Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*

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The yeast *Candida albicans* has a distinguishing feature, dimorphism, which is the ability to switch between two morphological forms: a budding yeast form and a multicellular invasive filamentous form. This ability has been postulated to contribute to the virulence of this organism. Studies on the morphological transition from a filamentous to a budding yeast form in *C. albicans* have shown that this organism excretes an autoregulatory substance into the culture medium. This substance was extracted and purified by normal-phase and reversed-phase HPLC. The autoregulatory substance was structurally identified as 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid) by NMR and mass spectrometry. Growth experiments suggest that this substance does not inhibit yeast cell growth but inhibits filamentous growth. These findings have implications for developmental signaling by the fungus and might have medicinal value in the development of antifungal therapies.

Candida albicans undergoes reversible morphogenetic transitions among budding, pseudohyphal growth forms mainly in response to environmental conditions; the switch from a yeast-like form to a filamentous form often correlates with pathogenicity (1–3). Morphogenesis is triggered by various signals *in vitro*. Serum causes single yeast cells to sprout true hyphae. High temperature, high ratio of CO_2 to O_2, neutral pH, and nutrient-poor media stimulate hyphal growth. *N*-Acetylglucosamine, a poor source of carbon and nitrogen, induces hyphae as a single environmental inducer (4). Conversely, low temperatures, air, acidic pH (pH 4 to pH 6), and enriched media promote yeast cell growth. Supplementing serum or *N*-acetylglucosamine media with amino acids and glucose causes hyphae to revert to yeast cells (5, 6). In addition, *C. albicans* has been reported to respond to exogenous human hormones such as estradiol and progesterone (7–9), suggesting that hyphal induction could be modulated by other host factors *in vivo*.

The first morphogenetic regulators identified from *C. albicans* are homologues of the *Saccharomyces cerevisiae STE* genes (10–13): the *C. albicans* genes *CPH1*, *HST7*, and *CST20* are homologues of the *S. cerevisiae STE12*, *STE7*, and *STE20* genes, respectively. *C. albicans* strains mutant in any of these genes show retarded filamentous growth but no impairment of serum-induced germ tube and hyphae formation (2, 11, 12). These results suggest that a mitogen-activated protein (MAP) kinase pathway related to that of *Saccharomyces* also operates in *Candida* to regulate filamentous growth and virulence. Negative regulators of hyphal development in *C. albicans* have also been described. A MAP kinase phosphatase mutant has been described that shows increased hyphal development, which is apparently due to an effect on Cek1, a Kss1p-related MAP kinase (14). Cek1p is thought to activate Cph1p, which is responsible for the transcriptional activation of the targets of a *STE*-like pathway. A second morphogenetic pathway in *C. albicans* is defined by Efg1p, which belongs to a family of important basic helix–loop–helix transcription factors that regulate development in fungi and vertebrates (15). Epistasis analysis has suggested that Efg1p and Cph1p lie on distinct but parallel pathways that activate hyphal development (16). *C. albicans tup1* mutants form hyphae under conditions that normally promote growth of the yeast form; hence Tup1p is a negative regulator of hyphal development (17). This observation is significant because it highlights the importance of negative regulation in the control of morphogenesis and reveals a mechanistic difference in morphogenetic regulation between *S. cerevisiae* and *C. albicans*: *S. cerevisiae tup1* mutants display reduced pseudohyphal growth.

The regulatory networks that control morphogenesis of *C. albicans* are being elucidated. However, despite these investigations, the mechanisms of dimorphism are still unclear, possibly because environmental factors that induce the dimorphic transition have many effects on cells, making it difficult to identify which factors are essential for the transition. In this paper we demonstrate that specific signal(s) induce the dimorphic transition of cells. We describe the purification of an autoregulatory substance (ARS) with the formula $C_{15}H_{24}O_2$, which represents a novel type of signal molecule required for the morphological transition in *C. albicans*. The structure of this compound, 3,7,11-trimethyl-2,6,10-dodecatrienoate, was determined by NMR and mass spectrometry.

Materials and Methods

Organism and Culture Conditions. *C. albicans* ATCC 10231 was maintained on Sabouraud's glucose agar slants and transferred at regular intervals. To prepare cells for the experiments, *C. albicans* cultures were grown at 23°C with shaking in the glucose salts (GS) medium (18) (5 g of glucose/0.26 g of $Na_2HPO_4{\cdot}12H_2O$/0.66 g of KH_2PO_4/0.88 g of $MgSO_4{\cdot}7H_2O$/0.33 g of NH_4Cl/16 μg of biotin per liter) for 48 h (early stationary phase). Under these conditions over 95% of *C. albicans* cells remained in the yeast form. The yeast cells were washed three times with sterile distilled water before use.

Continuous-Culture Flow Assay. In the course of studies on *C. albicans* dimorphism, we observed that when *C. albicans* grew in GS medium at 37°C, more than 95% of the inoculum yeast cells converted into hyphae (18). However, after about 8 h of incubation, yeast growth was favored and hyphal tips reverted to yeast-form cells. To learn more about the presence of a possible regulatory substance for morphological transition in the growth medium, a continuous-culture flow cell [glass, $1 \times 1 \times 4$ cm^3 (H × D × W)] was used to monitor hyphal growth with or without medium flow. The inner surface of the cell was coated

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Abbreviations: MAP, mitogen-activated protein; ARS, autoregulatory substance; GS, glucose salts.

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with poly-L-lysine, to which yeast cells and hyphae adhered throughout the experiment (19). Yeast cells (5×10^5 cells) were inoculated and allowed to form hyphae at 37°C under a constant flow rate (400 μl/min) or without flow. At each time point during the experimental period, the percentage of yeast and hypha was determined microscopically as described below. To investigate the possibility of nutrient depletion or change in pH in the growth medium, 24-h culture filtrates were neutralized (7.0) by pH adjustment or nutritionally restored by the addition of 20% fresh GS medium to neutralized filtrate and tested as described above. Holliday (20) has demonstrated that filament formation by *Ustilago maydis* is greatly enhanced in the presence of charcoal, which perhaps removes fungal pheromones. To ascertain the production of regulatory substances during the growth of *C. albicans* in this work, culture filtrate was passed through a charcoal filter and the filtrate was nutritionally restored by the addition of 20% of fresh GS medium. The charcoal-absorbed substances were also eluted with methanol-chloroform (1:1, vol/vol) and concentrated, and the residue was dissolved in fresh GS medium. Those medium samples were then subjected to continuous-culture flow assay.

ARS Activity Assay. For our bioassays, 5 ml of GS medium containing a prescribed concentration of ARS was added to 5×10^5 washed yeast cells in Petri dishes (diameter, 30 mm), mixed, and incubated for 8 h at 37°C. At each time point during the experiment, the percentage of yeast and hypha was determined microscopically by counting 200 cells from triplicate dishes. For the hypha-to-yeast transition assay, 5 ml of the GS medium was inoculated with yeast cells (5×10^5 cells per milliliter) and incubated for 4 h at 37°C. After we confirmed that more than 95% of the inoculum yeast cell converted into hyphal form, GS medium was replaced by GS medium containing a prescribed concentration of ARS, and the measurement was continued for an additional 8 h. At each time point the morphology of hyphal tips was counted microscopically in 200 cells from triplicate dishes.

Production of the ARS. A 5-liter Erlenmeyer flask containing 2 liters of GS medium was inoculated with yeast cells from early stationary phase cultures that had been washed and resuspended in GS medium. The initial cell density was adjusted to 5×10^5 cells per milliliter and cultured aerobically at 37°C. Under these conditions, over 95% of yeast cells converted into hyphal form within 4–6 h. Incubation was continued for 2 days. After we confirmed that the tips of hyphae converted into the yeast form again, the cells were removed by centrifugation and the supernatant liquid was collected and lyophilized. The lyophilized material was dissolved in methanol and centrifuged at $12,000 \times g$ for 10 min. The methanol solution was removed by rotary evaporation at 35°C, and the remaining residue was extracted in hexane. The hexane solution was passed through a 0.2-μm-pore filter and then applied to a silica column (TSKgel silica-60, 250×4.6 mm, 5 μm; Tosoh, Tokyo) and fractionated by normal-phase HPLC at 1 ml/min with the use of a hexane:chloroform:methanol solution (50:50:2; vol/vol/vol) in a Tosoh SC-8020 system. Detection was carried out with an UV detector at 240 nm. Active fractions were pooled, solvents were removed, and the residue was dissolved in 0.1 ml of 90% acetonitrile. The solution was loaded onto a C_{18} column (TSKgel ODS-80, 150×4.6 mm, 5 μm) and fractionated by reversed-phase HPLC at 1 ml/min with a linear gradient of acetonitrile in water. ARS activity was recovered as a single peak in 80% acetonitrile. The purified ARS was dried and stored at −30°C.

Spectra. The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on an A500 Fourier transform NMR (JEOL) at 500 MHz and 125 MHz, respectively. Chemical shifts were given in δ (ppm) values relative to tetramethylsilane as an internal standard. The complete assignments of the ^{1}H and ^{13}C NMR spectra of the compound were based on one- and two-dimensional spectra, in particular ^{1}H correlated spectroscopy and heteronuclear multiple bond correlation spectroscopy. GC-MS spectra were measured with a Hewlett–Packard 5890 II gas chromatograph linked to a JMS-SX102A mass spectrometer (JEOL) [column: DB-1 column (J & W Scientific, Folsom, CA), 15 m × 0.25 mm; line: He flow at 1 ml/min; column temperature profile: 50°C for 1 min, increasing from 50 to 250°C at 10°C/min and hold at 250°C for 10 min; electron potential: 70 eV].

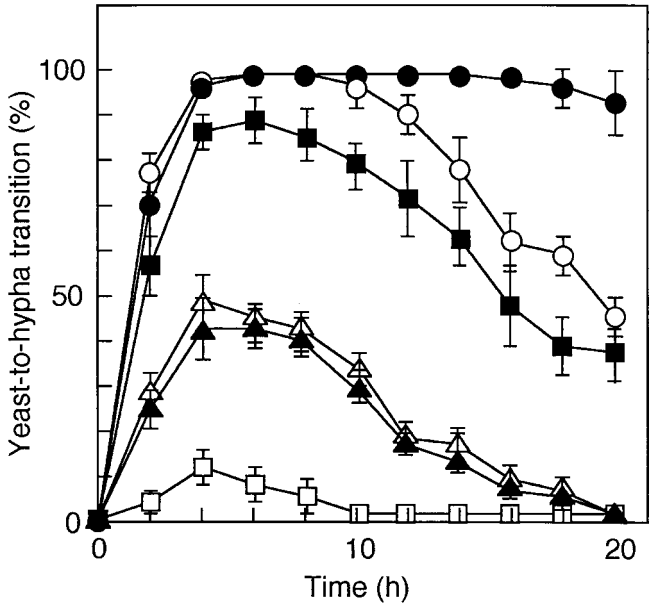

Fig. 1. Demonstration of the essential role of secreted extracellular factor(s) by continuous-culture flow assay. A continuous-culture flow cell was used to monitor hyphal growth with (●) or without (○) medium flow (400 μl/min). To investigate the possibility of nutrient depletion or change in pH in the growth medium, 24-h culture filtrates were neutralized by pH adjustment (7.0, ▲) or nutritionally restored by the addition of 20% fresh GS medium to neutralized filtrate (△). To ascertain the production of regulatory substances, culture filtrate was passed through a charcoal filter, and the filtrate was nutritionally restored by the addition of 20% fresh GS medium (■). The charcoal-absorbed substances also were eluted with methanol-chloroform (1:1, vol/vol) and concentrated, and the residue was dissolved in fresh GS medium (□). Those medium samples were then subjected to continuous-culture flow assay.

Results

Bioassay. The morphological transition of a low-density population of *C. albicans* cells (10^5 cells per milliliter) was inhibited by the addition of culture filtrates or extracts to GS medium in which the cells were placed. This inhibition was used as an assay for ARS activity and was quantified as the number of germ tubes observed after a specified time period with a precision of *ca.* ±5%. Cells in control experiments usually developed germ tubes only after about 1–2 h at the earliest.

Proof of the ARS Requirement by Continuous-Culture Flow Assay. The continuous-culture flow assay demonstrated the role of secreted extracellular factor(s) for the morphological transition in GS medium (Fig. 1). Without medium flow, yeast growth was favored and hyphal tips reverted to yeast cells after about 8 h of incubation. With medium flow, however, the time required for the hypha-to-yeast transition was markedly extended. The yeast-to-hypha transition percentages after 4 h of incubation in pH-neutralized filtrates and nutritionally reconstituted culture

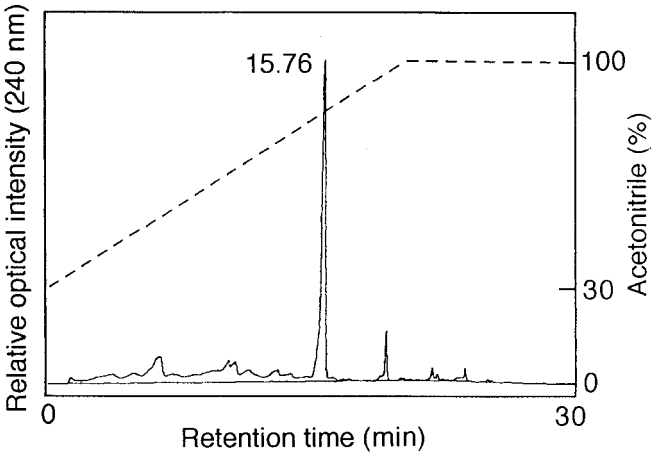

Fig. 2. Reversed-phase HPLC elute profile of ARS. A linear gradient of acetonitrile in water was used to obtain the elution of ARS activity at about 80% acetonitrile (column: TSKgel ODS-80; flow rate: 1 ml/min; UV detector: 240 nm).

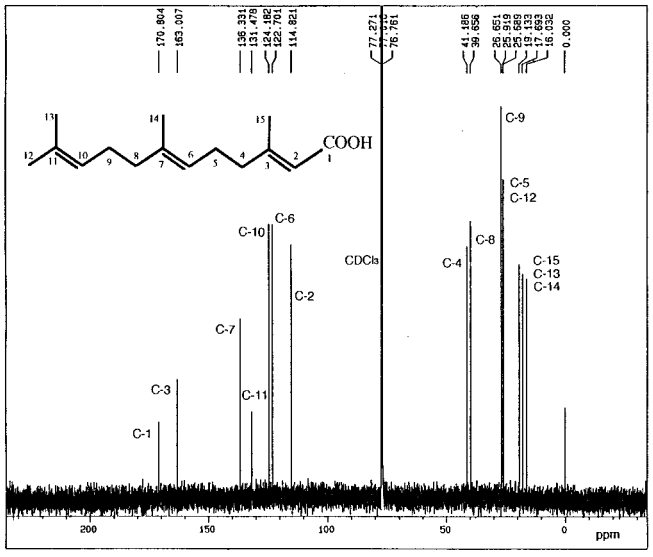

Fig. 3. ^{13}C NMR spectrum of the purified ARS, 3,7,11-trimethyl-2,6,10-dodecatrienoate (or farnesoic acid), in $CDCl_3$. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.60 (s, 6, C-7 CH_3 and C-11 CH_3), 1.68 (s, 3, C-11 CH_3), 1.98 (m, 2, H-9), 2.05 (m, 2, H-8), 2.18 (d, 3, $J = 1.5$ Hz, C-3 CH_3), 2.20 (s, 4, H-4 and H-5), 5.08 (m, 2, H-6 and H-10), and 5.70 ppm (s, 1, H-2). MS m/z (rel. int) 236 (M^+ 0.8%), 193 (23), 136 (40), 121 (43), 100 (72), 81 (75), and 69 (100).

filtrates were 43 and 48, respectively. Therefore, those differences were not due to nutrient depletion or change in pH without medium flow, because the yeast-to-hypha transition did not improve even when the culture medium was nutritionally supplemented and adjusted to pH 7.0. In contrast, the morphogenic regulatory substance appeared to absorb to charcoal because filament formation of *C. albicans* was greatly enhanced after charcoal treatment. In addition, ≈13% of the yeast-to-hypha transition activity was observed from the charcoal-absorbed substances acquired by MeOH-$CHCl_3$ elution. The evidence obtained here indicates that *C. albicans* produces a factor, which we will designate as ARS, that is capable of regulating the morphological transition.

Large-Scale Production and Purification of the ARS. Cultures of *C. albicans* were grown by shaking in GS broth at 37°C. After 2 days, cells were separated from the broth (total 150 liters) by centrifugation, and the supernatant liquid was collected and lyophilized (379 g). In the course of isolation, each fraction obtained at various steps was assayed for ARS activity by inhibition of the yeast-to-hypha transition of *C. albicans*. The lyophilized material was dissolved in methanol. The methanol-soluble fraction was recovered (four times, 1 liter each time), and the remaining residue (126 g) was extracted in hexane (four times, 500 ml each time). A crude sample obtained by hexane extract (0.13 g) showed marked activity. This hexane solution was loaded onto a silica column and fractionated by normal-phase HPLC. Active fractions were pooled and further purified by reversed-phase HPLC column. A linear gradient of acetonitrile in water was used to obtain the elution of ARS activity at about 80% acetonitrile. Almost all of the activity was recovered in a narrow time interval (Fig. 2). Material secreted in 150 liters of GS broth was processed to finally yield about 4 mg of purified ARS.

Analysis of the Purified ARS. The purified ARS (colorless oil) was analyzed by MS and GC. Only one GC peak was detected (data not shown). The molecular ion peak [M^+] appeared at m/z 236 in electron impact mass spectroscopy (EIMS) analysis. High-resolution EIMS analysis of this peak showed that m/z was 236.1786. $C_{15}H_{24}O_2$ was speculated to be a feasible molecular formula. The assignment of the purified compound was achieved without difficulty by comparison with methyl farnesoate and literature data (21–24). The complete assignments of the ^1H and ^{13}C NMR spectra of the compound were based on one- and two-dimensional spectra, in particular ^1H correlated spectroscopy and ^1H-^{13}C correlated spectroscopy. Signals for each vinyl Me appeared in the 16- to 25-ppm regions and were easily assignable to C-12 (25.6 ppm), C-13 (17.6 ppm), C-14 (16.0 ppm), and C-15 (19.1 ppm), respectively. Signals due to each of the olefinic carbons in the 114- and 131-ppm regions appeared throughout all spectra and were assigned to C-2 and C-11, respectively. In general, a disubstituted olefinic carbon [$(CH_3)_2C{=}C$] resonates at a lower field than a monosubstituted olefinic carbon (—$CH{=}C$) (25). In the case of farnesol and squalene, the C-10 carbon resonates at a somewhat lower field (125.6 ppm in farnesol, 125.5 ppm in squalene), compared with the C-6 carbon (124.9 ppm in farnesol, 125.5 ppm in squalene). Therefore, in the isolated compound, the 124.1-ppm signal at the lower field can be assigned to C-10 and the 122.7-ppm signal can be attributed to C-6. The proton NMR spectrum did not contain —CO_2CH_3 protons (a singlet at 3.70 ppm) but contained —COOH carbon (170.8 ppm in carbon NMR). Those spectral data indicated that the purified compound (ARS) was 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid), as shown in Fig. 3.

The effects of purified ARS on the morphological transition in *C. albicans* were investigated, and the results were surprising. As expected, the addition of 3.12 μg/ml of ARS resulted in inhibition of the yeast-to-hypha transition (Fig. 4). In contrast, ARS had no detectable effect on yeast cell growth at concentrations below 200 μg/ml (data not shown). In addition, hyphal tips reverted to yeast cells by the addition of 3.12 μg/ml of ARS after the inoculum yeast cells transformed into hyphal form, suggesting that ARS is required for inhibition of hyphal growth (or activation of the hypha-to-yeast transition) (Fig. 5). The time course for the appearance of ARS in the large-scale production system was analyzed by reversed-phase HPLC (Fig. 6). The production of ARS increased in concert with the yeast-to-hypha transition, reached a maximum about 4 h after the transition began, and decreased to a plateau level at *ca.* 16 h. With pure ARS, the threshold for inhibition of the yeast-to-hypha transition in our bioassay was *ca.* 0.4–0.8 μg/ml, and IC_{90} was obtained at *ca.* 3.12 μg/ml (Fig. 7).

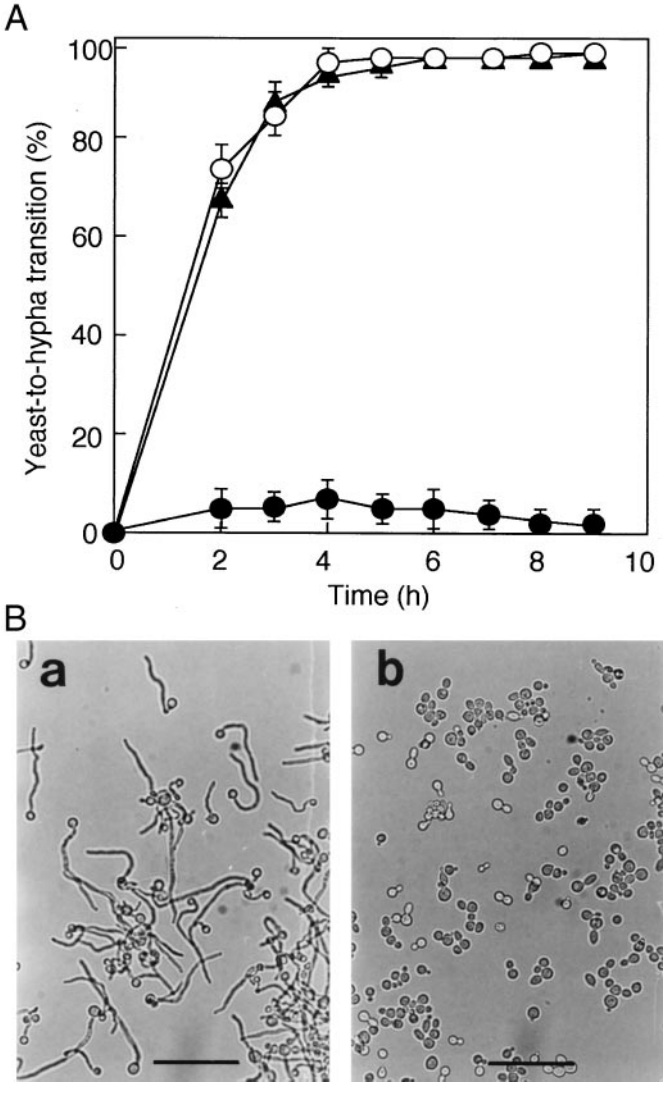

Fig. 4. Effect of purified ARS on cell growth of *C. albicans*. (*A*) Yeast-to-hypha transition. ○, GS medium (control); ▲, GS medium + 0.5% DMSO; ●, GS medium + 0.5% DMSO + 3.12 μg/ml of ARS. (*B*) Yeast cells were incubated without (*a*) or with (*b*) 3.12 μg/ml of ARS in GS medium for 4 h at 37°C. (The bar indicates 35 μm.)

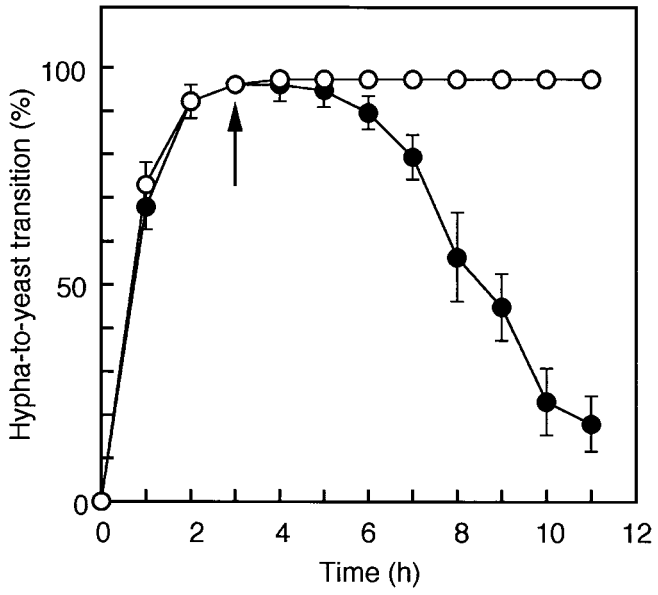

Fig. 5. Effect of purified ARS on the hypha-to-yeast transition of *C. albicans*. At the time indicated by the arrow, GS medium was replaced by GS medium containing 3.12 μg/ml of purified ARS, and the morphology of hyphal tips was counted microscopically for 200 cells from each one of triplicate dishes. ○, GS medium + 0.5% DMSO (control); ●, GS medium + 0.5% DMSO + 3.12 μg/ml of ARS.

Discussion

The evidence presented here indicates that *C. albicans* produces a factor capable of regulating a morphological transition. The production of ARS in a simple defined GS medium appears to be due to *de novo* synthesis and not simply to release from a storage depot in the cells because (*i*) GS does not contain an ARS-like factor and (*ii*) breakage of cells used to inoculate the GS medium does not cause release of ARS (data not shown). From NMR and mass spectrometry data, we conclude that the ARS is 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid), an isoprenoid compound. The time course studies for the appearance of ARS in the large-scale production system showed that the concentration of ARS increased in concert with the yeast-to-hypha transition, reached a maximum about 4 h after the transition began, and decreased to a plateau level (Fig. 6). On the other hand, the hypha-to-yeast transition started about 12 h after the yeast-to-hypha transition began. These results indicate that hyphal cells produce ARS, which then subsequently stimulates the hyphal cells to revert to the yeast form. These findings may be important for understanding developmental signaling mechanisms of the fungus.

It has been reported that isoprenoid compounds play an important role as precursors of protein prenylation (26, 27). Prenylated proteins are posttranslationally modified by the formation of cysteine thioethers with the isoprenoid lipids farnesyl (C-15) or geranylgeranyl (C-20) at or near the carboxy terminus. Many of the proteins that undergo these modifications have been identified and participate in important cell regulatory functions, particularly signal transduction pathways (28, 29). In *S. cerevisiae*, four components of the MAP kinase pathway that signals the mating pheromone response are also required for

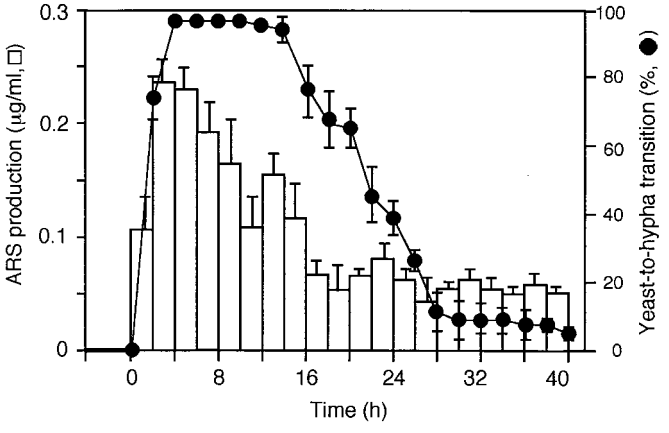

Fig. 6. Time course of ARS appearance in the large-scale production system. Culture filtrates of *C. albicans* were collected every 2 h and lyophilized. A crude sample obtained by methanol extract (see *Materials and Methods*) was loaded onto a C_{18} column (TSKgel ODS-80, 150 × 4.6 mm, 5 μm) and analyzed by reversed-phase HPLC (flow rate: 1 ml/min; detector: UV 240 nm). A linear gradient of acetonitrile in water was used to obtain elution of ARS at about 80% acetonitrile.

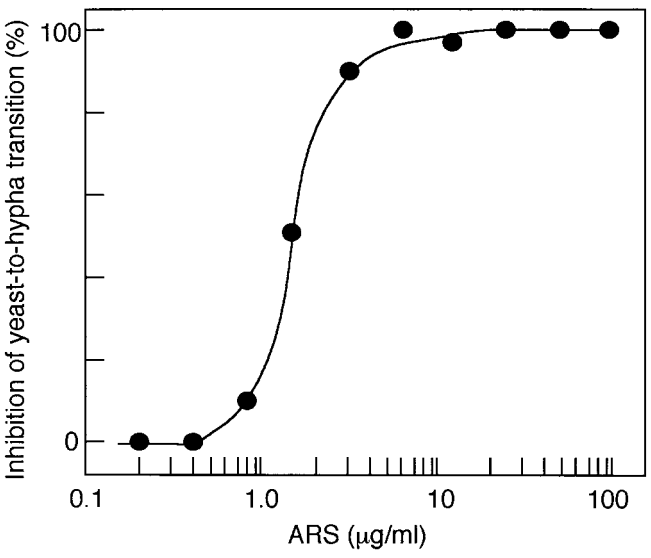

Fig. 7. Dose response of *C. albicans* morphology to purified ARS. Data were obtained with our standard assay as inhibition of yeast cells converted to hyphae after 4 h of incubation at 37°C in the GS medium.

filamentous growth of diploid cells and the invasive growth of haploid cells (10, 30). Despite the lack of an identified sexual cycle in *C. albicans*, homologues of MAP kinase cascade components have been isolated largely by their ability to complement the mating defects of *Saccharomyces* mutants (2, 11, 12, 31). Thus a MAP kinase pathway related to that of *Saccharomyces* also operates in *Candida* to regulate filamentous growth. Perhaps ARS regulates this pathway.

The ability of *C. albicans* to elaborate a germination inhibitor has been investigated previously. It has been suggested that autoinhibition of germination by *C. albicans* cells at closely apposed sides of two parallel streaks on agar-coated slides (32) may be due to the release of substances containing free SH groups into the growth medium (33). In accordance with these observations, Nickerson and Van Rij (34) have demonstrated that cysteine at a concentration of 10 mM inhibits germination. Tryptophol and phenethyl alcohol have been reported to act as "autoantibiotics" on *C. albicans* because they inhibit growth and germination (35). We did not obtain these effects at the reported concentrations. GC further showed that these substances, if present, are at undetectable concentrations in a 2-day culture filtrate.

Other fungi have been studied for the ability to control morphological conversions. Autoregulation of germination has been demonstrated with the urediniospores of the bean rust *Uromyces phaseoli* (36) and with *Glomerella cingulata* (37). Methyl-3,4-dimethoxycinnamate inhibits urediniospore germination of *U. phaseoli* (36). The pathogenic dimorphic fungus *Histoplasma capsulatum* has been speculated to control the yeast-to-hypha conversion through an inhibitor of RNA polymerase (38). Although the elaboration of germination regulatory substances by *C. albicans* has been refuted by some investigators (39, 40), it is not unreasonable to expect *C. albicans* to have this ability because other fungi have been shown to possess this characteristic (41, 42).

The role of ARS in the pathogenesis of candidiasis remains to be determined. Mackenzie (43) has speculated that the presence of either yeast or hyphae in a candidal lesion may be related to the environmental conditions within the host as well as the nature of the host's defenses. The ability of *C. albicans* to regulate its morphology via ARS may further indicate that the metabolic state of the organism also plays an important role in the development of disease. Thus we would like to identify the cellular control mechanisms involved in timing the synthesis and release of farnesoic acid and to elucidate the regulatory networks controlling *C. albicans* morphogenesis with ARS as a primary signal.

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