INFECTION AND IMMUNITY, Oct. 1997, p. 4360–4364 Vol. 65, No. 10
0019-9567/97/$04.00+0

Hemin Induces Germ Tube Formation in *Candida albicans*

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Received 21 March 1997/Returned for modification 19 April 1997/Accepted 22 May 1997

Hemin induced germination of *Candida albicans* blastoconidia when cells grown up to the early exponential phase were shifted from 28 to 37°C (70 to 75% of cells exhibited germ tubes). *N*-Acetyl-D-glucosamine (GlcNAc), another inducer of myceliation in this fungus, caused a similar effect. The combination of hemin and GlcNAc resulted in a higher percentage (95%) of blastoconidial germination. These results suggest that in addition to temperature, hemin levels and carbon source may coordinately regulate the expression of subsets of genes involved in the yeast-to-mycelium transition in *C. albicans*.

Candida albicans is one of the most pervasive species of pathogenic fungi, since it is able to invade virtually all kinds of animal tissues and to opportunistically infect a wide variety of hosts with impaired immunity. *C. albicans* exhibits the ability to grow in either a yeast-like (blastoconidial) or a mycelial (germ tube) form in response to different environmental factors. In vivo, the yeast-to-mycelium transition appears to play a role in pathogenesis and is considered an important virulence factor (5, 16). In vitro, the morphological transition can be easily induced by environmental and/or nutritional (i.e., temperature, pH, and carbon source) shifts in growing conditions (1, 4, 21). However, the physiological mechanisms involved in the morphogenetic process and their molecular regulation in *C. albicans* are still essentially unknown.

Recently, *C. albicans* genes coding for elements of the mating mitogen-activated protein (MAP) kinase signalling pathway have been cloned by their ability to suppress the mating defects of *Saccharomyces cerevisiae* mutants in the *ste20* and *ste7* genes (11, 12). Phenotypic analysis of *C. albicans* cells from which these genes were deleted suggests that more than one signalling pathway can trigger hyphal development, since null mutants are defective in hyphal formation on some media; however, hyphal development is still induced in some other media (e.g., serum). One of these pathways involves a protein kinase cascade that is analogous to the mating response pathway in *S. cerevisiae*, but other signalling pathways are independent of the MAP kinase cascade (11, 12).

In the present work, we have determined the effect of exogenous hemin as an inducer of germination in *C. albicans* since (i) signal transduction pathways in *S. cerevisiae* such as the mating response pathway may have become adapted to the control of mycelial formation in *C. albicans*, as mentioned above; (ii) heme is a signal molecule that plays a role as a mediator of oxygen induction in yeast (i.e., expression of genes coding for proteins involved in oxidative phosphorylation are regulated by heme) (besides, yeasts are known to be responsive to the addition of exogenous heme [6]); and (iii) morphological and biochemical changes associated with differentiation processes in higher eukaryotic cells appear to be induced in vitro by hemin, the oxidized form of heme (17). Therefore, it seemed of interest to determine whether pathways regulated by heme are involved in morphogenic change in *C. albicans*.

Strain ATCC 26555 (serotype A) of *C. albicans*, which was used in this study, was maintained by subculturing on 2% (wt/vol) Bacto Agar slants of Sabouraud dextrose medium at 28°C. Cells were propagated in liquid salts-proline-biotin (SPB) medium, which is a modification of the medium described by Lee et al. (13) with the following composition (per liter): 5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 2.5 g of K_2HPO_4, 1.8 g of KH_2PO_4, 5 g of NaCl (basal medium; pH 6.5), 0.5 g of proline, and 1 mg of biotin. The basal medium was sterilized at 120°C for 20 min in the autoclave, whereas the stock proline and biotin solutions were sterilized by filtration through Millipore filters (0.45-μm pore size). As a carbon source, aliquots from stock solutions of glucose and *N*-acetyl-D-glucosamine (GlcNAc [also sterilized by filtration as described above]) were added to the medium to reach final concentrations of 12.5 and 1 g per liter, respectively. When required, hemin was added to the SPB medium from a freshly prepared stock solution (sterilized by filtration) containing 1 mg of hemin (Sigma) per ml. The compound was dissolved in 1 ml of 0.5 N NaOH, and the final volume of the solution was adjusted up to 10 ml by adding 8.5 ml of distilled water and then 0.5 ml of 1 M phosphate buffer (pH 6.5).

A loopful of cells from a 48-h-grown slant culture on Sabouraud agar was inoculated into 2-liter Erlenmeyer flasks containing 500 ml of SPB medium, and the flasks were incubated at 28°C in a gyratory incubator (250 rpm) to a final density of 10^6 cells per ml, which corresponded to the early-exponential-growth phase (optical density at 600 nm, 0.2 to 0.3). This was found to be an essential experimental condition, since *C. albicans* blastoconidia reaching the stationary phase of growth at 28°C are able to form germ tubes by simple thermal shifting (4, 22) in the absence of any other nutritional or environmental inducer when they are diluted into fresh prewarmed medium at 37°C. Cells from the 28°C precultures were collected by centrifugation, washed with sterile glass-distilled water, and diluted at a concentration of 5×10^6 cells per ml into flasks containing 20 ml of prewarmed fresh SPB medium with different combinations of hemin (at various concentrations), carbon sources, and pHs, and the flasks were incubated at either 28 or 37°C (see Fig. 1A and Table 1) with shaking as described above. After 5 h of incubation (for all experiments), cells from samples withdrawn from the cultures were sedimented by cen-

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trifugation, washed with glass-distilled water, resuspended into 1 N NaOH containing 10 mM EDTA and 1% (vol/vol) of β-mercaptoethanol, subjected to vigorous vortexing for 60 s to eliminate clusters of cells that would hamper accurate microscopic cell counting (see below), washed again, fixed with 0.5% formaldehyde, and observed (wet mounts) in a Photomicroscope III (Zeiss) equipped with phase-contrast optics and for epifluorescence (UV filter no. 487702; excitation line, 365-366 nm). Pictures of randomly selected microscopic fields were taken to measure germ tube formation by counting the numbers of individual cells showing a definite outgrowing tube under the different experimental conditions tested and by expressing these as percentages of the total cell population. The percentages of myceliation were determined in three independent experiments for each particular combination of experimental conditions (approximately 3,000 cells were counted for each condition). Indirect immunofluorescence (IIF) staining of cells with a monoclonal antibody (MAb 4C12) which recognizes an epitope present in a mycelium-specific cell wall glycoprotein (2, 3, 8) as a probe was performed as previously described (2, 3).

As shown in Fig. 1A and B, exponentially growing blastoconidia were unable to develop germ tubes when they were diluted into prewarmed SPB medium and the medium was incubated for as much as 5 h at 37°C in the absence of hemin; only a few rare-germinated elements and a certain extent of blastoconidial clumping were observed in this case (Fig. 1B). In contrast, more than 70% of cells formed well-defined germ tubes when they were diluted into hemin-containing SPB medium and the medium was incubated at 37°C (Fig. 1A and C). Concentrations of hemin tested that were higher than 50 μg per ml did not cause a substantial increase in the percentage of germinated blastoconidia (Fig. 1A); hence, a concentration of 50 μg of this compound per ml was used in all subsequent experiments in combination with other substances or environmental conditions that induce germ tube formation in *C. albicans* (see below). Mycelial filaments formed in hemin-containing SPB medium at 37°C were specifically labelled by MAb 4C12 in IIF assays, whereas nongerminated yeasts or mother blastoconidia from which germ tubes emanate remained unlabelled (Fig. 1C). This IIF pattern is identical to that previously reported (2, 3, 9). Since hemin had no effect on germ tube induction in the absence of thermal shifting (Table 1), these results indicate that both thermal shifting (37°C) and the presence of hemin are simultaneously required to stimulate germination and hyphal development in *C. albicans*, suggesting that exponentially growing blastoconidia are not endowed with a specific competence to germinate. To discern whether the observed effect on myceliation was due to hemin or to any of its components, the effects of iron and protoporphyrin IX (deferrated hemin) were assayed independently. Iron ($FeCl_3$) added in molar concentrations equivalent to those provided by hemin did not show any effect on germ tube induction under the above-mentioned experimental conditions, whereas protoporphyrin IX at a concentration of 50 μg/ml induced the formation of pseudohyphae, with most (about 80%) of the cells in the culture being regular budding yeasts and with an almost total absence of true mycelial filaments; higher concentrations of protoporphyrin IX (100 μg/ml) induced formation of pseudohyphal morphology at higher levels, but still no significant amount of yeast cells bearing mycelial filaments was observed (not shown). These observations indicate that only the hemin is able to induce true germ tube formation in *C. albicans.*

It has been reported that pH changes (1) and the presence of GlcNac in the culture medium (21) also induce the yeast-mycelium conversion in *C. albicans*. When exponentially growing blastoconidia (taken from the precultures incubated at 28°C) of strain ATCC 26555 were diluted into prewarmed (37°C) SPB medium containing GlcNAc instead of glucose and the dilution was incubated for as much as 5 h at 37°C, more than 70% of the cells formed well-defined germ tubes; however, once again, no germination was observed when incubation was carried out in this same medium at 28°C (Table 1). As stated above, a similar response was observed when exponentially growing blastoconidia were incubated in hemin-containing SPB medium, both at 28 and 37°C (Table 1). The percentage of germination induced by GlcNAc in strain ATCC 26555 (this work) was found to be similar to those described previously for other *C. albicans* strains (21). How GlcNAc induces the morphologic transition in *C. albicans* is still unknown, although it has been postulated that this effect could be related to the regulation of the biosynthesis of chitin, a polymer whose proportion is increased in the walls of mycelial cells (21). Interestingly, the combined effect of hemin and GlcNAc resulted in the higher percentage of blastoconidial germination detected in the present study; thus, more than 90% of exponentially growing yeast cells incubated for 5 h at 37°C in SPB medium containing both compounds exhibited definite mycelial filaments (Table 1), which appeared to be longer (Fig. 2A) than those observed under the other experimental conditions tested.

When stationary-phase cells were diluted into fresh medium, the yeast phase was favored at 25°C and acidic pH (4.5), whereas incubation at 37°C and a medium pH near neutrality (6.5) favored hyphal growth (1, 16). We have examined the combined effect of pH and GlcNAc on the ability of exponentially growing blastoconidia to form germ tubes. The levels of germination after 5 h of incubation at 37°C in GlcNAc-containing SPB medium at an acidic pH (4.5) were 20 to 25% (Table 1). Although the lower percentage of yeast cells exhibiting hyphal filaments obtained under these experimental conditions may be related to a requirement for a cytosolic pH gradient during apical growth, as has been pointed out by other authors (7, 20), these results suggested that the germination-inducing effect of GlcNAc was superimposed on the effect of an acidic pH that, as stated above, favored yeast-phase growth (1, 16). It has to be stressed that growth in an acidic environment may cause other unknown physiological effects, since all nongerminating blastoconidia and mother blastoconidia from which germ tubes emanated exhibited a large, central vacuole (Fig. 2B [arrows]) that was never observed in cells grown at pH near neutrality (Fig. 1B to C and 2A). In all cases, specific labelling by MAb 4C12 (Fig. 1D) of mycelial filaments generated under the different experimental conditions tested here (Fig. 2; Table 1) was observed (not shown), which is consistent with previous observations from our group indicating that expression of the cell wall antigen bearing the epitope recognized by MAb 4C12 is specifically associated with the formation of genuine hyphal extensions by *C. albicans* yeast cells (2, 8, 9, 14).

Overall, the data presented in this work are in line with previous reports from other authors showing that the intrinsic capacity of *C. albicans* to form germ tubes is not an exclusive property of stationary-phase cells (15). When temperature is the only critical factor for dimorphic transition, only stationary-phase cells may undergo mycelial development (4, 22); however, given suitable chemical inducers, exponentially growing cells will also germinate efficiently (15). In agreement with our results, the concentration of cells in liquid cultures has been described to be essential for germ tube formation (18, 19). Hazen and Cutler (10) reported that germ tube formation was suppressed in shake cultures of blastoconidia when the cell

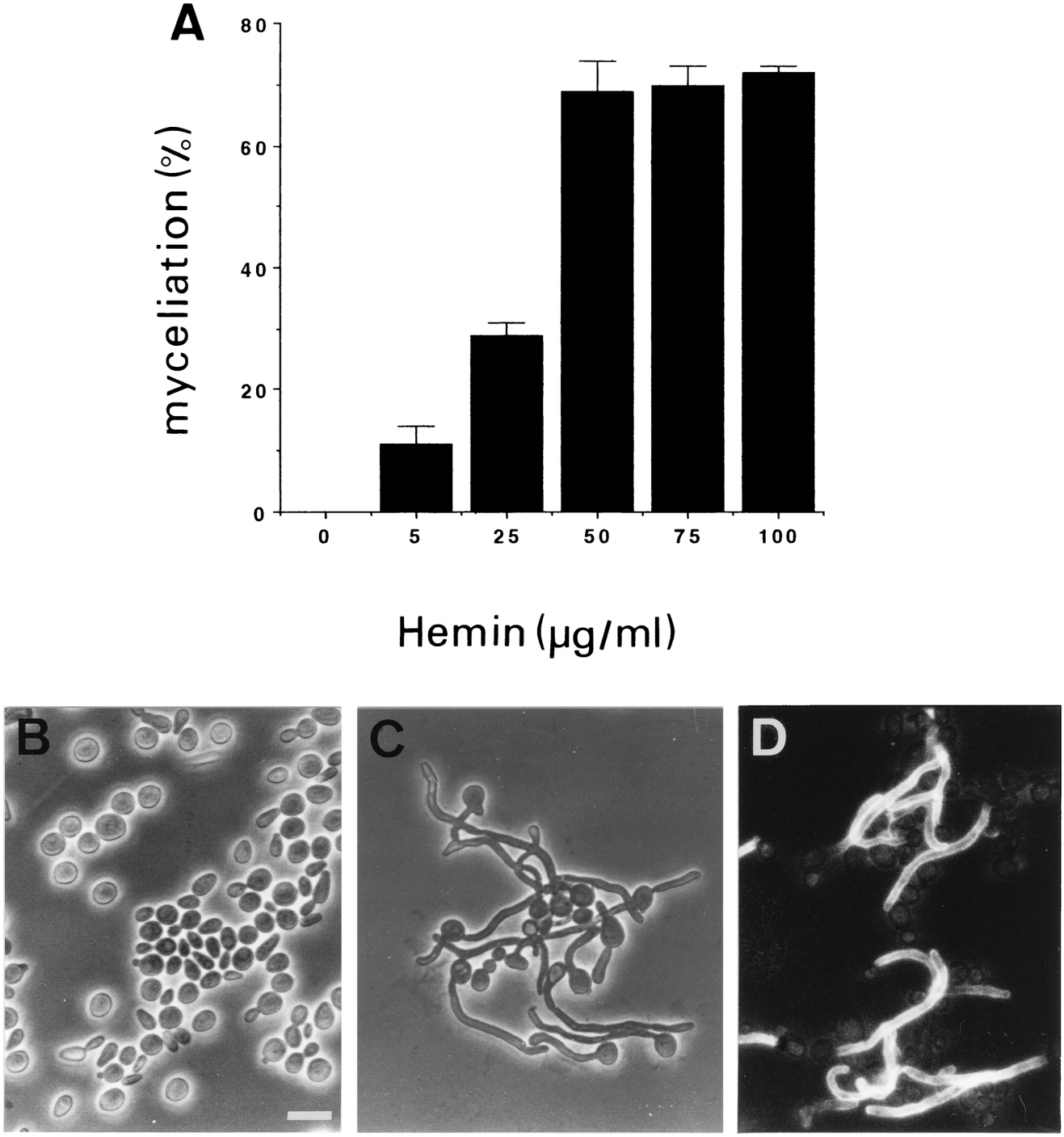

FIG. 1. Inducibility of germ tube formation in *C. albicans* by hemin. (A) Histogram showing the effect of increasing hemin concentrations on blastoconidial germination. Cells were grown at 37°C in SPB medium containing hemin at the indicated concentrations, and the amounts of blastoconidia exhibiting mature hyphal filaments (expressed as percentages of myceliation ± standard deviations) were determined by microscopic counting (see text for further details). Phase-contrast microscopy (B and C) and immunofluorescence (D) of *C. albicans* cells grown at 37°C in SPB medium in the absence (B) or presence (C and D) of 50 μg of hemin per ml are shown. The cells shown in panel D were reacted with MAb 4C12 (1:400 dilution). Bar, 10 μm.

concentration exceeds 5×10^5 yeast cells/ml. This may account for the observed absence of myceliation following thermal shifting in the absence of inducer. Thus, hemin may cause a bypass of this regulation of morphogenesis.

Phase transition in dimorphic fungi may involve differential gene expression, since certain genes get turned on or off as morphogenesis proceeds. The signal for triggering the morphogenetic change may be (i) shifts in environmental factors, (ii) depletion of essential nutrients, or (iii) increments in the concentration of some metabolites. Our results suggest that there are at least three physiological signals involved in the yeast-to-mycelium transition in *C. albicans*: temperature (in-

TABLE 1. Effects of pH, temperature, carbon source, and presence of hemin on germ tube formation in *C. albicans*

pH	Experimental condition[a]			Myceliation (%)[b]
	Temp (°C)	Carbon source	Hemin[c]	
6.5	28	Glucose	−	0
6.5	28	Glucose	+	0
6.5	37	Glucose	−	0
6.5	37	Glucose	+	70–75
6.5	28	GlcNAc	−	0
6.5	37	GlcNAc	−	70–75
6.5	37	GlcNAc	+	90–95
4.5	37	Glucose	−	0
4.5	37	GlcNAc[d]	−	20–25

[a] SPB medium and exponentially growing yeast cells (28°C precultures) were used in all experiments.
[b] Values are the ranges of myceliation measured in three independent experiments.
[c] When present, a hemin concentration of 50 μg per ml was used.
[d] Inducibility of germ tube formation by the combined effect of GlcNAc and hemin was not tested because low pH values caused hemin precipitation.

cubation at 37°C), the presence of hemin, and the carbon source (GlcNAc). Induction of germ tube formation may involve (i) a temperature-responsive element, since a thermal shift is required to induce germ tube formation even in stationary-phase cells; and (ii) a hemin-mediated regulation of specific genes in addition to those regulated by the carbon source. Both hemin and the carbon source exert an additive effect on germ tube induction in exponentially growing yeast, although in all cases a thermal shift is essential for germination of blastoconidia.

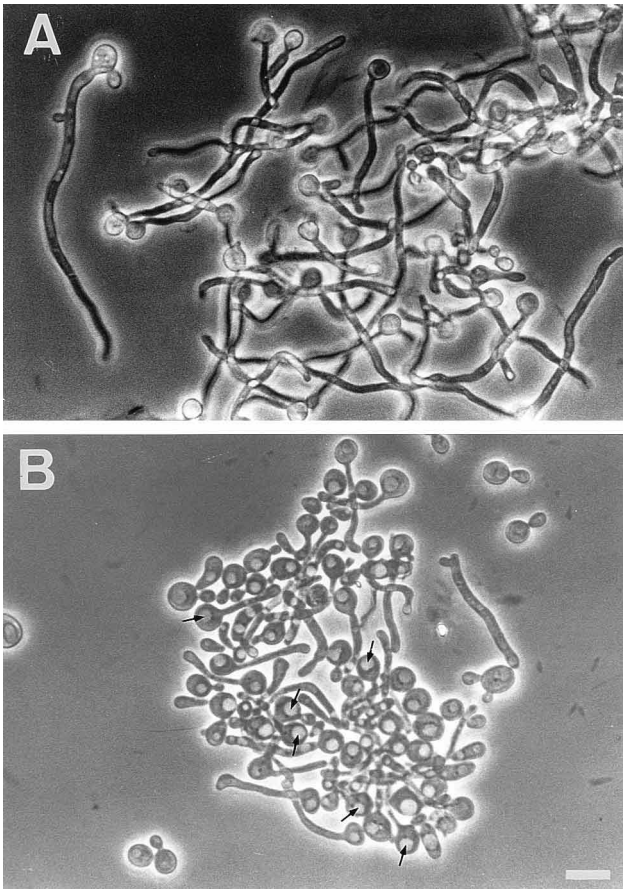

FIG. 2. Phase-contrast photographs of *C. albicans* grown at 37°C in SPB medium containing GlcNAc (1 mg/ml) and hemin (50 μg/ml) at pH 6.5 (A) and in SPB medium containing GlcNAc (1 mg/ml) at pH 4.5 (B). Arrows in panel B, the large, single vacuole present in nongerminated yeast and in mother blastoconidia from which germ tubes emanated. Bar, 10 μm.

As a final conclusion, germ tube induction in *C. albicans* may involve different signalling pathways triggered by distinct environmental factors which regulate different or overlapped subsets of gene systems controlling dimorphism. The functionality of these pathways may depend on both culture conditions and the growth phase, in a such a way that the response to distinct inducers depends on these factors. The fact that thermal shifting is the only requirement for inducing germination in stationary-phase cells whereas early-exponential-phase cells require the presence of other factors (e.g., hemin and GlcNAc) suggests the presence in the latter of negative controls repressing dimorphism which may be bypassed by the effect of inducers, as suggested by Hazen and Cutler (10).

The basic process of gene transcription and its regulation are conserved in mechanistic detail in all eukaryotes. In fact, *C. albicans* possesses a MAP kinase cascade analogous to the mating response pathway in *S. cerevisiae* which is involved in myceliation (11, 12). Therefore, it could be suggested that *C. albicans* has a system analogous to the HAP system of *S. cerevisiae* that regulates gene expression by heme and carbon source (6, 17, 23) which has become adapted to control dimorphism.

Whether heme present in plasma proteins arising from intravascular hemolysis of senescent erythrocytes plays a role as an inducer of germ tube formation in *C. albicans* during infection is unknown.

The support of grant SAF95-0595 from the CICyT (Plan Nacional de Salud y Farmacia), Ministerio de Educación y Ciencia, to J.P.M. and of Projecte d'Investigació Precompetitiu grant UV96-1778 (Universitat de València) to M.C. is acknowledged. A.M.C. was supported by a predoctoral fellowship from the Conselleria d'Educació i Ciencia, Generalitat Valenciana.

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Editor: T. R. Kozel