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Identification of protein and mannoprotein antigens of *Candida albicans* of relevance for the serodiagnosis of invasive candidiasis

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Summary. Antigens from *Candida albicans* blastoconidia and germ tubes were identified by two-dimensional electrophoresis and Western blotting and characterized by microsequencing, reactivity with concanavalin A, and a panel of human sera. Antigens identified included a polydispersed area in the acidic high-molecular-mass regions of blastoconidium and germ-tube extracts, and 16 antigens varying in molecular masses and isoelectric points (pIs). The majority of the detected antigens, especially those in the polydispersed region, showed mannosyl groups, as determined by concanavalin A reactivity. Antibodies present in sera from patients with invasive candidiasis showed high reactivity with a number of antigens not detected with sera from blood donors. Eight of the 16 antigens could be identified by reactivity with monoclonal antibodies or by microsequencing. Five antigens showed homology with five enzymes previously described as antigens in *C. albicans*: enolase, phosphoglycerate kinase, malate dehydrogenase, and two isoforms of the fructose biphosphate aldolase. However, to our knowledge, this is the first report of the immunogenic activity of a kexin precursor, a mitochondrial complex I chaperone, and a diacylglycerol kinase catalytic domain from *C. albicans*. Antigens described in this study may be of potential interest for the serodiagnosis of invasive candidiasis. [**Int Microbiol** 2007; 10(2):103-108]

Key words: *Candida albicans* · antigens · mannoproteins · proteome · serology

Introduction

The increasing prevalence of yeast infections is a cause of concern among clinicians, researchers, and patients. *Candida albicans* has become a relevant cause of nosocomial infections [11,28]; however, despite the growing clinical importance of *Candida* species, many types of yeast-host interactions are poorly understood. Diagnosis of invasive *Candida* infections may be difficult due to the variability and lack of specificity of clinical presentations. A definitive diagnosis is often not reached until late in the infection, with subsequent delays in the initiation of therapy. This, in turn, results in substantial morbidity and mortality.

Several non-culture approaches have been investigated in the search for a method to obtain an early diagnosis of invasive candidiasis [27]. These techniques include the detection of β(1,3)D-glucan [20], D-arabinitol [5], enolase [30], DNA [16], antigens, and antibodies [27]. Nevertheless, each technique has its limitations and none has found widespread clinical use. The clinical application of antibody detection in invasive candidiasis has been limited by problems of specificity and sensitivity [27]. One strategy to increase specificity has been the detection of antibodies to complex antigens of *C. albicans* germ tubes [7,9]. In previous studies, we described the use of SDS-PAGE to identify and characterize

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a high-molecular-mass component of the germ-tube cell wall [2,25,26], and we demonstrated that detection of antibodies against this antigen is useful in the serodiagnosis of invasive candidiasis [7,9].

Two-dimensional electrophoresis (2-DE) has been used to study the protein composition and antigenicity of extracts from *C. albicans* blastoconidia and germ tubes [1,15,22,23]. Although many proteins have been identified as antigenic for humans [24], little attention has been devoted to characterizing the antigenic differences between germ tubes and blastoconidia, although these differences may be useful in the serodiagnosis of invasive candidiasis. In this study, we report the use of 2-DE to identify several mannoprotein antigens specifically expressed in one of the two growth phases of *C. albicans*. The approach may be of interest for the serodiagnosis of invasive candidiasis.

Materials and methods

Strain and culture conditions. *Candida albicans* Universidad del País Vasco (UPV) reference strain no. 1413 [1] was used in all the experiments. Blastoconidia were grown at 24°C in Sabouraud dextrose broth for 24 h in a rotary shaker (120 rpm). Harvested cells were washed twice and the pellet was stored at –40°C until used. For germ-tube induction, blastoconidia were grown at a concentration of 10^6 cells/ml in medium 199 at 37°C for 4 h. Under these conditions, at least 95% of the blastoconidia germinated.

Antigen extraction. Suspensions of 10^{10} cells/ml in 500 ml of sample buffer (9 M urea, 2% β-mercaptoethanol, 2% pharmalyte 3–10, and 2% NP40) were sonicated in an ice bath at intervals of 20 s for 5 min at 60 W and centrifuged for 20 min at 11,000 ×*g*. Supernatants containing the antigenic mixtures were stored at –80°C. Total protein was determined by the Bradford assay [3].

Human sera. Sera from ten patients with microbiologically proven systemic candidiasis and six sera from blood donors were used in the experiment [7].

Isoelectric focusing and 2-D PAGE. Isoelectric focusing (IEF) was carried out according to Barea et al. [1] in an immobilized linear pH 3-10 gradient (IPG strips of 18 cm, GE Healthcare, Uppsala, Sweden) by a modification of the method described by Görg et al. [8]. The IPG strips were equilibrated in 10 ml of 0.05 M Tris-HCl buffer, pH 6.8, containing 6 M urea, 1% (w/v) SDS, 30% (w/v) glycerol, and 2% β-mercaptoethanol. After 15 min, the procedure was repeated with another 10 ml of equilibration buffer containing 240 mM iodoacetamide and 0.5% (w/v) bromophenol blue solution. Gels were loaded with 600 mg of protein in sample buffer containing 2% pharmalyte pH 3–10. IEF conditions were 500 V for 2 h and then 3500 V to obtain 55 kV/h at 20°C. For the second dimension, IPG gels were transferred to a 12–14% SDS-polyacrylamide gradient gel (Excel-gel, 24.5 × 18 cm, GE Healthcare) and electrophoresed at 20 mA, 1000 V and 40 W at 15°C. 2-DE gels were stained with Coomasie blue.

Western blotting. Proteins separated in the 2-D PAGE gels were electrotransferred according to Barea et al. [1] to an Immobilon P membrane (Millipore, Billerica, USA) using a semidry transfer system at 400 mA for 2 h. The membrane was treated with blocking solution containing 5% non-fat skim milk powder in PBS for 1 h at 37°C. After blocking, the samples were incubated with the blood donor or patient sera diluted 1/1000 or 1/5000 in PBS, respectively, for 1 h at 37°C. Antigen-antibody reactions were visualized after the addition of anti-human IgG-horseradish peroxidase (HRP) conjugate (Sigma, St. Louis, MO, USA) at a dilution of 1/5000 in PBS and detected by the chemiluminescence of peroxidase activity (ECL, GE Healthcare), according to the manufacturer's instructions. The blot was then wrapped in plastic and exposed to X-ray film (Curix RP-2, Agfa-Gevaert NV, Mortsel, Belgium). Monoclonal antibodies A2C7 (Sigma) and anti-heat-shock protein 70 (Sigma) were used to identify enolase [1] and heat-shock protein of 71 kDa. Anti-glyceraldehyde-3-phosphate dehydrogenase of *Saccharomyces cerevisiae* [4] and pyruvate kinase polyclonal antibodies were used to identify glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, respectively.

Mannoprotein detection by Con A-HRP. After electrotransfer, membranes were incubated in 10% BSA in TBS (150 mM NaCl in 50 mM Tris-HCl, pH 7.5) for 1 h at 37°C and then washed twice with buffer A (TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$). After incubation with Con A-HRP (Sigma) in buffer A (0.2 mg/ml), mannoproteins were visualized by chemiluminescence detection (ECL).

Protein microsequencing. Protein spots were excised and identified by Edman degradation (in the Proteomics Laboratory of the Centro Nacional de Biotecnología, Cantoblanco, Madrid) or by LC-MS/MS using a Q-Tof micro mass spectrometer (Waters, Milford, MA, USA) at the Proteomics facility of the University of the Basque Country, Bilbao, Spain). Identities between microsequencing data and protein sequences were either searched in SWISS-PROT, TrEMBL or were used to search protein databases for homologous proteins with NCBI's Basic Local Alignment Search tool (BLAST) or FASTA programs in Candida DB (Pasteur Institute, Paris, France).

Image analysis. X-ray films and silver-stained gels were analyzed with the BioImage 50S system and the 2D Protein Analyzer software (Millipore) in a Sparc 4 station (SUN Microsystems, Madrid, Spain). Each gel was reproduced at least five times, including biological and technical replicates. Differences in individual quantification of proteins showed a standard error <10%.

Results and Discussion

Two-dimensional electrophoresis is an optimal technique to characterize protein profiles of complex extracts. When used in combination with Western blot and enhanced chemiluminescence, 2-DE is a powerful tool to establish antigen-antibody reactions. In this study, 12–14% 2-DE was used to discriminate about 500 protein spots in blastoconidium and germ-tube extracts of *C. albicans* (Fig. 1A,B). In general, the molecular masses of the peptides ranged from 10 to 116 kDa, and isoelectric points (pI) from 4 to 8. Protein profiles obtained in this study were similar to those described in *C. albicans* blastoconidium extracts by our group [1] and by Pitarch et al. [23], although in the latter study the extraction procedure was different and a non-linear pH gradient was used.

Data obtained in this study showed that there are protein spots specifically or preferentially associated with each morphological phase (squares in Fig. 1A,B). However, other authors have not been able to identified antigenic differences

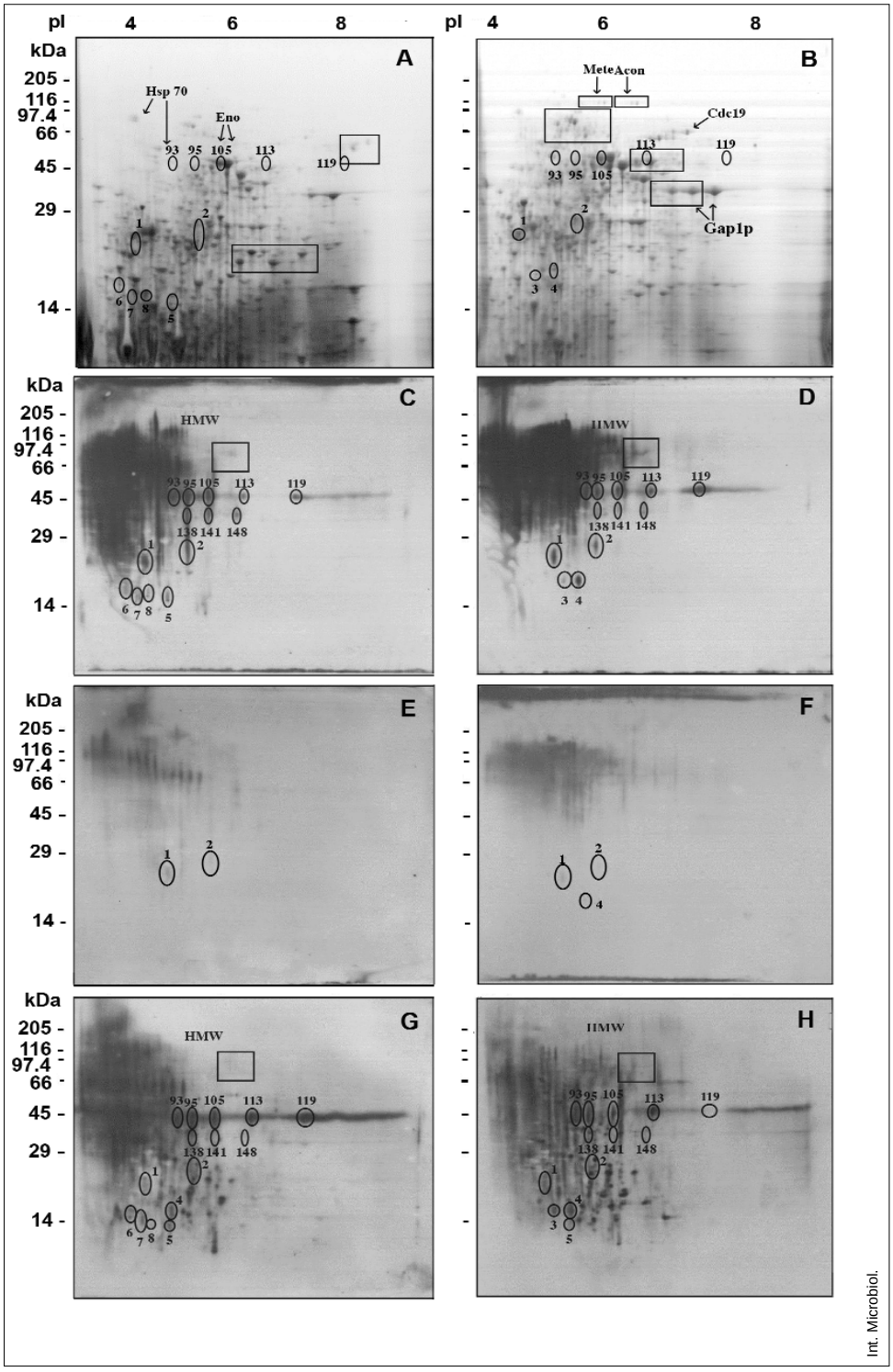

Fig. 1. Two-dimensional electrophoresis (2-DE) of *C. albicans* blastoconidia (**A, C, E, G**) and germ-tube (**B, D, F, H**) extracts stained with Coomassie blue (**A, B**), serum from a patient with systemic candidiasis (**C, D**), serum from a control blood donor (**E, F**), and Concanavalin A (**G, H**). Identified proteins are circled and numbered. Squares indicate proteins that showed differential expression in one of the Candida growth phases. Mete, methionine synthase; Acon, aconitate hydratase; Cdc19, pyruvate kinase; Gap1p, glyceraldehyde-3-phosphate dehydrogenase.

between blastoconidium and germ-tube extracts using 2-DE [15,19]. Spots overexpressed in our study were identified as methionine synthase (Mete), aconitate hydratase (Acon), glyceraldehyde-3-phosphate dehydrogenase (Gap1p), pyruvate kinase (Cdc19), and enolase (Eno). Although these enzymes have been described as immunogens by other authors [22–24,29], they did not show immunoreactivity in this study, with the exception of enolase (antigen no. 105).

Very different patterns of reactivity were observed between sera from patients with invasive candidiasis (Fig. 1C,D) and

from blood donors (Fig. 1E,F), as determined by Western blotting. Antibodies present in sera from patients with invasive candidiasis showed higher reactivity than control sera, although the concentrations of control sera were five times higher than those of patient sera. Control sera showed a very weak reaction in three spots (1, 2, 4), thus allowing good discrimination between control and patient sera. The most striking difference between the two groups of sera concerned a polydispersed area in the acidic high- to medium-molecular-mass regions of both extracts (Fig. 1C,D). Similar results were obtained with the ten sera from patients with candidiasis (data not shown). Sixteen antigens were characteristically recognized, ten of them in both extracts, with relative molecular masses of 22–24, 44–48, and 38–39 kDa and pI between 5.4 and 8 (spots 1, 2, 93, 95, 105, 113, 119, 138, 141, 148). Antigens 5–8 were found in blastoconidia extracts only and showed relative molecular masses between 14 and 17 kDa and pI of 4.3–5.3. Antigens 3 and 4 were found only in the germ-tube extracts and had molecular masses of 16–17 kDa and pI of 4.9–5.3.

The number of antigenic regions revealed in the reactions between sera from patients with invasive candidiasis and the extracts was higher than that found by Pitarch et al. [23] in their blastoconidium cytoplasmic extracts. The differences are probably due to the type of extract used in each study, since we used a complete extract. In addition, our extract was enriched in cell-wall mannoproteins, as revealed by Con A staining, and mannans and mannoproteins are highly immunogenic for humans [27]. Most antigens recognized by sera from patients with invasive candidiasis had a mannoprotein nature (Fig. 1G,H). In general, the blastoconidium extract showed a higher reactivity with Con A than the germ-tube extract, which suggested that the latter should contain more mannose groups. The detection by patient sera and Con A of antigens 3 and 4 in the germ-tube extract and antigens 6–8 in the yeast extract suggested an immunogenic role of the mannosylation of these peptides. Also, mannosylation of the polydispersed area seems to be related to antigenicity because no reaction with patient sera was detected after metaperiodate oxidation (data not shown).

The identification and characterization of antigens relevant for the serodiagnosis of invasive candidiasis is an important step in the development of future diagnostic tests. In this study, eight immunodominant antigens were identified by their reactivity with monoclonal antibodies or by microsequencing (Table 1). Spot 105 was identified as enolase by its reactivity with monoclonal antibody A2C7. Enolase is an immunodominant antigen of *C. albicans* that was recognized in our study by 80% of the sera from patients with invasive candidiasis and by none of the control sera. Detection of anti-enolase antibodies has been shown to be useful in the diagnosis [13] and prognosis of invasive candidiasis [24].

Only seven of the antigens submitted to protein microsequencing produced a sequence that could be used to search the protein databases. Pardo et al. [22] attributed the lack of a conclusive identification by MALDI-TOF mass spectrometry of *C. albicans* cytoplasmic proteins to the fact that this yeast is poorly characterized at the protein level. However, the complete genome sequence of *C. albicans* is now available, which will certainly improve protein characterization [14]. The N-terminal sequence data from the spots or mass spectrometry identification are summarized in Table 1.

Table 1. Sequence and homologies of identified spots from the *Candida albicans* protein extract

Spot	Sequence[a]	Accession number[b] -Protein name	Immunodetection	Mr[c]	pI[c]
2	ALENPERP	P46273 - Phosphoglycerate kinase	Human sera	23.9	5.7
3	XS/LEKXTK	CA4991 - Diacylglycerol kinase catalytic domain	Human sera	16.6	4.9
4	TLDVLRAA	CA5164 - Mitochondrial malate dehydrogenase	Human sera	17	5.3
5	XIFAKXL	Q5A8Y1 - Mitochondrial complex I chaperone	Human sera	14.4	5.3
8	XLENKEFP/L	013359 - Kexin precursor	Human sera	14.8	4.8
105		P30575 - Enolase	Human sera and MAb	45–50	5.5–6.0
141–148	APPAVLSK	Q9URB4 - Fructose biphosphate aldolase	Human sera	38–40	6.1–6.7

[a]Peptide sequence obtained by Edman degradation.
[b]Accession number of homologous protein in protein databases (SWISS-PROT, TrEMBL, NCBI or Candida DB).
[c]Molecular mass (Mr) and Isoelectrical point (pI) were obtained by 2D Analyzer Software (Millipore) on 2-DE gels.

A search of the databases with the sequence ALENPERP from spot 2 revealed 100% homology with the phosphoglycerate kinase from *C. albicans*. This glycolytic enzyme is a cytoplasmic and cell-wall protein known to elicit an immune response [23,29]. Peptide number 3 yielded the sequence XS/LEKXTK, which showed homology with the diacylglycerol kinase catalytic domain of *Candida albicans*. Ours is the first report of the immunogenic properties of this peptide, which showed immunogenic activity in germ-tube extracts only. Since the peptide was detected by ConA, mannosylation may be related to its antigenicity. Sequence TLDVLRAA, from antigen number 4, showed 100% homology with *C. albicans* malate dehydrogenase. This enzyme, which is inducible and has a pI of 5.5, plays an important role in the transport system (malate-aspartate shuttle) of NADH generated by the oxidation of methanol in the mitochondria [31]. Malate dehydrogenase has been reported to be a major allergen in *Malassezia* [21] and is immunogenic in a murine model [24].However, our study reports, for the first time, that this enzyme is recognized by sera from patients with invasive candidiasis but not by control sera.

Antigen number 5 yielded the sequence XIFAKXL, which showed homology with a potential mitochondrial complex I assembly chaperone of *Candida albicans* [12]. Sequence XLENKEFP/L, from peptide number 8, showed homology with a kexin precursor of *C. albicans*. This membrane protein is a Kex2 protease that belongs to peptidase family S8; it has been shown to influence *C. albicans* proteinase secretion and hyphal formation [18]. To our knowledge, this is the first report describing an immunogenic role for this protein. A number of *Candida* antigens with protease activity have been used as targets for antibody detection, including a secreted aspartyl proteinase [17] and a metallopeptidase [6]. The sequence XAPPAVLSK, from spots 141–148, showed complete homology with fructose biphosphate aldolase. This enzyme reacts with IgE antibody from allergic patients [10] and was immunodetected by sera from infected mice [24]. All sera from patients with invasive candidiasis but none of the controls used in this study recognized this glycolytic enzyme.

The reactivity of the control sera was very homogeneous against most of the antigens described. Only two control sera showed very limited recognition of antigens 1, 2, 3, and 4. While sera from patients with invasive candidiasis showed higher variability, antigens 1, 2, 4, 138, and 141 were recognized by all sera from patients with invasive candidiasis.

In conclusion, we identified eight antigens of potential interest for the serodiagnosis of invasive candidiasis. As far as we know, this is the first time that three of those antigens (kexin precursor, a mitochondrial complex I chaperone, and a diacylglycerol kinase catalytic domain) have been described as immunogenic for humans.

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