

Original article

Characterization of a novel *Candida albicans* 29 kDa IgE-binding protein – purification, cDNA isolation and heterologous expression of Cand a 3

Background: *Candida albicans* has been implicated in human allergic disorders. However, many of its immunoglobulin E (IgE)-reacting components have not yet been identified. The purpose of the present study is to characterize a novel 29 kDa IgE-binding protein from *C. albicans*.

Methods: The 29 kDa protein was partially purified and its tryptic digests subjected to mass spectrometric analysis. The cDNA encoding this protein was isolated and heterologously expressed in *Escherichia coli*. Monoclonal antibodies (MoAbs) were raised against the 29 kDa protein purified from *C. albicans* extracts.

Results: We isolated a 29 kDa IgE-reacting component from *C. albicans*. The protein was digested on-gel with trypsin and the masses of the resulting fragments were determined in a MALDI-TOF mass spectrometer. The data were searched against protein sequences deduced from the *C. albicans* genome. An open reading frame that possibly encodes the 29 kDa IgE-reacting component was identified. The cDNA corresponding to the open reading frame was isolated. It encodes a 236 residues protein that has 62% sequence identity to that of a hypothetical protein (YDR533c) from *Saccharomyces cerevisiae*. Conserved domain search suggests that the encoded protein belongs to the ThiJ/PfpI family. The cDNA isolated was inserted into a pQE-30 vector for protein expression in *Escherichia coli*. The recombinant protein can react with IgE antibodies in sera from asthmatic patients and two MoAbs that were generated against the purified native 29 kDa protein from *C. albicans*.

Conclusions: We identified and cloned a novel 29 kDa IgE-reacting component (Cand a 3) from *C. albicans*. The recombinant proteins produced from this clone and the MoAbs prepared may be useful in the standardization of diagnostic extracts. They are also instrumental in elucidating the role of *C. albicans* in clinical allergy.

H. Chou¹, M. F. Tam², C.-Y. Chang¹,
H.-Y. Lai¹, M.-H. Huang¹, C.-T. Chou³,
S.-S. Lee⁴, H.-D. Shen¹

¹Departments of Medical Research and Education and ³Internal Medicine, Veterans General Hospital-Taipei; ²Institute of Molecular Biology, Academia Sinica; ⁴Department of Internal Medicine, Cheng Hsin Rehabilitation Medical Center, Taipei, Taiwan

Key words: 29 kDa IgE-binding protein; *Candida albicans*; cDNA isolation; monoclonal antibodies (MoAbs); peptide mapping by mass spectrometry.

Horng-Der Shen PhD
Department of Medical Research and Education
Veterans General Hospital-Taipei
Shih-Pai,
Taipei,
Taiwan 11217

Accepted for publication 21 April 2003

Molds including yeasts are causative agents of human allergic disorders. *Candida albicans* has been proposed as one of the molds that play a role in bronchial asthma, allergic rhinitis, chronic urticaria and atopic dermatitis (1–8). *Candida albicans* is a representative allergen in routine diagnostic panels that detect the presence of immunoglobulin E (IgE) antibodies to allergenic yeasts. However, *C. albicans* is a common human commensal and its role as an allergen is still controversial. To better understand the allergic significance of *C. albicans*, it is essential to differentiate IgE-binding proteins of *C. albicans* and to prepare well-characterized and standardized allergenic extracts.

Immunoblotting results indicate that *C. albicans* has over 40 components that react with human IgE antibodies

(5, 9–14). The 46 kDa enolase is an important allergen of *C. albicans* (9, 10, 12, 15, 16) and its cDNA has been isolated. A 44 kDa acid protease (8, 17), a 40 kDa alcohol dehydrogenase (18), a 43 kDa phosphoglycerate kinase, a 37 kDa aldolase (12), and two components of 27 and 29 kDa (9, 11) have also been reported as allergens of *C. albicans*. Among these *Candida* proteins, the 29 kDa component is a major allergen with an IgE-binding frequency of 64% (11, 14). However, detailed information about this major *Candida* allergen is still lacking. In this study, we partially purified a 29 kDa IgE-binding protein from *C. albicans*. The cDNA encoding this protein was isolated and expressed in *Escherichia coli*. In addition, two monoclonal antibodies (MoAbs) against

this IgE-reacting *Candida* component were also generated. In addition to identify a new gene product of *C. albicans*, information at molecular level, recombinant proteins prepared and MoAbs generated will be useful in clarifying the allergenic significance of *C. albicans*.

Material and methods

Serum samples

Serum samples were collected from adult patients with bronchial asthma and attended the allergy clinics at Veterans General Hospital-Taipei. Their mean age was 53 with a range of 18–74 years. These asthmatic subjects had a clinical history of intermittent and reversible airway obstruction and were diagnosed according to the statement provided by the American Thoracic Society (19). The presence of IgE antibodies in these serum samples against *C. albicans* was determined by the Pharmacia CAP system (RAST FEIA; Pharmacia AB, Uppsala, Sweden). The results were expressed as RAST class ranging from 0 to 6 according to the suggestions of the manufacturer.

Crude preparations of *C. albicans*

Candida albicans strain CCRC21538 was obtained from the Food Industry Research & Development Institute, Hsinchu, Taiwan. Crude extracts of *C. albicans* were prepared essentially as described by Farley et al. (20). Briefly, the *C. albicans* strain was grown in a liquid medium containing glucose (15 g/l), (NH₄)₂SO₄ (1.0 g/l), KH₂PO₄ (2.0 g/l), CaCl₂·2H₂O (0.05 g/l), MgSO₄·7H₂O (0.05 g/l), biotin (25 µg/l), and peptone (1.0 g/l). Incubation was carried out with shaking at 28 °C for 18 h. The yeast culture was diluted (1 : 40) with the liquid medium and cultured under the same condition for another 18 h. The yeast cells were collected and immersed in chloroform for about 12 min and mixed with an equal volume of water. The suspension was adjusted to pH 7.5 and stored at 4°C for 24 h. After centrifugation (9000 g for 10 min), the supernatant was adjusted to pH 5.0 and incubated at 28°C for 15 h with constant stirring. The preparation was then subjected to centrifugation (9000 g for 10 min) and the clear supernatant was designated as the crude extracts.

Purification of the 29 kDa IgE-binding protein

The crude extracts of *C. albicans* were dialyzed against 50 mM Tris-HCl, pH 8.0, for 18 h at 4°C. The sample was loaded onto a DEAE cellulose column (DE SeptraSorb; Sepragen Corporation, Hayward, CA) that has been pre-equilibrated in the same buffer. The column was eluted with a step gradient of 0.1, 0.2 and 0.3 M NaCl. Fractions containing IgE-reacting proteins with a molecular mass of 29 kDa were pooled. The protein content of the partially purified preparation was determined by the dye-binding method of Bio-Rad (Richmond, CA).

Identification of the 29 kDa *C. albicans* protein

Proteins in the combine fractions from the DEAE cellulose column were further separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 29 kDa *C. albicans* protein was excised from the gel and digested with trypsin as outlined by Chern et al. (21). The resulting peptides were extracted from the gel slice accordingly and further desalted with a ZipTip pipette tip (Millipore, Bedford, MA) before MALDI-TOF mass spectrometric analysis.

The masses of the peptides were determined on a Bruker (Bruker-Daltonics, Bremen, Germany) REFLEX III time-of-flight mass spectrometer equipped with a SCOUT source and delayed extraction. The peptide ions were detected in positive ion reflector mode. The instrument was calibrated with angiotensin II (1045.53 amu), human adrenocorticotrophic hormone fragment 18–39 (2464.19 amu) and somatostatin 28 (3145.52 amu) as external standards. The samples were prepared with nitrocellulose and α-cyano-4-hydroxycinnamic acid as matrix (22). Peptide masses obtained were searched against the data bank of *C. albicans* at the Stanford Genome Technology Center using the Mascot program (23).

Isolation of the 29 kDa protein cDNA

The cDNA of the 29 kDa *C. albicans* protein was isolated with reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from mid-log-phase (A540, ~0.5) *C. albicans* cells grown with constant shaking at 25°C in a liquid synthetic medium (24) containing glucose (1.25%, wt/vol) as the carbon source. Cells were disrupted by sonification (six 1-min pulses) in the presence of a Trizol-reagent (Life Technologies, Inc., Grand Island, NY) and kept on ice between pulses. Total RNA was extracted according to the instructions provided by the manufacturer. The first-strand cDNA was synthesized with reverse transcriptase [Moloney murine leukemia virus reverse transcriptase (MMLV-RT); Stratagene, La Jolla, CA] and oligo-(dT) as primer. The primers (Table 1) were designed according to the sequence of orf6.1991.prot (nucleotides 3815–4525, 711 bp, 236 aa) within contig6–2034 (11561 bp) that is in the databank of *C. albicans*. Primer 273 contains the BamHI restriction site plus nucleotides that encode the putative N-terminal eight amino acid residues (VKVLLALT) of the 29 kDa protein (Fig. 2). Primer 275 contains a SalI restriction site and sequence that encompasses nucleotide 713–738 of the cDNA encoding the *C. albicans* 29 kDa protein (Fig. 2). The PCR product was gel purified, sub-cloned into the pGem-Teasy vector (Promega, Madison, WI) and transformed into *E. coli* JM109 competent cells. The plasmid DNA was purified and the nucleotide sequence of the cDNA insert was determined with an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA).

The initiation codon of the 29 kDa cDNA was confirmed by RT-PCR. The template cDNA in the reaction was synthesized with MMLV-RT and primer 294. Primer 294 was constructed according to the internal sequence (nucleotides 541–558 in Fig. 2) of the product obtained in the previous PCR reaction. The RT-PCR experiment was carried out with primers 293 and 284. The forward primer 293 was synthesized according to nucleotides 3788–3806 of the contig6–2034 (nucleotides –27 to –9 from the ATG codon in Fig. 2). The reverse primer 284 was constructed according to the

Table 1. Primers for polymerase chain reactions

Name	Nucleotide sequence in 5' to 3'-end orientation
Primer 273	5'-CGGGATCCGTCAAAGTTTACTCGCTCTTAC-3'
Primer 275	5'-ACGCGTCGACTTAATTACATTCAAAGCAGCAATTA-3'
Primer 294	5'-CATAATGTGCGGTAACACC-3'
Primer 293	5'-ACTTCCAATCAATAATAAC-3'
Primer 284	5'-TATTATAACAGTAGTAG-3'
AP	5'-GGCCACGCGTCGACTAGTACT-(dT)16-3'
Primer 282	5'-GGTGTTACCGACATTATG-3'
5BamHI-Eno-Ca	CGGGATCCTCTTACGCCACTAAAATC
3PstI-Eno-Ca	AACTGCAGTTACAATTGAGAAGCCCTTTTG

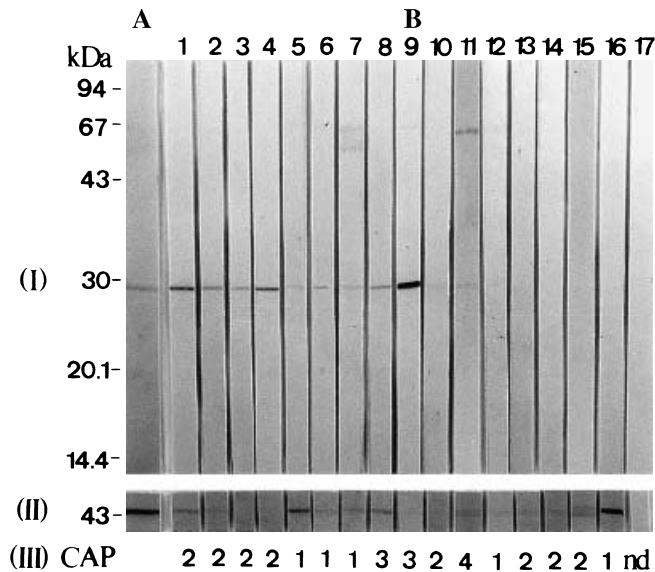


Figure 1. Immunoglobulin E immunoblot reactivity in sera from 16 asthmatic patients (panel B, strips one to 16) against the 29 kDa (part I) and the enolase (part II) proteins from *Candida albicans*. The Coomassie blue-stained protein profiles of the purified native 29 kDa protein and the recombinant enolase allergen from *C. albicans* on polyvinylidene difluoride blots were shown in panel A of part I and part II, respectively. Results using serum sample from a normal healthy control were shown in strip 17 of part I and part II. Results of Pharmacia CAP test of these 16 serum samples to *C. albicans* were shown in part III. nd, Not done.

internal sequence (nucleotides 392–409 in Fig. 2) of the cDNA fragment obtained in the previous PCR.

The template cDNA used for the 3'-rapid amplification of cDNA end (RACE) was synthesized by MMLV-RT using oligo-(dT) as primer. The primers used in the reaction were primers AP and 282. Primer AP (Table 1) contains the sequence of oligo-(dT). Primer 282 corresponds to nucleotides 541–558 of the cDNA obtained in the previous PCR. Products from the PCR and the RACE reaction were purified individually, sub-cloned, transformed and sequenced as described above.

Preparation of recombinant proteins

The 29 kDa protein of *C. albicans* was expressed as 6xHis-tagged protein according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). The coding region of the 29 kDa protein (excluding the N-terminal methionine) was amplified through PCR as described above. The PCR experiment was carried out with primers 273 and 275. The PCR product was separated by agarose gel electrophoresis and purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). It was then restricted with BamHI and SalI then ligated into the pQE-30 vector after re-purification. The resulting vector was transformed into *E. coli* JM109 and M15. The recombinant protein expressed in *E. coli* M15 was affinity-purified under denaturing condition with a Ni-NTA resin column (Qiagen) according to the manufacturer's instructions.

In this study, the enolase of *C. albicans* was also prepared as a 6xHis-tagged protein essentially as described above. The first-strand

cDNA of *C. albicans* was synthesized as mentioned above. The primers used in the PCR amplification of the cDNA for the *C. albicans* enolase were 5BamHI-Eno-Ca and 3PstI-Eno-Ca. The forward primer 5BamHI-Eno-Ca contains the BamHI restriction site in addition to the sequence encoding the N-terminal six amino acid residues (SYATKI) of the *C. albicans* enolase (25). The reverse primer 3PstI-Eno-Ca has a PstI restriction site, a stop codon and encodes the C-terminal six amino acid residues (nucleotide number 1302–1322) of the *C. albicans* enolase (25).

Development of MoAbs

Monoclonal antibodies against the 29 kDa protein were prepared essentially as previously described (26). The purified 29 kDa protein in complete Freund adjuvant (Difco Laboratories, Detroit, MI) was used in intra-peritoneal immunization of BALB/c mice. A booster immunization with the same antigen preparation in incomplete Freund adjuvant (IFA; Difco) was administered 2 weeks later. Two more booster immunizations with antigens in IFA were delivered 1 week apart. Three days before fusion, the mice received a tail vein injection of antigens prepared in phosphate buffer (10 mM, pH 7.2). Spleen cells from immunized animals were then fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (MW 1500 Da; Boehringer Mannheim, Mannheim, Germany). Hybrid cells secreting antibodies were screened by enzyme-linked immunosorbent assay (ELISA) and/or SDS-PAGE-immunoblot using the purified protein as antigen. Hybridomas producing antibodies against the purified protein were sub-cloned at least twice by limiting dilutions and further expanded. The isotype of the MoAbs was determined with ELISA and class-specific antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL).

Gel electrophoresis and immunoblotting

Purified native and recombinant 29 kDa proteins and crude *C. albicans* extracts were separated by SDS-PAGE. The proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μ m, Millipore, Bedford, MS). The membranes were blocked with 1% skim milk, then incubated either with diluted human serum samples (1 : 5 dilution in Tris-buffered saline, pH 7.5 with 0.05% Tween 20 and 1% skim milk) for 16 h at 4°C or with culture supernatant from hybridoma cells at room temperature for 1 h. The human IgE antibodies were probed with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (PharMingen, San Diego, CA) for 1 h at room temperature. The blot was then developed with a substrate solution of carbonate buffer (0.1 mol/l NaHCO₃, pH 9.8, and 1.0 mmol/l MgCl₂) containing 0.35 mmol/l 5-bromo-4-chloro-3-indolyl-phosphate and 0.37 mmol/l p-nitroblue tetrazolium chloride for about 30 min. Serum samples from healthy laboratory personnel were used as negative controls. Low molecular weight standards (Pharmacia, Uppsala, Sweden) were used to estimate the molecular mass of the protein components.

Monoclonal antibodies from hybridoma cells were detected with horseradish peroxidase labeled goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The MoAbs on the PVDF membrane were visualized by developing the blot in a substrate solution of acetate buffer (50 mmol/L, pH 5.0) containing 2 mmol/l 3-amino-9-ethyl-carbazole (Sigma, St Louis, MO) and 0.01% (v/v) hydrogen peroxide for about 5 min. A culture supernatant containing MoAb against house dust mite allergen Der p 7 (26) was used as a negative control.

ACTTCCAATCAATAATACTATTCAAA																27
ATG	GTC	AAA	GTT	TTA	CTC	GCT	CTT	ACT	TCT	TAT	AAC	GAA	ACT	TTT		72
M	V	K	V	L	L	A	L	T	S	Y	N					
<u>E</u>	<u>T</u>	<u>F</u>														
TAC	AGT	GAC	GGT	AAA	AAG	ACT	GGT	GTT	TTC	GTT	GAA	GCT	TTA			117
<u>Y</u>	<u>S</u>	<u>D</u>	<u>G</u>	<u>K</u>		K	<u>T</u>	<u>G</u>	<u>V</u>	<u>F</u>	<u>V</u>					<u>V</u>
<u>E</u>	<u>A</u>	<u>L</u>														
CAT	CCT	TTT	GAA	GTA	TTC	AGA	AAA	AAG	GGG	TAT	GAA	ATC	CAA	CTT		162
<u>H</u>	<u>P</u>	<u>F</u>	<u>E</u>	<u>V</u>	<u>F</u>	<u>R</u>		K	K	G	Y	E				
I	Q	L														
GCT	TCT	GAA	ACA	GGT	ACT	TTT	GGA	TGG	GAT	GAT	CAT	AGT	GTC	GTC		207
A	S	E	T	G	T	F	G	W	D	D	H					
S	V	V														
CCA	GAT	TTT	TTG	AAT	GGT	GAA	GAT	AAA	GAA	ATT	TTC	GAT	AAT	GTC		252
P	D	F	L	N	G	E	D	K	E	I	F					
D	N	V														
AAT	TCT	GAA	TTC	AAT	GTT	GCT	TTG	AAA	AAT	TTG	AAA	AAA	GCT	TCT		297
N	S	E	F	N	V	A	L	K	N	L	K					
K	A	S														
GAT	CTT	GAT	CCA	AAT	GAT	TAT	GAT	ATC	TTT	TTC	GGT	AGT	GCT	GGT		342
D	L	D	P	N	D	Y	D	I	F	F	G					
S	A	G														
CAT	GGT	ACT	TTA	TTT	GAT	TAT	CCA	CAT	GCT	AAA	GAT	TTA	CAA	AAA		387
H	G	T	L	F	D	Y	P	H*	A	K	D					
L	Q	K														
ATT	GCT	ACT	ACT	GTT	TAT	AAT	AAA	GGT	GGT	GTT	GTT	TCT	GCT	GTT		432
<u>I</u>	<u>A</u>	<u>T</u>	<u>T</u>	<u>V</u>	<u>Y</u>	<u>N*</u>	<u>K</u>	<u>G</u>	<u>G</u>	<u>V</u>						<u>V</u>
<u>S</u>	<u>A</u>	<u>V</u>														
TGT	CAT	GGT	CCT	GCC	ATT	TTT	GAA	AAT	TTG	AAC	GAT	CCA	AAA	ACT		477
<u>C</u>	<u>H</u>	<u>G</u>	<u>P</u>	<u>A</u>	<u>I</u>	<u>F</u>	<u>E</u>	<u>N</u>	<u>L</u>	<u>N</u>	<u>D</u>					
<u>P</u>	<u>K</u>	<u>T</u>														
GGT	GAA	CCA	TTA	ATT	AAA	GGT	AAA	AAA	ATC	ACT	GGA	TTC	ACT	GAT		522
<u>G</u>	<u>E</u>	<u>P</u>	<u>L</u>	<u>I</u>	<u>K</u>		G	K	K	<u>I</u>	<u>T</u>	<u>G</u>				
<u>F</u>	<u>T</u>	<u>D</u>														
ATT	GGA	GAA	GAT	ATT	TTA	GGT	GTT	ACC	GAC	ATT	ATG	AAG	AAA	GGT		567
<u>I</u>	<u>G</u>	<u>E</u>	<u>D</u>	<u>I</u>	<u>L</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>D</u>	<u>I</u>	<u>M</u>					
<u>K</u>	K	G														
AAT	TTG	TTG	ACC	ATT	AAA	CAA	GTT	GCT	GAA	AAG	GAA	GGT	GCT	ACT		612
N	L	L	T	I	K	Q	V	A	E	K	<u>E</u>					
<u>G</u>	<u>A</u>	<u>T</u>														
TAT	ATT	GAA	CCA	GAA	GGT	CCA	TGG	GAT	AAT	TTC	ACC	GTC	ACT	GAT		657
<u>Y</u>	<u>I</u>	<u>E</u>	<u>P</u>	<u>E</u>	<u>G</u>	<u>P</u>	<u>W</u>	<u>D</u>	<u>N</u>	<u>F</u>	<u>T</u>					
<u>V</u>	<u>T</u>	<u>D</u>														
GGT	AGA	ATT	GTT	ACC	GGT	GTC	AAC	CCA	CAA	TCT	GCT	GTC	AAA	ACT		702
<u>G</u>	<u>R</u>	<u>I</u>	<u>V</u>	<u>T</u>	<u>G</u>	<u>V</u>	<u>N</u>	<u>P</u>	<u>Q</u>	<u>S</u>	<u>A</u>					
<u>V</u>	<u>K</u>	T														
GCA	GAA	GAT	GTA	ATT	GCT	GCT	TTT	GAA	TGT	AAT	TAA	CTA	ACT	AAATAA		750
A	E	D	V	I	A	A	F	E	C	N	*					
CTAATAAATAGGGCATACAAAGGGTGTGGATTAGTTAGTAAAGAAATATAAAT																811
AATCTATCACAGTATTTTATTCTTGGTGTGGTAGTAGTTAAAAA																872

Figure 2. The nucleotide and the deduced amino acid sequences (GenBank accession number AY136739) of the 29 kDa immunoglobulin E-binding protein from *C. albicans*. Amino acid residues correspond to those depicted from mass spectrometric analyses are in bold letters and underlined. Two amino acid residues that are different from those deduced from the databank at the Stanford Genome Technology Center are marked with asterisks.

Results

Protein purification

A 29 kDa component that reacts with IgE antibodies in sera from asthmatic patients was partially purified from the crude extracts of *C. albicans* with a DEAE column. The 29 kDa IgE-reacting proteins were eluted from the anionic column with 0.1–0.2 M NaCl. Fractions containing this protein were pooled and analyzed with SDS-PAGE (panel A of part I, Fig. 1). The 29 kDa protein is the major component in the preparation. A panel of 16 serum samples from asthmatic patients was used to demonstrate the IgE-binding reactivity of the 29 kDa protein. All these samples showed positive Pharmacia

CAP test to *C. albicans* (Fig. 1, part III), and nine (56%) of them demonstrated IgE-binding to the 29 kDa protein (strips one to nine, panel B of part I, Fig. 1). Among these nine positive serum samples, sera no. 1, 5, 7 and 8 also have IgE-immunoblot reactivity to recombinant enolase (strips 1, 5, 7 and 8 of panel B, part II, Fig. 1). In addition, serum no. 9 showed strong IgE-binding to the 29 kDa protein but negative IgE reactivity to the enolase (strip 9, panel B of parts I and II, respectively). However, serum no. 16 has strong IgE-binding to enolase and negative reactivity to the 29 kDa protein (strip 16, panel B of parts II and I, respectively). Serum from a normal laboratory volunteer showed negative IgE reactivity to both antigens tested (strip 17 of panels I and II, part B).

Identification of the 29 kDa IgE-reacting protein

Components in the partially purified fractions recovered from the DEAE column were further separated by SDS-PAGE. The 29 kDa protein that has been shown to be positive for human IgE-binding was excised from the gel and subjected to trypsin digestion. The molecular masses of the resulting peptides were determined by MALDI-TOF mass spectrometry and the results were searched against the *C. albicans* genomic sequence deposited at the Stanford Genome Technology Center. We identified the 29 kDa protein as the gene product of orf6.1991.prot (nucleotides 3815–4525 of contig6-2034). The mass spectrometric data are summarized and compared with the deduced amino acid sequence derived from orf6.1991.prot in Table 2. We have detected eight peptide fragments encompassing 121 residues. With the exception of two fragments, the derivations between the observed and theoretical molecular masses of the peptides are well below 100 ppm. Therefore, although the peptides recovered represent only 51% of the deduced sequence, we can easily pick out orf6.1991.prot as the gene encoding the 29 kDa protein that we observed on the polyacrylamide gel. Tentatively, we conclude that the gene product from orf6.1991.prot is the protein that reacts with human IgE antibodies.

cDNA isolation and sequencing

An 872 bp cDNA (GenBank accession number AY136739) corresponding to the 29 kDa *C. albicans* protein was isolated. Its nucleotide and deduced amino acid sequences are shown in Fig. 2. The 'AATATA' and the 'AATAAT' sequences are located 44–49 and 38–43 nucleotides upstream of the poly A tail, respectively. They possibly represent the polyadenylation signal of the cDNA isolated. Translation of this transcript probably initiates at the first AUG codon as shown in Fig. 2. The open reading frame encodes a 236 amino acids polypeptide with a calculated molecular mass of 25 834 Da including the initiation methionine. The theoretical molecular mass is lower than that estimated by electrophoretic mobility. Amino acid residues corresponding to those depicted from

MALDI-TOF analysis are in bold type and underlined (Fig. 2). Between the sequence in the databank and that determined by us, we observed nucleotide substitution at three bases resulting in transposition of two amino acid residues (asterisked residues in Fig. 2). They are N and D deduced from the databank and H and N determined in our sequence, respectively. Two cysteines and two potential N-glycosylation sites were also identified on the 29 kDa *C. albicans* protein (Fig. 3).

Protein databank search with the deduced amino acid sequence revealed that the 29 kDa *C. albicans* protein has 62% sequence identity with that of the hypothetical protein YDR533c from the yeast *Saccharomyces cerevisiae* (accession no. S69588) (Fig. 3). One cysteine was conserved in both sequences. The *Saccharomyces* YDR533c protein has a potential N-glycosylation site that was found to be different from those in the 29 kDa *Candida* protein. Conserved domain search indicated that the 29 kDa protein has significant alignments to the ThiJ/PfpI family (pfam01965).

Characterization of the recombinant polypeptide

The 29 kDa protein was expressed as a His-tagged recombinant protein. The protein was recovered from inclusion bodies and purified under denaturing condition. The apparent molecular mass of the purified recombinant protein was 30 kDa (Fig. 4, strip 1 of panel B). It was recognized in immunoblotting by IgE antibodies in two serum samples from asthmatic patients (Fig. 4, strips 2 and 3 of panel B). Both sera also showed IgE-immunoblot reactivity to the purified native 29 kDa protein (Fig. 4, strips 2 and 3 of panel A). Enzyme color reactivity was not detected when a normal control serum sample was used (Fig. 4, strip 4 of panels A and B). The Allergen Nomenclature Sub-Committee has designated this IgE-reacting 29 kDa protein of *C. albicans* as Cand a 3 allergen.

Development of MoAbs

Two stable hybridoma clones (MoAb 1 and MoAb 23) were selected after limited dilution. Both antibodies have positive ELISA (data not shown) and immunoblot reactivity to the native and the recombinant 29 kDa protein (Fig. 4, strips 5 and 6 of panels A and B, respectively). Enzyme color reactivity was not detected when a culture supernatant with a MoAb against mite allergen Der p 7 was used (Fig. 4, strip 7 of panels A and B). The isotype of MoAbs 1 and 23 determined with ELISA are IgM, κ and IgG1, κ , respectively (data not shown).

Identification of the 29 kDa protein in *C. albicans* extracts

Monoclonal antibodies 1 and 23 reacted with a 29 kDa component in the *C. albicans* extracts prepared in our

Table 2. MALDI-TOF mass spectrometric analysis of the tryptic digests from the 29 kDa IgE-reacting protein

Residues	Sequence	Monoisotopic mass of molecular ion		
		Theoretical	Observed	Deviation (ppm)
150–156	TGEPLIK	757.40	758.39	13
121–128	IATTVYNK	909.50	909.46	44
213–224	IVTGVNPQSAVK	1212.69	1212.48	66
22–37	TGVFVVEALHPFEVFR	1846.98	1846.65	43
4–20	VLLALTSYNETFYSDGK	1920.95	1921.57	323
160–178	ITGFTDIGEDILGVTDIMK	2037.13	2037.59	226
129–149	GGVVSAVCHGPAIFENLNDPK	2124.05	2123.94	47
192–212	EGATYIEPEGPWDNFTVDGR	2354.05	2353.97	34

Cand a 3	MV--KVLALTSYNETFYSDGKKTGVPVVEALHPFEVFRKKGYEIQ ^{LASE}	48
YDR533c	MAPK.....DV.....A.....NT...E.F.VDFV..	50
Cand a 3	TGTFGWDDHSVVPDFLNGEDKEIFDNVNSEFNVALKNLKKASDLD ^{PNDYD}	98
YDR533c	..K...E..LAK.....Q.ETD.K.KD.D.. <u>KT</u> .AKI.TPKEVNAD..Q	100
Cand a 3	IFFGSAGHGTLFDYPHAKDLQKIATTVYNKGGVVS ^{AVCHG} PAIFENLNDP	148
YDR533c	...A.....K.....D..SEI.AN...A.....DG.T.K	150
Cand a 3	KTGEPLIKGKKITGFTDIGEDILGVTDIMKKGNLLTIKQVAEKEGATYIE	198
YDR533c	...R...E..S.....V..T....DS.L.AK..A.VED..K.Y..K.LA	200
Cand a 3	PEGPWDNFTVTDGRIVTGVNPQSAVKTAEDVIAAFECN	236
YDR533c	.V....DYSI....L.....A..HS..VRS.D.LKN	237

Figure 3. Alignment of the amino acid sequences of Cand a 3 (the 29 kDa protein from *Candida albicans*) and YDR533c (a hypothetical protein from *Saccharomyces cerevisiae*, accession no. S69588). Amino acid residues on YDR533c that are identical to those of Cand a 3 are presented with dots. The cysteine residues are in bold type letters. The potential N-glycosylation sites are underlined.

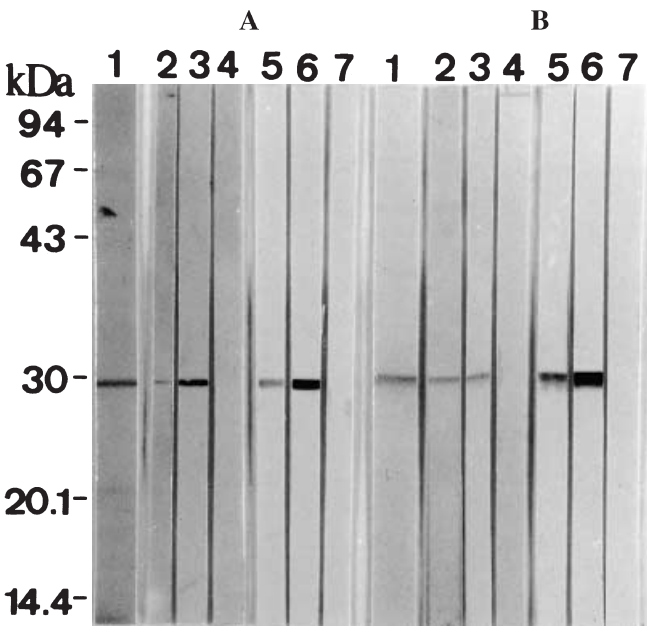


Figure 4. Reactivity of immunoglobulin E (IgE) antibodies and monoclonal antibodies (MoAbs) to the purified native (panel A) and the recombinant Cand a 3 (panel B). Strips 1 show the Coomassie blue-stained protein profiles of the native (panel A) and the recombinant (panel B) Cand a 3. Strips 2 and 3 represent IgE-immunoblot reactivity to the 29 kDa proteins in sera from two asthmatic patients. Strips 5 and 6 represent immunoblot reactivity of MoAbs 1 and 23 to Cand a 3, respectively. Strips 4 and 7 in both panels represent negative controls using serum obtained from a healthy laboratory volunteer and a culture supernatant with a MoAb against Der p 7 mite allergen, respectively.

laboratory as shown in strips 2 and 3 of Fig. 5, respectively. Sera from two asthmatic patients with IgE immunoblot reactivity against the purified native 29 kDa *Candida* protein (data not shown) also have IgE-binding reactivity to the 29 kDa component in the

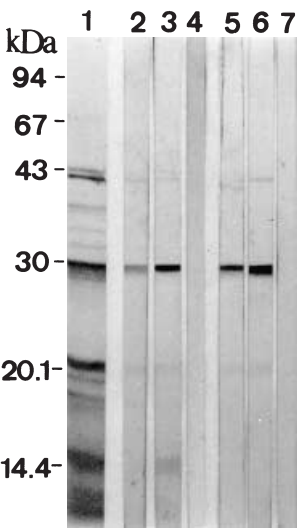


Figure 5. Immunoblot reactivity of monoclonal antibodies (MoAbs) 1 and 23 and immunoglobulin E antibodies to components in the *Candida albicans* crude extracts. Strip 1 shows the Coomassie blue-stained protein profile of the *C. albicans* crude extracts. Strips 2 and 3 are immunoblot reactivity of MoAbs 1 and 23 to components of the *C. albicans* extracts, respectively. Immunoglobulin E reactivity in sera from two asthmatic patients is shown in strips 5 and 6. Strips 4 and 7 represent negative controls using a culture supernatant with a MoAb against Der p 7 allergen and serum obtained from a healthy laboratory volunteer, respectively.

crude *C. albicans* extracts (Fig. 5, strips 5 and 6). However, immunoblot reactivity of MoAbs 1 and 23 to components in a commercially available *C. albicans* extract (Greer, Lenoir, NC) was not detectable (data not shown). For negative controls, immunoblot reactivity was not detected when a culture supernatant with a MoAb against Der p 7 mite allergen (Fig. 5, strip 4) and a serum sample from a normal volunteer (Fig. 5, strip 7) were used.

Discussion

The contributory role of *C. albicans* to clinical symptoms of allergy still remains unclear. Inadequate knowledge on the molecular identity of IgE-reacting proteins is undoubtedly one of the major reasons. In this study, we isolated a 29 kDa IgE-binding protein from the crude extracts of *C. albicans*. The purified protein reacted with IgE antibodies in nine (56%) of 16 asthmatic sera with positive CAP tests to *C. albicans*. It indicates that the 29 kDa component may be an important allergen of *C. albicans*. It correlates with the findings of Savolainen et al. (11, 14) that a 29 kDa protein in the crude fungal extracts of *C. albicans* appeared to be a major IgE-binding component (64% frequency) as analyzed by immunoblotting.

Data from mass spectrometric analysis of the tryptic peptides from the 29 kDa protein were searched against the *C. albicans* genomic sequence. A cDNA encoding for this protein was identified, isolated and expressed in *E. coli*. The open reading frame of this cDNA encodes a 236 amino acids polypeptide with a calculated average molecular mass of 25 834 Da including the initiator methionine. Kozak (27) has identified ACCATGG as the optimal sequence for translation initiation by eukaryotic ribosomes. The most highly conserved position in that motif is the purine at the -3 position (three nucleotides upstream from the ATG codon). Approximately 97% of vertebrate mRNAs have a purine, most often A, in that position (27). In addition, a G at the +4 position appears to be important for efficient translation (28). In this study, the AUG codon is embedded in the Kozak sequence. In augmentation, we observed a tryptic peptide with molecular mass match with that of the sequence covering residues four to 20 (Table 2). These data imply that protein translation initiates at the first AUG and the protein does not have a signal peptide. Discrepancies between the nucleotide sequences obtained in this study and those in the data bank can be the results of sequencing errors or the presence of isoforms of this 29 kDa protein.

Sequence alignment revealed that the deduced amino acid sequence of the 29 kDa *Candida* protein has a 62% sequence identity to a *S. cerevisiae* hypothetical protein (YDR533c). Conserved domain search indicates that the 29 kDa protein belongs to the ThiJ/PfpI family protein. Members of the ThiJ/PfpI family include proteins involved in RNA-protein interaction regulation, thiamine biosynthesis, Ras-related signal transduction and protease activity. We have not detected proteolytic activity when casein (0.5%) was used as substrate for the 29 kDa *Candida* protein (data not shown). Thus, the biological function of this newly identified *Candida* protein is presently unknown and needs further study.

In this study, we generated two MoAbs by immunizing BALB/c mice with the purified 29 kDa *Candida* protein. The antibodies generated reacted with the purified and the recombinant proteins, and also a 29 kDa IgE-reacting protein in crude extracts of *C. albicans* prepared in our laboratory. However, these MoAbs cannot detect a

29 kDa band in a commercially available *C. albicans* extract used for clinical diagnostic purposes. Consequently, the MoAbs generated and the recombinant 29 kDa protein prepared in this study are useful as reagents in the standardization of clinical diagnostic extracts.

The clinical impact of this 29 kDa protein remains unknown. Savolainen et al. (11, 14) observed a prevailing allergen of 29 kDa among adolescent/adult atopic subjects with predominantly atopic eczema and asthma. The 29 kDa protein reported by these authors might be the same Cand a 3 characterized in this study.

Savolainen et al. (10) also reported that the 46 kDa enolase protein is the most important allergen of *C. albicans* in a population of asthmatic children. Ten (77%) of 13 patients analyzed have IgE reactivity against the 46 kDa allergen (10). However, IgE reactivity to the 29 kDa *C. albicans* protein was detectable only in one (8%) of these 13 patients (10). As for adolescent/adult atopics (11, 14), the frequency of IgE reactivity to the 29 kDa allergen was 64% while the frequency of IgE binding to the 46 kDa *C. albicans* allergen was in the 41–50% range. A similar finding was observed in this study. We found IgE antibodies in nine (56%) of 16 serum samples obtained from adult asthmatic patients when purified 29 kDa protein was used as antigen. Among the same 16 samples, only five (31%) showed apparent IgE reactivity to the recombinant enolase protein (Fig. 1).

Similar findings were observed in studies of IgE responses to mite allergens. O'Brien and Thomas (29) examined IgE-specific immune reactivity to the purified Der p 1 and Der p 2 allergens by radioimmune dot-blot, and to components of *Dermatophagoides pteronyssinus* by immunoblotting. Their results showed that positive IgE responses to the purified Der p 1 and Der p 2 were found in 79% of children, whereas reactivity was only present in 23 and 19%, respectively, of adults (29). In children, the majority of the IgE reactivity was directed to Der p 2 (17 of 19, 89%) or a 100 kDa fraction. However, reactivities were generally directed to other fractions in adults and Der p 2 was recognized in only 58% (15 of 26) of the patients. The significance of the discrepancy observed in IgE reactivity to allergens in different age groups needs further study.

In conclusion, we identified a novel *C. albicans* gene product that reacts with IgE antibodies. Results obtained at molecular level suggest that the 29 kDa IgE-reacting protein belong to the ThiJ/PfpI protein family. Recombinant 29 kDa proteins prepared and specific MoAbs generated in the present study will provide reagents that are important for research and clinical purposes.

Acknowledgments

This work was supported by grants from the National Science Council (NSC90-2320-B-075-008) and the Veterans General Hospital-Taipei, Taiwan, Republic of China.

References

1. GUMOWSKI P, LECH B, CHAVES I, GIRARD JP. Chronic asthma and rhinitis due to *Candida albicans*, epidermophyton and trichophyton. *Ann Allergy* 1987;**59**:48–51.
2. ITKIN IH, DENNIS M. Bronchial hypersensitivity to extract of *Candida albicans*. *J Allergy* 1966;**37**:187–194.
3. KEENEY EL. *Candida* asthma. *Ann Int Med* 1951;**34**:223–226.
4. JAMES J, WARIN RP. An assessment of the role of *Candida albicans* and food yeasts in chronic urticaria. *Br J Derm* 1971;**84**:227–237.
5. SAVOLAINEN J, LAMMINTAUSTA K, KALIMO K, VIANDER M. *Candida albicans* and atopic dermatitis. *Clin Exp Allergy* 1993;**23**:332–339.
6. KURIMOTO Y. Relationship among skin tests, bronchial challenge and serology in house dust and *Candida albicans* allergic asthma. *Ann Allergy* 1975;**35**:131–141.
7. AKIYAMA K, YUI Y, SHIDA T, MIYAMOTO T. Relationship between the results of skin, conjunctival and bronchial tests and RAST with *Candida albicans* in patients with asthma. *Clin Allergy* 1981;**11**:343–351.
8. AKIYAMA K, SHIDA T, YASUEDA H, MITA H, YAMAMOTO T, YAMAGUCHI H. Atopic asthma caused by *Candida albicans* acid protease: case reports. *Allergy* 1994;**49**:778–781.
9. SAVOLAINEN J. A standardized desitometric immunoblotting analysis of *Candida albicans* protein allergens. *Clin Exp Allergy* 1995;**25**:357–363.
10. SAVOLAINEN J, VIANDER M, KOIVIKKO A. IgE, IgA and IgG antibody responses to carbohydrate and protein antigens of *Candida albicans* in asthmatic children. *Allergy* 1990;**45**:54–63.
11. SAVOLAINEN J, KOIVIKKO A, KALIMO K, NIEMINEN E, VIANDER M. IgE, IgA and IgG antibodies and delayed skin response towards *Candida albicans* antigens in atopics with and without saprophytic growth. *Clin Exp Allergy* 1990;**20**:549–554.
12. ISHIGURO A, HOMMA M, TORII S, TANAKA K. Identification of *Candida albicans* antigens reactive with immunoglobulin E antibody of human sera. *Infect Immun* 1992;**60**:1550–1557.
13. SHEN HD, CHOO KB, TANG RB, LEE CF, YEH JY, HAN SH. Allergenic components of *Candida albicans* identified by immunoblot analysis. *Clin Exp Allergy* 1989;**19**:191–195.
14. SAVOLAINEN J, KORTEKANGAS-SAVOLAINEN O, NERMES M, VIANDER M, KOIVIKKO A, KALIMO K, TERHO EO. IgE, IgA and IgG responses to common yeasts in atopic patients. *Allergy* 1998;**53**:506–512.
15. ITO K, ISHIGURO A, KANBE T, TANAKA K, TORII S. Detection of IgE antibody against *Candida albicans* enolase and its cross-reactivity to *Saccharomyces cerevisiae* enolase. *Clin Exp Allergy* 1995;**25**:522–528.
16. BREITENBACH M, SIMON B, PROBST G, OBERKOFER H, FERREIRA F, BRIZA P et al. Enolases are highly conserved fungal allergens. *Int Arch Allergy Immunol* 1997;**113**:114–117.
17. AKIYAMA K, SHIDA T, YASUEDA H, MITA H, YANAGIHARA Y, HASEGAWA M et al. Allergenicity of acid protease secreted by *Candida albicans*. *Allergy* 1996;**51**:887–892.
18. SHEN HD, CHOO KB, LEE HH, HSIEH JC, LIN WL, LEE WR, HAN SH. The 40-kilodalton allergen of *Candida albicans* is an alcohol dehydrogenase: molecular cloning and immunological analysis using monoclonal antibodies. *Clin Exp Allergy* 1991;**21**:675–681.
19. DANTZKER DR, PINGLETON SK. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis* 1987;**136**:225–244.
20. FARLEY PC, SHEPHERD MG, SULLIVAN PA. The purification and properties of yeast proteinase B from *Candida albicans*. *Biochem J* 1986;**236**:177–184.
21. CHERN MK, CHANG KN, LIU LF, TAM T-CS, LIU YC, LIANG YL, TAM MF. Yeast ribosomal protein L12 is a substrate of protein-arginine methyltransferase 2. *J Biol Chem* 2002;**277**:15 345–15 353.
22. LANDRY F, LOMBARDO CR, SMITH JW. A method for application of samples to matrix-assisted laser desorption ionization time-of-flight targets that enhances peptide detection. *Anal Biochem* 2000;**279**:1–8.
23. PERKINS DN, PAPPIN DJC, CREASY DM, COTTRELL JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999;**20**:3551–3567.
24. LEE KL, BUCKLEY HR, CAMPBELL CC. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* 1975;**13**:148–153.
25. MASON AB, BUCKLEY HR, GORMAN JA. Molecular cloning and characterization of the *Candida albicans* enolase gene. *J Bacteriol* 1993;**175**:2632–2639.
26. SHEN HD, CHUA KY, LIN WL, HSIEH KH, THOMAS WR. Characterization of the house dust mite allergen Der p 7 by monoclonal antibodies. *Clin Exp Allergy* 1995;**25**:416–422.
27. KOZAK M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucl Acids Res* 1987;**15**:8125–8132.
28. KOZAK M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 1986;**44**:283–292.
29. O'BRIEN RM, THOMAS WR. Immune reactivity to *Der p I* and *Der p II* in house dust mite-sensitive patients attending paediatric and adult allergy clinics. *Clin Exp Allergy* 1994;**24**:737–742.