Cytoplasmic localization of the white phase-specific *WH11* gene product of *Candida albicans*

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Cells of *Candida albicans* WO-1 switch frequently, spontaneously and reversibly between a white and opaque phase. The white–opaque transition involves the regulation of phase-specific genes. In the white budding phase, cells express the white phase-specific gene *WH11*, which encodes a protein with homology to the heat shock protein Hsp12 of *Saccharomyces cerevisiae*. A recombinant Wh11 protein has been synthesized, purified to apparent homogeneity and used to generate a rabbit polyclonal antiserum. The antiserum was used to localize the Wh11 protein in white phase cells. Wh11 is distributed throughout the cytoplasm but appears to be excluded from vesicles, plasma membrane and nucleus. An analysis by Western blotting of Wh11 expression in a number of *C. albicans* strains and related species suggests a correlation between round budding cell shape and expression.

Keywords: *Candida* switching, white–opaque transition, bud–hypha transition, recombinant Wh11 protein, cell shape

INTRODUCTION

Most strains of *Candida albicans* can switch between a limited number of general phenotypes distinguishable by colony morphology (Slutsky *et al.*, 1985; Pomés *et al.*, 1985; Soll, 1992). Switching occurs spontaneously, is reversible and has a pleiotropic effect on phenotype. It can affect phenotypic characteristics as diverse as cellular morphology (Slutsky *et al.*, 1987; Anderson & Soll, 1987), antigenicity (Anderson *et al.*, 1989, 1990), secretion of acid proteinase (Morrow *et al.*, 1992; White *et al.*, 1993; Hube *et al.*, 1994), wall morphology (Anderson & Soll, 1987; Anderson *et al.*, 1990), pathogenicity in mouse models (Ray & Payne, 1990), sensitivity to white blood cells (Kolotila & Diamond, 1990) and adherence (Kennedy *et al.*, 1988; Vargas *et al.*, 1994). Switching frequently and reversibly alters phenotypic traits in a combinatorial fashion, including traits involved in pathogenesis. Therefore, it has been suggested that switching provides a mechanism for rapid adaptation to changes in the environment.

In the case of *C. albicans* WO-1, cells switch reversibly and at high frequency (10^{-2} – 10^{-4}) between a white and opaque

colony-forming phenotype, which are distinguishable not only by colony morphology, but also by ultrastructure (Slutsky *et al.*, 1987). Switching in WO-1 has been followed back and forth through more than 100 sequential switches in a single lineage with no effects on either the alternative phenotypes or the subsequent frequency of switching (M. Rotman & D. R. Soll, unpublished observations), suggesting that switching is not a random event, but rather a highly programmed process. This interpretation has been reinforced, in recent years, by the identification of both a white-phase-specific gene (Srikantha & Soll, 1993) and several opaque-phase-specific genes (Morrow *et al.*, 1992, 1993; White *et al.*, 1993; Hube *et al.*, 1994). In addition, the promoter of the white-phase-specific gene, *WH11*, has been functionally characterized and demonstrated to contain two *cis*-acting transcription activation domains which function synergistically in the activation of *WH11* transcription in the white phase (Srikantha *et al.*, 1995). Finally, gel retardation experiments have demonstrated the presence of white-phase-specific factors which bind to the two transcription activation domains of *WH11* (Srikantha *et al.*, 1995). These results demonstrate that switching involves a precise programme of phase-specific gene regulation.

The regulation of *WH11* gene expression is particularly interesting for a number of reasons. Firstly, as noted, it is regulated by the white–opaque transition (Srikantha &

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Soll, 1993). It is selectively transcribed in the white budding phenotype, but not in the opaque budding phase. Secondly, it is also regulated by the bud-hypha transition. It is expressed in the white budding phase, but not in the white hyphal phase (Srikantha & Soll, 1993) and, therefore, represents the first bud-specific gene in *C. albicans*. Its expression correlates with a round cell phenotype (i.e. it is expressed in the round budding phase, but is deactivated in both the elongate opaque budding phase and the elongate hyphal phase). Thirdly, it is homologous to the glucose and lipid-regulated protein Glp1 of *Saccharomyces cerevisiae* (Stone *et al.*, 1990), which has also been shown to be heat-shock protein Hsp12 (Praekelt & Meacock, 1990; Varela *et al.*, 1995). To begin to understand the role of the *WH11* gene product in switching, we have generated a rabbit polyclonal antiserum against a recombinant Wh11 protein and have used this antiserum to localize the *WH11* gene product in white budding cells. In addition, we have screened the protein extracts of budding cells from a number of medically important *Candida* species to test the relationship between cell shape and the presence of a Wh11 homologue.

METHODS

Strains. *Candida albicans* strains 3153A and WO-1 represent highly characterized laboratory strains from the Soll laboratory (e.g. Slutsky *et al.*, 1985, 1987). Isolates *Candida tropicalis* 996-28, *Candida guilliermondii* 1228-32, *Candida lusitanae* 1225-10, *Candida parapsilosis* 1345-22, *Torulopsis glabrata* 7549, *Candida kefyr* 881-02, *Candida krusei* 1225-24 and *Trichosporon beigelii* 1265-15 were generous gifts of Dr Michael Pfaller, University of Iowa, IA, USA. *C. tropicalis* J941810 was a generous gift from Dr Frank Odds, Janssen Research Foundation, Beerse, Belgium. *Candida shebatae* 34887 was purchased from the American Type Culture Collection, Bethesda, MD, USA. *Candida famata* 101 was from the Soll laboratory collection. *Saccharomyces cerevisiae* LA93-89 was a generous gift from Dr Jack Sobel, Wayne State University, Detroit, MI, USA. *Dictyostelium discoideum* Ax3 clone RC3 represents a highly characterized strain from the Soll laboratory (Soll, 1979; Wessels *et al.*, 1996). The atypical *C. albicans* strain was isolated in Montpellier, France by C. Pujol and characterized for isoenzyme patterns, Southern blot hybridization with specific probes and sugar assimilation pattern. It was similar in all respects to *Candida dubliniensis* NCPF 3108 (C. Pujol, S. Joly, M. Tibayrenc & D. R. Soll, unpublished observations), obtained from the British National Collection of Pathogenic Fungi, PHLS, Colindale, UK. *C. albicans* CAI-4 was a generous gift from Dr W. Fonzi, Georgetown University, School of Medicine, Washington, DC, USA.

Growth and maintenance of yeast strains. *C. albicans* WO-1 was maintained on nutrient agar slants containing modified Lee's medium (Bedell & Soll, 1979). For experimental purposes, cells were plated at low density and cells from a white or from an opaque colony were inoculated into separate flasks containing modified Lee's liquid medium. Cultures were shaken at 250 r.p.m. at 25 °C. For experimental use, cells were harvested at mid-exponential phase. Cell cultures were always assessed for phenotypic homogeneity (i.e. percentage white or opaque cells) prior to use in an experiment. All white or opaque cultures were over 99% homogeneous for the respective phenotype. Other yeast species employed in this study were maintained either on Sabouraud's agar slants (1%, w/v, yeast extract, 4%, w/v, glucose) or YPD agar slants (1% yeast extract, 2%, w/v, Bacto-

peptone, 2% glucose). For experimental purposes, cells of these species were grown to mid-exponential phase in YPD broth.

Construction of the recombinant plasmid. The *WH11* ORF (Srikantha & Soll, 1993) was fused to the glutathione-S-transferase gene (*GST*) in pGEX-2T (Smith & Johnson, 1988) (Pharmacia) by the following procedure. A PCR product of 201 bp was generated using a pair of oligonucleotide primers (primer I: 5' ATGTCCGACTTAGGTAG 3'; primer II: 5' GGATCCATTTGGAATCACCAAA 3'), complementary to the 5' and 3' ends of the *WH11* ORF, and pWhgP.4 (Srikantha & Soll, 1993), containing the *WH11* gene, as a template. The PCR product was gel-purified, digested with *Bam*HI and *Hinc*II and end-repaired using T4 DNA polymerase to ensure in-frame fusion. Plasmid pGEX-2T was digested with *Eco*RI followed by end repair with Klenow DNA polymerase. The PCR product was then inserted between the filled-in *Eco*RI sites of pGEX-2T to generate plasmid pWHOR.2. The in-frame fusion of the *GST* ORF and the *WH11* ORF in pWHOR.2 was confirmed by dideoxy sequence analysis (Sanger *et al.*, 1977) using Sequenase II according to the manufacturer's specifications (USB).

Expression and purification of recombinant Wh11 protein.

Escherichia coli LE392 was transformed with pWHOR.2 and an ampicillin-resistant clone was inoculated into 5 ml LB broth containing 100 µg ampicillin ml⁻¹. This culture was grown overnight at 37 °C and used as an inoculum for 250 ml LB medium. The cells were grown at 37 °C to an OD₆₀₀ of 1.0 prior to the induction of fusion protein synthesis. To induce fusion protein synthesis, IPTG was added to the culture to a final concentration of 150 µM and the culture was incubated for an additional 4 h. The bacteria were harvested, washed in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1% Triton X-100, lysed using a French press at 20000 p.s.i. and the lysate was centrifuged at 20000 g for 15 min. Glutathione-sephadex was added to the supernatant containing the rWh11 fusion protein (Pharmacia). The glutathione-Sepharose had previously been equilibrated with TBS/0.1% Triton X-100 and the mixture incubated for 60 min at 4 °C. After incubation for 1 h, the Sepharose beads were pelleted by low speed centrifugation, washed twice with thrombin cleavage buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2.5 mM CaCl₂) and the pellet resuspended and incubated for 90 min at 37 °C in an equivalent volume of thrombin cleavage buffer containing 50 units of thrombin (Sigma). NaCl was then added to the reaction mixture to a final concentration of 1.5 M and the mixture was centrifuged at 5000 g for 10 min. The supernatant was mixed with an equal volume of 2× SDS-PAGE loading buffer to final concentrations of 4% (w/w) SDS, 12% (w/v) glycerol, 50 mM Tris/HCl, pH 6.8, 2% (v/v) 2-mercaptoethanol and 0.01% Serva Blue G. The mixture was heated to 80 °C for 25 min, loaded on a preparative 12% tricine-SDS polyacrylamide gel (Schägger *et al.*, 1987) and electrophoresed at 80 V until the dye reached the bottom of the gel. The rWh11 fusion protein was localized by incubating the gel in ice-cold 250 mM potassium acetate. The identified rWh11 band was excised and protein electroeluted for 25 h at 200 V using the Elutrap System (Schleicher & Schuell). The eluted rWh11 protein was dialysed against PBS (3 mM KCl, 137 mM NaCl, 2 mM KH₂PO₄, 7 mM Na₂HPO₄, pH 7.4) diluted to 800 µg protein ml⁻¹ and stored at 4 °C.

Generating an anti-rWh11 antiserum. A 0.5 ml sample containing 400 µg protein was emulsified with 0.5 ml Freund's complete adjuvant and injected intradermally at 10 independent sites in the back of a New Zealand White rabbit. Subsequent injections with Freund's incomplete adjuvant were performed after 30, 60, 93 and 152 subsequent days at respective protein doses of 400, 400, 100 and 100 µg into the hip muscle of the

animal. Fourteen days after the last injection, 5 ml blood was collected and allowed to clot at 4 °C. The clot was removed by centrifugation and cell-free supernatant was stored at -20 °C. The presence of rWh11-specific antibodies in the serum was tested by Western blot analysis.

Preparation of cell-free protein extracts. Cells were harvested from broth cultures and washed twice in a solution containing 150 mM NaCl and 50 mM Tris/HCl, pH 8.3. Approximately 10^9 cells were transferred to 2 ml microfuge tubes and resuspended in 1 ml lysis buffer (150 mM NaCl, 50 mM Tris/HCl, pH 8.3, 0.1% 2-mercaptoethanol, 1 mM PMSF, 2 mM ϵ -aminocaproic acid, 2 mM pepstatin, 0.6 μ M leupeptin, 2 μ g chymostatin ml⁻¹, 50 μ g aprotinin ml⁻¹) containing 1 vol. 0.4 mm glass beads. Cells were disrupted in a Bead Beater II (Biospec Products) through 10 cycles of 15 s duration interspersed with 30 s periods of cooling in an ice bath. Lysates were clarified by high speed centrifugation at 10000 g for 20 min at 4 °C. Samples were stored at -70 °C. Protein concentration was determined by the method of Bradford (1976) (Bio-Rad).

Western blot analysis. Cell-free extract containing 20–25 μ g protein was heated to 80 °C for 25 min in SDS-PAGE loading buffer, loaded on a 12% (w/v) tricine-SDS polyacrylamide gel (Schägger *et al.*, 1987) and electrophoresed at 80 V until the dye reached the bottom of the gel. The gel was then either stained with Coomassie Blue R250 or transferred to Immobilon-P membrane (Millipore) using a semi-dry blotting technique according to the manufacturer's specifications. Prior to transfer, the gel and membrane were soaked in cathode transfer buffer (25 mM Tris/HCl, pH 9.4, 40 mM glycine, 20%, v/v, methanol). The transfer of separated proteins from the gel to membranes was performed for 30 min at 0.33 mA cm⁻², for 30 min at 0.50 mA cm⁻² and finally for 30 min at 0.66 mA cm⁻². Following transfer, the membrane was washed for 2 h in PBS and incubated either in a solution of 0.1% India Ink and 0.3% Tween-20 to visualize proteins, or in blocking solution (5%, w/v Carnation milk powder, 1%, w/v, BSA, 0.1%, v/v, Tween-20 in PBS) for 15 h at room temperature for immunostaining. For immunostaining, the membrane was transferred to fresh blocking solution containing a 1:2500 dilution of rabbit anti-rWh11 antiserum and incubated for 60 min at room temperature. The membrane was rinsed six times for 5 min in a solution containing PBS/0.2% Tween 20 and incubated in a solution containing a 1:2500 dilution of goat anti-rabbit antiserum conjugated with alkaline phosphatase (Life Technologies) in blocking solution for 30 min. The membrane was washed three times in a solution of PBS/0.2% Tween-20 and two times in TBS. For detection of immunostained proteins, the membrane was soaked in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) containing NBT-BCIP according to the manufacturer's recommendations (Promega). Staining of bands was terminated by rinsing in 100 μ M EDTA.

Indirect immunofluorescent staining of cells. Cells were washed in water and resuspended at a density of 10^7 ml⁻¹. One millilitre was mixed with 0.5 ml freshly prepared 12% (v/v) paraformaldehyde, pH 8.0. The cell mixture was incubated for 30 min at 25 °C, pelleted, resuspended in a solution containing 4% (w/v) paraformaldehyde and 5% (v/v) DMSO in PBS, pH 7.4, and incubated first for 30 min at 25 °C, then for 60 min at 37 °C. Fixed cells were then washed twice in a solution of PBS containing 5% DMSO, resuspended in 2.5 ml SCM (1.2 M sorbitol, 50 mM sodium citrate, pH 5.9, 0.1% mercaptoethanol, 5 mM MgCl₂) containing 3 mg Zymolyase 100T (Seikagaku America), 3 mg Novozyme 234 (Sigma) and 5 mg β -glucuronidase (Sigma) and incubated for 10–15 min in the case of opaque phase cells or 180 min in the case of white phase cells. Cell wall digestion was monitored by phase contrast micro-

scopy. Cells were washed twice in SCM and once in a solution containing 1.2 M sorbitol and 100 mM Tris/HCl, pH 8.3. Cells were permeabilized by incubating them in a solution containing PBS, 5% DMSO and 0.2% Triton X-100 for 10 min at 25 °C and washed in PBS, pH 8.2, containing 5% DMSO and 1% BSA. Cells were incubated in blocking solution (25%, v/v, goat serum, 10% Carnation milk powder, 5% DMSO in PBS) for 60 min and then in a 1:500 dilution of rabbit anti-rWh11 antiserum in blocking solution for 90 min, followed by five washes in a solution of PBS containing 5% (v/v) DMSO. Cells were incubated in a 1:300 dilution of FITC-labelled goat anti-rabbit IgG antiserum (Organon Teknika Corp.) in blocking solution for 45 min in a dark chamber. Cells were washed six times with PBS followed by staining with 4,6-diamino-2-phenylindole (DAPI) (0.02 μ g ml⁻¹) for 5 min. Cells were finally pelleted by centrifugation at 6000 g, resuspended in 50 μ l Vectashield Mounting medium (Vector Labs) and spread on slides for microscopic analysis. For visualizing cells stained with FITC-labelled goat anti-rabbit IgG antiserum, a blue I 450 MM excitation filter was employed and for DAPI-stained cells a UV 365 MM excitation filter was employed. Cells were photographed using epiluminescent fluorescence in a Zeiss ICM405 inverted microscope. The same preparations were examined with a Bio-Rad 600 MRE confocal microscope.

RESULTS

Expression and purification of recombinant Wh11 protein and characterization of polyclonal anti-rWh11 antiserum

The entire protein-coding region of *WH11* cDNA was fused in-frame to *GST* in pWHOR.2 and the resulting plasmid was used to transform *E. coli* LE392. After induction with IPTG, the fusion protein was affinity-purified with glutathione cross-linked to Sepharose (Smith & Johnson, 1988). The affinity-purified preparation was enriched for a protein of 32 kDa, the estimated size of the GST-Wh11 fusion protein (Fig. 1, lane 2). The

Fig. 1. Affinity purification of rWh11 protein. The figure shows a polyacrylamide gel containing Coomassie-stained proteins following separation by SDS-PAGE. Lanes: 1, total protein from *E. coli* LE392 transformed with pWHOR.2; 2, protein affinity-purified with glutathione-Sepharose; 3, flow-through after affinity purification; 4, GST and rWh11 after cleavage with thrombin; 5, rWh11 after preparative SDS-PAGE; 6, molecular mass markers. Lane 5 demonstrates the relatively pure rWh11 subsequently used to immunize a rabbit.

Fig. 2. Analysis of the antiserum generated against the rWh11 protein. (a) Polyacrylamide gel containing Coomassie-stained proteins following separation by SDS-PAGE. Lanes: M, molecular mass markers; WH, total protein from white phase cells; OP, total protein from opaque phase cells; rWh11, purified rWh11 protein. (b) Western blot of protein extract from white phase cells (WH), protein extract from opaque phase cells (OP) and rWh11 probed with rabbit pre-immune serum. Note that both white and opaque phase protein extracts show similar patterns of lightly stained high molecular mass proteins. (c) Western blot of protein extract from white phase cells (WH), protein extract from opaque phase cells (OP) and rWh11 probed with rabbit immune serum (anti-rWh11 antiserum). Note the intense band of approximately 7 kDa in the white phase protein extract but not in the opaque phase protein extract. Note also the similar molecular mass of rWh11 and the major white phase antigen.

fusion protein was then cleaved with thrombin, resulting in a protein of approximately 25 kDa, the size of the GST polypeptide, and in a protein of approximately 7 kDa, the predicted size of the rWh11 protein (Fig. 1, lane 4). After preparative SDS-PAGE, the rWh11 protein was purified to apparent homogeneity (Fig. 1, lane 5). This protein preparation was used to immunize a rabbit.

The polyclonal antiserum raised against the rWh11 recombinant polypeptide stained purified rWh11 in Western blots (Fig. 2c). To demonstrate the specificity of the anti-rWh11 antiserum, the cell-free protein extracts of white and opaque phase cells were stained with preimmune and immune serum in a Western blot. Preimmune serum exhibited non-specific staining of the same array of high molecular mass protein bands in both white and opaque cell-free protein extracts (Fig. 2b). In neither case did the preimmune serum stain a protein of ~7 kDa. Preimmune serum also did not stain the purified rWh11 protein (Fig. 2b). Although the serum obtained from the rabbit immunized with rWh11 protein stained an identical array of high molecular mass proteins in both white and opaque cell extracts, it selectively stained a major protein of approximately 7 kDa in the white cell extract but not in the opaque cell extract (Fig. 2c). The level of staining of the 7 kDa protein was dramatically greater than that of the higher molecular mass proteins. Absorption with protein from bacteria transformed with control plasmid pGx2T without the *WH11* insert, did not

remove antibody reacting with the high molecular mass proteins. These bands, therefore, represent cross-reacting antigens which are not phase-specific. The white-phase-specific antigen exhibited the same molecular mass as the rWh11 protein (Fig. 2c). These results demonstrate that the anti-rWh11 antiserum selectively stains the *WH11* gene product. The similarity of the molecular size of rWh11 protein and the antiserum-identified antigen in white cell extract suggests that Wh11 is not subject to major post-translational modification.

The anti-rWh11 antiserum selectively stains the cytoplasm of white phase cells in a punctate fashion

To assess the cellular location of the *WH11* gene product in white phase budding cells, fixed white phase cells were treated with anti-rWh11 antiserum and then stained with FITC-labelled goat anti-rabbit IgG antiserum. Examples of the phase contrast images are presented in Fig. 3a–d, and respective fluorescently stained white phase budding cell images are presented in Fig. 3a'–d'. White budding cells stained in a punctate fashion throughout the cytoplasm. There was no indication of either cortical or membrane localization. When stained white cells were examined by confocal microscopy, a similar pattern was observed (Fig. 3e–j). Staining appeared throughout the cytoplasm and, again, no specific localization was evident either in the outer cortex of the cytoplasm or plasma

Fig. 3. White phase budding cells stained with anti-rWh11 antiserum. Staining was accomplished by labelling anti-rWh11 antiserum-treated preparations with FITC-conjugated goat anti-rabbit IgG antiserum. (a–d) and (a'–d') Phase contrast and anti-rWh11 antiserum-stained epifluorescent images, respectively, of four white phase cells in the budding phase of growth. (e–j) Confocal images of anti-rWh11 antiserum-stained white phase cells in the budding phase of growth. Regions in the cell excluding Wh11 antigen are noted in panels (c'), (e), (f) and (h) by white arrows. In (c') there is an insert of the DAPI-stained nucleus of the cell; the cell perimeter in the insert is distinguished by white dots. Bars, 5 μ m.

membrane. Staining appeared to be excluded from a single region in the cell interior (e.g. Fig. 3c', e, f, h), and double staining with anti rWh11 antiserum and DAPI

(Fig. 3c') suggested that these exclusion regions probably corresponded to cell nuclei. These results were obtained with four separate cell preparations.

Fig. 4. Opaque phase cells and white phase hyphae do not stain with anti-rWh11 antiserum. (a, a', a'', b, b', b'') Phase contrast, DAPI-stained and anti-rWh11 antiserum-stained images, respectively, of two opaque phase cells in the budding phase of growth. (c, c', c'') Phase contrast, DAPI-stained and anti-rWh11 antiserum-stained images, respectively, of a white phase cell which has formed a hypha. (d, d', d'') Phase contrast, DAPI-stained and anti-rWh11 antiserum-stained images,

The anti-rWh11 antiserum stains neither opaque phase cells nor white phase budding cells which have formed hyphae

It was previously demonstrated that neither opaque phase cells nor white phase cells forming hyphae contained significant levels of the *WH11* transcript (Srikantha & Soll, 1993; Srikantha *et al.*, 1995). To test whether opaque phase cells in the budding phase or white phase cells induced to form hyphae by pH-regulated dimorphism (Buffo *et al.*, 1984; Soll, 1986) contained the Wh11 protein, these two cell types were subjected to indirect immunofluorescent staining with anti-rWh11 antiserum and FITC-labelled goat anti-rabbit IgG antiserum. The same cells were also stained with DAPI to visualize nuclei. Phase contrast micrographs in Fig. 4a and b demonstrate the elongate shape and prominent vacuole of budding opaque cells, and DAPI-stained images of the same cells in Fig. 4a' and b' demonstrate the eccentrically located nucleus (Fig. 4a and b). Anti-rWh11 antiserum did not stain opaque phase cells (Fig. 4a'' and b''). This was verified by examining several stained preparations containing more than 500 opaque cells.

Hypha formation was induced by allowing white phase budding cells to enter stationary phase at 25 °C in modified Lee's liquid medium, where they accumulate as unbudded singlets in G1 (Soll, 1986), and then diluting them into fresh medium at 37 °C, in which they synchronously form germ tubes (Buffo *et al.*, 1984). In Fig. 4c, the phase contrast image is presented of a white phase budding cell which has formed a long hypha under this induction regime. DAPI staining (middle panel, Fig. 4c') reveals a nucleus in the original mother cell. Anti-rWh11 antiserum stained neither the mother cell, which contained Wh11 protein during exponential growth in the budding phase, nor the daughter hypha (Fig. 4c''). In Fig. 4d, a phase contrast micrograph is presented of the distal end of a hypha. DAPI staining revealed a nucleus midway along the hypha (Fig. 4d), but again anti-Wh11 antiserum did not stain the hypha (Fig. 4d''). The absence of Wh11 antigen in mother cells and hyphae was verified by examining multiple stained preparations containing more than 500 mother cells and daughter hyphae.

Wh11 expression and cellular morphology in other strains of *C. albicans* and other species of *Candida*

Using the anti-rWh11 antiserum, we tested whether the *WH11* gene product is expressed in the basic o-smooth phenotype of *C. albicans* 3153A, two additional strains of *C. albicans*, CAI-4 (Fonzi & Irwin, 1993) and an atypical strain of *C. albicans*, which biotyped as *Candida dubliniensis* (Sullivan *et al.*, 1995). All strains were analysed in the budding phase and all strains expressed an antigen at ~ 7 kDa which stained strongly with anti-rWh11 antiserum (Fig. 5a).

We next tested whether *WH11* was expressed in the

budding phase of other *Candida* species by Western blot analysis. The cell-free protein extract of budding cells of two isolates of *C. tropicalis* and one isolate each of *C. guilliermondii*, *C. lusitanae* and *C. famata* contained an antigen of approximately 7 kDa which stained strongly with the anti-rWh11 antiserum (Fig. 5b). The cell-free protein extract of *C. parapsilosis* contained an antigen of approximately 9 kDa which stained strongly with the anti-rWh11 antiserum (Fig. 5b). However, cell-free protein extract of *T. glabrata* (also referred to as *Candida glabrata*), *C. kefyr* and *C. krusei* contained no detectable cross-reacting antigen (Fig. 5c). In addition, the more distantly related species *Trich. beigeli* and *S. cerevisiae*, and the cellular slime mould *D. discoideum* contained no detectable cross-reacting antigen (Fig. 5c).

The expression of *WH11* in WO-1 appeared to correlate with a round cell morphology, since it was expressed in the round white budding cell, but not in the elongate opaque budding cell or the elongate hypha formed by white cells (Srikantha & Soll, 1993). We therefore assessed the expression of Wh11 in the tested *C. albicans* strains, the various *Candida* species and the related yeast genera in relation to their budding cell morphology. Cellular morphology was designated as either round (rnd) or elongate (elong) based upon microscopic analysis. The correlation between the presence of the Wh11 antigen and round cell morphology was suggestive, but not definitive in all cases. All *C. albicans* strains which showed a strongly staining Wh11 antigen in Western blots (*C. albicans* WO-1 WH, 3153A, CAI-4, and the atypical *C. albicans* strain) exhibited a round cell morphology in the budding phase of growth. The majority of other *Candida* species which showed a strongly staining Wh11 antigen in Western blots (*C. tropicalis* strains 1 and 2, *C. guilliermondii*, *C. lusitanae*, *C. famata* and *C. parapsilosis*) also exhibited a round cell morphology in the budding phase of growth. In the case of *C. guilliermondii*, the cell population contained primarily round, but also elongate cell morphologies and, interestingly, Western blots of this species showed a slightly weaker signal (Fig. 5b). All *Candida* species with very low (*C. shehatae*) or no (*C. kefyr*, *C. krusei*) reactivity in Western blots (Fig. 5b) exhibited an elongate morphology in the budding phase of growth. *C. glabrata* was the only exception. It exhibited a round cell morphology in the budding phase of growth and no reactivity with anti-rWh11 antiserum (Fig. 5c). Species of the two other yeast genera analysed, *Trich. beigeli* and *S. cerevisiae*, did not show any reactivity with the antiserum (Fig. 5c), but did show round cell morphologies. Two independent preparations of every species were stained and in every case the same staining results were obtained.

DISCUSSION

The white-phase-specific gene *WH11* is regulated at the level of transcription by both the white-opaque switching

respectively, of a white phase hypha. Hyphae were induced by pH-regulated dimorphism (Buffo *et al.*, 1984; Soll, 1986). Note that neither opaque phase cells, hyphae nor the white phase mother cell which formed the hypha in (c, c', c'') stain for Wh11 protein. Bar, 3.5 µm.

Fig. 5. Expression of Wh11 in other strains of *C. albicans* (a) and in unrelated *Candida* and other fungal species (b, c). The panels show Western blots of protein extracts of budding cells separated by SDS-PAGE using the anti-rWh11 antiserum. Cell morphologies were analysed microscopically and scored as either round (rnd) or elongate (elong). In the case of *D. discoideum*, a slime mould amoeba, the morphology is irrelevant (-).

system and the bud-hypha transition in *C. albicans* WO-1 (Srikantha & Soll, 1993). It contains an ORF encoding a putative protein of 65 amino acids with a molecular mass

of approximately 7.0 kDa. Amino acids 1–59 of the glucose/lipid-regulated protein Glp1 of *S. cerevisiae* (Stone *et al.*, 1990), which has also been identified as a heat shock

protein referred to as Hsp12 (Praekelt & Meacock, 1990; Varela *et al.*, 1995), exhibits 47% homology to Wh11 (Srikantha & Soll, 1993). Hsp12 accumulates to high levels upon heat shock, osmotic stress, oxidative stress and alcohol (Varela *et al.*, 1995). Unlike Hsp12, the expression of Wh11 is regulated neither by glucose nor lipid, and does not appear to be induced by heat shock alone. Rather, expression of this gene is regulated by the two developmental programmes of *C. albicans* (switching and dimorphism), and deactivation of this gene in both programmes correlates with the formation of an elongate morphology. Recently, we demonstrated that *WH11* transcription in the white phase is regulated through two transcription activation domains (Srikantha *et al.*, 1995). A comparison of the DNA-protein complexes formed by white or opaque cell protein extracts and the individual activation domains using a gel retardation assay has revealed the existence of one or more white-phase-specific binding factors (Srikantha *et al.*, 1995).

To begin to understand the physiological role of the *WH11* gene product in both the white-opaque transition and the bud-hypha transition, we first had to map the localization of the Wh11 protein within the cell. Since the *WH11* message appears to be of low abundance (Srikantha & Soll, 1993), purifying the *WH11* gene product did not seem practical. We therefore generated a recombinant protein with a molecular mass similar to the native Wh11 protein, purified it to apparent homogeneity and used it to generate a polyclonal antiserum in a rabbit. The specificity of the antiserum has been verified by several criteria, including specific Western blot staining of a white-phase-specific antigen with the same molecular mass as the *WH11* gene product. This antiserum was then used to immunostain white and opaque phase budding cells and white cells induced to form hyphae through the regime of pH-regulated dimorphism (Buffo *et al.*, 1984; Soll, 1986). Anti-rWh11 antiserum stained the cytoplasm of white phase budding cells, but did not stain either the elongate opaque phase budding cell or hyphae. In white phase budding cells induced to form hyphae, the antigen was absent not only in the hypha, but also in the mother cells, which contained the antigen prior to hypha induction. This result demonstrates that the Wh11 antigen is lost sometime between entry into stationary phase and the formation of a germ tube, and suggests that it is metastable and present only during the growth of a budding cell.

The anti-rWh11 antiserum stains the cytoplasm of white phase budding cells in a punctate fashion and labelled cells viewed both by epifluorescent microscopy and confocal microscopy exhibit patterns which suggest that the antigen is either coating cytoplasmic vesicles or is distributed in the cytoplasm but excluded from vesicle interiors. Double staining of the Wh11 gene product of white budding cells with anti-rWh11 antiserum and nuclei with DAPI suggests that the Wh11 protein was excluded from the nucleus, and confocal images of anti-rWh11 antiserum-stained cells revealed an exclusion zone in each white phase cell which very likely represents the cell nucleus. The plasma membrane did not stain and there was no indication of localized staining in the cell cortex.

In addition, anti-rWh11 antiserum did not stain the cell wall. This staining pattern is similar to that of *S. cerevisiae* stained with antiserum against Hsp26, a small heat shock protein related to Hsp12, which has been demonstrated to be a chaperone protein (Rossi & Lindquist, 1989).

We have explored further the suggested relationship between expression of *WH11* and cell shape by analysing a number of different strains of *C. albicans* and different *Candida* and non-*Candida* species for correlations between expression of the *WH11* gene product and budding cell morphology. We initially demonstrated that *C. albicans* 3153A expressed *WH11* in the budding but not in the hyphal growth phase (Srikantha & Soll, 1993), as was the case for white budding cells of WO-1. However, *WH11* was not deactivated in three tested variant phenotypes of the 3153A switching system, even though these variant phenotypes differentially expressed the opaque-phase-specific gene *OP4* (Morrow *et al.*, 1994). Therefore, although *WH11* is differentially expressed in the white-opaque transition, it is not differentially expressed in the high frequency phenotypic switching system of 3153A. However, unlike the variant opaque phenotype in the switching system of WO-1, all three 3153A switching variants grew in the budding phase with round cell morphologies, again correlating *WH11* expression with a round budding cell morphology. Here, we have demonstrated that three *C. albicans* strains and one atypical *C. albicans* strain express the Wh11 antigen in the budding growth phase and, in all cases, the budding cells exhibited a round cell morphology. We have also found that in addition to *C. albicans*, five additional *Candida* species (*C. tropicalis*, *C. guilliermondii*, *C. lusitanae*, *C. famata* and *C. parapsilosis*) which exhibit strong 7 or 9 kDa bands in Western blots stained with the anti-rWh11 antiserum, also exhibit a round budding cell morphology. In contrast, three *Candida* species (*C. kefyr*, *C. shehatae* and *C. krusei*) which exhibit no stained 7 kDa band or a very low signal, exhibit an elongate cell morphology. Species from three additional yeast genera (*T. glabrata*, *Trich. beigellii* and *S. cerevisiae*) grew with a round cell morphology but exhibited no 7 kDa signal with anti-rWh11 anti-serum. We assume that the absence of a signal in these cases is due to genetic unrelatedness. We therefore believe that the association of *WH11* expression and the round cell morphology remains intact and deserves further investigation. To this end, we are in the process of generating *WH11* misexpression mutants in which *WH11* is under the control of an opaque promoter and is expressed in opaque cells and gene-knockout mutants which cannot express *WH11*.

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