Biochemical and Biophysical Research Communications 375 (2008) 671–674

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Lys89, Lys90, and Phe91 are critical core amino acid residues of the Pen ch 18 major fungal allergen recognized by human IgE antibodies

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ARTICLE INFO

Article history:
Received 6 August 2008
Available online 28 August 2008

Keywords:
IgE epitope mapping
Pen ch 18 allergen

ABSTRACT

A vacuolar serine protease (Pen ch 18) has been identified as a major allergen of *Penicillium chrysogenum*. The molecular features of antigenic determinant(s) on Pen ch 18 recognized by human IgE antibodies, however, have remained unclear. Here, we show that a dominant IgE epitope on the N-terminally processed Pen ch 18 allergen was narrowed down to residues 83–91. In addition, Lys89, Lys90, and possibly Phe91 were identified as the core residues. Substitution of Lys89, Lys90, or Phe91 with alanine can significantly reduce IgE-binding to Pen ch 18. Immunoblot inhibition confirmed that Lys89 and Phe91 played a significant role in IgE-binding against Pen ch 18. Molecular modeling suggests they are located on a loop-like structure at or near the surface of the major fungal allergen.

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Airborne fungi are important causative agents of human atopic respiratory disorders [1]. Alkaline and/or vacuolar serine proteases have been identified as a major pan-fungal allergen group of prevalent fungi including *Penicillium*, *Aspergillus*, *Cladosporium*, *Curvularia*, and *Rhodotorula* species [2,3].

Identification and characterization of IgE-binding epitopes provide basis in understanding the allergenicity of major allergens and their interaction with human IgE antibodies at molecular level [4]. It will also facilitate the development of safer strategies in allergy therapeutics [5,6]. Hence, we reported the major linear IgE-binding epitope (^{48}GHADFGGR^{55}) of the major Pen ch 13 alkaline serine protease allergen from *Penicillium chrysogenum* [7] which forms a loop-like structure at the surface of the allergen. Substitution of His49 and/or Phe52 with methionine significantly reduced IgE-binding to Pen ch 13 [7].

Pen ch 18 is a vacuolar serine protease and a major allergen from *P. chrysogenum* [8]. About 82% of *P. chrysogenum*-hypersensitive patients showed IgE reactivity against the major Pen ch 18 allergen [2,8]. We have previously mapped a dominant linear IgE-reacting epitope on Pen ch 18. It encompasses residues 73–95 of the N-terminally processed allergen [8]. However, the critical core amino acid residues and the spatial organization of the allergenic epitope still remain to be elucidated. The aim of the present study is to characterize the critical features of the major IgE epitope on Pen ch 18. Results obtained will advance our understanding of the molecular basis of allergenicity for the Pen ch 18 allergen and facilitate the design of hypoallergenic peptides and derivatives for immunotherapy of atopic disorders.

Materials and methods

Patients' sera. Sera used in the present study were collected from 12 patients with a clinical history of bronchial asthma and previously shown to contain IgE antibodies against the Pen ch 18 allergen. Serum samples used as controls were collected from non-atopic laboratory volunteers. This study has been approved by the Institutional Review Board of Taipei Veterans General Hospital.

Peptide synthesis and peptide mapping. Peptides were custom synthesized on derivatized cellulose sheets (Genesis Biotech, Taipei, Taiwan, ROC). Immunoassay of peptides reacting with IgE antibodies was performed essentially as described [7,8]. The membranes were blocked with 1% skimmed milk and incubated, for 16 h at 4 °C, with serum samples that previously showed positive IgE reaction against Pen ch 18. The membranes were washed and incubated for 2 h with horseradish peroxidase labeled monoclonal anti-human IgE antibodies (AbD Serotec, Kidlington, Oxford, UK) at room temperature. The membranes were washed and developed by using an enhanced chemiluminescence (ECL) kit (Amersham Bioscience Ltd., Piscataway, NJ, USA).

Site-directed mutagenesis and immunoblotting. A plasmid (pQE30, Qiagen, Hilden, Germany) carrying the coding sequence of Pen ch 18 is available in the laboratory [8] and used as template for polymerase chain reactions (PCR). Pen ch 18 mutants carrying single alanine substitute at K89, K90, or F91 were generated by PCR based overlap extension using oligonucleotide primers with

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doi:10.1016/j.bbrc.2008.08.097

mismatches [9]. Primers employed in the generation of these mutants are listed in Table 1. For each substitution, a forward mutagenic primer with the pQE30-r primer and a reverse mutagenic primer with the pQE30-f primer were employed to generate two PCR products using Super-Therm polymerase (JMR Holdings, London, UK). These products with overlapping sequences were then purified with Qiagen MinElute gel extraction kit (Qiagen, Hilden, Germany) and used as templates in a subsequent overlap extension reaction using primers pQE30-f and pQE30-r. Following PCR, the newly synthesized DNA carrying the point mutation was purified and restricted with BamHI and HindIII then subcloned into the pQE30 expression vector as described [8]. The resulting vector was transformed into *Escherichia coli* M15 for protein expression [8]. All mutant constructs were confirmed by DNA sequencing. The recombinant proteins were affinity-purified with a Ni–NTA resin column (Qiagen) under denaturing condition according to the manufacturer's instructions.

Wild-type Pen ch 18 and mutants purified in denatured form were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μm; Millipore, Bedford, MS, USA). The membranes were blocked with 1% skimmed milk and incubated, for 16 h at 4 °C, with serum samples that previously showed positive IgE reaction against Pen ch 18. The membranes were washed, incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, CA, USA) and substrate solution essentially as described [7,8].

Immunoblot inhibition. In this study, immunoblot inhibition was performed to test the antigenicity of the Pen ch 18 mutants. A serum sample with IgE positivity against the wild-type Pen ch 18 and various degrees of IgE reactivity against Pen ch 18 mutants was used. The serum sample was pre-incubated, individually, at 4 °C for 16 h with PVDF membranes blotted with 5 μg of recombinant Pen ch 18 or its mutants before immunoblotting against the wild-type Pen ch 18. As a control, the same positive serum sample was pre-incubated with 5 μg of bovine serum albumin (BSA, Pierce, Rockford, IL, USA) before immunoblotting against Pen ch 18. The blots were washed and incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies and developed with enzyme substrates.

Homology-based modeling of antigenic epitopes recognized by IgE antibodies. Molecular modeling of Pen ch 18 was performed using Swiss-Model programs in first approach mode [10,11]. The coordinates (lic6A) of a serine protease (proteinase K from *Tritirachium album*) with 42% amino acid sequence identity with the mature Pen ch 18 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 WebLab Viewer version 3.1 (Accelrys, San Diego, CA, USA) or VMD molecular visualization program (VMD1.8.6) (http://www.ks.uiuc.edu/Research/vmd/) [12]. Solvent accessibility of surface residues was calculated with the GETAREA program (http://www.scsb.utmb.edu) [13].

Table 1
Primers used in PCR

Name	Nucleotide sequence in 5′–3′-end orientation
$Pn18K89A_{forward}$	5′-^{248}CTGGCACTATTGCCGGCGCGAAGTTCGGTGTCGCC^{282}-3′
$Pn18K89A_{reverse}$	5′-^{282}GGCGACACCGAACTTCGCGCCGGCAATAGTGCCAG^{248}-3′
$Pn18K90A_{forward}$	5′-^{250}GGCACTATTGCCGGCAAGGCGTTCGGTGTCGCCAAGAAG^{288}-3′
$Pn18K90A_{reverse}$	5′-^{288}CTTCTTGGCGACACCGAACGCCTTGCCGGCAATAGTGCC^{250}-3′
$Pn18F91A_{forward}$	5′-^{252}CACTATTGCCGGCAAGAAGGCCGGTGTCGCCAAGAAGG^{289}-3′
$Pn18F91A_{reverse}$	5′-^{289}CCTTCTTGGCGACACCGGCCTTCTTGCCGGCAATAGTG^{252}-3′
pQE30-f	5′-CCCGAAAAGTGCCACCTG-3′
pQE30-r	5′-GTTCTGAGGTCATTACTGG -3′

Results and discussion

IgE Epitope mapping of Pen ch 18

We have reported previously a preliminary determination of the Pen ch 18 epitope recognized by human IgE antibodies. Our results demonstrate that 11 sera from asthmatic patients with IgE reactivity against Pen ch 18 showed IgE-binding to the PN1 fragment of Pen ch 18 [8] (Fig. 1). Only two of the 11 serum samples tested showed weak IgE-immunoblot reactivity to the PN2 and the PN3 fragments [8]. In addition, 12 overlapping peptides (Fig. 1) were constructed to map the linear epitopes within PN1 and PN2. Among six PN1-positive asthmatic serum samples, four have IgE reactivity to a peptide covering amino acid residues 73–95 of Pen ch 18 [8].

Linear B-cell epitopes are typically 5–10 residues in length [14–16]. In this study, two overlapping peptides (Fig. 1) covering residues 78–86 (^{78}HGTHCSGTI^{86}) and 83–91 (^{83}SGTIAGKKF^{91}) of this major linear epitope were synthesized to pinpoint the IgE-binding determinant. Two serum samples with IgE reactivity against a peptide covering residues 73–95 were used. Both sera showed IgE-binding to the peptide fragment covering amino acid residues 83–91 of Pen ch 18. A representative result with one of the serum samples tested is shown in Fig. 1.

The IgE-binding determinant(s) on this fragment was further mapped with four overlapping peptides synthesized on cellulose membranes. The overlapping peptides are 9, 10, 11, and 12 amino acids long and cover amino acid residues 79–87, 79–88, 79–89, and 79–90 of Pen ch 18, respectively. Results generated with one of the two serum samples with IgE-binding against peptide 83–91 indicate that IgE antibodies react with ^{79}GTHCSGTIAGK^{89} and ^{79}GTHCSGTIAGKK^{90} of Pen ch 18 (data not shown). The reactivity decreased drastically in the absence of Lys89 (peptides 79–87 and 79–88) (data not shown). Thus, the IgE antibodies from the serum sample react with the peptide covering residues 83–91 but do not recognize peptides covering residues 79–88 (this study) or 91–102 [8]. However, we cannot exclude the possibility that some N-terminal amino acids from the peptide covering residues 91–102 are part of the epitope. With all these considerations, we tentatively concluded that Lys89, Lys90, and possibly Phe91 are important for IgE recognition.

IgE reactivity against recombinant Pen ch 18 mutants

To understand further the IgE-binding epitope of Pen ch 18, residues Lys89, Lys90, or Phe91 identified with the peptide immunoassay were substituted singly with alanine by site-directed mutagenesis. The mutated proteins were then interacted, by immunoblotting, with 12 sera from asthmatic patients with positive IgE against the Pen ch 18 allergen (Fig. 2). The IgE reactivity against recombinant Pen ch 18 and its mutants was then compared. The degree of reduction in IgE-binding was determined by measuring the immunoblot with the AlphaEaseFC™ software (version 4.0.0, Alpha Innotech Corpration, San Leandro, CA, USA) (Fig. 2). Pen ch 18 with Lys89 to alanine exchange showed a decrease in IgE-binding in ten of the 12 sera tested (Fig. 2). An IgE inhibition of $\geqslant$70% was observed in eight of these 10 serum samples (Fig. 2). In addition, mutants with substitution at residues Lys90 or Phe91 caused a decrease in IgE-binding in seven or nine of the 12 sera tested, respectively (Fig. 2). These three mutants did not change the IgE-binding reactivity among two of these 12 patients (Fig. 2, serum Nos. 4 and 5 of B). For control, serum from a non-atopic laboratory volunteer showed negative reactivity against Pen ch 18 and its mutants (data not shown).

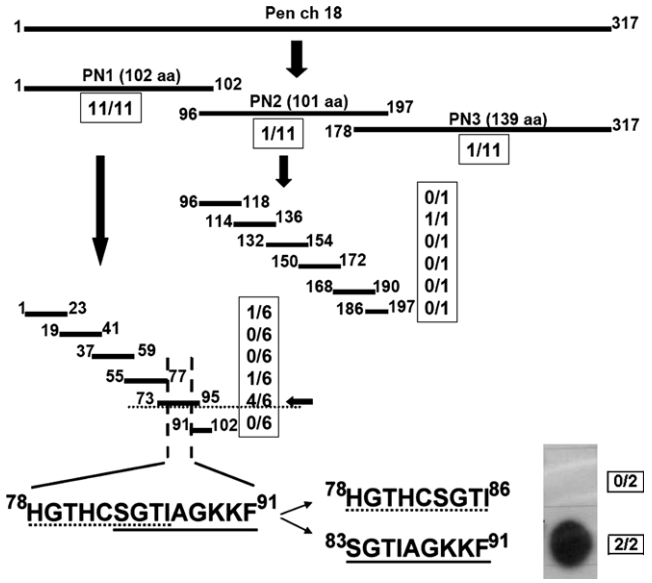
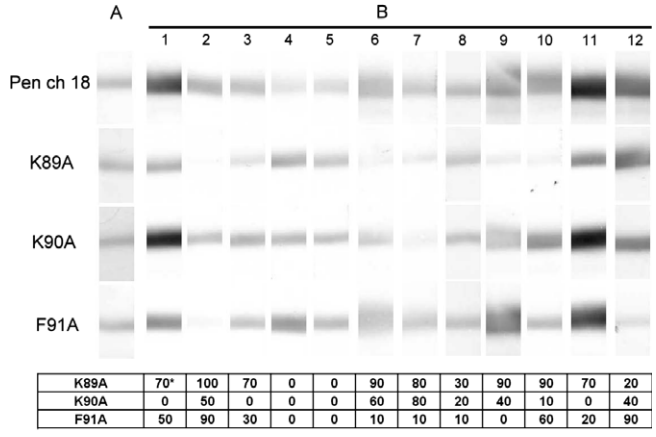


Fig. 1. Immuno-reactivity of IgE antibodies against different fragments and synthetic peptides with overlapping sequences of the Pen ch 18 allergen. The numbers in the boxes show the frequency of IgE recognition.



K89A	70*	100	70	0	0	90	80	30	90	90	70	20
K90A	0	50	0	0	0	60	80	20	40	10	0	40
F91A	50	90	30	0	0	10	10	10	0	60	20	90

Fig. 2. Coomassie blue-stained protein profiles of the wild type and the Pen ch 18 mutants on PVDF membranes (A) and their immunoblot reactivities against IgE antibodies in 12 sera from asthmatic patients (B). The table below shows data on the percent reduction of IgE-binding against Pen ch 18 mutants compared with that against the wild-type Pen ch 18 determined with the AlphaEaseFC™ software (version 4.0.0, Alpha Innotech Corpration, San Leandro, CA, USA).

In this study, some, but not all, patients tested have reduced IgE-binding to each of the three single-point mutants investigated. Similar results with respect to patient-to-patient variations were also found in earlier reports [17,18]. Each of the six Bet v 1a single amino acid substitution mutant efficiently decreased IgE-binding for some, but not all, patients tested [18]. The substitution at position 10 of Bet v 1a reduced IgE-binding in 87% of the patients tested [18]. It was also found that in one case, every single amino acid exchange abolished IgE-binding [18].

In this study, our results showed that mutants with Lys89 or Lys90 to alanine replacement have reduced reactivity against human IgE antibodies (Fig. 2). It indicates that both amino acid positions are involved in the formation of IgE-binding epitope on Pen ch 18. The results also imply that positively charged residues may be needed at these positions for antigen-antibody interaction. Similarly, the four single mutants of Der f 13 allergen (E41A, K63A, K91A, K103A) have significantly lower IgE-binding reactivity than that of the wild-type Der f 13 [6]. All four residues are charged and exposed on the surface of the protein [6]. Our results also showed that IgE-immunoblot reactivity can be reduced when Phe91 of Pen ch 18 was substituted with alanine (Fig. 2). The results suggest an aromatic side-chain at this position possibly facilitates the interaction between Pen ch 18 and human IgE antibodies.

Immunoblot inhibition

Immunoblot inhibition was performed to confirm the contribution of Lys89, Lys90, and Phe91 in the IgE-binding of Pen ch 18. Results in Fig. 3 were obtained with serum No. 10 in Fig. 2. The data show clearly that after the serum was pre-absorbed with 5 μg of wild-type (WT) Pen ch 18 or its K90A mutant, approximately 91–96% IgE-binding inhibition against Pen ch 18 were detected. A 53% inhibition against Pen ch 18 was detected when the serum was pre-absorbed with 5 μg of the Pen ch 18 F91A mutant. Only 3% inhibition of IgE-binding against Pen ch 18 was detected when the same serum sample was pre-absorbed with 5 μg of the Pen ch 18 K89A mutant or with BSA.

The results show a close correlation between the effectiveness of these mutants in inhibiting IgE-binding to Pen ch 18 (Fig. 3) and the reactivity of these mutants against human IgE (Fig. 2). It affirms the contribution of Lys89 and Phe91 in the interaction between Pen ch 18 and the IgE antibodies of this patient.

Homology modeling

The IgE-reacting epitope on Pen ch 18 vacuolar serine protease allergen and the critical core amino acid residues that are involved in IgE-binding are illustrated as 3D models (Fig. 4). They are constructed by homology modeling with the structure of a serine protease (lic6A, proteinase K from *Tritirachium album*) as template. The critical core Lys89 (yellow), Lys90 (blue) and Phe91 (red) residues locate on a loop-like structure of the Pen ch 18 protein (Fig. 4B). In addition, the Lys89 critical core amino acid locates on the surface of the Pen ch 18 allergen (Fig. 4A). It is solvent exposed (ratio: 60.2%) as analyzed by the GETAREA program (data not shown) [13].

We have shown previously that a major linear IgE-binding epitope formed a loop-like structure at the surface of the Pen ch 13 allergen [7]. Trevino et al. also reported that B-cell epitopes are mainly located on loops [19]. The putative IgG and IgE epitopes of Ole e 9 were found to be mainly located in coil-structure and solvent-accessible regions [19]. In addition, five of the six IgE-binding regions in the soybean allergen glycinin G2 are located on the surface of the molecule [20]. To date, unique structural features of linear IgE epitopes of allergens have not been reported. However, results obtained all reveal the importance of surface charged residues and loop-like structure on IgE-binding and allergenicity of allergens.

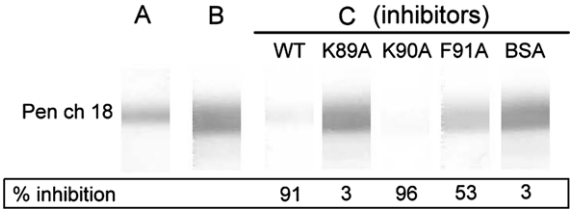


Fig. 3. Inhibition of IgE-immunoblot reactivity against Pen ch 18 with purified wild-type (WT) recombinant Pen ch 18, K89A, K90A, F91A mutants, or BSA as inhibitors (C). The strip in (A) shows the Coomassie blue-stained protein profile of recombinant Pen ch 18 protein. IgE-binding against Pen ch 18 using serum No. 10 of Fig. 2 is shown in (B). The intensity of IgE-binding against Pen ch 18 is quantified by the AlphaEaseFC™ software (version 4.0.0).

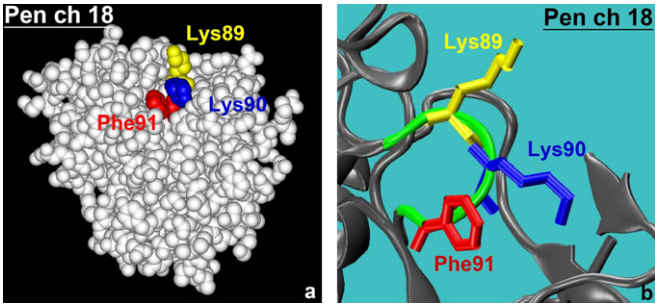


Fig. 4. A space filled 3D model of Pen ch 18 allergen is shown in (A). A region of the same model corresponding to the major IgE epitope is presented as ribbon diagram in (B). The three critical core amino acid residues (Lys89, Lys90, and Phe91) are colored in yellow, blue, and red, respectively. The models were constructed with Swiss-Model programs, viewed and displayed with the WebLab Viewer (Accelrys, San Diego, CA, USA) (A) and the VMD molecular visualization program (VMD1.8.6) (http://www.ks.uiuc.edu/Research/vmd/) (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In conclusion, a dominant linear IgE epitope of Pen ch 18 was characterized in this study. We identified residues 83-91 on Pen ch 18 as an immunodominant epitope. Lys89, Lys90 and Phe91 are among critical core amino acid residues of Pen ch 18 recognized by human IgE antibodies. They are located on a loop-like structure at or near the surface of Pen ch 18. Results obtained from the present study provide information on molecular and structural features that define the allergenicity of the Pen ch 18 allergen and can be used for developing effective therapeutics.

Acknowledgments

This work was supported by grants from the National Science Council (Grant NSC95-2320-B-075-005-MY3) and the Taipei Veterans General Hospital (Grant 97C1-041), Taipei, Taiwan, Republic of China.

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