

Complementary DNA cloning and immunologic characterization of a new *Penicillium citrinum* allergen (Pen c 3)

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Background: *Penicillium citrinum* has been identified as the most prevalent airborne *Penicillium* species in the Taipei area. It is important to understand the allergenic composition of this ubiquitous fungal species.

Objective: The complementary DNA (cDNA) clone of an allergen from *P. citrinum* was isolated and expressed in *Escherichia coli* as a fusion protein. mAbs were prepared with the recombinant protein as antigen. The corresponding natural allergen in the fungal extracts was identified with the mAbs.

Methods: A Uni-Zap XR *P. citrinum* cDNA library was screened with sera from asthmatic patients. An IgE-binding cDNA clone was isolated and expressed as a glutathione-S-transferase fusion protein. The frequency of IgE binding to the expressed protein was analyzed by immunoblotting. Spleen cells from BALB/c mice immunized with the recombinant protein were fused with NS-1 cells for mAb generation.

Results: A *P. citrinum* cDNA library was screened with a mixture of serum samples from 4 asthmatic patients. An IgE-binding cDNA clone was obtained and designated as PCE2. PCE2 has a 694-bp insert that contains a 167 amino acids open reading frame. The deduced amino acid sequence of the encoded protein has 82.6% (138 amino acids) identity with an *Aspergillus fumigatus* peroxisomal membrane protein allergen (Asp f 3). PCE2 was expressed in *E. coli* as a fusion protein and designated as Pen c 3. Sera from 13 (46%) of the 28 *Penicillium*-sensitized asthmatic patients demonstrated IgE binding to Pen c 3. In addition, 11 of the 13 Pen c 3-positive serum samples have IgE immunoblot reactivity to recombinant Asp f 3. The presence of IgE cross-reactivity between Pen c 3 and

Asp f 3 was also detected by immunoblot inhibition. Four of the 6 mAbs generated against Pen c 3 cross-react with Asp f 3. The presence of the corresponding 18-k natural allergens in the crude extracts of *P. citrinum* and *A. fumigatus* were detected by immunoblot with use of the mAbs and sera from asthmatic patients.

Conclusion: Results obtained suggest that the peroxisomal membrane protein (Pen c 3) is an important allergen of *P. citrinum*. PCE2 is a full-length cDNA clone encoding this allergen. In addition, the mAbs generated may be useful in standardizing the diagnostic allergenic extracts. (J Allergy Clin Immunol 2000;105:827-33.)

Key words: *Penicillium citrinum*, allergen, peroxisomal membrane protein, cDNA cloning, mAbs

Penicillium is a prevalent indoor airborne fungus that might cause extrinsic bronchial asthma.¹⁻³ More than 150 different species have been identified in the genus *Penicillium*.⁴ We have shown previously that *Penicillium citrinum* is the most prevalent airborne *Penicillium* species in the Taipei area.⁵ Muilenberg et al⁶ have also demonstrated that *P. citrinum* is one of the 5 most frequently recovered species of *Penicillium* in Topeka, Kan. We analyzed and have shown that a 33-kD alkaline serine proteinase is a major allergen of this prevalent *Penicillium* species.⁷ The alkaline/vacuolar serine proteinases have also been identified as major allergens in 5 other prevalent *Penicillium* and *Aspergillus* species (*Penicillium notatum*, *Penicillium brevicompactum*, *Penicillium oxalicum*, *Aspergillus oryzae*, *Aspergillus flavus*).⁷⁻¹¹ IgE cross-reactivity was detected among these serine proteinase allergens.⁸⁻¹¹ In addition, results from our previous studies also showed that a heat-shock protein in the hsp70 family is an allergen of *P. citrinum*.¹² Recently, more than 10 different *Aspergillus fumigatus* allergens have been isolated through molecular approaches.^{13,14} Among them, an 18.5-kD peroxisomal membrane protein (Asp f 3) has been identified.¹³⁻¹⁵

In this study a clone encoding an allergen of *P. citrinum* was isolated by screening a Uni-Zap XR complementary DNA (cDNA) library with sera from asthmatic patients. This cDNA was expressed in *Escherichia coli*. mAbs were generated against the recombinant protein and used in the identification of the corresponding natural allergen. Results obtained suggest that the peroxisomal

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Supported by grants from the National Health Research Institutes (No. DOH88-HR-508), the National Science Council (No. NSC88-2314-B-075-006), and the Veterans General Hospital-Taipei, Taiwan, Republic of China.

Received for publication May 12, 1999; revised Dec 22, 1999; accepted for publication Dec 22, 1999.

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0091-6749/2000 \$12.00 + 0 1/1/105220

doi: 10.1067/mai.2000.105220

Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
BCIP:	5-Bromo-4-chloro-3-indolyl-phosphate
cDNA:	Complementary DNA
GST:	Glutathione-S-transferase
MW:	Molecular weight
NBT:	<i>p</i> -Nitro blue tetrazolium chloride
PVDF:	Polyvinylidene difluoride

membrane protein may be an important allergen of *P citrinum*. IgE cross-reactivity between the peroxisomal membrane proteins of *P citrinum* and *A fumigatus* was also detected.

METHODS**Serum samples**

Serum samples were collected from adult patients with bronchial asthma and attending the allergy clinics at the Veterans General Hospital, Taipei. These patients have clinical histories consistent with intermittent and reversible airway obstruction. They were diagnosed according to the statement provided by the American Thoracic Society.¹⁶ After collection, sera were stored in aliquots at -70°C . The presence of IgE antibodies in these samples against components of *P citrinum* was analyzed by immunoblotting. The occurrence of IgE reactivity to components of *P notatum* was determined by the Pharmacia CAP system (RAST FEIA, Pharmacia AB, Uppsala, Sweden). The results were expressed as radioallergosorbent test class ranging from 0 to 6 according to the manufacturer's suggestions.

Preparation of allergenic extracts

P citrinum strain 52-5 was isolated from the air in the Taipei area.⁵ *A fumigatus* strain CCRC33476 was a kind gift from Professor Shean-Shong Tzean of the National Taiwan University and has been verified by the Food Industry Research and Development Institute, Hsinchu, Taiwan. Fungal species were grown with shaking for 3 days at 30°C . The liquid medium contains Czapek concentrate⁴ (10 mL/L), potassium phosphate (1.0 g/L), yeast extract (Difco, Detroit, Mich; 5g/L), and sucrose (30 g/L). The collected fungal mats comprise both spores and mycelia. They were ground under liquid nitrogen with mortar and pestle and then resuspended in a PBS (150 mmol/L, pH 7.2). The suspension was loaded into an MSK cell homogenizer (B. Braun, Melsungen, Germany) and subjected to 8 cycles of intermittent 1-minute homogenization and cooling on ice. The cell debris was removed by centrifugation and the supernatant was dialyzed extensively against phosphate buffer (10 mmol/L, pH 7.2) for 24 hours at 4°C . The dialysate was sterilized by filtration (0.2 μm , Sartorius, Hayward, Calif) and stored in aliquots at -20°C . The protein content of the extracts was determined by the dye-binding method of Bio-Rad (Richmond, Calif).

In the current study allergenic extracts of *A fumigatus* (GM3-50B-3A5) and *P notatum* (GM19-45B-3A6) obtained from Greer Laboratories (Lenoir, NC) were also used.

Cloning and molecular techniques

Total RNA was extracted from a 2-day-old fungal mat culture of *P citrinum*¹² for the construction of a lambda-Uni-ZAP XR-based cDNA library (custom-made by Stratagene, La Jolla, Calif). The cDNA clones were screened by plaque immunoassay of the isopropyl- β -D-thiogalactopyranoside library with use of a serum mixture from 4 asthmatic patients.¹² IgE reactivity was detected with alkaline phosphatase-labeled monoclonal antihuman IgE (Pharmin-

gen, San Diego, Calif) and developed with the alkaline phosphate conjugate substrates kit (containing 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine [BCIP] and *p*-nitro blue tetrazolium chloride [NBT] solutions, Bio-Rad, catalog No. 170-6432). An IgE-binding cDNA clone (PCE2) was obtained and its insert was excised from the ZAP vector by superinfecting the *E coli* SOLR strain with helper phage (ExAssist helper phage, M13, Stratagene). The resulting phagemid was replicated in *E coli* and the nucleotide sequence of the insert was determined by dideoxy sequencing.¹²

Preparation of recombinant proteins

PCE2 was expressed as a glutathione-S-transferase (GST) fusion protein.¹² The cDNA insert derived from clone PCE2 was amplified through PCR. The forward primer used in the reaction contains the enterokinase and the *Bam*HI-recognizing sites in addition to the 5' sequence of the cDNA insert (5'-GCGGATCCGATGACGATGACAAAATGTCTCTTAAGGCCGGC-3'). The reverse primer contains an *Eco*RI restriction site, the stop codon (TAA), and the sequence covering the 3' end of the cDNA insert (5'-CGGAATTCTTACAGCTGCTTAAGGACGTG-3'). The PCR product was isolated by agarose gel electrophoresis and purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). It was then digested with *Bam*HI and *Eco*RI restriction enzymes and ligated into the pGEX-2T¹⁷ vector after repurification. The resulting vector was transformed into *E coli* DH5 α . The recombinant fusion protein was purified with a glutathione agarose affinity column.¹⁷

The cDNA of Asp f 3 was obtained through PCR. Total RNA was extracted from a 4-day-old *A fumigatus* (strain CCRC33476) fungal mat. The forward (5'-GCGGATCCGATGACGATGACAAAATGTCTGGACTCAAGGCCG-3') and reverse (5'-CGGAATTCTTACAGGTGCTTGAGGACGGT-3') primers used in the reaction contain nucleotide sequences covering the termini of the Asp f 3 gene¹⁵ and restriction sites as described above. After digestion with *Bam*HI and *Eco*RI, the PCR product was inserted into a similarly restricted pGEX-2T vector and transformed into *E coli* DH5 α . The Asp f 3-GST fusion protein was affinity purified with a glutathione agarose column.¹⁷ The reactivity of the recombinant proteins with IgE or mAbs was analyzed by SDS-PAGE immunoblot.

Preparation of mAbs

mAbs against recombinant PCE2 were prepared essentially as described.⁷ BALB/c mice were immunized intraperitoneally with 20 μg of recombinant PCE2 in CFA (Difco Laboratories, Detroit, Mich). Intraperitoneal booster immunizations were then performed with 20 μg of the same antigen prepared in incomplete Freund's adjuvant (Difco) 2 and 3 weeks after the first immunization. Three days before fusion mice were injected with another 20 μg of antigen in phosphate buffer (10 mmol/L, pH 7.2) through the tail vein. Spleen cells from immunized animals were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol¹⁸ (molecular weight [MW] 1500 d; Merck, Boehringer Mannheim, Mannheim, Germany). Antibody-producing hybrid cells were screened by ELISA and SDS-PAGE immunoblotting with use of GST and PCE2-GST fusion protein as antigens. Hybridomas producing mAbs against PCE2 were subcloned at least twice by limiting dilutions and then further expanded. Class-specific antisera (Southern Biotechnology Associates, Birmingham, Ala) were used in ELISA to determine the isotype of these mAbs.

ELISA

The antigens were suspended in 0.05 mol/L carbonate buffer, pH 9.6, and coated onto the wells of polyvinyl microtiter plates. After being blocked with 1% skimmed milk, the wells were incubated with culture supernatant from hybridoma cells for 1 hour at 37°C . The wells were then washed with PBS containing 0.05% Tween 20. An affinity-purified horseradish peroxidase-labeled goat antimouse IgG

(H+L) (Jackson ImmunoResearch Laboratories, West Grove, Pa) was added to the wells and incubated for 1 hour at 37°C. After being washed, the presence of enzyme-labeled antibodies in the wells was detected with 2,2 di-(3-ethylbenzthiazoline-6-sulfonic acid) and hydrogen peroxide according to the manufacturer's protocol (Bio-Rad, peroxidase substrate kit 172-1064). The color change was detected by measuring the absorbance at 415 nm on a SPECTRA-max 190 instrument (Molecular Devices, Sunnyvale, Calif). The culture supernatant from hybridoma cells producing mAb against group 7 house dust mite allergen¹⁹ was used as negative control.

The presence of IgE antibodies in patient serum was detected as above with slight modifications. The serum samples were diluted 5-fold with PBS containing 1% skimmed milk before incubation with the antigens in the microtiter plates for 16 hours at 4°C. Antibodies that were bound onto the antigens coated on the microtiter plates were detected with alkaline phosphatase-labeled monoclonal antihuman IgE (Pharmingen). The color reaction was measured at 405 nm after it was developed with a 1 mg/mL solution of *p*-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) for 30 minutes at 37°C. An average of 2 readings from separate determinations was calculated. Two cord serum samples and sera from 2 mite-allergic patients were used as negative controls. ELISA index values of serum samples tested were calculated as the mean absorbance at 405 nm divided by the average absorbance value of the 4 negative control sera.

SDS-PAGE and immunoblotting

Recombinant proteins or crude allergenic extracts were separated by SDS-PAGE and transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (0.45 µm, Millipore, Bedford, Mass).²⁰ The membrane was blocked with 1% skim milk. It was then incubated with culture supernatant from hybridoma cells at room temperature for 1 hour or with diluted human serum samples (1:5 dilution in TRIS-buffered saline solution, pH 7.5, 0.05% Tween 20 and 1% skim milk) for 16 hours at 4°C. After being washed, the mAbs and the human IgE antibodies on the blot were detected with horseradish peroxidase-labeled and alkaline phosphatase-conjugated secondary antibodies, respectively, as in ELISA. For the visualization of mAbs, the blot was developed in a substrate solution of acetate buffer (50 mmol/L, pH 5.0) containing 1 mmol/L 3-amino-9-ethyl-carbazole (Sigma) and 0.015% (vol/vol) hydrogen peroxide for about 5 minutes. The human IgE on the blot was developed with a substrate solution of carbonate buffer (0.1 mol/L sodium bicarbonate, pH 9.8, and 1.0 mmol/L magnesium chloride) containing 0.35 mol/L BCIP and 0.37 mol/L NBT for about 30 minutes. The same set of negative controls for ELISA was used in the immunoblotting experiments.

RESULTS

cDNA cloning and sequencing

Four asthmatic sera with positive IgE reactivity to *P notatum* (2 to 3 classes) in CAP test were selected for screening a *P citrinum* cDNA library. A clone designated as PCE2 was selected by its strong IgE-binding activity by plaque immunoassay. The nucleotide (GenBank accession No. AF144753) and the deduced amino acid sequences of PCE2 are shown in Fig 1. The typical "AATAAA" polyadenylation signal for mammalian genes was omitted from PCE2. The 694-bp cDNA insert encoded a 167 amino acids polypeptide with a calculated molecular mass of 18,240 d. Two cysteines and 1 putative N-glycosylation site were identified on the protein (Fig 1). Protein database search with the deduced amino acid sequence revealed that PCE2 has 83% (138

residues) identity with the 18.5-kd peroxisomal membrane protein allergen (Asp f 3) of *A fumigatus*¹⁵ (Fig 2).

Characterization of recombinant PCE2 and Asp f 3

PCE2 and Asp f 3 were expressed as GST-fusion proteins (see Methods). The resultant PCE2 has an apparent MW of about 44 kd (data not shown) and a yield of approximately 2.5 mg/L culture. The IgE reactivity of these recombinant proteins was tested with sera from 28 asthmatic patients with positive IgE immunoblot reactivity to *P citrinum* or positive CAP reactivity to *P notatum* (Fig 3). Thirteen (46%) (serum No. 1-13 in Fig 3) of these 28 asthmatic sera demonstrated IgE binding to PCE2 (Fig 4, B). Immunoblot reactivity to GST alone was not detected (data not shown). Results similar to those shown in Fig 4 were observed when IgE binding to PCE2 was analyzed by dot immunoassay (data not shown). Data in Fig 4 also demonstrated that 11 of the 13 positive serum samples to PCE2 have IgE binding to recombinant Asp f 3. Serum No. 3, 5, 8, and 12 demonstrated relatively strong IgE binding to both PCE2 and Asp f 3. Weak IgE binding to both recombinant proteins was observed for serum No. 6 and 9. Serum No. 4 and 10 demonstrated weak and negative IgE binding to PCE2 and Asp f 3, respectively. Serum No. 1 and 7 have relatively stronger IgE binding to PCE2 than that to Asp f 3, whereas opposite results were observed for serum No. 2, 11, and 13 (Fig 4). A cord serum was included as a negative control for these experiments (Fig 4, serum No. 14).

The IgE reactivity of PCE2 and Asp f 3 was further analyzed by ELISA. The ELISA index values of IgE binding to PCE2 for serum No. 1 to 13 in Fig 4 are 1.4, 1.2, 6.1, 1.3, 16.5, 1.8, 4.2, 8.4, 1.2, 1.4, 6.0, 6.3, and 1.1, respectively. The ELISA indexes of IgE binding to recombinant Asp f 3 are 1.1, 1.1, 4.2, 1.0, 16.2, 1.3, 1.6, 9.9, 1.1, 1.0, 1.6, 6.8, and 1.5, respectively, for these 13 serum samples.

The presence of IgE cross reactivity between PCE2 and Asp f 3 was analyzed by immunoblot inhibition with use of a mixture of serum samples (serum No. 1, 3, 5, 7, 8, 11, and 12 in Figs 3 and 4). The results are presented in Fig 5 for PCE2 (A) and Asp f 3 (B). The intensities of the bands were analyzed in Gel Doc 2000 equipped with Quantity one 4.0.3 software (Bio-Rad Laboratories, Hercules, Calif). The serum mixture was preincubated with either 1 or 5 µg of PCE2 (strips 3 and 4) and Asp f 3 (strips 5 and 6) before immunoblotting. For PCE2, IgE binding to this protein was inhibited by 94%, 94%, 78%, and 83%, respectively, after the serum mixture was preincubation with 1 and 5 µg of PCE2 and 1 and 5 µg of Asp f 3. IgE binding to Asp f 3 was inhibited by 84%, 90%, 95%, and 95%, respectively, with the same set of inhibitors. Inhibition of IgE immunoblotting was not detected when 1 (strip 7) and 5 µg (strip 8) of BSA (Pierce, Rockford, Ill) were used as inhibitors. In addition, IgE immunoblot reactivity to recombinant PCE2 and Asp f 3 was not inhibited when 5 µg of a *P citrinum* allergen-GST fusion protein¹² (heat-shock protein 70) was used as an inhibitor (data not shown).

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ttcattccacatcactcccgctttaatacaac ATG TCT CTT AAG GCC GGC 49
Met Ser Leu Lys Ala Gly
GAC AGC TTC CCC GAG GGT GTT ACC TTC TCC TAC ATC CCC TGG 91
Asp Ser Phe Pro Glu Gly Val Thr Phe Ser Tyr Ile Pro Trp
GCT GAG GAT GCT AGC GAG ATC ACC TCT TGT GGT ATC CCC ATC 133
Ala Glu Asp Ala Ser Glu Ile Thr Ser Cys Gly Ile Pro Ile
AAC TAC AAC GCC TCC AAG GAG TTC GCC AAC AAG AAG GTC GTT 175
Asn Tyr Asn Ala Ser Lys Glu Phe Ala Asn Lys Lys Val Val
CTC TTC GCT CTG CCC GGT GCT TTC ACC CCC GTC TGC TCC GCC 217
Leu Phe Ala Leu Pro Gly Ala Phe Thr Pro Val Cys Ser Ala
AAC CAC GTC CCC GAG TAC ATC CAG AAG CTC CCT GAG CTC CGC 259
Asn His Val Pro Glu Tyr Ile Gln Lys Leu Pro Glu Leu Arg
GCC AAG GGT GTC GAC CAG GTC GCC GTT CTG GCC TAC AAC GAT 301
Ala Lys Gly Val Asp Gln Val Ala Val Leu Ala Tyr Asn Asp
GCC TAC GTC ATG AGC GCC TGG GGT AAG GCC AAC GGT GTC ACC 343
Ala Tyr Val Met Ser Ala Trp Gly Lys Ala Asn Gly Val Thr
GCC GAC GAC ATT CTC TTC CTG TCT GAC CCC GAG GCT AAG TTC 385
Gly Asp Asp Ile Leu Phe Leu Ser Asp Pro Glu Ala Lys Phe
TCC AAG TCC ATC GGC TGG GCT GAT GAG GAG GGC CGT ACC TAC 427
Ser Lys Ser Ile Gly Trp Ala Asp Glu Glu Gly Arg Thr Tyr
CGT TAC GTC CTG GTC ATC GAC AAC GGC AAG ATC ATC TAC GCC 469
Arg Tyr Val Leu Val Ile Asp Asn Gly Lys Ile Ile Tyr Ala
GCC AAG GAG GCT GCT AAG AAC TCT CTG GAG CTC TCC CGC GCC 511
Ala Lys Glu Ala Ala Lys Asn Ser Leu Glu Leu Ser Arg Ala
GAC CAC GTC CTT AAG CAG CTG TAA gcgctagtgtgggacacaattg 558
Asp His Val Leu Lys Gln Leu *
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ggcctctgatgcttctgcttctgtctcatccaatagaggatcacatcctaattg 668
aagctaagaaaaaaaaaaaaaaaaa 694

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FIG 1. Nucleotide and inferred amino acid sequences of cDNA clone PCE2. Numbers to the right are nucleotide positions. Putative glycosylation site (Asn-Ala-Ser) and TAA stop codon are underlined.

PCE2	MS-LKAGDSFPPEGVTFSYIPWAEDASEITSCGIPINYNAS	39
Asp f3	..G.....SD.V.....S..KG...A.....	40
PCE2	KEFANKKVVFLFALPGAFTPVCSANHVPEYIQKLPELRAKG	79
Asp f3	..W.D...I.....R.....E....I....	80
PCE2	VDQVAVLAYNDAYVMSAWKGKANGVTGDDILFLSDPEAKFS	119
Asp f3	..V.....Q.....D.R..	120
PCE2	KSIGWADEEGRTYRYVLVDINGKIIYAAKEAANKNSLELSR	159
Asp f3	K..A....H...T...L.P...H...F.S	160
PCE2	ADHVLKQL	167
Asp f3	.ET...H.	168

FIG 2. Comparison of deduced amino acid sequence of PCE2 and peroxisomal membrane protein allergen (Asp f 3) of *A fumigatus*. Residues identical to PCE2 sequence are indicated by dot. Dashes, Spaces that have been introduced to optimize alignment. Putative N-glycosylation site is underlined.

Characterization of mAbs to PCE2

After dilution was limited, 6 stable hybridoma clones (PCE2-3, PCE2-7, PCE2-11, PCE2-15, PCE2-19, PCE2-21) were selected. mAbs from these hybridomas are not reactive toward the GST portion of the fusion protein by ELISA (Table I) or immunoblotting (data not shown).

They all have positive immunoblot reactivity to the recombinant PCE2 protein (Fig 6, *I-B*, strips 1-6). Among these mAbs, 4 of them (PCE2-3, PCE2-7, PCE2-11, and PCE2-15) have positive immunoblot reactivity against recombinant Asp f 3 (Fig 6, *I-D*, strips 1-4). An mAb (WH20) raised against Der p 7 (the group 7 house dust mite allergen) does not react with PCE2 or Asp f 3 by immunoblotting (Fig 6, strips 7 in *I-B* and *I-D*, respectively). The isotypes of these mAbs were determined with ELISA with use of class-specific antibodies. PCE2-7 belongs to IgG1, λ . The other 5 mAbs are IgG1, κ .

Characterization of the natural protein corresponding to clone PCE2

mAbs generated above were used to identify the natural form of PCE2 in the crude allergenic extract. Results from immunoblotting indicate that these mAbs react with an 18-kd component in the *P citrinum* extract (Fig 6, strips 1-6 in *II-B*). In addition, mAbs PCE2-3, PCE2-7, PCE2-11, and PCE2-15 can also react with an 18-kd component in the crude extract of *A fumigatus* (Fig 6, strips 1-4 in *II-D*). These mAbs do not have immunoblot reactivity toward 2 commercially available *P notatum* (GM19-45B-3A6) and *A fumigatus* (GM3-50B-3A5) extracts obtained from Greer Lab (data not shown). IgE antibodies in serum No. 8 of Figs 3 and 4 reacted with the same 18-kd components in the *P citrinum* and *A fumigatus* extracts prepared in our laboratory (strips 8 of Fig 6, *II-B* and *II-D*, respectively). For negative controls, mAb WH20, and a cord serum sample were included in the immunoblotting experiments (Fig 6, strips 7 and 9 in *II-B* and *II-D*, respectively).

DISCUSSION

Molecular cloning of allergen genes with sera from asthmatic patients has been used successfully to characterize allergens. We have previously identified allergens from house dust mite¹⁹ and *P citrinum*¹² through such an approach. We report here the isolation of another IgE-binding clone, PCE2, from a *P citrinum* cDNA library. PCE2 has 83% amino acid sequence identity with that of the peroxisomal membrane protein allergen (Asp f 3)¹⁵ of *A fumigatus*. The result suggests that PCE2 may also encode a peroxisomal membrane protein from *P citrinum*. The encoded protein has been designated as Pen c 3 as recommended by the Allergen Nomenclature Subcommittee.

The frequency of IgE binding to recombinant Pen c 3 was about 46% (13/28) when immunoblotting analysis was carried out with sera from 28 asthmatic patients sensitized to *Penicillium*. Eleven of the 13 Pen c 3-positive sera also have positive IgE binding to recombinant Asp f 3 (Fig 4). We also analyzed the interaction between IgE and these 2 allergens by ELISA. A good correlation was observed between the ELISA IgE index value and the intensity of IgE immunoblot reactivity of the serum samples. Some asthmatic sera tested have shown strong IgE binding to Pen c 3-GST fusion protein. The results suggest that the peroxisomal protein may be an important allergen in these patients.

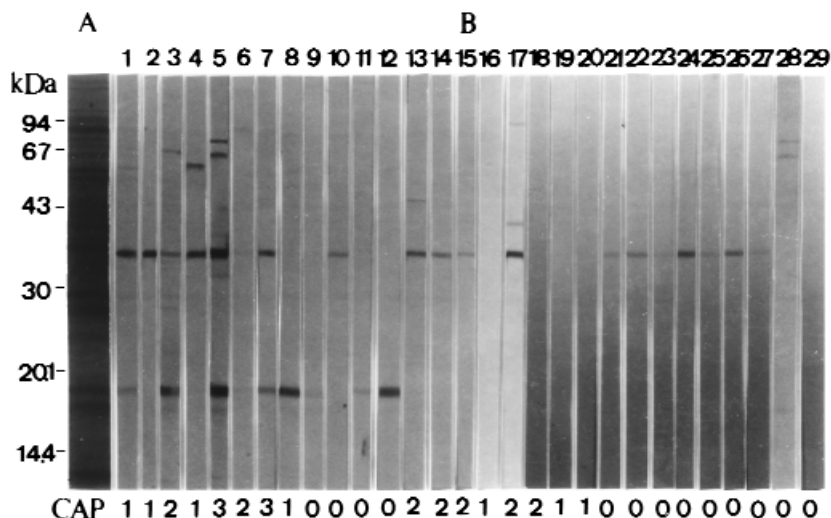


FIG 3. IgE immunoblot reactivity to components of *P citrinum* in asthmatic serum samples. **A**, Coomassie blue-stained protein profile of *P citrinum* extract on PVDF membrane. Immunoblots generated from 29 asthmatic serum samples are presented in **B**. Sera 16, 18, 19, and 20 have negative IgE immunoblotting. Serum 29 was a negative control obtained from mite-sensitive asthmatic patient. Molecular mass standards (Pharmacia, Uppsala, Sweden) used were phosphorylase b (94 kd), BSA (67 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), soybean trypsin inhibitor (20.1 kd), and α -lactalbumin (14.4 kd). Results of commercial Pharmacia CAP test to *P notatum* of individual serum sample tested are expressed as RAST classes and are shown in **B**.

Asp f 3 has been identified as a major allergen of *A fumigatus*. Serum IgE reactivity to recombinant Asp f 3 could be detected in 72% of 89 individuals sensitized to *A fumigatus*.¹⁵ According to Hemmann et al,²¹ all 11 patients with allergic bronchopulmonary aspergillosis (ABPA) and 5 of 8 *A fumigatus*-sensitized asthmatic patients without ABPA exhibited a type I skin reaction to Asp f 3. Asp f 3 also shares IgE binding epitopes with two *Candida boidinii* peroxisomal proteins (PMPA and PMPB).^{15,22} IgE cross-reactivity between Pen c 3 and Asp f 3 was detected in the current study by immunoblot inhibition. Thus IgE cross-reactivity between Pen c 3 and the 2 *C boidinii* peroxisomal proteins might also be present.

Results from immunoblot inhibition with use of a mixture of positive serum samples demonstrated a high degree of IgE cross-reactivity between Pen c 3 and Asp f 3. Recently, an IgE cross-reactivity among the 34/32-kd alkaline/vacuolar serine proteinases that are the major allergens of prevalent *Penicillium* and *Aspergillus* species, has been detected.⁸⁻¹¹ We presented data here that 9 (serum No. 1-8 and 13 in Fig 3) of the 13 Pen c 3-positive serum samples have also positive IgE reactivity in the Pharmacia CAP test against *P notatum*. Recently, 2 novel allergens (Mal f 2 and Mal f 3) from the lipophilic yeast *Malassezia furfur* were identified.²³ Database search revealed that sequence homology exists among Mal f 2, Mal f 3, PMPA, PMPB, and Asp f 3.²³ The presence and the significance of IgE cross-reactivity among the peroxisomal membrane protein allergens in *Penicillium* and *Aspergillus* species and in yeasts such as *C boidinii* and *M furfur* needs further study.

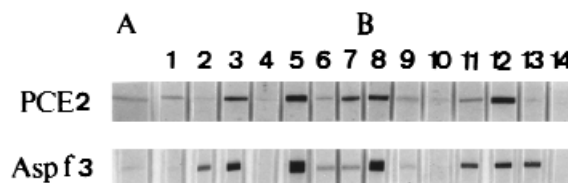


FIG 4. Immunoblot reactivity of PCE2-GST and Asp f 3-GST fusion proteins to IgE antibodies in asthmatic serum samples. **A**, Coomassie blue-stained SDS-PAGE protein profiles of affinity-purified PCE2-GST and Asp f 3-GST fusion proteins. Strips in **B** show immunoblot reactivity of recombinant proteins to IgE antibodies in sera from 13 asthmatic patients (serum No. 1-13 in Fig 3). Strip No. 14 represents result of cord serum sample.

TABLE I. Reactivity of 6 mAbs against recombinant PCE2 analyzed by ELISA

mAbs	ELISA activity (A415)	
	PCE2-GST fusion protein	GST
PCE2-3	3.88	0.10
PCE2-7	3.67	0.10
PCE2-11	3.97	0.10
PCE2-15	3.95	0.10
PCE2-19	3.94	0.11
PCE2-21	3.94	0.12
WH20*	0.12	0.11

*WH20 represents mAb against group 7 mite allergen (Der p 7¹⁹).

In this study 6 mAbs against Pen c 3 were generated. Among them, PCE2-3, PCE2-7, PCE2-11, and PCE2-15 cross-react with Asp f 3. These mAbs react with an 18-kd component in the *P citrinum* extract. Of the 4 mAbs that

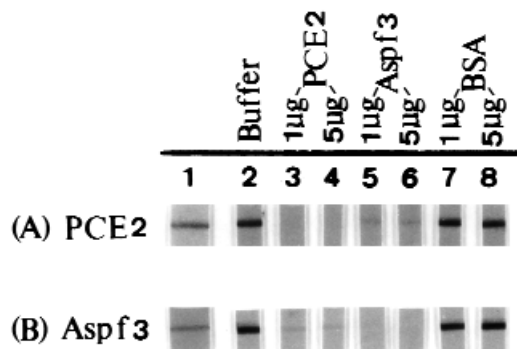


FIG 5. Inhibition of IgE immunoblot reactivity of a mixture of serum samples (serum No. 1, 3, 5, 7, 8, 11, and 12 in Figs 3 and 4) that has positive reactivity to PCE2 (**A**) and Asp f 3 (**B**). Strips 1 in both panels show Coomassie blue-stained protein profiles of recombinant PCE2 and Asp f 3 on PVDF membranes. Strips 2 show IgE immunoblot reactivity of serum mixture against individual recombinant allergen. Strips 3 to 8 represent results of immunoblot obtained by incubating PVDF blots with same serum mixture that has been mixed and preincubated with 1 and 5 μ g, respectively, of PCE2 (strips 3 and 4), Asp f 3 (strips 5 and 6), and BSA (strips 7 and 8).

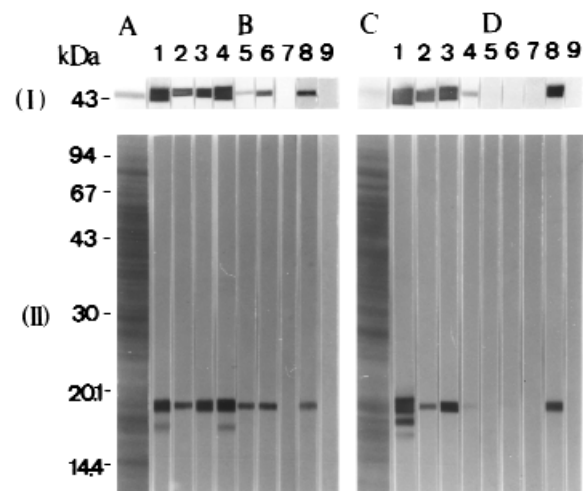


FIG 6. Immunoblot reactivity of 6 mAbs to recombinant PCE2 (**I-B**), Asp f 3 (**I-D**), crude fungal extracts of *P citrinum* (**II-B**) and *A fumigatus* (**II-D**). **I-A**, **I-C**, **II-A**, and **II-C**, Coomassie blue-stained SDS-PAGE protein profiles of the PCE2-GST, Asp f 3-GST, *P citrinum*, and *A fumigatus* fungal extracts, respectively. Strips 1-6 in **B** and **D** summarize immunoblot reactivity of mAbs PCE2-3, PCE2-7, PCE2-11, PCE2-15, PCE2-19, and PCE2-21 to recombinant proteins (**I**) and crude fungal extracts (**II**), respectively. Culture supernatant containing mAb WH20 against house dust mite allergen Der p 7 shows negative result (strips 7). Strips 8 and 9 in all panels show immunoblot results with serum No. 8 in Figs 3 and 4 and a cord serum sample, respectively.

cross-react with Asp f 3, they all can react with an 18-kd component in the *A fumigatus* extract. IgE antibodies in asthmatic serum samples that demonstrated IgE binding to PCE2 and Asp f 3 also bound onto these 18-kd components. Results obtained suggest that the 18-kd components in both fungal extracts may be the corresponding peroxisomal membrane protein allergens in *P citrinum* and *A fumigatus*.

These two 18-kd proteins were electrophoretically separated and subjected to peptide sequence analysis. However, the sequencer did not yield any signal for the *P citrinum* protein, even after the protein has been treated with trifluoroacetic acid vapor at 65°C for 30 minutes. This treatment has been used to remove the acetyl group from N-terminal serine and threonine residues.²⁴ Therefore the 18-kd *P citrinum* protein was either presented in an amount below the sensitivity of the sequencer or has an amino acid other than acetylated serine or threonine at the N-terminus. The identity of this protein was confirmed by partial internal sequence analysis. The protein was treated with endoproteinase Glu-C (Promega, Madison, Wis) and the digests were separated by gel electrophoresis. A peptide with a sequence of YIQKLPELRAK that encompasses the region between residues 68-78 of PCE2 was recovered. The 18-kd component of *A fumigatus* was pretreated with trifluoroacetic acid before being inserted into the sequencer. We obtained a sequence of SGLKAGDS, which is identical to that of residues 2-9 of Asp f 3 (Fig 2). Thus these two 18-kd components from *P citrinum* and *A fumigatus* correspond to PCE2 and Asp f 3, respectively.

The fungal extracts used in the identification of the natural allergens were prepared from fungal mats that were grown at 30°C with shaking. In addition, the mats were ground under liquid nitrogen and further homogenized before extraction. According to our experience, we could not detect the peroxisomal membrane protein allergens in extracts prepared from fungal mats grown at 25°C without shaking and homogenization. We would also like to point out that our 6 mAbs against Pen c 3 cannot react with any component in an *A fumigatus* extract obtained from Greer Labs with SDS-PAGE immunoblot (data not shown). The result suggests that Asp f 3 may not be present in the diagnostic fungal extract. mAbs PCE2-3 and PCE2-15 also have immunoblot reactivity to a 16-kd component in the fungal extracts of *P citrinum* and *A fumigatus* prepared from fungal mats grown at 25°C without shaking and homogenization (data not shown). The 16-kd component in the *A fumigatus* extract has been identified through Edman degradation of the isolated protein. It has an N-terminal amino acid sequence of AYAQWVTVHLINSMS identical to that of the *A fumigatus* Asp hemolysin.²⁵ The reason for the binding of Pen c 3-specific mAb to Asp hemolysin in the crude extract from *A fumigatus* might be the result of sequence homology. Asp f 3 has a sequence of NASKEW for residues 38-43. The corresponding sequence (residues 28-33) on the *A fumigatus* hemolysin is NASLSW. Whether PCE2-3 can recognize NASKEW as its epitope remains to be investigated.

Results from studies on Asp f 3 showed that specific serum IgE titers have reliable predictive value for immediate type I skin reactions after challenge with recombinant Asp f 3.²¹ The authors suggest the possibility of relying on serologic data obtained with recombinant allergens to diagnose sensitization to *A fumigatus*.²¹ An obvious hindrance to reliable diagnosis of fungal allergy

is the lack of well-defined allergen preparations. The production of recombinant allergens and mAbs is of particular importance for organisms whose extracts are difficult to standardize, such as fungi. The recombinant protein and mAbs prepared in the current study may provide useful tools in the preparation and standardization of diagnostic fungal reagents.

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