Identification of Critical Amino Acids in an Immunodominant IgE Epitope of Pen c 13, a Major Allergen from *Penicillium citrinum*

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Abstract

**Background:** Pen c 13, identified as a 33-kDa alkaline serine protease, is a major allergen secreted by *Penicillium citrinum*. Detailed knowledge about the epitopes responsible for IgE binding would help inform the diagnosis/prognosis of fungal allergy and facilitate the rational design of hypoallergenic candidate vaccines. The goal of the present study was to characterize the IgE epitopes of Pen c 13.

**Methodology/Principal Findings:** Serum samples were collected from 10 patients with mold allergy and positive Pen c 13 skin test results. IgE-binding epitopes on rPen c 13 were mapped using an enzymatic digestion and chemical cleavage method, followed by dot-blotting and mass spectrometry. A B-cell epitope-predicting server and molecular modeling were used to predict the residues most likely involved in IgE binding. Theoretically predicted IgE-binding regions were further confirmed by site-directed mutagenesis assays. At least twelve different IgE-binding epitopes located throughout Pen c 13 were identified. Of these, peptides S16 (A148–E166) and S22 (A243–K274) were recognized by sera from 90% and 100% of the patients tested, and were further confirmed by inhibition assays. Peptide S22 was selected for further analysis of IgE-binding ability. The results of serum screening showed that the majority of IgE-binding ability resided in the C-terminus. One Pen c 13 mutant, G270A (T261–K274), exhibited clearly enhanced IgE reactivity, whereas another, K274A, exhibited dramatically reduced IgE reactivity.

**Conclusions/Significance:** Experimental analyses confirmed *in silico*-predicted residues involved in an important antigenic region of Pen c 13. The G270A mutant of Pen c 13 has the potential to serve as an additional tool for the diagnosis/prognosis of mold allergy, and the K274A mutant, as a hypoallergenic form of the epitope, may provide a framework for the design and development of a safe and efficient therapeutic strategy for treating human allergic diseases.

Introduction

Fungi are ubiquitous in our environment, and many have been identified as causative factors in IgE-mediated allergy. Among the fungi associated with allergic disorders, *Penicillium* sp. is one of the most frequently encountered species [1–4]. Previous studies identified the major allergen Pen c 13, a highly prevalent allergen secreted by *Penicillium citrinum* among patients with mold allergy [5], as a 33-kDa alkaline serine protease, and showed that it might directly contribute to sensitization and is associated with the development of asthma [6–9]. These effects probably result from the pathophysiological changes in the lung microenvironment, the expression of thymic stromal lymphopoietin, and the migration of antigen-presenting cells to the draining lymph node under the influence of protease [10–12].

Understanding of IgE-binding epitopes on allergens should help elucidate allergen-antibody interactions and may contribute to the development of more effective immunotherapeutic strategies. It has been demonstrated that immunotherapy induces strong allergen-specific IgG responses and that protective IgG antibodies are important for the success of allergen-specific immunotherapy [13–17]. The effectiveness of recently developed genetically engineered and synthetic allergen derivatives that have been modified to reduce their allergenic activity demonstrates the potential use of specific epitopes in immunotherapeutic approaches to allergic disease [18–21].

Among the strategies for identifying allergen epitopes are fragmentation of allergens, either enzymatically [3,22] or synthetically [23,24]; phage display [25,26]; and various recombinant technologies [27–29]. Epitopes might be composed of sequential
residues along the polypeptide chain (linear epitopes) or nonsequential residues from segments of the chain brought together by the folded conformation of the protein (conformational epitopes) [30,31]. Many epitope-mapping studies of different allergens have shown that small linear epitopes bind specifically to IgE antibodies of allergic patients; moreover, the IgE-binding capacity of these allergens could be abolished by single amino acid mutations within each epitope [26,27,32–34]. Furthermore, the identification of specific residues involved in IgE antibody binding can be sped up using computer programs that predict B-cell epitopes [35–39].

Pharmacological agents have been the mainstay of allergy symptom management, but allergen-specific immunotherapy is the only long-lasting, allergen-specific approach for treating type I allergy and preventing its progression to severe disease manifestations [40–43]. An important element in the implementation of specific immunotherapy is developing a clearer understanding of the immunogenicity of allergen derivatives.

In the present study, we mapped the IgE-binding epitopes on recombinant Pen c 13 (rPen c 13) using enzymatic digestion and chemical cleavage methods, followed by dot-blotting and mass spectrometry. A concentration-response inhibition assay was performed to provide evidence of IgE binding. We also integrated results from a B-cell epitope-predicting server and molecular modeling to minimize the experimental validation process. Subsequent site-directed mutagenesis of the residues involved in the major epitope, S22, led to the identification of amino acids that are important for allergen-IgE interactions. Identification of immunodominant regions of Pen c 13 may facilitate the development of specific peptide-based diagnostics and possibly lead to therapies for mold allergy.

Materials and Methods

Patients’ sera

Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Committee of the National Taiwan University Hospital. Sera from 80 patients with mold allergy and control sera from 30 healthy volunteers were used in this study. Sera were collected at the National Taiwan University Hospital (Taipei, Taiwan) and stored in aliquots at -80°C until use. The allergic response was confirmed by clinical history and diagnosis, and characterized by measuring specific IgE reactivity using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). Mold-allergic patients were initially included on the basis of an ImmunoCAP score for the crude extract of *Penicillium notatum* from the major allergens, and the IgE-binding capacity of these sera on each plate.

Specific IgE quantification

The detection of specific IgE antibodies reactive with Pen c 13 was examined by enzyme-linked immunosorbent assay (ELISA) using sera from 80 patients and 30 healthy controls. Microtiter plates (Costar Inc., Cambridge, USA) were coated with rPen c 13 by incubating with a 0.5-µg/ml solution of rPen c 13 in PBS. Each serum sample was serially diluted in PBS/0.1% Tween-20 and added to plates, which were then incubated for overnight at 4°C. Bound IgE was detected by incubating sequentially with alkaline phosphatase-labeled anti-human IgE antibodies (Biosource) and the CDP-Star detection system. The optical density (OD) of the test samples was calculated by dividing the mean of the 30 controls. To adjust for inter-plate variability, normalized ELISA values were calculated by dividing the optical density (OD) of the test samples by the mean OD of the reference sera on each plate.

Peptide purification and identification

Pen c 13 peptides were obtained through enzymatic and chemical cleavage. rPen c 13 was digested overnight at 37°C with lysyl endopeptidase (EC 3.4.21.50), trypsin (EC 3.4.21.2), chymotrypsin (EC 3.4.21.1), or Glu-C endopeptidase (EC 3.4.21.19) at an enzyme/substrate ratio of 1:50. Lysyl endopeptidase, trypsin, and chymotrypsin digestions were carried out in 0.1 M pyridine/acetate/collidine (pH 8.2), and Glu-C endopeptidase digestions were performed in 0.1 mM calcium chloride and 0.1 M pyridine/acetate (pH 6.5). The resulting peptides were fractionated by reversed-phase high-performance liquid chromatography (HPLC) on a Beckman ODS (C18) column (300 Å, 4.6 x 250 mm) using a linear gradient of 0–44% acetonitrile in 0.06% trifluoroacetic acid and a flow rate of 1 mL/min. Cyanogen bromide (CNBr) cleavage was performed at 37°C for 24 h in 70% formic acid with a 100-fold molar excess of CNBr over methionine residues. After cleavage, the fragments were separated by HPLC on a YMC-Pack C4 column (300 Å, 4.6 x 150 mm) using the same elution system as for the Beckman column. Peptide elution was monitored at 220 nm, and all fractions were collected and analyzed for peptide mass and purity using a Procise ABI 494 protein sequencer (Applied Biosystems, Foster City, CA, USA) and a QSTAR XL mass spectrometer (Applied Biosystems).

Dot-blot immunoassays

Approximately 30 pmol each of lysyl endopeptidase-, trypsin-, chymotrypsin-, Glu-C endopeptidase-, and CNBr-cleaved peptides from rPen c 13 was spotted onto a polyvinylidene fluoride (PVDF) membrane. A total of 24 peptides covering most of the Pen c 13 sequence were screened for their ability to bind human IgE in individual serum samples from allergic subjects. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-human IgE (Biosource) and the CDP-Star detection...
system (GE Healthcare), according to the manufacturer’s instructions.

IgE-binding inhibition assay

To evaluate the major epitopes S16 and S22, using three individual patient sera (patient 1, 7, and 10), with a high, medium and low ranges of the ELISA OD values were previously incubated overnight at 4°C with different concentrations of S16, S22, or both. For inhibition control, a pool of sera from the 10 individual patient sera (patient 1, 7, and 10), with a high, medium and low ranges of the ELISA OD values were previously incubated overnight at 4°C with different concentrations of rPen c 13 as a positive control or albumin as the negative one. Microtiter plates (Costar Inc., Cambridge, USA) were coated with rPen c 13 by incubating for overnight at 4°C with 100 μl of a 5-μg/ml solution of rPen c 13 in PBS, then incubated for overnight at 4°C with aliquots of separate sera (diluted 1:10) that had previously been incubated with different concentrations of peptides or proteins as mentioned above. The amount of specific IgE bound to the wells was determined as described above.

Computational matching of epitopes

To assist in the identification of potential residues that are critical in IgE binding, we utilized an epitope conservancy analysis tool. This tool was implemented as a component of the Immune Epitope Database and Analysis Resources (IEDB) [39] and was used for predicting B-cell epitopes. Amino acid property scales for predicting antigenic determinants were developed based on Bepipred Linear Epitope Prediction (BepiPred), which predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method [35].

Molecular modeling of Pen c 13

Pen c 13 was modeled based on the structure of the alkaline serine protease from Leucosporidium flouccifer, Ver112 (Protein Data Bank code 3F7M), which is the mostly closely related to Pen c 13 (highest BLAST score value) in the structure database [44]. To further examine the Pen c 13 structure, we generated a three-dimensional (3D) model structure using the SWISS-MODEL structure homology-modeling server [45]. The images were obtained and rendered with PyMOL software.

Expression and purification of the GST fusion rPen c 13 peptides, (A243–K274), (A243–A260) and (T261–K274)

The region of the Pen c 13 targeted for (A243–K274), (A243–A260) and (T261–K274), is amplified using pQE-30/Pen c 13 as a template and the PCR with oligonucleotide primers (F243-BamHI, 5’-GGATCCGCTCTCGAGGGCGTGTCAGCT-3’, R274-EcoRI, 5’-GAATTCCTTAAAGCGGAGCTGACATTACGG-3’; R260-EcoRI, 5’-GAATTCCTTAAAGCGGAGCTGACATTACGG-3’; F261-BamHI, 5’-GGATCCACCTTGGCTCAATCTTGAGGCGCTG-3’ designed specifically for amplification of the piece of cDNA of interest. Incorporating restriction sites into the primers facilitates subcloning into the multiple cloning site of the pGEX-2T vector. The PCR products were gel-purified and then ligated into the pGEX-2T expression vector (GE Healthcare) at BamHI and EcoRI sites. The fidelity of the cloned sequence was confirmed by DNA sequencing. For production of recombinant proteins, all plasmids were transformed into Escherichia coli strain BL-21 (DE3) (Stratagene, La Jolla, CA). The recombinant proteins were purified using the GST tag and then subjected to SDS-PAGE and western blot analysis. The purified recombinant proteins were further analyzed by mass spectrometry to confirm the identity of the recombinant proteins.

Table 1. Characterization of sera from patients with positive IgE binding to rPen c 13.

| Patient No. | Sex | Age (y) | Symptoms | Serum total IgE (kU/L) | Pen c 13 ELISA (OD) | ImmunoCAP score |
|-------------|-----|---------|----------|-----------------------|---------------------|-----------------|
| 1           | M   | 66      | AS       | 951                   | 1.45                | +3              |
| 2           | M   | 56      | AS, AR   | 1565                  | 1.28                | +2              |
| 3           | M   | 41      | AS, UR   | 411                   | 1.22                | +2              |
| 4           | M   | 24      | AR       | 120                   | 1.19                | +2              |
| 5           | M   | 22      | AD       | >2000                 | 1.16                | +4              |
| 6           | M   | 28      | AS, AR, AD | 782                   | 1.07                | +2              |
| 7           | F   | 22      | AR, AD   | >2000                 | 1.04                | +2              |
| 8           | F   | 10      | AR, AD   | >2000                 | 1.01                | +3              |
| 9           | M   | 33      | AS, AR, AD | >2000                 | 0.96                | +3              |
| 10          | M   | 20      | AS, AR, AD | >2000                 | 0.94                | +3              |

AD, atopic dermatitis; AR, atopic rhinitis; AS, asthma; UR, urticaria. doi:10.1371/journal.pone.0034627.t001
Jolla, CA). Cultures of transformed cells were grown to an OD₆₀₀ value of 0.6, induced with 1 mM IPTG, and harvested after 4 h. Cells were resuspended in binding buffer (PBS, pH 7.4, containing 1% Triton X-100) and sonicated on ice for 5 min, after which the suspension was centrifuged and the supernatant was loaded onto a Glutathione Sepharose 4 Fast Flow column (GE Healthcare) pre-equilibrated in binding buffer. After adsorption, the non-bound material was removed by washing with binding buffer, and bound protein was eluted using elution buffer (50 mM Tris/HCl, pH 8.0, containing 10 mM reduced glutathione). Eluted fractions were collected and analyzed by SDS-PAGE.

Figure 2. Mapping of IgE-binding epitopes by dot-blotting. Twenty-four overlapping peptides spanning almost the entire sequence of Pen c 13 were tested for reactivity toward serum IgE from 10 mold-allergic patients (A–J) and nonatopic individual (K). The diagram on the top-left shows the pattern of peptides on the blots. doi:10.1371/journal.pone.0034627.g002
Mutant clones

The gene fragment for wild-type (WT) Pen c 13 (T261–K274) was cloned into the pGEX-2T vector as described above and used as a template for the preparation of all Pen c 13 (T261–K274) mutants. These mutants were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutated genes were confirmed by direct DNA sequencing. The primers used for mutagenesis were as follows:

S269A: 5'-ATCTCTAGGGCTCCGCTGACCTACAGG-3'
G270A: 5'-TCTAGGGCTCCCTCTGCAAGTAAGAA-3'
T271A: 5'-AGGGGCTCCCTCTGCAAGTAAGAA-3'
T272A: 5'-GCTCCCTCTGCAAGTAAGAA-3'
S273A: 5'-CCCTCTGCAAGTAAGAAATC-3'
K274A: 5'-GCTGCAAGTAAGAAATC-3'

The nucleotide sequences were obtained from GenBank (accession no. AF084546); the mutated sites are underlined. The nucleotide sequences of all clones were confirmed by DNA sequencing. All GST fusion rPen c 13 (T261–K274) mutants were expressed and purified as described above.

SDS-PAGE and immunoblotting with IgE

GST fusions of WT rPen c 13 (A243–K274), (A243–A260) and (T261–K274), and mutants of rPen c 13 (T261–K274) were separated by SDS-PAGE along with GST only (control). For specific IgE immunodetection, resolved GST fusion peptides were transferred to a PVDF membrane, which was then blocked with skim milk, and incubated with serum (diluted 1:10) from mold-allergic patients. Bound human IgE was detected using horseradish peroxidase-conjugated goat anti-human IgE antibodies (BioSource). Bands were visualized using enhanced chemiluminescence reagents (Millipore) and autoradiography. Densitometric analysis was performed using the LabWorks software (UVP BioImaging Systems, Upland, CA, USA). The relative density of the bands normalized to loading control.

Results

Determination of Pen c 13 IgE reactivity in patient sera by ELISA

We have previously reported that Pen c 13 is a major allergen in mold-allergic patients [6]. To obtain sera contained higher specific IgE titers, we first analyzed 80 patients with mold allergy for Pen c 13-binding IgE by ELISA. The sera from 10 patients displaying high IgE reactivity were used to identify potential B-cell epitopes in Pen c 13. The distribution of Pen c 13-specific IgE values in all

Table 2. Characteristics of peptide fragments used for human IgE-binding dot-blot assays.

| Peptide No. | IgE reactivity (%) | N-terminal sequence | Mass (Da) [M+H]+ | Position in Pen c 13 |
|-------------|-------------------|---------------------|-----------------|----------------------|
| S1          | 10                | ANVVQ               | 2013.0          | Ala1-Lys79           |
| S2          | –                 | TGTTTS              | 1008.4          | Thr41-Asp79          |
| S3          | –                 | TYDST               | 1336.5          | Thr42-Tyr59          |
| S4          | 70                | STAGE               | 2154.0          | Ser25-Asp51          |
| S5          | 10                | GVDTG               | 1362.6          | Gly40-Phe52          |
| S6          | –                 | FGGRA               | 1406.4          | Phe42-Asp54          |
| S7          | 10                | WGTNV               | 2814.0          | Trp70-Lys90          |
| S8          | 10                | YGVAK               | 537.4           | Tyr86-Lys90          |
| S9          | –                 | KATLV               | 829.0           | Lys91-Lys98          |
| S10         | 60                | VAVKV               | 2220            | Val23-Trp117         |
| S11         | 50                | VLGAD               | 2121            | Val93-Lys120         |
| S12         | 20                | AKSRG               | 1970            | Ala121-Glu140        |
| S13         | –                 | YVMNM               | 1462.0          | Tyr131-Lys143        |
| S14         | –                 | SKAVN               | 1691.0          | Ser142-Phe158        |
| S15         | –                 | AVNDA               | 1071.0          | Ala144-Lys154        |
| S16         | 90                | AAANV               | 1818.0          | Ala148-Glu166        |
| S17         | –                 | NASNS               | 1105.1          | Asn169-Glu140        |
| S18         | 50                | VCTIA               | 2442.0          | Val93-Asp104         |
| S19         | 60                | TNFGS               | 2771.0          | Thr92-Leu104         |
| S20         | –                 | SGTSM               | 1489.0          | Ser123-Tyr130        |
| S21         | –                 | AAPHV               | 1222.2          | Ala126-Met132        |
| S22         | 100               | ALEGV               | 3438.0          | Ala127-Lys136        |
| S23         | –                 | IVQLA               | 1174.0          | Ile128-Arg130        |
| S24         | –                 | LLYNG               | 905.5           | Leu127-Val132        |

The purity of the peptides was assessed by N-terminal sequencing. N-terminal residues were determined by Edman degradation. Mass was determined using a QSTAR XL mass spectrometer. doi:10.1371/journal.pone.0034627.t002
patients is shown in Figure 1. Seventy-six percent (61/80) of mold-allergic patients showed IgE binding to rPen c 13 at levels exceeding the two-fold mean of controls. Some characteristics of 10 patients selected for further evaluation and associated serum total IgE levels are shown in Table 1.

Identification of IgE-binding epitopes of rPen c 13

Peptide fractions corresponding to 24 peptides covering almost the entire amino acid sequence of Pen c 13 were produced by cleavage for use in dot-blot screens of sera from 10 Pen c 13-allergic individuals (A–J) (Fig. 2). The intensity of IgE binding and epitope recognition by serum IgE varied substantially among individuals. Twelve different IgE-binding determinants were found among the 24 peptides. Antibody-binding regions were identified in peptides S1, S4, S5, S7, S8, S10, S11, S12, S16, S18, S19 and S22, corresponding to residues 1–19, 30–51, 40–52, 58–85, 86–90, 95–117, 99–120, 122–140, 148–166, 197–224 and 243–274, respectively. Table 2 summarizes the IgE reactivity of these 12 IgE-binding epitopes and their respective positions in the Pen c 13 molecule. Epitopes S16 and S22 were recognized by serum IgE from 90% (9/10) and 100% (10/10) of the mold-allergic patients, and both epitopes were classified as dominant epitopes in the sampled population. Other epitopes were recognized by serum IgE in 10–70% of the 10 patients tested. The control serum sample (K) showed no IgE reactivity (Fig. 2).

Confirmation of the major IgE-binding epitopes by inhibition ELISA

To analyze the relevance of IgE-binding sequences identified by dot-blotting, we performed IgE ELISA inhibition experiments. Three serum samples that recognized the epitopes S16 and S22 of Pen c 13 in dot-blots were used for ELISA inhibition analysis. Sera from patients preincubated with different concentrations of the peptide (S16, S22 or S16+S22) were evaluated for IgE binding to rPen c 13. The results showed that IgE reactivity with Pen c 13 was inhibited in a concentration-dependent manner by epitope S16 and/or S22 (Fig. 3A–C). For inhibition control, using Pen c 13 as a positive control and albumin as a negative one with a pool of sera were carried out to compare the inhibition capacity. As shown in Fig. 3D, maximal inhibition reaching 69.15% was found at 3 μM concentration of rPen c 13. Taken together, these results demonstrate that S16 and S22 are the major epitopes of Pen c 13.
Molecular modeling of the location of IgE-binding epitopes in Pen c 13

According to the results of IEDB prediction, shown in Table 3, the most critical residues involved in IgE binding are located in the C-terminus of the S22 peptide, especially the final six-residue region, which had higher prediction scores. To obtain more detailed information on the binding of IgE to Pen c 13, we built a 3D structure of Pen c 13 using the crystal structure of the cuticle-degrading protease, Ver112, secreted by Lecanicillium psalliotae, as a suitable template (PDB ID: 3F7M). The identity and similarity between the target and template were 48% and 67%, respectively. As shown in Figure 4A, the model consists of an α/β core flanked by several amphipathic helices and anti-parallel β sheets. IgE reactivities is depicted by colors based on their intensity as follows: negative (blue), weak (green), intermediate (yellow), and strong (red). Epitope S1 is situated on the N-terminal β-sheet, one turn, and an α-helix, whereas S18 and S19 are located on one turn and four β-sheets close to the C-terminus. Epitope S4 consists of two turns and one β-sheet adjacent to the N-terminal region, whereas S5, situated similarly, lacks one turn and part of the β-sheet, accounting for its marked reduction in IgE reactivity. Epitopes S7 and S8 consist of two turns and one α-helix. Epitopes S10, S11, and S12 consist of three turns, two β-sheets, and one α-helix; epitope S10/S11 is considered to contain the same allergenic sites (one turn and one helix), but S10 contains an extra β-sheet in its N-terminus. Of the two immunodominant IgE epitopes, S16 forms α-helices and a β-sheet in the middle part of the protein, and S22 forms α-helices and turns in the extreme C-terminal domain. The current model of Pen c 13 reveals that the six residues predicted from IEDB are situated on a turn and are located on the protein surface (Fig. 4B). Thus, we suggest that the final six-residue region is an important antigenic region of Pen c 13.

Modulation of IgE reactivity by site-directed mutagenesis

To validate the IgE-binding region of Pen c 13 (A243–K274) and narrow down the candidate epitope residues predicted by the in silico-based method, we expressed and isolated the GST fusion peptides, rPen c 13 (A243–A260) and rPen c 13 (T261–K274). Immunoblotting unambiguously showed that IgE reactivity is located in the rPen c 13 (T261–K274) (Fig. 5A), consistent with in silico predictions. Using site-directed mutagenesis assays, we further confirmed the theoretical prediction of potential IgE-binding regions, which presumably exhibit both a protuberant local surface and an electrostatically active local molecular domain. Consistent with this presumption, the side chains of

Figure 4. Molecular models and the immunodominant IgE-binding epitope of Pen c 13. A, Surface and ribbon diagrams of the Pen c 13 model. The different strengths of IgE-binding epitopes are colored green (weak), yellow (intermediate), and red (strong). Different levels of IgE-binding intensity are defined as: weak (10–20%), intermediate (50–70%), and strong (90–100%). B, Stick diagram of Pen c 13 residues 269–274.

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These predicted residues are all located on the polar half of the surface, as shown in Figure 4B. We therefore created the alanine (A) substitution mutants, S269A, G270A, T271A, T272A, S273A and K274A, corresponding to these six residues within the rPen c 13 (T261–K274) GST fusion, and assayed their ability to bind IgE. The result of the immunoblot strip in patient 10 was shown in Fig. 5A. The bands were quantified by densitometric analysis and shown as histogram (Fig. 5B). Of the six individual substitutions, the K274A mutation fully abolished IgE recognition; in contrast, the G270A mutation substantially increased IgE-binding capacity. In addition, pooled serum IgE from 10 patients was analyzed in the same manner, and the critical amino acids for IgE-binding were found to be the same as in patient 10 (data not shown). These results suggest that G270 and K274 are important for the IgE-binding activity of the Pen c 13 allergen. In order to validate the reduced IgE reactivity, we performed dot-blot immunoassay using two synthetic peptides (TSSISRAPSGTTSK and TSSIS-RAPSGTTSA), followed by probing with pool serum from 10 patients. The result is shown in Fig. 5C; no binding of the serum IgE was observed when the alanine was substituted at the lysine position.

**Discussion**

Identification of IgE-binding epitopes might provide a better understanding of the functional role that allergens play in the disease and could have implications for immunodiagnosis and immunotherapy. A number of approaches are currently used to predict linear epitopes, including sequence-based prediction.

| Table 3. BepiPred Linear Epitope Prediction scores of each residue in the S22 peptide (residues 243–274 of Pen c 13). |
|---|
| Position | Residue | Score | Position | Residue | Score |
| 1 | A^{243} | −0.092 | 17 | L^{259} | −0.487 |
| 2 | L^{244} | 0.21 | 18 | A^{260} | −0.582 |
| 3 | G^{245} | 0.25 | 19 | T^{261} | −0.222 |
| 4 | G^{246} | 0.444 | 20 | S^{262} | 0.155 |
| 5 | V^{247} | 0.531 | 21 | S^{263} | 0.309 |
| 6 | S^{248} | 0.568 | 22 | Y^{264} | 0.757 |
| 7 | A^{249} | 0.734 | 23 | S^{265} | 1.057 |
| 8 | G^{250} | 0.511 | 24 | P^{266} | 1.263 |
| 9 | N^{251} | 0.331 | 25 | A^{267} | 1.336 |
| 10 | A^{252} | 0.106 | 26 | P^{268} | 1.397 |
| 11 | C^{253} | −0.321 | 27 | S^{269} | 1.758 |
| 12 | A^{254} | −0.388 | 28 | Y^{270} | 1.789 |
| 13 | R^{255} | −0.85 | 29 | T^{271} | 1.855 |
| 14 | I^{256} | −0.979 | 30 | T^{272} | 1.938 |
| 15 | V^{257} | −0.896 | 31 | S^{273} | 1.981 |
| 16 | E^{258} | −0.728 | 32 | K^{274} | 1.824 |

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Figure 5. Analysis of IgE reactivity to GST fusion peptides and synthetic peptides. A, The data shown represent the results for patient 10. Immunoblots of purified GST fusion peptides Pen c 13 (A^{243}–K^{274}), (A^{243}–A^{260}) and (T^{261}–K^{274}); GST fusion Pen c 13 (T^{261}–K^{274}) mutant peptides S269A, G270A, T271A, T272A, S273A or K274A; and GST only, probed with serum from a Pen c 13-allergic patient. The protein blots were stained with Fast Green as a loading control. B, The histogram shows the quantitative densitometry of the bands. C, The critical amino acid for IgE-binding in Pen c 13 (T^{261}–K^{274}) was validated further using two synthetic peptides and tested for IgE-binding reactivity with pool serum from 10 allergic patients. The sequences of two synthetic peptides are listed, and the residue that was changed to alanine is underlined. Alanine substitution is shown in boldface letter.

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Mapping of IgE-Binding Epitopes on Pen c 13

software and 3D structure-based prediction [46]. Such approaches, most of which are based on the physicochemical properties of the amino acids in the molecule, may provide a better understanding of antigen-antibody binding. Linear epitopes of antigens share some common properties: they are exposed to the surface of the allergenic molecule, making them easily accessible; they are localized to flexible regions; and they are composed of polar residues.

In the present study, we mapped IgE-binding epitopes using enzymatically/chemically cleaved fragments to obtain more detailed information on the binding of IgE to the Pen c 13 molecule. Cleaved peptides covering most of the sequence of Pen c 13 were analyzed by dot-blotting using sera from mold-allergic patients. Twelve distinct IgE-binding epitopes distributed throughout the entire molecule were found, although additional epitopes may also exist. Only a few epitopes may have been missed because epitopes are located on cleavage sites and small gaps in the sequence coverage. In molecular modeling studies, these regions were predicted to lie on the protein surface and contained mostly solvent-exposed residues. The results suggest that IgE predominantly recognizes linear epitopes, the S22 peptide (residues 243–274), accounts for a high percentage of Pen c 13-specific IgE binding. In a 3D model of Pen c 13 based on the structure of Lecanicillium psalliotae Ver112, the IgE-binding determinant 261TSSIRAPSGTTSK274 assumes a loop-like structure on the surface of the protein. This finding is consistent with the known importance of loop-like structures in IgE binding and the reported allergenicity of other allergens, such as transaldolase, a fungal allergen whose IgE-reacting fragment is located in a loop-like structure of the C-terminal region [47]; the dust mite allergen Der f 7, whose IgE-binding epitope forms a loop-like structure on the surface of the protein [48]; the olive pollen allergen Ole e 9, whose B-cell epitopes within the C-terminal domain are mainly located in loops [49]; the mugwort pollen allergen Art v 1, whose non-linear IgE epitopes are found within exposed loop regions harboring lysine residues [50]; and the bovine milk allergen β-lactoglobulin, whose strongest IgE epitope is located in a loop [51]. Furthermore, IgE interactions with hyaluronidase, a major bee venom allergen, may also involve a surface-exposed loop-like IgE determinant [52].

Using computational prediction, we further narrowed the responsible sequence to a six-residues region (260SGTTSK274) and generated six mutants with single alanine substitutions to test the role of the predicted residues. The K274A mutation largely abolished IgE-binding ability, reflecting charge and/or size differences between lysine and alanine, implying that K274 is a critical core amino acid of Pen c 13 recognized by human IgE antibodies. These residues are located on a loop-like structure at or near the surface of Pen c 13. The results obtained from the present study provide information on the molecular and structural features that define the allergenicity of the Pen c 13 allergen and can be used for developing better diagnostic tools and effective immunotherapeutic strategies for the clinical management of fungal allergy.

Supporting Information

Method S1 Skin prick test (SPT). An SPT was performed on 10 patients who had serum IgE reactive with Pen c 13 in a specific IgE ELISA and a history of mold allergy. The skin testing was performed using 50 µl of purified Pen c 13 from Penicillium citrinum at concentrations of 10 mg/ml and 100 mg/ml in physiological saline (0.9% NaCl) or physiological saline solution alone as a negative control. Skin reactions (wheels and erythema) were recorded 15 min after prick. Wheals with a diameter of at least 1.5 mm greater than that produced by the negative control were regarded as positive responses. (DOC)

Author Contributions

Conceived and designed the experiments: LPC. Performed the experiments: JCC LLC WNH JGC HKL. Analyzed the data: JCC. Contributed reagents/materials/analysis tools: KLL. Wrote the paper: JCC LPC.

References

1. Horner WE, Helbling A, Salvaggio JE, Lehrer SB (1995) Fungal allergens. Clin Microbiol Rev 8: 161–179.
2. Kurup VP, Shen HD, Banejeje B (2000) Respiratory fungal allergy. Microbes Infect 2: 1101–1110.
3. Yu CJ, Chen YM, Su SN, Forouhar F, Lee SH, et al. (2002) Molecular and immunological characterization and IgE epitope mapping of Pen n 18, a major allergen of *Penicillum notatum*. Biochem J 363: 707–713.

4. Simon-Nobbe B, Denk U, Föll V, Riel R, Breitenbach M (2008) The spectrum of major allergens. Arch Immunol Ther Exp 56: 88–96.

5. Shen HD, Lin WL, Liaw SF, Tam MF, Han SH (1997) Characterization of the 33-kilodalton major allergen of *Penicillum citrinum* by using MoAbs and N-terminal amino acid sequencing. Clin Exp Allergy 27: 79–86.

6. Su NY, Yu CJ, Chen HD, Pan FM, Chow LP (1999) Pen e 1, a novel enzymic allergen protein from *Penicillum citrinum*. Purification, characterization, cloning and expression. Eur J Biochem 261: 113–123.

7. Chiu LL, Perreg DW, Yu CH, Su SN, Chow LP (2007) Mold allergen, Pen e 13, induces B-cell expression in human airway epithelial cells by activating protease-activated receptor-1 and 2. J Immunol 178: 5237–5247.

8. Chiu LL, Lee KL, Lin YF, Chu CY, Su SN, et al. (2008) Secretome analysis of novel IgE-binding proteins from *Penicillum citrinum*. Proteomics Clin Appl 2: 343–352.

9. Chen JC, Chuang JG, Su YY, Chiang BL, Lin YS, et al. (2011) The protease allergen Pen e 13 induces allergic airway inflammation and changes in epithelial barrier integrity and function in a murine model. J Biol Chem 286: 26670–26678.

10. Sokol CI, Barton GM, Farr AG, Medzhitov R (2008) A mechanism for the initiation of allergen-induced T helper type 2 responses. Nat Immunol 9: 310–318.

11. Kmiecik H, O'Grady SM, Lawrence CB, Kita H (2009) Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptors-2. J Immunol 183: 1437–1438.

12. Paul WE, Zhu J (2010) How are T1H2-type immune responses initiated and amplified? Nat Immunol 10: 225–233.

13. Vrtala S, Ball T, Spitzauer S, Pandjaitan B, Suphioglu C, et al. (1998) Immunization with purified natural and recombinant allergens induces mouse IgG1 antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basal degranulation. J Immunol 160: 6171–6174.

14. Mothes N, Heinzkll M, Drachenberg KJ, Sperr WR, Krauth MT, et al. (2003) Allergen-specific immunotherapy with a monophospholipid lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basal histamine release by therapy-induced blocking antibodies. Clin Exp Allergy 33: 1198–1208.

15. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, et al. (2002) Identification of epitope assembly and mimotope screening. Int Arch Allergy Immunol 153: 133–140.

16. Larche M, Akdis CA, Valenta R (2006) Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 6: 761–771.

17. Yang X, Yu X (2009) An introduction to epitope prediction methods and analysis of the immune response database. *Proteomics Clin Appl* 3: 1021–1028.

18. Chou H, Tam MF, Chou CT, Tai HY, et al. (2011) Transdallosolides are novel and immunoglobulin E cross-reacting fungal allergens. Clin Exp Allergy 41: 739–749.

19. Chou H, Tam MF, Lee SS, Tang RB, Lin IH, et al. (2011) Asp159 is a critical residue governing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. J Mol Biol 415: 58–66.

20. Larche M, Akdis CA, Valenta R (2006) Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. *PLoS One* 5: e9892.

21. Salome N, Bleri W, Peters A (2010) Design and optimization of epitope-based databases and predictive tools. Immunogenetics 62: 185–196.

22. Durham SR, Till SJ (1998) Immunologic changes associated with allergen immunotherapy. J Allergy Clin Immunol 102: 157–164.

23. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, et al. (1999) Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 341: 465–475.

24. Moller C, Dreborg S, Ferdoussi HA, Halken S, Hau A, et al. (2002) Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). J Allergy Clin Immunol 109: 251–256.

25. Larche M, Akdis CA, Valenta R (2006) Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 6: 761–771.

26. Yang X, Yu X (2009) The Swiss-Model workbench: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195–201.

27. Yang X, Yu X (2009) An introduction to epitope prediction methods and analysis of the immune response database. *Proteomics Clin Appl* 3: 1021–1028.

28. Chou H, Tam MF, Chou CT, Tai HY, et al. (2011) Transdallosolides are novel and immunoglobulin E cross-reacting fungal allergens. Clin Exp Allergy 41: 739–749.

29. Chou H, Tam MF, Lee SS, Tang RB, Lin IH, et al. (2011) Asp159 is a critical residue governing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. J Mol Biol 415: 58–66.

30. Larche M, Akdis CA, Valenta R (2006) Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. *PLoS One* 5: e9892.

31. Salome N, Bleri W, Peters A (2010) Design and optimization of epitope-based databases and predictive tools. Immunogenetics 62: 185–196.

32. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workbench: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195–201.

33. Yang X, Yu X (2009) The Swiss-Model workbench: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195–201.
58. Campbell JD, Buckland KF, McMillan SJ, Kearley J, Oldfield WL, et al. (2009) Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. J Exp Med 206: 1535–1547.

59. Holm J, Gajhede M, Ferreras M, Henriksen A, Ipsen H, et al. (2004) Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. J Immunol 173: 5258–5267.

60. Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, et al. (1998) Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 101: 747–754.

61. Oldfield WL, Kay AB, Larche M (2002) Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. Lancet 356: 47–53.

62. Karamloo F, Schmid-Grendelmeier P, Kussebi F, Akdis M, Salagianni M, et al. (2005) Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes. Eur J Immunol 35: 3268–3276.