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Role of Hyphal Formation in Interactions of *Candida albicans* with Endothelial Cells

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The ability to change from yeast to hyphal morphology is a major virulence determinant of *Candida albicans*. Mutants with defined defects in filamentation regulatory pathways have reduced virulence in mice. However, is it poorly understood why hyphal formation is critical for *C. albicans* to cause hematogenously disseminated infections. We used recently constructed mutants to examine the role of hyphal formation in the interactions of *C. albicans* with endothelial cells in vitro. These interactions included the ability of the mutants to invade and injure endothelial cells. Because the formation of hyphae may influence the host inflammatory response to *C. albicans*, we also investigated the capacity of these mutants to stimulate endothelial cells to express E-selectin and intercellular adhesion molecule 1. We infected endothelial cells with *C. albicans* strains containing homozygous null mutations in the following filamentation regulatory genes: *CLA4*, *CPH1*, *EFG1*, and *TUP1*. Whereas the wild-type strain formed true hyphae on endothelial cells, we found that neither the Δ*efg1* nor the Δ*cph1* Δ*efg1* double mutant germinated. The Δ*tup1* mutant formed only pseudohyphae. We also found that the Δ*efg1*, Δ*cph1* Δ*efg1*, and Δ*tup1* mutants had significantly reduced capacities to invade and injure endothelial cells. Therefore, Efg1p and Tup1p contribute to virulence by regulating hyphal formation and the factors that enable *C. albicans* to invade and injure endothelial cells. With the exception of the Δ*cph1* Δ*efg1* mutant, all other mutants stimulated endothelial cells to express at least one of the leukocyte adhesion molecules. Therefore, the combined activities of Cph1p and Efg1p are required for *C. albicans* to stimulate a proinflammatory response in endothelial cells.

Candida species are the fourth most common cause of nosocomial bloodstream infections (5). In humans with hematogenously disseminated candidiasis, it has been found that the microabscesses contain both blastospores and hyphae (6). This observation has prompted investigations into the role of hyphal formation during the development of candidal infections. Over the past several decades it has been recognized that the transition from yeast to hyphal morphology is a major virulence factor of *Candida albicans*.

Because the transition from a yeast to a hyphal morphology is important for candidal virulence, the signal transduction pathways that regulate this transition have been the subject of intense investigation. Multiple different pathways that regulate this process have been identified (reviewed in reference 2). These pathways include the mitogen-activated protein kinase (MAPK) and the cyclic AMP (cAMP)-protein kinase A (PKA) pathway. Tup1p is a general transcriptional repressor that may act on the MAPK pathway, and it is currently unknown if it acts on the cAMP-PKA pathway.

In *C. albicans*, the MAPK pathway terminates in the transcription factor Cph1p (23, 24). Homozygous *cph1* mutants exhibit reduced filamentation on some solid media, yet they retain normal virulence in the murine model of hematogenously disseminated infection (Table 1). Disruption of the genes encoding most proteins in the MAPK pathway that are upstream of Cph1p results in similar defects in hyphal formation in vitro (3, 18, 19). Strains carrying some of these mutations have decreased virulence in mice; however, none of these mutants have been shown to have diminished hyphal formation in vivo (3, 19). Thus, the MAPK pathway likely regulates other virulence factors in addition to hyphal formation. Although Cla4p is almost certainly a MAPK, deletion of *CLA4* results in a unique phenotype. Unlike other MAPK pathway mutants, homozygous *cla4* mutants form aberrantly shaped cells that produce only short germ tubes in both liquid and solid media (Table 1). Also, these mutants have reduced virulence in mice (20). Therefore, Cla4p may regulate hyphal formation by a pathway that is independent of Cph1p.

The cAMP-PKA pathway likely terminates in the transcription factor Efg1p. Homozygous *efg1* mutants form only pseudohyphae on solid media and do not germinate at all in liquid media (Table 1) (24, 30). These mutants exhibit a moderate reduction in virulence in mice. Strains with the greatest defect in filamentation are those with homozygous deletions of both *efg1* and *cph1*. These mutants form short rod-like cells and do not germinate under most conditions. They have very low virulence in mice (24).

Tup1p functions as a general transcriptional repressor and is required to maintain the organism as a blastospore. Disruption of both alleles of *TUP1* results in cells that grow only as pseudohyphae, even under conditions that normally induce the organism to form blastospores (Table 1). A preliminary study indicates that homzygous *tup1* mutants have decreased virulence in mice (1).

Although the signal transduction pathways that regulate the transition from yeast to hyphal morphology are clearly essential for full virulence in *C. albicans*, it is less well understood why this transition is critical for the development of a hematogenously disseminated infection. One hypothesis is that the formation of hyphae is necessary for the organism to invade and injure host cells. In addition, the transition from yeast to hyphal morphology may alter the host inflammatory response to the organism. To investigate these hypotheses, we used

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TABLE 1. Strains of *C. albicans* used in these experiments

Strain	Genotype	In vitro phenotype	Virulence in mice	Source	Reference
SC5314	Wild type	Forms hyphae on liquid and solid media	High	W. A. Fonzi	11
CLJ1	*ura3*::*1imm434*/*ura3*::*1imm434 cla4*::*hisG*/*cla4*::*hisG-URA3-hisG*	Forms short, aberrantly shaped hyphae on liquid and solid media	Low	M. Whiteway	20
CAN16	*ura3*::*1imm434*/*ura3*::*1imm434 cph1*::*hisG*/*cph1*::*hisG-URA3-hisG*	Does not germinate on some solid media	High	G. R. Fink	23
CAN33	*ura3*::*1imm434*/*ura3*::*1imm434 efg1*::*hisG*/*egf1*::*hisG-URA3-hisG*	Forms pseudohyphae on solid media, does not germinate on liquid media	Low	G. R. Fink	24
CAN37	*ura3*::*1imm434*/*ura3*::*1imm434 efg1*::*hisG*/*efg1*::*hisG EFG1 URA3*	Similar to that of SC5314	Not reported	G. R. Fink	24
CAN34	*ura3*::*1imm434*/*ura3*::*1imm434 cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	Does not germinate on liquid or solid media	Very low	G. R. Fink	24
BCa2-10	ura3::*1imm434*/*ura3*::*1imm434 tup1*::*hisG*/*tup1*::*hisG-URA3-hisG*	Grows as pseudohyphae on both liquid and solid media	Low	B. R. Braun	1
BCa2-11	*ura3*::*1imm434*/*ura3*::*1imm434 tup1*::*hisG*/*tup1*::*hisG TUP1 URA3*	Similar to that of SC5314	Not reported	B. R. Braun	1

mutants with defined defects in the above-named filamentation regulatory pathways to examine the role of hyphal formation in the interactions of *C. albicans* with vascular endothelial cells in vitro. These interactions included the ability of different mutants to invade and injure endothelial cells. We also investigated the capacity of these mutants to stimulate a proinflammatory response in endothelial cells, as manifested by the expression of the leukocyte adhesion molecules E-selectin and intercellular adhesion molecule 1 (ICAM-1).

MATERIALS AND METHODS

Organisms. The strains of *C. albicans* used in this study are listed in Table 1. All mutants were constructed in CAI4, which is a homozygous *ura3* mutant of SC5314 (11). For use in the experiments, the organisms were grown overnight at 20°C on a rotating drum in yeast nitrogen base (YNB) broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% glucose as described previously (8, 10). The organisms were collected by centrifugation, washed twice in Dulbecco's phosphate-buffered saline (PBS) (Irvine Scientific, Santa Anna, Calif.), and enumerated using a hemacytometer.

In some experiments, blastospores of *C. albicans* SC5314 were pregerminated by incubation in RPMI 1640 medium (Irvine Scientific) for 90 min in a shaking incubator at 37°C (9). Greater than 90% of the organisms germinated under these conditions. To kill either blastospores or the germinated cells, the organisms were incubated in methanol for 2 min and then washed extensively in PBS prior to being added to the endothelial cells.

Endothelial cells. The endothelial cells were harvested from human umbilical cord veins by the method of Jaffe et al. (16). They were cultured in M-199 (Gibco, Grand Island, N.Y.) containing 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, Calif.), 10% defined bovine calf serum (Hyclone, Logan, Utah), and 2 mM L-glutamine with penicillin and streptomycin (Irvine Scientific). The cells were grown to confluence on fibronectin (Collaborative Biomedical Products, Bedford, Mass.), either in multiwell tissue culture plates (Falcon, Lincoln Park, N.J.) or on 12-mm-diameter glass coverslips. All incubations were at 37°C in 5% CO_2.

Endocytosis assay. The number of organisms internalized by the endothelial cells was determined using a differential fluorescence assay (12, 13). Briefly, endothelial cells on glass coverslips were infected with 10^5 cells of each strain of *C. albicans* in RPMI 1640 medium. After incubation for 3 h, the cells were fixed with 3% paraformaldehyde. The noninternalized cells were stained with anti-*C. albicans* rabbit serum (Biodesign International, Kennebunk, Maine) that had been conjugated with Alexa 594 (Molecular Probes, Eugene, Oreg.). Next, the endothelial cells were permeablized in 0.1% (vol/vol) Triton X-100 in PBS, after which both the internalized and the noninternalized organisms were stained with 1% (vol/vol) Uvitex (22). The coverslips were mounted inverted on a microscope slide and viewed under epifluorescence. The number of organisms endocytosed by the endothelial cells was determined by subtracting the number of noninternalized organisms (which fluoresced red) from the total number of organisms (which fluoresced blue). At least 100 organisms were counted on each coverslip, and all experiments were performed in triplicate on at least three separate occasions.

Measurement of endothelial cell injury. The extents of endothelial cell injury caused by the different strains of *C. albicans* were measured by the release of ^{51}Cr using a slight modification our standard assay (8, 10). Endothelial cells were grown to confluence in 96-well plates containing detachable wells. These cells were incubated overnight with 1 μCi of $Na_2{}^{51}CrO_4$ (ICN Biomedicals, Irvine, Calif.) per well. The following day, the unincorporated tracer was aspirated and the wells were rinsed twice with Hanks' balanced salt solution (Irvine Scientific). Next, the endothelial cells were infected with 4×10^4 *C. albicans* cells per well in 150 μl of RPMI 1640 medium. After a 3-h incubation, the upper 50% of medium was removed from each well and then the wells were manually detached from one another. The amounts of ^{51}Cr in the aspirates and the wells were determined by gamma counting. To measure the spontaneous release of ^{51}Cr, uninfected endothelial cells exposed to medium alone were processed in parallel. After we corrected for differences in the levels of incorporation of ^{51}Cr in the wells, the specific release of ^{51}Cr was calculated using the following formula: $(2 \times$ experimental release $- 2 \times$ spontaneous release$)/($total incorporation $- 2 \times$ spontaneous release$)$. Each experiment was performed in triplicate at least three different times.

Detection of leukocyte adhesion molecule expression. The surface expression of E-selectin and ICAM-1 by endothelial cells infected with the different strains of *C. albicans* was determined by whole-cell enzyme-linked immunosorbent assay by the method of Noel et al. (25) as described previously (26). The endothelial cells were grown in 96-well tissue culture plates, and inocula of both 4×10^4 and 8×10^4 organisms per well were tested. Because the endothelial cells were exposed to the organisms for 8 h, the RPMI 1640 medium was supplemented with 1% pooled human serum (Gemini Bio-Products, Inc.) to maintain endothelial cell viability. E-selectin expression was detected using the monoclonal antibody clone 1.2B6 (Biodesign International), and ICAM-1 expression was measured using the monoclonal antibody clone 15.2 (Biodesign International). The experiments were performed in triplicate using endothelial cells from at least three different umbilical cords.

Statistical analysis. Differences among the various strains of *C. albicans* were assessed using analysis of variance with the Bonferroni correction for multiple comparisons. P values of ≤ 0.05 were considered to be significant.

RESULTS

Different filamentation mutants had various morphologies after exposure to endothelial cells. We first examined the morphologies of the different mutants after they had been in contact with the endothelial cells for 3 h in RPMI 1640 medium. All strains of *C. albicans* except for the Δ*tup1* mutant (which grows only as pseudohyphae) were added to the endothelial cells as blastospores. The wild-type strain, SC5314, germinated and produced long hyphae with almost no branches (Fig. 1A). The Δ*cla4* mutant also formed hyphae. However, these hyphae tended to clump together and were generally shorter than

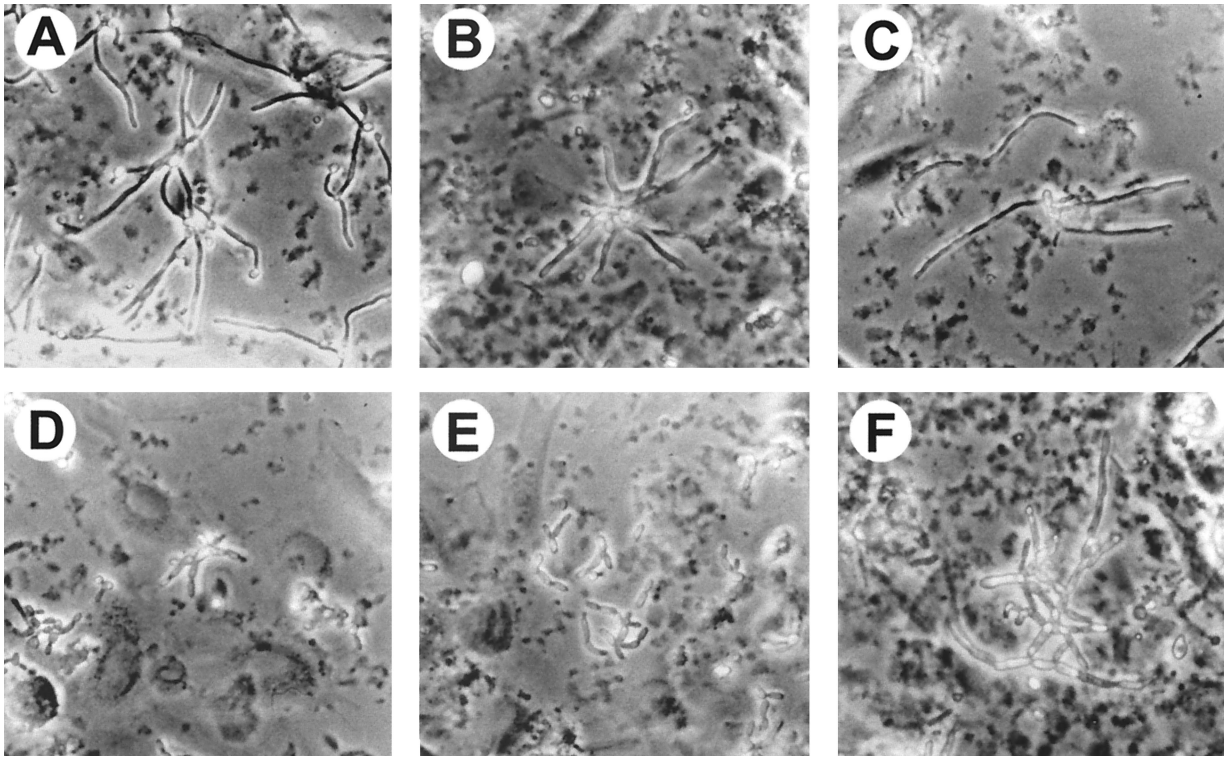

FIG. 1. Photomicrographs of the different strains of *C. albicans* after growth on endothelial cells in RPMI 1640 medium for 3 h. (A) SC5314; (B) Δ*cla4* mutant; (C) Δ*cph1* mutant; (D) Δ*efg1* mutant; (E) Δ*cph1* Δ*efg1* mutant; (F) Δ*tup1* mutant. The original magnification of all photomicrographs was ×150.

those of the parent strain, SC5314 (Fig. 1B). In addition, occasional bizarrely shaped cells were seen. Hyphae of the Δ*cph1* strain appeared to be identical to those of the wild-type parent (Fig. 1C). Both the Δ*efg1* mutant and the Δ*cph1* Δ*efg1* double mutant formed short chains of rod-like cells (Fig. 1D and E). Neither of these mutants produced germ tubes while they were in contact with the endothelial cells. Finally, the Δ*tup1* mutant grew as branching pseudohyphae, which in aggregate were much longer than the hyphae of the wild-type strain (Fig. 1F).

Mutants with abnormal filamentation had reduced capacities to invade endothelial cells. We have shown previously that the predominant method by which *C. albicans* invades endothelial cells in vitro is by inducing its own endocytosis (10). Therefore, the ability of the different strains to induce their own endocytosis was determined. In general, all mutants that had visible abnormalities in filamentation had at least some diminution in their ability to induce endocytosis (Fig. 2). The mutants with the greatest reduction were the Δ*efg1*, Δ*cph1* Δ*efg1*, and Δ*tup1* strains. Endothelial cell endocytosis of the Δ*cla4* mutant was only slightly reduced, and the endocytosis of the Δ*cph1* strain was not significantly different from that of strain SC5314. These findings suggest that factors regulated by Efg1p and Tup1p are important for *C. albicans* to induce its own endocytosis by endothelial cells.

Hyphal formation occurs via the protrusion of a germ tube from the blastospore. Because germ tube formation is the earliest phase in the transition from yeast to hyphal morphology, we investigated the link between germination and endocytosis. We germinated *C. albicans* SC5314 by incubating blastospores of this strain in RPMI 1640 medium at 37°C for 90 min. Next, we fixed the germ tubes in methanol to prevent them from progressing to hyphae. These germ tubes were approximately two blastospore diameters in length. We then investigated the uptake of these fixed germ tubes.

We found that almost 70% of the germinated organisms were taken up by the endothelial cells (Fig. 3). In contrast, there was very little endocytosis of blastospores of strain SC5314 that had been fixed after overnight growth in YNB broth at 20°C. We also examined the possibility that heat shock rather than germination caused the change(s) in the candidal

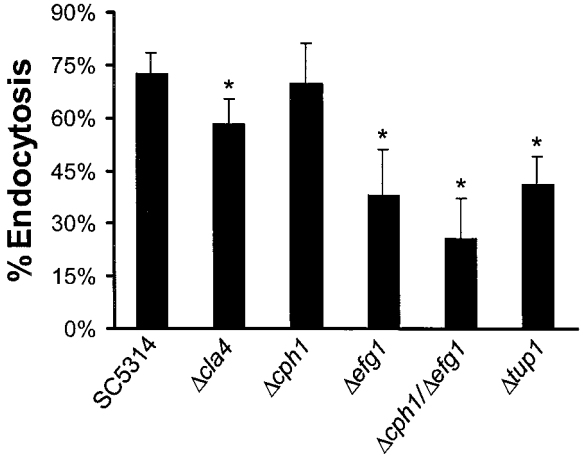

FIG. 2. Endothelial cell endocytosis of the different strains of *C. albicans*. Results are means ± standard deviations of results of at least three independent experiments, each performed in triplicate. *, $P < 0.001$ compared to values for SC5314.

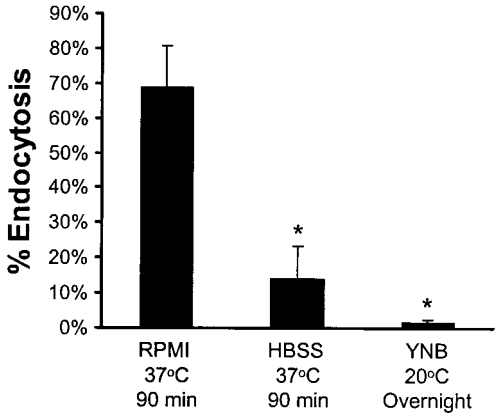

FIG. 3. Effects of germination on the endocytosis of methanol-fixed *C. albicans*. *C. albicans* cells were grown under the indicated conditions and then fixed in methanol prior to being added to the endothelial cells. Results are means ± standard deviations of results of three independent experiments. *, $P < 0.0001$ compared to values for organisms grown in RPMI 1640 medium at 37°C for 90 min. HBSS, Hanks' balanced salt solution.

cell wall that enabled the organism to induce its own endocytosis (17, 27). We incubated blastospores in Hanks' balanced salt solution at 37°C for 90 min prior to fixing them. These organisms did not germinate, and significantly fewer of them were endocytosed (Fig. 3). From these results, we conclude that the endocytosis of *C. albicans* can be induced by factors that are expressed as early as germ tube formation in the yeast-to-hypha transition.

The poorly endocytosed mutants had a reduced ability to injure endothelial cells. Next, the ability of the different mutants to injure endothelial cells was investigated. We found that the extent of endothelial cell injury caused by these strains paralleled the extent of endothelial cell endocytosis (Fig. 4). However, even though all of the mutants were endocytosed to some extent, the Δ*efg1*, Δ*cph1* Δ*efg1*, and Δ*tup1* strains caused virtually no damage to the endothelial cells. These findings suggest that the Efg1p and Tup1p signaling pathways may also regulate the synthesis and/or release of factors required for *C. albicans* to injure endothelial cells.

To confirm that disruption of *EFG1* and *TUP1* was the cause of the decreased ability of the mutants to injure endothelial cells, we assessed the phenotypes of *EFG1* and *TUP1* revertant strains. These strains had been constructed by reintroducing a copy of the gene that had been disrupted back into the homozygous null mutant (Table 1). When added to the endothelial cells, both revertants produced hyphae that were indistinguishable from those of the wild-type strain, SC5314. In these experiments, the specific release of ^{51}Cr induced by strain SC5314 was 23% ± 3%. The *EFG1* revertant caused slightly less endothelial cell injury, and the *TUP1* revertant induced slightly more injury than did strain SC5314 (specific releases of ^{51}Cr, 17% ± 4% and 30% ± 4%, respectively; $P < 0.001$ versus the specific release for SC5314 for both comparisons).

Most mutants stimulated normal leukocyte adhesion molecule expression on endothelial cells. Endothelial cells respond to contact with *C. albicans* by expressing leukocyte adhesion molecules and secreting proinflammatory cytokines (9, 26). These immunomodulatory factors have the potential to influence the host response to *C. albicans*. Because the magnitude of the host inflammatory response determines the outcome of a hematogenously disseminated candidal infection, we examined the ability of the different mutants to stimulate the endothelial cells to express E-selectin and ICAM-1.

We found significant day-to-day variation in the absolute amount of E-selectin that was expressed by endothelial cells infected with the different strains. Despite this variation, the relative amounts of E-selectin expression induced by the different mutants were consistently observed. When the inoculum was 4×10^4 organisms per well, the amounts of E-selectin expression induced by the mutants were not significantly different from that induced by SC5314 (Fig. 5A). When the endothelial cells were infected with 8×10^4 organisms per well, both the Δ*cph1* Δ*efg1* and the Δ*tup1* mutant stimulated significantly less E-selectin expression than did SC5314.

The only strain with a significantly decreased ability to stimulate ICAM-1 expression was the Δ*cph1* Δ*efg1* double mutant (Fig. 5B). There was a trend towards reduced ICAM-1 expression by the endothelial cells infected with the Δ*efg1* mutant, but this difference was not significant ($P = 0.2$). Interestingly, even though the Δ*tup1* mutant had a reduced capacity to stimulate E-selectin expression, its ability to induce ICAM-1 expression was normal. Therefore, endothelial cells react with a vigorous proinflammatory response to all mutants of *C. albicans* except those with the most severe defects in hyphal formation.

DISCUSSION

Our results demonstrate that the Efg1p and Tup1p signal transduction pathways are particularly important in the interactions of *C. albicans* with endothelial cells in vitro. In contrast, the MAPK pathway is less significant in these interactions.

The Δ*efg1* mutant did not germinate on endothelial cells, was only weakly endocytosed, and caused virtually no endothelial cell injury. Therefore, one or more factors that are regulated by Efg1p contribute significantly to the ability of *C. albicans* to invade and damage endothelial cells.

The Δ*tup1* mutant was also markedly deficient in its ability to invade and injure endothelial cells. However, this mutant formed extensive pseudohyphae on endothelial cells. These findings indicate that the ability to assume an elongated morphology per se is not sufficient for *C. albicans* to be endocytosed by and cause damage to endothelial cells under the conditions tested. One notable difference between the Δ*tup1* mutant and SC5314 was that the former strain grew as pseudohyphae on endothelial cells rather than as hyphae. Thus, one or more factors associated with the formation of true hyphae

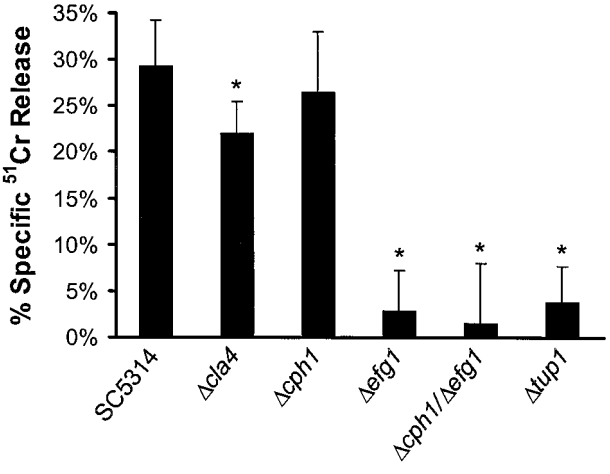

FIG. 4. Amount of endothelial cell injury caused by the different strains of *C. albicans*. Results are means ± standard deviations of results of at least three separate experiments. *, $P < 0.001$ compared to values for SC5314.

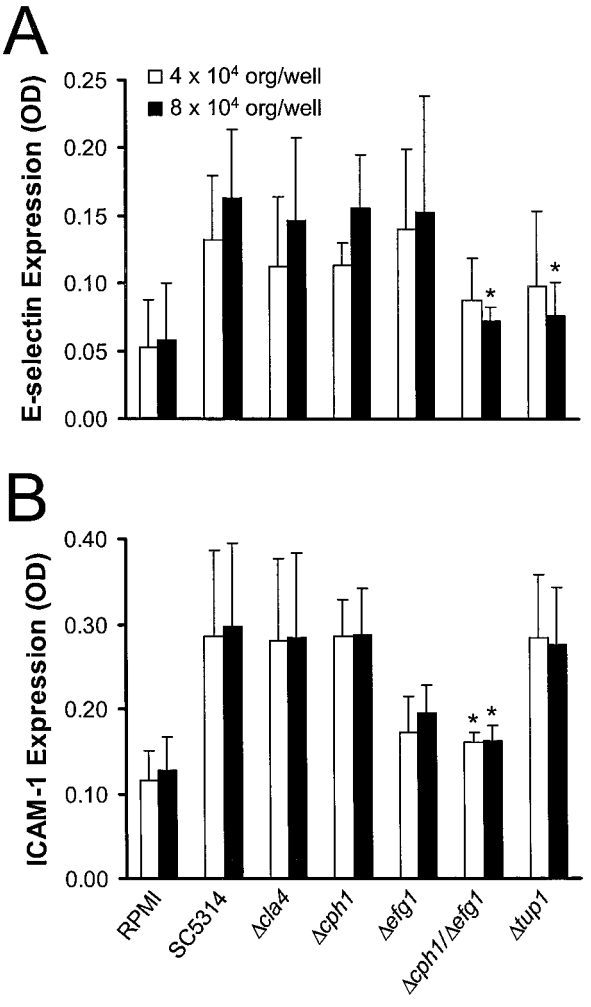

FIG. 5. Stimulation of endothelial cell expression of E-selectin (A) and ICAM-1 (B) by the different strains of *C. albicans*. Results are means ± standard deviations of results of at least three different experiments. *, $P < 0.001$ compared to values for SC5314. OD, optical density; org, organisms.

are likely required for *C. albicans* to invade and injure endothelial cells in vitro.

We also determined that both the *EFG1* revertant and the *TUP1* revertant were able to cause significant endothelial cell injury. Because *C. albicans* must be endocytosed by endothelial cells in order to injure them, the capacity of the revertants to injure the endothelial cells indicates that these strains were also able to induce their own endocytosis by these cells. Although there were minor differences in the amounts of injury induced by the revertants, compared to that induced by strain SC5314, these differences were likely of little biological significance. We therefore conclude that Efg1p and Tup1p are important for *C. albicans* to invade and injure endothelial cells in vitro.

The Δ*tup1* mutant stimulated endothelial cells to express ICAM-1 but not E-selectin. Previously, we determined that *C. albicans* induces the expression of these two leukocyte adhesion molecules via different mechanisms. E-selectin expression is mediated by the endothelial cell synthesis of tumor necrosis factor alpha, whereas ICAM-1 expression occurs independently of this cytokine (26). Our current results provide additional evidence for the differential regulation of E-selectin and ICAM-1 expression in response to *C. albicans*. Moreover, they suggest that Tup1 regulates the candidal factor(s) required to induce endothelial cells to express E-selectin but not ICAM-1.

The MAPK signal transduction pathway was less important in regulating the interactions of *C. albicans* with endothelial cells. The Δ*cph1* mutant was indistinguishable from SC5314 in all endothelial cell interactions studied. Also, the Δ*cph1* Δ*efg1* double mutant was similar to the Δ*efg1* mutant in its diminished capacity to germinate on, invade, and injure endothelial cells. One difference between the Δ*cph1* Δ*efg1* and Δ*efg1* mutants was in their abilities to stimulate endothelial cells to express leukocyte adhesion molecules. The Δ*cph1* Δ*efg1* mutant induced significantly less expression of E-selectin and ICAM-1 than did SC5314, whereas the Δ*efg1* mutant did not. These findings suggest that a candidal factor controlled by the MAPK pathway is important in stimulating endothelial cells to express leukocyte adhesion molecules.

Interestingly, although the Δ*cph1* Δ*efg1* mutant causes no mortality in mice following tail vein injection (24), the kidneys from these mice actually contain greater numbers of organisms than do the kidneys of mice infected with SC5314 (Woods Hole Molecular Mycology Course 1999, unpublished data). The weak proinflammatory response induced by the Δ*cph1* Δ*efg1* strain in vitro suggests that the high number of organisms in the murine kidneys may be the result of diminished clearance of the organisms due to a reduced host inflammatory response. However, additional studies are required to evaluate this possibility.

The Δ*cla4* mutant formed aberrantly shaped cells when it was exposed to endothelial cells. However, the majority of the filaments appeared to be hyphae rather than pseudohyphae. This finding may explain why the Δ*cla4* mutant had an only slightly decreased ability to invade and injure endothelial cells. The relatively minor defects of the Δ*cla4* mutant in vitro do not correspond to its markedly reduced virulence in the murine model of disseminated infection (20). A possible explanation for these divergent results is that candidal factors regulated by Cla4p may be important during the interaction of the organism with host cells other than endothelial cells. An additional explanation may be that our in vitro model of the interactions of *C. albicans* with endothelial cells does not completely mimic the conditions that exist in vivo. However, the Cla4p mutant did exhibit a similar morphology upon exposure to endothelial cells in vitro, as it does in the murine kidney during a hematogenous infection (20).

Taken together, our results suggest that the signal transduction pathways that regulate the yeast-to-hypha transition, especially those containing Efg1p and Tup1p, significantly influence the expression of candidal factors required for the organism to invade, injure, and stimulate endothelial cells. How these pathways regulate these putative candidal virulence factors is unclear. Furthermore, the candidal factors that mediate endothelial cell invasion, injury, and stimulation have not been delineated completely.

We have shown previously that secreted aspartyl proteinase 2 contributes to endothelial cell injury by *C. albicans* (15). However, whether Egf1p and Tup1p regulate the expression of the various secreted aspartyl proteinase genes is unknown. Other lytic enzymes, such as phospholipases may also play a role in causing cellular damage (7, 21). One candidal phospholipase that probably does not contribute significantly to endothelial cell injury under the conditions tested is encoded by *PLB1*. Hoover et al. reported that this gene is highly expressed in a Δ*tup1* mutant (14), whereas we found that this mutant caused virtually no damage to endothelial cells. Nevertheless,

other phospholipases of *C. albicans* may well be a cause of endothelial injury.

Although endothelial cell injury is likely caused by factors secreted by *C. albicans*, induction of endocytosis is not. We have found previously that killed *C. albicans* cells are endocytosed by endothelial cells but that they do not cause detectable injury to these cells (10). Our present results suggest that the candidal factors that induce endothelial cell endocytosis are expressed by *C. albicans* cells shortly after they germinate. Because the yeast-to-hypha transition is accompanied by a significant alteration in the candidal cell surface (4, 31), it is probable that the germinated organisms express molecules on their surfaces that trigger the process of endocytosis.

In the near future, the ability to use DNA microarrays to measure gene expression on a genome-wide scale will provide much information about which virulence factor genes are expressed during the different phases of the yeast-to-hypha transition (28, 29). Applying this technique to the different signal transduction pathway mutants will also yield important data on how these virulence factors are regulated. Finally, combining the gene expression data with the findings of in vitro studies similar to the ones performed here will very likely result in the identification of additional virulence factors of *C. albicans*.

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