The role of microfilaments and microtubules during pH-regulated morphological transition in *Candida albicans*

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Yeast cells of *Candida albicans* produced germ tubes in a salt-glucose medium containing 4% calf serum at pH 7 and 37°C. Hyphal growth continued for 24 h and the filaments did not revert to yeast cells. When cells were grown at pH 4, reversion to yeast growth was observed, despite the presence of serum. The elongation of hyphae was inhibited within 30 min. The distribution of microtubules and microfilaments during pH-regulated morphological transition was studied by an immunofluorescence technique using an antitubulin antibody with a FITC-conjugated secondary antibody, and by staining with tetramethylrhodaminyl phalloidin for filamentous actin and actin granules. After changing to acidic conditions, microtubules were distributed normally in the cytoplasm; however, microfilaments disappeared from hyphal cells, and actin granules were localized at the site of budding. These results show that microfilaments play an important role during pH-regulated morphological transition.

**Keywords:** *Candida albicans*, microtubules, microfilaments, pH regulation, morphological transition

**INTRODUCTION**

*Candida albicans* is a pathogenic and polymorphic fungus having four major morphologies, namely yeast cells, pseudohyphae, hyphae and chlamydospores. There are several major differences between the growth of yeast and filamentous forms of *C. albicans* (Yokoyama & Takeo, 1983). The reciprocal transformations observed between the yeast and filamentous forms seems to be related to the pathogenicity of this fungus. The study of dimorphism of this fungus is useful for elucidating the mechanisms of morphogenesis, growth and differentiation.

Environmental factors are vital for control of the morphology of *C. albicans*. Temperature and pH produce morphological changes in amino acid-containing defined media (Lee *et al.*, 1975; Buffo *et al.*, 1984). The characteristics of pH-regulated dimorphism of this fungus have been reported when defined amino acid-containing media are used (Buffo *et al.*, 1984). Lee’s medium (lacking glucose) induced germ tubes at pH 3 (Pollack & Hashimoto, 1987). Changes of intracellular pH correlated with the dimorphic transition of this organism (Stewart *et al.*, 1988, 1989; Kaur *et al.*, 1988). Plasma membrane H+-ATPase activity was monitored during pH-regulated dimorphism of *C. albicans* by Kaur & Mishra (1991). They suggested that the changes of intracellular pH and ATPase may play a regulatory role in dimorphism of this fungus. However, since Gupta & Prasad (1993) reported that levels of plasma membrane H+-ATPase do not change during growth and morphogenesis of *C. albicans*, post-translational modification(s) of enzyme protein is suggested to account for the variation in PM-ATPase activity during morphogenesis. The correlation between intracellular pH change and morphological change is not yet clear.

We previously reported (Yokoyama *et al.*, 1990) that cytoplasmic microtubules are not essential for the elongation of filamentous cell tips but that microfilaments are. In this paper, we report that microfilaments play a leading role in pH-regulated dimorphism of *C. albicans*.

**METHODS**

**Organisms.** The *Candida albicans* strains used, IFM (Research Center for Pathogenic Fungi and Microbial Toxicses) 40009 (ATCC-40130), 40100, 40101 and 40102, were all clinical isolates.
Fig. 1. Conversion of hyphal growth to yeast growth on changing the pH of the medium from 7 to 4 at 37°C. Microphotographs were taken after germ tube formation at 2 h [(a, i), pH 7; (ii), pH 4], 6 h [(b, i), pH 7; (ii), pH 4] and 21 h [(c, i), pH 7; (ii), pH 4] for 3 h at 37°C in HFM7. Bar, 50 μm.

Media and culture. Preincubation liquid medium was YPD, containing 1% (w/v) yeast extract, 2% (w/v) polypeptone (Wako) and 2% (w/v) glucose. Hypha-forming medium (HFM) contained the following components per litre: glucose, 5 g; Na₂HPO₄, 12H₂O, 0.26 g; KH₂PO₄, 0.66 g; MgSO₄, 7H₂O, 0.08 g; NH₄Cl, 3.3 g; biotin 16 μg; calf serum (Gibco) 4% (v/v). The pH of HFM7 and of HFM4 was adjusted to 7.0 and 4.0 with 3 M NaOH and 1 M HCl, respectively, and filter-sterilized. Plastic Petri dishes (30 mm diameter) were coated with a solution (200 μl) of poly-L-lysine (100 μg ml⁻¹; poly-L-lysine hydrobromide, M, 70000-150000, Sigma). After 5 min, the solution was removed by aspiration and the treated surface was rinsed twice with 1.5–2.0 ml sterile, distilled and deionized water. The culture dishes were air-dried before use.

Induction of germ tubes or yeast growth. C. albicans was preincubated in YPD medium at 25°C and magnetically stirred (about 60 r.p.m.) for 24–38 h (early stationary phase; about 10⁶ cells ml⁻¹). Germ tubes were induced within 60 min after preincubated cells were inoculated (final 10⁴ cells ml⁻¹) in HFM7 at 37°C. Preincubated yeast cells did not form germ tubes at 37°C in HFM4 medium. HFM7 medium was removed after 3 h incubation from the culture dish and HFM4 medium was added. Hyphal growth converted to yeast growth when
pH-regulated morphological transition in *C. albicans*

*Fig. 2.* Time-lapse microphotographs of *C. albicans* IFM 40009 initiated after conversion of the incubation medium to HFM4. Cells were photographed at 15 min intervals. M, filamentous mother cell; D₁, first daughter cell from M; D₂, second daughter cell from D₁; D₃, third daughter cell from D₂; MD₂, other daughter cell from M; D₁D₂, new daughter cell from D₁. Inhibition of hyphal extension was observed within 30 min and yeast growth appeared at 75 min. Bar, 10 μm.

HFM7 medium was changed to HFM4 at 37 °C. Microphotographs were taken at 2, 6 and 21 h after hypha formation for 3 h at 37 °C in HFM7 using a Nikon TMD inverted phase-contrast microscope with Plan ×10 or ×20 BM objective lenses (Nippon Kogaku).

**Time-lapse photomicrography.** Preincubated yeast cells were incubated for 3 h in HFM7 at 37 °C, and germ tubes elongated to about 50 μm. Time-lapse photomicrography was initiated just after hypha formation for 3 h and the change of the incubation medium to HFM4. The process of morphological change was observed by a Nikon TMD inverted phase-contrast microscope with a Plan ×20 BM objective lens. Cells were photographed at 15 min intervals.

**Observation of microtubules and microfilaments.** Pre-
incubated yeast cells were incubated in HFM7 using poly-l-lysine-coated plastic Petri dishes at 37 °C for 2.5 h. Medium was subsequently changed to HFM4 and cells were incubated for 30 min, fixed with 4% paraformaldehyde in 5% DMSO-PBS (PBS containing 0.8% NaCl, 0.02% KCl, 0.29% Na₃H₂PO₄·12H₂O and 0.02% KH₂PO₄, adjusted to pH 8.2 with 3 M NaOH) for 30 min at 37 °C and 1 h at room temperature. Fixed cells were washed twice in 5% DMSO-PBS (pH 7.2).

These cells were stained by monoclonal anti-yeast-tubulin YOLI/34 IgG (Serotec) and affinity-purified FITC-conjugated anti-rat IgG (Jackson Immunoresearch Laboratories) for tubulin labelling, by tetramethylrhodaminyl (Rh-)phalloidin (Molecular Probes) for observation of F-actin (microfilaments and actin granules) and by 4',6-diamidino-2-phenylindole (DAPI, Sigma) for nuclei. The triple-stained cells in the Petri dish were observed and photographed with an epi-illumination fluorescence microscope-photometer SPM-RFL-II (Nikon), according to Yokoyama et al. (1990).

**RESULTS**

**Change from hyphal to yeast growth elicited by external pH**

For all yeast cells preincubated in YPD, germination was observed within 60 min after inoculation into HFM7 at 37 °C. Extension of germ tubes continued for 24 h in this medium (Fig. 1; c, i). When germ tubes were incubated for the first 3 h in HFM7 (pH 7) and incubated for 2 h (Fig. 1; a, ii) or 6 h (Fig. 1; b, ii) after external pH was converted from 7 to 4 at 37 °C, the elongation of apical cells stopped and buds appeared on the apical cell and hyphal cells (Fig. 1; a, ii; b, ii; c, ii). These observations show that this medium supported hyphal growth for a long time; despite the presence of 4% serum, the extension of hyphal cells did not continue at pH 4.

Time-lapse microphotography was used to observe the
growth conversion progress. Fig. 2 shows inhibition of the extension of the apical hypha within 30 min. The first daughter cell (D1) produced the second daughter cell (D2, Fig. 2, 90 min) simultaneously with, or slightly after, the mother cell (M) produced another daughter cell (MD2) (Fig. 2, 90 min). This is characteristic of yeast growth. During hyphal growth, the daughter cells always produced other daughter cells faster than the mother cells produced other daughter cells. The inhibition of hyphal extension was observed within 30 min and yeast growth appeared 75 min (one generation time) after change in external pH.

Effect of external pH on the distribution of microtubules, microfilaments and actin granules

Microtubules, microfilaments and nuclei were observed in hyphal cells incubated in HFM7 medium at 37 °C (Fig. 3). Microtubules were widely distributed in the cytoplasm (Fig. 4a) and microfilaments were observed in growing hyphae (Figs 3b, 4). Long microfilaments were distributed along the lines of cell growth (Fig. 4a, b) and a network of microfilaments was observed in the tips of apical cells, often connected with actin granules in HFM7 medium at 37 °C (Figs 3b, 4c). Actin granules were localized at the tips of apical cells and septum-forming sites (Figs 3b, 4).

When the external pH was changed from 7 to 4, microtubules were observed in the hyphal cell within 30 min at 37 °C (Fig. 5a). This distribution of microtubules was the same as in hyphal growth (Figs 3a, 5a). Microtubules distributed regularly according to nuclear migration and the cell cycle. The decrease of external pH did not affect microtubules directly, but long microfilaments disappeared from hyphal cells within 30 min after changing the external pH at 37 °C (Fig. 5b). Localization of actin granules, concentrated at the tip of apical cells in normal hyphal growth (Figs 3b, 4), was not observed (Fig. 5b). Rather, actin granules were dispersed throughout the apical cells and rearranged at the budding and septum-forming sites (Fig. 5b).

We obtained the same results in all four strains used. These results suggest that the effect of low external pH particularly affects long microfilaments, which disappear from the cytoplasm, and consequently trigger the reversion from hyphal growth to yeast growth.

DISCUSSION

In our previous paper (Yokoyama et al., 1990), we showed by using a microfilament inhibitor that microfilaments play an important role in the elongation of apical cells and hyphal growth. In this work we attempted to elucidate the role of microfilaments in fungal morphogenesis.

By using time-lapse microphotography (Fig. 2), we observed a change of hyphal to yeast growth at a low external pH (pH 4), although the medium contained serum. Microtubules were distributed in the cytoplasm in a similar fashion as during hyphal growth; however, long microfilaments disappeared from the cytoplasm within 30 min due to the change of external pH (Fig. 5b). These distributions coincided with the result obtained by using...
Fig. 5. Triple staining of germ tube of *C. albicans* IFM 40009 at an external pH of 4. Cells were incubated for 3.5 h in HFM7 at 37 °C and the medium then changed from HFM7 to HFM4 at 37 °C. Cells were fixed after 30 min incubation in HFM4 medium. (a) Staining with anti-yeast-tubulin antibody and FITC-conjugated secondary antibody; (b) staining with Rh-phalloidin; (c) staining with DAPI. Microtubules were distributed normally in filamentous cells (a); however, long microfilaments disappeared (b). Bar, 2.5 μm.

It has been reported that glucose, but not pH, plays an important role, in directly regulating dimorphism in *C. albicans* (Pollack & Hashimoto, 1987): germ tubes were induced by proline or absence of glucose when the pH was between 3 and 9. HFM7 medium (containing 0.5% glucose) induced germ tube formation (100% of pre-incubated yeast cells) and HFM4 inhibited it at 37 °C, which make these media favourable for the study of dimorphism of this fungus. In other studies on pH-regulated dimorphism, it has been reported that the formation of buds or germ tubes (Kaur et al., 1988; Kaur & Mishra, 1991) and induction of germ tube formation is accompanied by a steep rise in internal pH and activity of H⁺-ATPase (Stewart et al., 1988, 1989). Recently, Gupta & Prasad (1993) reported that levels of plasma membrane H⁺-ATPase do not change during growth and morphogenesis of this fungus.
Ca²⁺ and calmodulin have been reported to be involved in differentiation of fungi such as *Ceratocystis ulmi* (Muthukumar & Nickerson, 1984), *Dictyostelium discoideum* (Lydan & O’Day, 1988), *Physarum polycephalum* (Uyeda & Furya, 1986) and yeast (Muthukumar et al., 1987). Protein phosphorylation is also reported to be involved in the growth and differentiation of *Mucor* (Orlowski & Sypherd, 1978), *D. discoideum* (Sinclair & Rickwood, 1985) and *P. polycephalum* (Fronk & Toczko, 1987). Recently, it has been reported that Ca²⁺ and calmodulin-mediated protein phosphorylation play a role in germination of *Mucorhiizm anisopliae* (St Leger et al., 1989) and *C. albicans* (Paranjape et al., 1990).

External pH may affect internal pH, concentration of Ca²⁺ and activity of calmodulin, triggering depolymerization or polymerization of microfilaments. Microfilaments support the assembly of actin granules that probably participate in cell wall synthesis and may induce morphological changes as a result.

**REFERENCES**


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