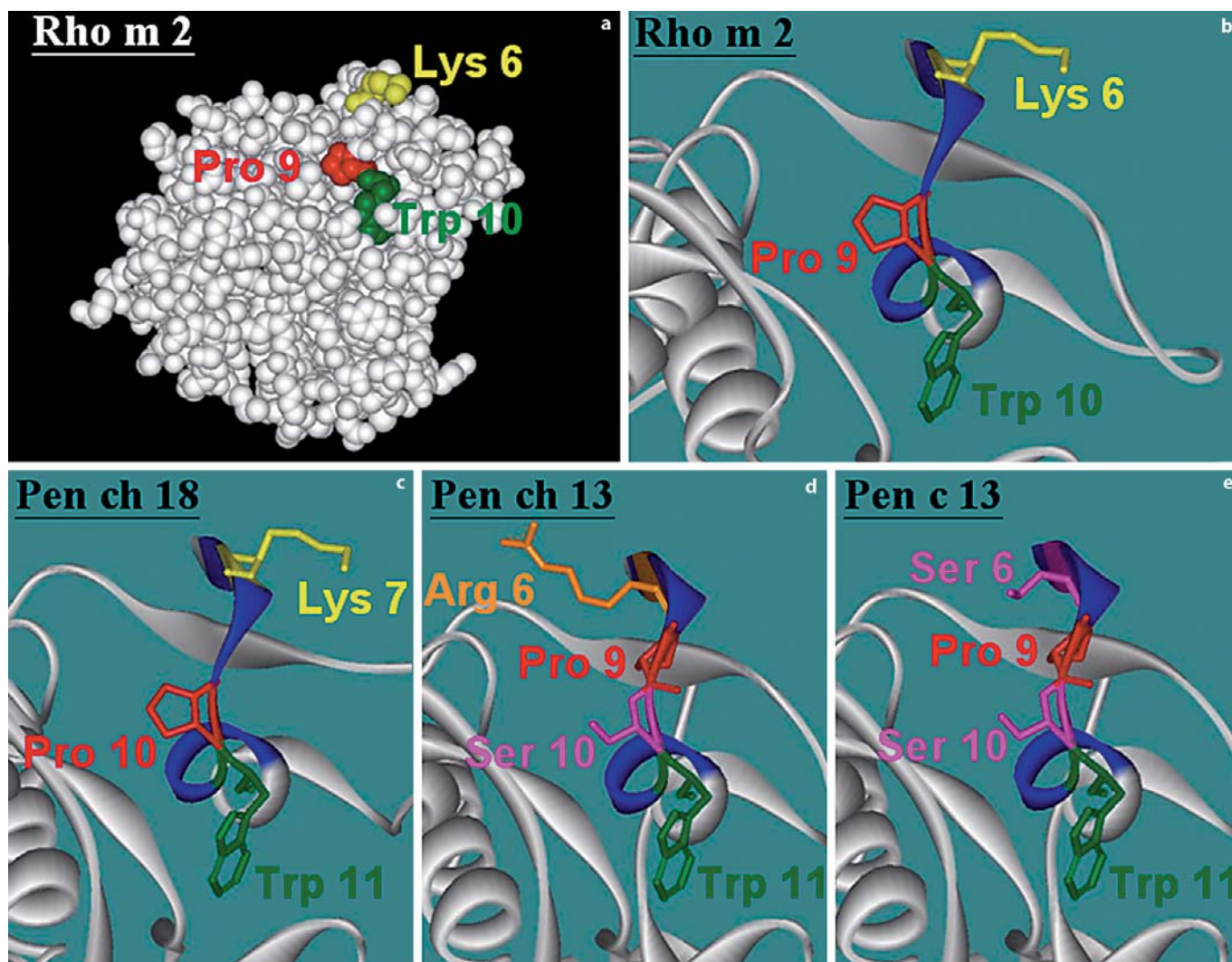


Fig. 4. A space filled 3D model of Rho m 2 is shown in **a**. A region of the same model corresponding to the antigenic epitope against FUM20 is presented as ribbon diagram in panel **b**. The three critical core amino acid residues (Lys6, Pro9 and Trp10) are colored in yellow, red, and green, respectively. **c** A ribbon diagram of the antigenic epitope and critical core amino acid residues on Pen ch 18 that are involved in FUM20 binding. **d, e** Ribbon diagrams of the corresponding region of Pen ch 13 (**d**) and Pen c 13 (**e**). The models were constructed using Swiss-Model programs, viewed and displayed with the WebLab Viewer (Accelrys, San Diego, Calif., USA).

Discussion

mAbs against major allergens are important tools in characterizing allergens and standardizing the crude allergenic extracts. In the present study, we identified and characterized the antigenic epitopes of fungal serine protease major allergens recognized by mAb FUM20.

Using overlapping synthetic peptides covering the N-terminal 23-amino acid residues, the antigenic determi-

nant of Pen ch 18 recognized by FUM20 was narrowed down to a stretch of 9 residues (fig. 1, ⁶EKNAPWGLA¹⁴). FUM20 also reacts with the corresponding peptide ⁵AKGAPWGLA¹³ derived from Rho m 2 (fig. 2, peptide 7). These two peptides differ in only the first (E/A) and third (N/G) positions, and they are not critical for FUM20 binding.

We used peptides with single substitutions (fig. 2) and show that residues K, P and W located near the N-termi-

nal region are important for the interaction between serine protease allergens and mAb FUM20. In addition, results obtained with synthetic peptides (fig. 2) agree closely with those derived previously with recombinant proteins as shown in table 1. Firstly, the N-terminal sequences of Pen c 18 and Pen o 18 are identical to that of the antigenic peptide on Pen ch 18, while Asp f 18 has a single residue substitution at position 3. All four proteins can react with FUM20. The corresponding region of Asp f 13 and Asp fl 13 differs from that of Pen ch 18 or Rho m 2 at positions 1, 3 and 9. These *Aspergillus* serine proteases can still be recognized by FUM20. Therefore, amino acids at position 1, 3 and 9 are not critical residues for FUM20 binding.

In this study, G7, G11 or L12 on the Rho m 2 antigenic peptide (⁵AKGAPWGGLA¹³) were replaced with alanine, and immunodot reactivity of FUM20 against these peptides was still detected (fig. 2). Even though G11 and L12 are conserved among fungal serine proteases, as shown in table 1, results obtained suggest that residues G7, G11 and L12 are also not important in immune reaction between fungal serine proteases and FUM20.

The corresponding peptides of Pen ch 13 and Pen c 13 (fig. 2, peptides 4 and 6, respectively) showed negative reactivity against FUM20. It affirms our previous findings that FUM20 cannot recognize Pen ch 13 or Pen c 3 (table 1) [10]. Among the nine fungal serine protease allergens presented in table 1, Pen ch 13 and Pen c 13 differ from others by having an additional Ser between the critical residues Pro and Trp. Adding a Ser between Pro and Trp abolished the immunodot reactivity of FUM20 against the Pen ch 18 peptide (fig. 2, peptide 2). Deletion of the corresponding Ser from the Pen ch 13 peptide resulted in weak immunoreactivity against FUM20 (fig. 2, peptide 3). We have to point out that after removing Ser from the Pen ch 13 peptide, it has a sequence similar to that of the Pen ch 18 peptide with glutamine and arginine (Q5 and R6 of Pen ch 13) to glutamate and lysine (E6 and K7 of Pen ch 18) replacements. However, removing the same Ser from Pen c 13 did not convert the peptide to a positive reactivity against FUM20 (fig. 2, peptide 5). Noticeably, Pen ch 13 has an R6 while Pen c 13 has a S6. Results obtained suggest that either R or K is needed at position 6/7 for antibody-antigen recognition. However, the peptide with a K at position 6/7 is a much better antigenic determinant for FUM20. Even though both R and K side chains are cationic in nature, they are structurally quite different. The guanidino group on arginine cannot faithfully replace the ϵ -amino side chain of lysine in antibody-antigen recognition.

The peptide results were further confirmed by Rho m 2 mutants. Replacement of K6 on Rho m 2 with alanine abolished its reactivity against FUM20. However, replacing K6 with arginine retained a positive but weaker immunoblot reactivity of Rho m 2 against FUM20 (fig. 3). The data affirm the results we obtained with synthetic peptides. Therefore, a charged residue is definitely needed at this position for antigen-antibody interaction.

Immunoblot reactivity against FUM20 was undetectable when P9 of Rho m 2 was substituted with either residue A or G. We speculate that a proline is needed at this position to bend the peptide backbone into a conformation for optimal protein-protein interaction. Putting an A or G at this position changes the conformation of the protein significantly that affects protein binding. However, we cannot exclude the possibility that FUM20 does specifically recognize and interact with P9.

The side chain of W10 is pointed towards the interior of the protein and partially buried. Immunoblot reactivity of FUM20 was not detectable when W10 of Rho m 2 was substituted by either residue A or F. The results conclude an aromatic side chain is insufficient for FUM20 interaction.

In the analysis of IgE-binding components in crude fungal extracts, it was found that both the alkaline and the vacuolar serine protease allergens were detected in *P. chrysogenum* (*P. notatum*) extracts prepared in our laboratory [16]. However, the 32-kDa vacuolar serine proteinase (Pen ch 18) major allergen was not detectable in a commercially available *P. chrysogenum* extract obtained from Greer Laboratories (Lenoir, N.C., USA) [16]. FUM20 may thus be utilized as a useful tool in the standardization of major serine protease allergens in allergenic fungal extracts used clinically.

In summary, results obtained in this study using mAb FUM20 confirmed our previous results and provide valuable information for understanding the interaction of protein antigens with reactive antibodies. In addition, the value of using cross-species homologues to pinpoint epitope regions and elucidate key features recognized by allergen-specific and/or cross-reactive antibodies was demonstrated.

Acknowledgments

This work was supported by grants from the National Science Council (grant NSC91-2320-B-075-008) and the Taipei Veterans General Hospital (grant 94-370C), Taipei, Taiwan, ROC.

References

- 1 Kurup VP, Shen HD, Banerjee B: Respiratory fungal allergy. *Microbes Infect* 2000;2:1101–1110.
- 2 Bush RK: Fungal extracts in clinical practice. *Allergy Proc* 1993;14:385–390.
- 3 Esch RE: Manufacturing and standardizing fungal allergen products. *J Allergy Clin Immunol* 2004;113:210–215.
- 4 Chapman MD: Allergen specific monoclonal antibodies: new tools for the management of allergic disease. *Allergy* 1988;43(suppl 5):7–14.
- 5 Jimeno L, Duffort O, Serrano C, et al: Monoclonal antibody-based ELISA to quantify the major allergen of *Artemisia vulgaris* pollen, Art v 1. *Allergy* 2004;59:995–1001.
- 6 Shen HD, Tam MF, Chou H, et al: The importance of serine proteinases as aeroallergens associated with asthma. *Int Arch Allergy Immunol* 1999;119:259–264.
- 7 Shen HD, Tam MF, Chou H: *Penicillium* allergy; in Kurup VP (ed): *Mold Allergy, Biology and Pathogenesis*. Kerala, Research Signpost, 2005, pp 69–75.
- 8 Kurup VP, Shen HD, Vijay H: Immunobiology of fungal allergens. *Int Arch Allergy Immunol* 2002;129:181–188.
- 9 Hong C, Tam MF, Lee SS, et al: A vacuolar serine protease (Rho m 2) is a major allergen of *Rhodotorula mucilaginosa* and belongs to a class of highly conserved pan-fungal allergens. *Int Arch Allergy Immunol* 2005;138:134–141.
- 10 Lin WL, Chou H, Tam MF, et al: Production and characterization of monoclonal antibodies to serine proteinase allergens in *Penicillium* and *Aspergillus* species. *Clin Exp Allergy* 2000;30:1653–1662.
- 11 Shen HD, Chou H, Tam MF, et al: Molecular and immunological characterization of Pen ch 18, the vacuolar serine protease major allergen of *Penicillium chrysogenum*. *Allergy* 2003;58:993–1002.
- 12 Shen HD, Chua KY, Lin WL, et al: Characterization of the house dust mite allergen Der p 7 by monoclonal antibodies. *Clin Exp Allergy* 1995;25:416–422.
- 13 Swiss-Model: An Automated Comparative Protein Modelling Server. <http://swissmodel.expasy.org>.
- 14 Guex N, Peitsch MC: SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997;18:2714–2723.
- 15 Fraczekiewicz R, Braun W: Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comput Chem* 1998;19:319–333.
- 16 Shen HD, Lin WL, Tam MF, et al: Characterization of allergens from *Penicillium oxalicum* and *Penicillium notatum* by immunoblotting and N-terminal amino acid sequence analysis. *Clin Exp Allergy* 1999;29:642–651.