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Identification of superficial *Candida albicans* germ tube antigens in a rabbit model of disseminated candidiasis. A proteomic approach

Aranzazu Sáez-Rosón,[1] María-Jesús Sevilla,[2] María-Dolores Moragues[3*]

[1]Dept. of Immunology, Microbiology and Parasitology, School of Medicine and Odontology, Univ. of the Basque Country UPV/EHU, Leioa, Spain. [2]Dept. of Immunology, Microbiology and Parasitology, School of Sciences and Technology, Univ. of the Basque Country UPV/EHU, Leioa, Spain. [3]Department of Nursing 1, Univ. of the Basque Country UPV/EHU, Leioa, Spain

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Summary. The diagnosis of invasive candidiasis remains a clinical challenge. The detection by indirect immunofluorescence of *Candida albicans* germ-tube-specific antibodies (CAGTA), directed against germ-tube surface antigens, is a useful diagnostic tool that discriminates between colonization and invasion. However, the standardization of this technique is complicated by its reliance on subjective interpretation. In this study, the antigenic recognition pattern of CAGTA throughout experimental invasive candidiasis in a rabbit animal model was determined by means of 2D-PAGE, Western blotting, and tandem mass spectrometry (MS/MS). Seven proteins detected by CAGTA were identified as methionine synthase, inositol-3-phosphate synthase, enolase 1, alcohol dehydrogenase 1,3-phosphoglycerate kinase, 14-3-3 (Bmh1), and Egd2. To our knowledge, this is the first report of antibodies reacting with Bmh1 and Egd2 proteins in an animal model of invasive candidiasis. Although all of the antigens were recognized by CAGTA in cell-wall dithiothreitol extracts of both germ tubes and blastospores of *C. albicans*, immunoelectron microscopy study revealed their differential location, as the antigens were exposed on the germ-tube cell-wall surface but hidden in the inner layers of the blastospore cell wall. These findings will contribute to developing more sensitive diagnostic methods that enable the earlier detection of invasive candidiasis. [**Int Microbiol** 2014; 17(1):21-29]

Keywords: *Candida albicans* · germ tube antibodies · invasive candidiasis · rabbit model

Introduction

Species of *Candida* are major fungal pathogens in humans, causing a wide variety of surface or mucocutaneous candidiases as well as systemic or invasive candidiasis (IC) in both immunocompromised and immunocompetent patients. Although new antifungal agents active against most *Candida* species have recently been introduced, the morbidity and mortality rates associated with IC remain high (10–49 %) [28]. Between 95 and 97 % of invasive candidiasis are caused by five species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. Of these, *C. albicans* is the most prevalent species involved in IC, but other species, such as *C. krusei*, which is intrinsically resistant to azoles, and *C. glabrata* are increasingly being isolated [10].

The diagnosis of IC is difficult because there are no specific signs and it is difficult to distinguish between colonization and invasion. Furthermore, the available diagnostic techniques are of low sensitivity and specificity, and the aggressive methods sometimes required to obtain samples are often not feasible because of the critical condition of affected patients [37]. As a result, therapy is often implemented late or

*Corresponding author: M.D. Moragues
Departamento de Enfermería I
Universidad del País Vasco
Barrio Sarriena, s/n
48940 Leioa, Spain
Tel. +34-946015599. Fax +34-946013300
E-mail: lola.moragues@ehu.es

not at all, which in part explains the high mortality associated with IC [26].

The limitations of traditional microbiological techniques have led to the search for new diagnostic methods based on the detection of biomarkers, most notably structural and metabolic components of *Candida* such as mannan, β-1,3-D-glucan, D-arabinitol, and DNA, as well as *Candida* antigen components and antibodies, but none of these methods are conclusive enough for application in clinical practice [27,37,40]. García-Ruiz et al. developed an indirect immunofluorescence (IIF) technique that detects specific antibodies to antigens located on the surface of the cell wall of *C. albicans* germ tubes (*C. albicans* germ tube-specific antibodies, CAGTA) [8]. Since the mycelial phase of *C. albicans* is associated with tissue invasion, this technique differentiates between colonization and invasion. CAGTA detection has proved to be useful for the diagnosis of IC in both immunocompetent and immunocompromised patients, with a sensitivity and specificity of 79–89 % and 91–100 %, respectively [8,11,41,42].

The sensitivity and specificity of the commercial kit Invasive Candidiasis (CAGTA) IFA IgG (Vircell Microbiologists, Granada, Spain) in the diagnosis of IC are 84.4 % and 94.7 %, respectively [22], and data for its specificity and negative predictive values have been corroborated in a recent study focused on the discrimination of deep-seated candidemias from transient or catheter-related *Candida* infections not involving deep tissues [20]. León et al. [17] reported that positive CAGTA titers combined with positive β-glucan values accurately differentiated *Candida* colonization from IC in intensive care unit (ICU) patients with severe abdominal conditions. Also, an additional role for CAGTA, as prognostic markers in critically ill patients in ICUs has been suggested [50,51], based on the association of an increase in positive titers of CAGTA with a significant decrease in mortality (22.7 % vs. 61.2 % for those with negative CAGTA titers), particularly in patients undergoing antifungal treatment. The authors of those studies therefore have recommended that antifungal therapy be considered in critically ill patients with increasing CAGTA titers. The advantages of the CAGTA method are that it is simple and fast, with good sensitivity and specificity. Nonetheless, an important limitation is that it has to be evaluated visually by IIF, which is a subjective method and thus difficult to standardize.

In the search for novel biomarkers in the development of new diagnostic tools for IC, immunoproteomics has received serious consideration [9,25,29,31–33,35]. Serum IgG profiles in patients with IC and in healthy controls have been compared in order to identify *Candida* antigens recognized only or mainly by sera from infected patients. This strategy has resulted in the identification of a large number of biomarkers with potential applications in the early diagnosis of IC [9,29,32,33] and in the development of vaccines or immunotherapies [7,25,31].

In this study an immunoproteomic strategy was used to determine the antigen recognition pattern of CAGTA throughout the course of a *C. albicans* infection in a rabbit animal model. Our results provide a time course and picture of the specific antibodies and marker antigens for IC and may serve as the basis for the development of new tools in the early and objective diagnosis of IC.

Materials and methods

Strain and culture conditions. All experiments were carried out with *C. albicans* NCPF 3153 (National Collection of Pathogenic Fungi, Bristol, UK). Yeast-phase cells were routinely grown on Sabouraud agar (Difco, Sparks, MD, USA) plates at 24 ºC for 48 h. To obtain germ tubes, cells grown on Sabouraud agar were inoculated into TC199 medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 24 ºC with shaking (120 rpm) overnight. The resulting blastospores were harvested and then suspended in four volumes of TC199 medium pre-heated to 37 ºC. The germ tubes were collected after 4 h of shake-incubation at 37 ºC and 120 rpm.

Rabbit model of disseminated candidiasis. Two female New Zealand White rabbits (Granja Cunícola San Bernardo S.L; Navarra, Spain) each weighing approximately 2 kg were injected through the ear marginal vein with 2×10^6 blastospores of *C. albicans* suspended in 0.2 ml of sterile saline solution (day 0); inoculation was repeated on days 28, 56, and 84. Preimmune sera were withdrawn prior to infection; immune sera were collected through the ear marginal vein every week after the onset of infection. Blood samples were left to clot at 4 ºC overnight, and sera were stored at –20 ºC until needed.The rabbits were maintained at the animal facilities of the University of the Basque Country UPV/EHU (Spain), according to animal welfare ethics policy.

Quantification and purification of CAGTA. Serum CAGTA levels were titrated by IIF according to Moragues et al. [22]. The antibodies were purified from sera previously adsorbed with an equal volume of a suspension of heat-inactivated *C. albicans* blastospores (10^{10}cell/ml) for 2 h at room temperature, to remove anti-mannan antibodies. Adsorbed sera were centrifuged at 2500 rpm for 5 min. The supernatants were mixed with an equal volume of a pellet of germ tubes washed with phosphate buffered saline (PBS; Sigma-Aldrich) and then incubated with gentle agitation at room temperature for 1 h. After centrifugation, the samples were washed with PBS and CAGTA bound to the mycelial surface were eluted in 5 ml of 2.5 M sodium iodide (Sigma-Aldrich) in PBS by gentle shaking at room temperature for 1 h. After centrifugation, the supernatants containing the eluted CAGTA were dialyzed against PBS (MWCO 12,000–4000 Da; Medicell International, London, UK) and concentrated with polyethylene glycol 20,000 (Merck, Hohenbrunn, Germany).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of dithiothreitol (DTT) cell-wall extracts from *Candida albicans*. Cell-wall proteins of *C. albicans* NCPF 3153 were extracted with DTT from germ tubes (DTT-GT) or blastospores (DTT-B), ac-

cording to Ponton and Jones [38]. Two hundred µg of DTT-extracted cell-wall proteins were processed for western blotting, and 500 µg for Coomassie staining as follows: Cell-wall extracts were suspended in rehydration buffer containing 7 M urea, 2 M thiourea, 4 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM DTT, 0.5 % IPG buffer pH 4–7 (v/v), and traces of bromophenol blue, and adsorbed onto 11-cm strips containing an immobilized pH gradient of 4–7 (IPG; GE Healthcare Bio-Sciences, Uppsala, Sweden), with active rehydration at 50 V for 12 h at 20 ºC. Isoelectric focusing was carried out on a Protean IEF cell electrophoresis unit (Bio-Rad, Hercules, CA, USA) under the following conditions: step 1, 250 V for 20 min; step 2, ramped to 8000 V over 2.5 h; and step 3, 8000 V for a total of 30,000 V/h. After focusing, the IPG strips were equilibrated for 15 min in a reducing solution of 1 % DTT (w/v) in 75 mM Tris-HCl pH 8.8, 6 M urea, 30 % glycerol (v/v), 2 % sodium dodecyl sulfate (SDS) (w/v) (TUG-SDS) and then in an alkylating solution of 2.5 % iodoacetamide (w/v) in TUG-SDS, with gentle shaking. The second dimension was run in a Criterion XT Bis-Tris 4–12 % gel (Bio-Rad) in a Protean II electrophoresis chamber (Bio-Rad) at a constant voltage of 200 V for 55 min. Protein spots were stained with the colloidal blue staining kit (Invitrogen, USA) or electroblotted onto a polyvinyl difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) using a semidry Fast-blot system (Biometra, Germany) and transfer buffer (25 mM Tris, 150 mM glycine, and 10 % methanol) at 0.25 mA/cm^2 for 1 h.

Western blotting. PVDF membranes were incubated with the eluted CAGTA in Tris-buffered saline (TBS; 10 mM Tris-HCl, 0.9 % NaCl [w/v], pH 7.3) containing 8 % skimmed milk (TBS-M) in a humid chamber at 37 ºC for 1 h with gentle shaking. After washing three times with TBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Sigma-Aldrich; 1:300 in TBS-M) under the same conditions. After washing with TBS, the membranes were processed with the Immun-Star HRP chemiluminescence kit (Bio-Rad) and the reactions were visualized with the Chemidoc Quantity One system (Bio-Rad). The reactive proteins were excised from parallel gels and identified by tandem mass spectrometry (MS/MS). At least two replicates of each sample were obtained.

Mass spectrometry analysis. Selected protein spots were excised manually from the gel and subjected to in-gel tryptic digestion according to Shevchenko et al.[44], with minor modifications. The gel pieces were swollen in digestion buffer (50 mM NH_4HCO_3 and 12.5 ng/ µl proteomics grade trypsin [Roche, Basel, Switzerland]) and the digestion was allowed to proceed at 37 °C overnight. The supernatant was recovered and peptides were extracted twice, first with 25 mM NH_4HCO_3 and acetonitrile (ACN, Thermo Scientific Pierce), and then with 0.1 % trifluoroacetic acid (Thermo Scientific Pierce) and ACN. The recovered supernatants and extracted peptides were pooled, dried in a SpeedVac, dissolved in 10 µl of 0.1 % formic acid (FA) (Thermo Scientific Pierce), and sonicated for 5 min.

MS/MS analysis was performed in a SYNAPT HDMS mass spectrometer (Waters, Milford, MA, USA) interfaced with a nanoAcquity UPLC system (Waters). An 8-µl aliquot of each sample was loaded into a Symmetry300 C18 (180 µm × 20 mm) precolumn (Waters) and washed with 0.1 % FA for 3 min at a flow rate of 5 µl/min. The precolumn was connected to a BEH130 C18 column (75 µm x 200 mm; Waters) equilibrated in 3 % ACN and 0.1 % FA. Peptides were eluted with a 30-min linear gradient of 3–60 % ACN directly into a nano-electrospray capillary tip. The capillary voltage was set to 3500 V and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3, or 4 over a survey *m/z* range of 350–1990.

Spectra were processed using the Virtual Expert Mass Spectrometrist (VEMS) software [21] and searched against the SwissProt database (restricted to "other fungi") using Mascot (Matrix Science, London, UK). Protein identification was based on the following variables: carbamidomethylation of cysteines as fixed modification, oxidation of methionines as variable modification, 50-ppm peptide mass tolerance, 0.1-Da fragment mass tolerance, and 1 missed cleavage.

Electron microscopy and immunocytochemistry. *Candida albicans* germ tubes or blastospores were washed in Sorenson's buffer [0.133 M Na_2HPO_4, 0.133 M KH_2PO_4 [4:1 v/v], pH 7.4], treated with fixing solution (0.5 % glutaraldehyde, 4 % formaldehyde in Sorenson's buffer) at 4 °C for 24 h, and washed with 88 mM sucrose in Sorenson's buffer. The cells were dehydrated using graded concentrations of acetone, embedded in Spurr resin (low-viscosity embedding medium Spurr's kit, Electron Microscopy Sciences, Hatfield, PA, USA), and polymerized at 56 ºC for 2 days. Sections 50- to 100-nm-thick were deposited on 200-mesh nickel grids coated with Formvar (Electron Microscopy Sciences) and blocked with blocking solution (10 % inactivated goat serum, 0.02 % sodium azide, 0.1 % Tween-20 in PBS, pH 8.2) at room temperature for 1 h with gentle shaking in a humid chamber. The samples were then incubated with the eluted CAGTA in the incubation solution (1 % inactivated goat serum, 1 % BSA, 0.02 % sodium azide, 0.1 % Tween-20 in PBS, pH 8.2) for 2 h under the same conditions. After washing with incubation solution, the samples were incubated with anti-rabbit IgG antibody labeled with 10-nm diameter colloidal gold (Sigma-Aldrich; 1:10 in incubation solution) under the same conditions. Finally, the samples were washed with blocking solution and distilled water, stained with 2 % uranyl acetate and lead citrate, and observed in a transmission electron microscope Philips EM208S. Negative controls without primary antibody were performed, and samples were obtained from two independent experiments.

Results

Fungal infection of rabbits with *Candida albicans*. The injection of four doses of 2×10^6 blastospores of *C. albicans* NCPF 3153 produced a disseminated fungal infection in the two rabbits, and their immune systems developed antibodies (CAGTA) that recognized antigens on the surface of *C. albicans* germ tubes, as determined by IIF (Fig. 1). CAGTA titers were estimated for each serum sample collected during the course of the experiment, and rabbits 1 and 2 showed a similar trend in their humoral immune responses to IC (Fig. 2). The levels of CAGTA rose moderately after the first injection but increased significantly after the second and third injections, reaching peak titers (1/640 and 1/1280) on day 72. Although differing in extent, the CAGTA titers for the two rabbits tended to stabilize by the end of the third round of infection (Fig. 2).

Antigens recognized by CAGTA. Since CAGTA serve as markers of invasive *Candida* infection, we identified the reacting antigens in a DTT cell-wall extract of *C. albicans* germ tubes (DTT-GT) separated by 2D-PAGE. The reproducibility of the gels allowed a comparison of the Coomassie-stained reference protein map (Fig. 3A) with the spots reacting with the CAGTA by immunoblotting (Fig. 3C), and thereby the identification of the corresponding proteins by MS/MS analysis.

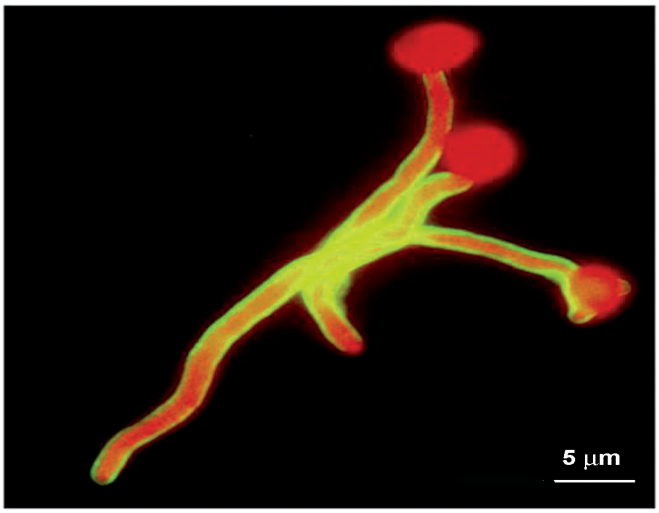

Fig. 1. Reaction of serum CAGTA from a rabbit with disseminated candidiasis with the cell-wall surface of *Candida albicans* germ tubes, as evidenced by indirect immunofluorescence [22]. The germ-tube surfaces stain brightly (light green in the color version of the image) after their reaction with a secondary FITC-conjugated anti-rabbit IgG antibody (Sigma); the blastospores are red following contrast staining with Evan's blue.

When analyzed by Western blotting, pre-immune sera (day 0) adsorbed with *C. albicans* blastospores were not reactive with *C. albicans* DTT-GT proteins. However, the eluted CAGTA purified from immune sera obtained on days 3, 17, 37, and 58 after the initial infection recognized several protein spots on the DTT-GT bidimensional map. Seven proteins were identified by MS/MS: methionine synthase (Met6), inositol-3-phosphate synthase (Ino1), enolase 1 (Eno1), alcohol dehydrogenase 1 (Adh1), 3-phosphoglycerate kinase (Pgk1), 14-3-3 (or Bmh1), and nascent polypeptide-associated complex, α subunit (or Egd2) (Fig. 3A,C). CAGTA reactivity was highly similar for the two rabbits although the intensities of the reactions oscillated over the course of the infection (Table 1). Antigens Eno1 and Ino1 showed a strong reactivity that began at the early stages of infection (Table 1) and remained stable over time. Met6 and Adh1 displayed an intermittent reactivity with either of the rabbit antisera (Table 1). CAGTA exhibited strong reactivity against the 14-3-3 protein in the sera of both rabbits three days after the initial infection (Table 1), even though CAGTA were not appreciable by IIF at that time. This reactiv-

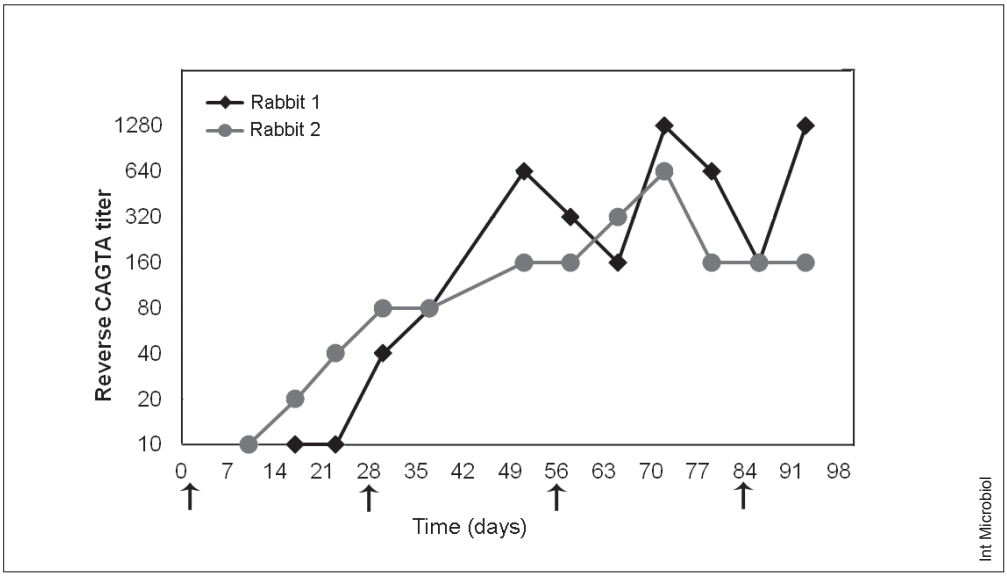

Fig. 2. Change over time of the serum CAGTA response of two rabbits infected with *Candida albicans* NCPF 3153. Arrows indicate the time (days 0, 28, 56, and 84) at which the rabbits were infected intravenously with 2×10^6 blastospores in saline.

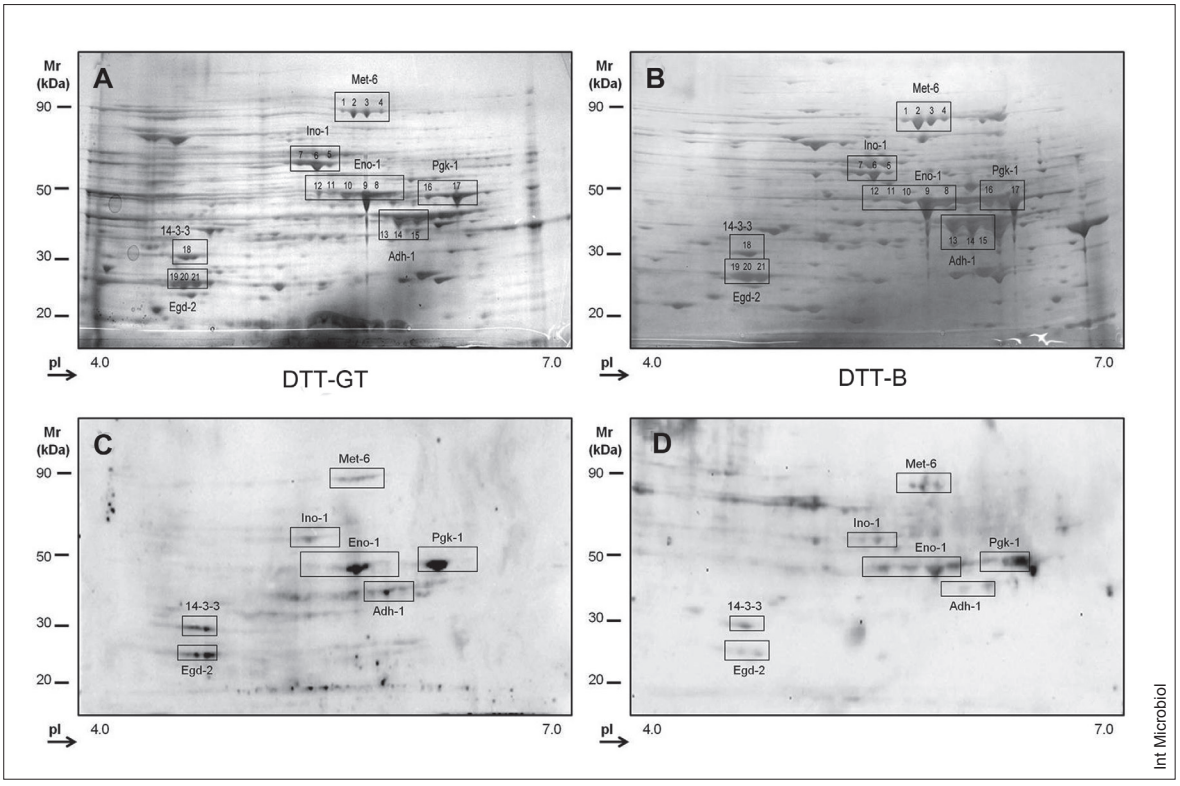

Fig. 3. Two-dimensional electrophoresis of DTT cell-wall extracts of germ tubes and blastopores of *Candida albicans* NCPF 3153. (**A**) Germ tubes (DTT-GT). (**B**) Blastopores (DTT-B). Gels were stained with the colloidal blue staining kit (Invitrogen). Immunoblot of DTT-GT (**C**) and DTT-B (**D**) extracts assayed with CAGTA eluted from the serum of rabbit 2 on day 58 of the IC model of infection.

ity gradually diminished over time but recovered after the second inoculation with *C. albicans*. A reaction with Egd2 was not observed until day 37 (Table 1), after which the reactivity remained strong with rabbit 2 sera but gradually faded in the sera of rabbit 1. Similarly, CAGTA reactivity against Pgk1 was weaker in sera from rabbit 1 (Table 1). In addition, as shown in the 2D profiles stained with Coomassie blue (Fig. 3A), most antigens had several isoforms such that their reactivity with CAGTA in the sera of both rabbits was rather heterogeneous.

In a different approach to characterize the antigens reacting with CAGTA, the 2D-PAGE protein pattern displayed by the DTT cell-wall extracts of *C. albicans* blastospores was compared with that of the germ tubes. The images from Coomassie stained 2D-gels were very similar for most of the proteins, although some of the bands differed in their relative concentrations and/or electrophoretic mobility (Fig. 3A,B). However, using CAGTA purified from the 58-day serum of rabbit 2, the main antigens recognized in the DTT-B extract were the same as those identified in the DTT-GT extract (Fig. 3C,D).

Location of antigens recognized by CAGTA. Immunoelectron microscopy, used to locate the identified antigens in the *C. albicans* cell wall, revealed that the CAGTA reacted with superficial elements of the germ-tube cell walls (Fig. 4A), although reactive components were also present in the inner layers. By contrast, CAGTA only reacted with compounds located in the inner layers of the blastospore cell wall (Fig. 4B).

Discussion

Because of the commensal nature of *C. albicans*, one of the challenges of the serological diagnosis of IC has been to differentiate between colonized and infected patients. The detection of CAGTA solves this diagnostic problem since the antibodies recognize specific antigens on the surface of *C. albicans* germ tubes, the morphological phase associated with invasion. However, immunofluorescence-based analysis requires subjective interpretation of the results, ruling out automation of the process and thus its standardization. In this study, we set out to characterize the antigen recognition pat-

Table 1. Major cell-wall proteins in DTT extracts of *Candida albicans* germ tubes reacting with CAGTA raised in two rabbits with experimental disseminated candidiasis

				CAGTA reactivity[e] (days of infection)									
				Rabbit 1					Rabbit 2				
Protein name (Accesion No.)[a]	Spot No.[b]	Mr (kDa)[c]	pI[d]	0	3	17	37	58	0	3	17	37	58
Methionine synthase (P82610)	1–4	85.8	5.44	–	–	++	–	+++	–	–	++	–	++
Inositol-3-phosphate synthase (P42800)	5–7	57.8	5.35	–	–	+++	+++	++	–	–	+++	++	++
Enolase-1 (P30575)	8–12	47.2	5.54	–	–	+++	+++	+++	–	+	++	++	+++
Alcohol dehydrogenase 1 (P43067)	13–15	37.2	6.02	–	–	++	–	++	–	–	+	–	++
Phosphoglicerate kinase 1 (P46273)	16–17	45.3	6.07	–	–	–	+	+	–	+	++	+	+++
Bmh1 (O42766)	18	29.6	4.75	–	+++	–	+++	+++	–	+++	+	++	+++
Nascent polypeptide-associated complex subunit alpha (Q5ANP2)	19–21	19.5	4.7	–	–	–	++	–	–	–	–	+++	+++
CAGTA titer[f]				0	0	10	80	320	0	0	20	80	160

[a]Protein name and accession number according to the UniProtKB database. [b]Spot numbers as indicated in the gel shown in Figure 3A.
[c]Protein molecular weight as shown in the SwissProt database. [d]Theoretical isoelectric point as shown in the SwissProt database.
[e]CAGTA reactivity levels (+++, very strong; ++, strong; +, mild; –, no reactivity). [f]CAGTA titer (inverse) of sera evaluated by IIF.

tern of CAGTA over the duration of IC using a rabbit animal model. The recognition in IC patients of at least a subset of these antigens by one or more CAGTA may provide the basis for the development of more sensitive and specific diagnostic techniques for IC, as reported by Clancy et al. [3].

Western blot analysis of *C. albicans* DTT-GT extracts separated by 2D-PAGE identified Met6, Ino1, Eno1, Adh1, Pgk1, 14–3–3 (Bmh1), and Egd2 as the major proteins recognized by the CAGTA of rabbits with IC. Although these proteins are involved in cellular processes carried out in the cytoplasm, they have also been detected in the cell wall of *C. albicans* blastospores [2,19,23,43,48] and mycelia [6,19,36,47,48]. Several of these proteins are involved in cellular metabolism, such as Eno1 and Pgk1, which are glycolytic enzymes. Eno1 has also been described as an allergen [12], while Pgk1 participates in the biogenesis and degradation of the *C. albicans* cell wall [1]. Adh1 is involved in glucose fermentation, by reducing acetaldehyde to ethanol, and is also an allergen. Note that these three proteins bind human plasminogen, generating the proteolytic enzyme plasmin, which could increase the capacity of *C. albicans* for tissue invasiveness and necrosis [5,13]. Ino1 catalyzes the conversion of glucose-6-phosphate to inositol-1-phosphate, the first step in the production of inositol-containing components such as phospholipids, which are essential for cell-membrane lipid bilayers [15]. Met6 is involved in the biosynthesis of methionine during *C. albicans* morphogenesis and is essential for growth, by limiting the toxicity of homocysteine [45].

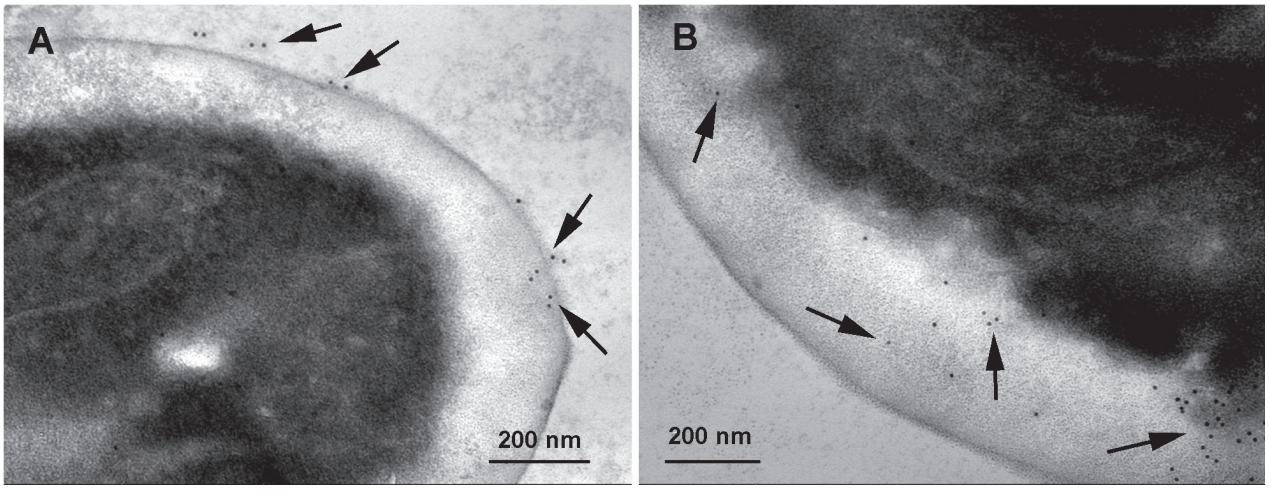

Fig. 4. Transmission electron microscopy images of the cell wall of *Candida albicans* NCPF 3153, showing the reaction of CAGTA purified from the serum of a rabbit infected with this fungal strain. Note that the colloidal gold particles are mainly located in the outermost cell-wall layers of the germ tubes (**A**), and in the inner cell-wall layers of blastospores (**B**). Arrows indicate the different positions of the colloidal gold particles.

Bmh1 and Egd2 are also involved in other cellular processes. Bmh1 is required for both the vegetative growth and the filamentation of *C. albicans*. In addition, it participates in processes such as the cellular response to stimuli, chlamydospore formation, pathogenesis, the regulation of carbohydrate metabolism, and signal transduction [4,14,24,49]. Therefore, this protein is likely to play a key role in colonization and invasion, by coordinating the necessary regulatory systems. Egd2 is the α subunit of the ribosomal nascent polypeptide-associated complex, which consists of Egd1 and Egd2. It participates in the transport of both cytoplasmic and mitochondrial proteins, binding to emerging polypeptides and thereby ensuring their proper orientation.

All of the above proteins have been described in both blastospores and mycelia of *C. albicans*, but quantitative studies have shown differences in their expression depending on the morphology of the fungus. Pitarch et al. [36] have found that, among others, Pgk1, Eno1, Adh1, and Ino1 are overexpressed after the induction of *C. albicans* germination. Martínez-Gomariz et al. [19] have added Met6 to this group of proteins. While Ebanks et al. [6] have also reported the expression of Adh1 protein in the mycelial phase, they found greater amounts of Egd2 and Eno1 in the yeast phase. In our study the protein map of DTT-GT cell-wall extract separated by 2D-PAGE also differed to some extent from that of DTT-B extracts. However, CAGTA eluted from germ tubes of *C. albicans* reacted mainly with the same antigens in the two extracts. Since in the IIF assay CAGTA reacted only with compounds of the cell surface of germ tubes, we asked whether the cellular location of the antigens depended on the morphology of *C. albicans*. Immunoelectron microscopy showed that CAGTA reacted with antigens that were mainly located on the outer layers of germ-tube cell walls, whereas in blastospores reactivity was in the inner layers of the cell wall. The morphological dependence of the position of some antigens in the *C. albicans* cell-wall structure has been reported by Pontón et al. [39], who described four types of antigens in the *C. albicans* cell wall based on their differential reactivity with several monoclonal antibodies. According to that system, the CAGTA antigens described in our study belong to type II, since they were located on the surface of the germ-tube cell wall and in the inner layers of the blastospore cell wall.

The location of at least some of the proteins on the outer germ-tube surface is in agreement with the development of CAGTA as a specific response of the host immune system during the invasive process. Our observations are consistent with those of other immunoproteomic studies that have attributed antigenic characteristics to most of the proteins identified using these antibodies, both in murine models of infection [30,46] and in patients with IC [9,25,29,31,32,34,35]. The immunogenicity of these proteins has allowed the development of ELISA tests to detect specific antibodies of diagnostic utility, with satisfactory results reported for recombinant Eno1 [16,18,32], Met6, and Pgk1 [3,33].

To the best of our knowledge, ours is the first study to identify Bmh1 and Egd2 in a study characterizing the CAGTA response in an animal infection model. In addition to this, CAGTA of both rabbits reacted with Bmh1 at a very early

stage of infection, only 3 days after the first inoculation, before CAGTA were detectable by IIF. The apparent absence of this reactivity in the preimmune sera may reflect previous sensitization to low levels of this protein, given the commensal nature of *C. albicans* in the yeast-phase morphology. Since Bmh1 belongs to a family of conserved regulatory proteins expressed in all eukaryotic cells, such that the rabbits may have been previously sensitized, the paradoxical behavior of CAGTA could be indicative of a quick response to the exposure of this antigen on the surface of the *C. albicans* germ tubes at the onset of fungal invasion. Because Bmh1 is involved in the filamentation process [14] and reacts with CAGTA of infected rabbits, the detection in serum of this protein and/or specific antibodies against it may allow its use as an early marker of IC. Further studies would be essential to confirm this relationship and whether it also pertains to IC in humans.

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Conflict of interest. None declared.

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