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Hydrogen Peroxide Induces Hyphal Differentiation in *Candida albicans*▽

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In this study, we demonstrate that hyphal differentiation is induced by the subtoxic concentration of exogenous H_2O_2 in *Candida albicans*. This finding is confirmed by the changing intracellular concentration of H_2O_2. In order to induce the same level of differentiation, low concentrations of exogenous H_2O_2 are required for the null mutants of the thiol-specific antioxidant and catalase, while higher concentrations are needed for cells treated with ascorbic acid, an antioxidant chemical.

Hydrogen peroxide (H_2O_2) directly affects various redox systems to regulate cell differentiation, proliferation, death, signal transduction, and ion transport (3, 12, 13, 19, 20) at subtoxic concentrations (23, 27–29). Therefore, the homeostatic maintenance of H_2O_2 at low levels should be tightly regulated (1, 9, 28).

The yeast *Candida albicans* is a pleomorphic human pathogen. An important virulence factor is the morphological transition involving hyphae formation (6, 16, 24), which is regulated by signaling pathways, including the cyclic AMP/protein kinase A and mitogen-activated protein kinase pathways (4, 7,

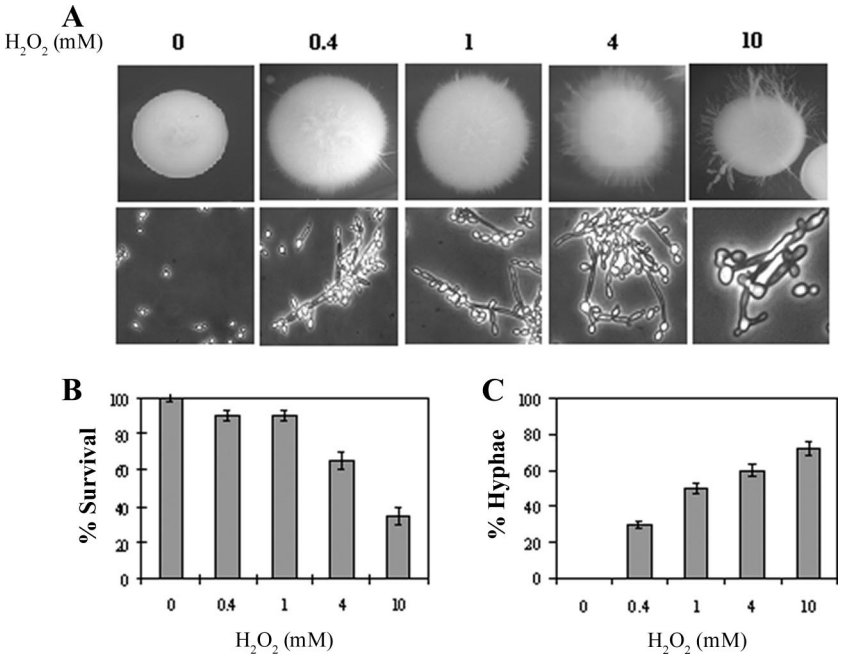

FIG. 1. Hyphal induction by exogenous H_2O_2. (A) Microscopic images of H_2O_2-induced hyphae. Wt cells were grown on YPD solid plates supplemented with the indicated concentrations of H_2O_2 at 30°C for 6 days. Representative colonies were photographed with a stereomicroscope (top). Cells in the mid-log phase were cultured in YPD liquid medium containing H_2O_2 for 6 h at 30°C and observed with a light microscope (bottom). (B) Cytotoxicity of H_2O_2. Standardized cell suspensions were challenged with the indicated concentrations of H_2O_2 for 30 min, plated onto YPD solid medium, and incubated at 30°C for 2 days. The survival rate was expressed as a percentage of the number of colonies in the presence of H_2O_2 divided by the number of colonies in the absence of H_2O_2. (C) Efficiency of hyphal differentiation. Cells were grown on YPD solid medium containing the indicated concentrations of H_2O_2 and incubated at 30°C for 6 days. The percentage of hyphal differentiation was expressed as the number of hyphal colonies divided by the total number of colonies.

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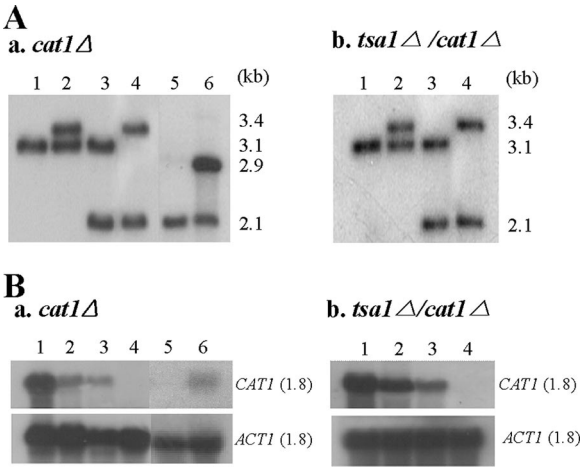

FIG. 2. Construction of *CAT1* null mutants and a revertant. The *CAT1* genes of the wt and the *tsa1*Δ mutant (30, 31) were disrupted using *URA3-dpl200* (32), yielding the *cat1*Δ and *tsa1*Δ *cat1*Δ mutants, respectively. The sense and antisense primers were nucleotide positions 754 to 823 and 2312 to 2381, respectively, of the *CAT1* open reading frame (ORF). To construct a revertant, the DNA fragment containing its own promoter, ORF, and terminator was cloned into pLUX, linearized with NheI, and transformed into the *cat1*Δ mutant. Southern (A) and Northern (B) analyses were performed to confirm the authenticity of the constructed strains, using the ^{32}P-labeled probe prepared from the MfeI fragment of the *CAT1* ORF. For the Southern analyses, genomic DNA was digested with NsiI and NcoI. Lanes 1, parental strains (CAI4 and the *tsa1*Δ mutant in panels A and B, respectively); lanes 2, strains with one allele disrupted; lanes 3, strains with *URA3* popped out from the lane 2 strains; lanes 4, null mutants (the *cat1*Δ and *tsa1*Δ *cat1*Δ mutants in panels A and B, respectively); lanes 5, strains with *URA3* popped out from the *cat1*Δ mutant; lanes 6, *CAT1*-reintroduced strains of the *cat1*Δ mutant.

18, 21, 22). Pathway triggers are varied (8) and include specific carbohydrates or amino acids (5, 26), serum (11), temperature (17), pH (10), N-acetylglucosamine (2), and starvation (7).

Following infection, *C. albicans* encounters macrophages but survives ingestion by rapidly adopting a hyphal morphology (25). Since the intracellular concentration of H_2O_2 in a macrophage is intrinsically high, it was presently germane to examine whether H_2O_2 can induce hyphal differentiation.

Hyphal differentiation by H_2O_2. When wild-type (wt) SC5314 cells were grown on YPD solid or liquid medium containing 0, 0.4, 1, 4, or 10 mM H_2O_2, the extent of differentiation was augmented in a dose-dependent manner (Fig. 1A). At the 10 mM concentration, however, the cells were severely swollen due to the cytotoxic effects of H_2O_2, which was inferred by the survival rate (35%) in contrast to the survival rate at 0.4 mM and 1 mM (90%) (Fig. 1B). Interestingly, undifferentiated colonies also appeared at all concentrations, enabling the evaluation of induction efficiency expressed as a percentage of the number of differentiated colonies in the total number of colonies. The induction efficiency was dose dependent as expected, but 100% differentiation did not occur even at 10 mM (Fig. 1C).

Next, we increased or decreased the endogenous intracellular H_2O_2. The increase was achieved by nullifying two H_2O_2-scavenging genes, the thiol-specific antioxidant *C. albicans TSA1* (30, 31) and the catalase *C. albicans CAT1*, individually (*tsa1*Δ or *cat1*Δ) or simultaneously (*tsa1*Δ *cat1*Δ) (Fig. 2). The growth of null mutants was impeded, and mutants were more H_2O_2 sensitive than the wt over the concentration range (data not shown). The decrease was achieved by the addition of ascorbic acid, an antioxidant chemical (14, 15) (see Fig. 4 and 5).

The enhanced sensitivity of the mutants to exogenous H_2O_2 was presumably caused by an increase in the concentration of intracellular H_2O_2. The relative amount of intracellular H_2O_2 was measured by visualizing fluorescent dichlorodihydrofluorescein (DCF) produced by esterase and H_2O_2 from 5-chloromethyl-2′,7′-DCF diacetate (CM-H2DCFDA) (Invitrogen, Carlsbad, CA). At exogenous H_2O_2 concentrations of 0.2 mM and 1 mM, fluorescent intensity was enhanced to some degree in the *tsa1*Δ and *cat1* mutants and in the *tsa1*Δ *cat1*Δ mutant (Fig. 3A). When the intensities were converted to arbitrary units for quantitative comparison, the intracellular H_2O_2 concentration increased about 1.5-fold in the *tsa1*Δ and *cat1*Δ mutants and about twofold in the *tsa1*Δ *cat1*Δ mutant compared with that of the wt (Fig. 3B).

The hyphal differentiation efficiencies of the wt and null mutants were compared using 0.2 mM exogenous H_2O_2. As shown in Fig. 3C, efficiency was considerably enhanced from 5% in the wt to about 25% in the *tsa1*Δ and *cat1*Δ mutants and to about 35% in the *tsa1*Δ *cat1*Δ mutant. This efficiency was obtained when the wt cells were treated with 1 mM H_2O_2 (Fig. 1C). The effective promotion of hyphal differentiation at a low concentration of exogenous H_2O_2 in mutants in which intracellular H_2O_2 increased indicated that H_2O_2 is a genuine inducer of *C. albicans* hyphal differentiation. When the functional *CAT1* gene was reintroduced, the percentage of hyphae reduced to the level between the wt and the *cat1*Δ mutant.

The effects of a decreased intracellular H_2O_2 concentration on hyphal differentiation were examined in the presence of ascorbic acid, which reduces the number of intracellular reactive oxygen species in some organisms. When wt cells were cultured under the full differentiation conditions (YPD plus 10% fetal bovine serum [FBS], 37°C), the level of intracellular H_2O_2 increased about sevenfold, from 8 to 65 arbitrary units (Fig. 4B and C). However, the addition of 50 mM or 100 mM ascorbic acid to the medium reduced the amount of intracellular H_2O_2 to the same or a lower level of serum depletion (Fig. 4B and C). Microscopic examination revealed that hyphal differentiation was markedly inhibited by ascorbic acid (Fig. 4A). Although the mechanisms of H_2O_2-induced hyphal transition are unclear, it is highly possible that increased intracellular H_2O_2 might be partly or completely involved. We further confirmed the above effects in the *tsa1*Δ, *cat1*Δ, and *tsa1*Δ *cat1*Δ mutants. When 50 mM ascorbic acid, an antioxidant chemical, was added to the medium 30 min after the treatment of mutant cells with different concentrations of exogenous H_2O_2, hyphal differentiation was induced even at otherwise toxic concentrations: 10 mM for the wt, 4 mM for the *tsa1*Δ and *cat1*Δ mutants, and 1 mM for the *tsa1*Δ *cat1*Δ mutant (Fig. 5). Thus, ascorbic acid lowered the intracellular concentration of H_2O_2 and inhibited hyphal differentiation. Also, efficient hyphal differentiation in the presence of ascorbic acid required exogenous H_2O_2.

The above results suggest that the mere increase of intracellular H_2O_2 is insufficient for complete hyphal differentiation. The intracellular H_2O_2 concentration of cells cultured in

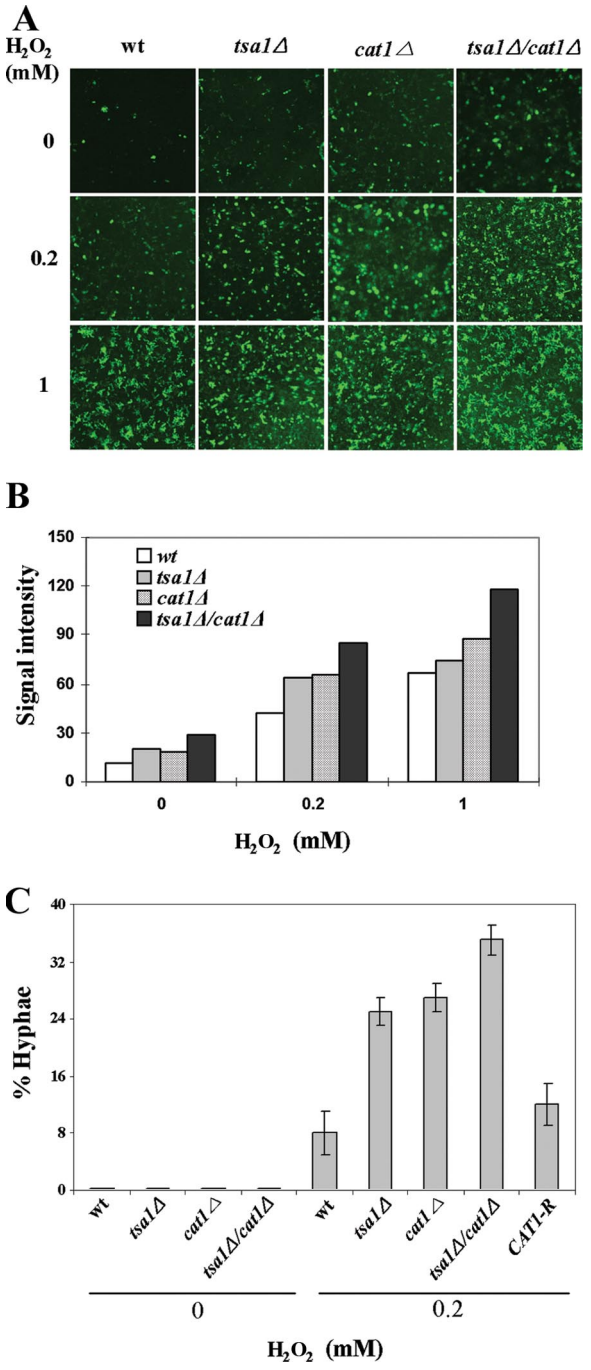

FIG. 3. Effects of increased intrinsic H_2O_2 on hyphal differentiation. Cells were grown in YPD medium containing 0.2 and 1 mM H_2O_2 for 6 h, washed, and resuspended in Hank's balanced salt solution. After the addition of CM-H2DCFDA (10 μM final), the cells were further incubated at RT for 10 min. (A) Images of DCF fluorescence were taken by using a confocal microscope with excitation and emission wavelengths at 488 nm and 520 nm, respectively. (B) Relative concentrations of intracellular H_2O_2 were derived from the confocal microscope-aided integration of fluorescence signal intensity within a scope. (C) Efficiency of hyphal differentiation at 0.2 mM H_2O_2 was determined as described in the legend to Fig. 1C. *CAT1*-R represents the strain into which the functional *CAT1* gene was introduced.

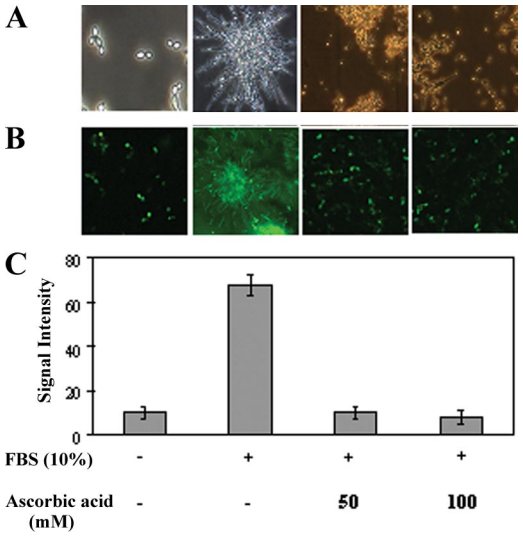

FIG. 4. Effects of ascorbic acid on hyphal differentiation in FBS-treated cells. Wt cells were grown in YPD in the absence (−) or presence (+) of 10% FBS for 30 min, followed by supplementation with 50 mM or 100 mM ascorbic acid. A portion of the cells was removed to take light microscopic images (A). For the rest of cells, fluorescence images (B) were taken, and the relative concentrations of intracellular H_2O_2 (C) were determined as described in the legend to Fig. 3.

FBS-supplemented YPD was identical to cells grown in YPD in the presence of exogenous 4 mM H_2O_2 (Fig. 4C and 5B), although differentiation was 100% and 60%, respectively (Fig. 1C). This indicates that some factors present in the serum are required for full hyphal differentiation in addition to increased intracellular H_2O_2. Based on these observations, we propose that hyphal differentiation in *C. albicans* occurs through two separate, but not mutually exclusive, steps: (i) initiation by

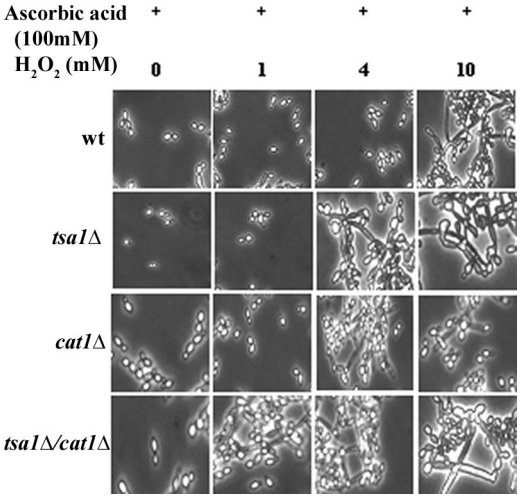

FIG. 5. Effects of ascorbic acid on hyphal differentiation. After the wt and *tsa1*Δ, *cat1*Δ, and *tsa1*Δ *cat1*Δ mutant cells were grown in YPD medium supplemented with 4 concentrations of exogenous H_2O_2 for 30 min at 30°C, 100 mM ascorbic acid was added, and the cells were further grown for 6 h at 30°C, the cultures were observed with a light microscope (magnification, ×400).

intracellular H_2O_2 above a certain concentration and (ii) promotion by currently unknown additional factors in serum.

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