N-cadherin Mediates Endocytosis of *Candida albicans* by Endothelial Cells*

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*Candida albicans* is the most common cause of fungal bloodstream infections. To invade the deep tissues, blood-borne organisms must cross the endothelial cell lining of the vasculature. We have found previously that *C. albicans* hyphae, but not blastospores, invade endothelial cells in *vitro* by inducing their own endocytosis. Therefore, we set out to identify the endothelial cell receptor that mediates the endocytosis of *C. albicans*. We determined that endocytosis of *C. albicans* was not mediated by bridging molecules in the serum and that it was partially dependent on the presence of extracellular calcium. Using an affinity purification procedure, we discovered that endothelial cell N-cadherin bound to *C. albicans* hyphae but not blastospores. N-cadherin also co-localized with *C. albicans* hyphae that were being endocytosed by endothelial cells. Chinese hamster ovary (CHO) cells expressing human N-cadherin endocytosed significantly more *C. albicans* hyphae than did CHO cells expressing either human VE-cadherin or no human cadherins. The expression of N-cadherin by the CHO cells resulted in enhanced endocytosis of hyphae, but not blastospores, indicating the selectivity of the N-cadherin-mediated endocytosis. Down-regulation of endothelial cell N-cadherin expression with small interfering RNA significantly inhibited the endocytosis of *C. albicans* hyphae. Therefore, a novel function of N-cadherin is that it serves as an endothelial cell receptor, which mediates the endocytosis of *C. albicans*.

Hematogenously disseminated candidiasis is a serious fungal infection that occurs in hospitalized patients. *Candida albicans* causes —60% of all cases of these infections, whereas the remainder are due to other species of *Candida* (1, 2). In susceptible hosts, the organisms enter the bloodstream either via an indwelling vascular catheter or by translocation across the gastrointestinal mucosa. Once inside the bloodstream, the organisms disseminate to virtually all of the organs in the body. To escape from the intravascular compartment and invade the deep tissues, the organisms must cross the endothelial cell lining of the blood vessels. Thus, the endothelial cell is one of the first host cells that *C. albicans* encounters as it establishes a hematogenously disseminated infection. We have been investigating the interactions of *C. albicans* with endothelial cells *in vitro* with the long-range goal of developing methods to prevent blood-borne organisms from traversing the vascular endothelium and invading the deep tissues.

*C. albicans* must first adhere to endothelial cells to cross the endothelial cell lining of the vasculature. Several different *C. albicans* adhesins that mediate the binding of this organism to endothelial cells *in vitro* have been identified. They include Als1p, Als3p, Als5p, hydrophobic cell wall proteins, and a α5β3 integrin-like receptor (3–7). The α5β3 integrin-like receptor probably binds to vitronectin or other Arg-Gly-Asp-containing proteins on the endothelial cell surface (6). However, the endothelial cell targets for the other candidal adhesins are unknown.

*C. albicans* adherence to endothelial cells is followed by invasion of the organism into these cells. We have found that endothelial cell adherence and invasion are distinct processes. For example, although *C. albicans* blastospores adhere avidly to endothelial cells, they invade these cells poorly (8). In contrast, *C. albicans* hyphae both adhere to and invade endothelial cells. *C. albicans* hyphae invade endothelial cells *in vitro* by inducing their own endocytosis (9, 10). The endocytosis of *C. albicans* hyphae by endothelial cells is independent of fungal viability and requires functional endothelial cell microfilaments and microtubules (8, 9). Moreover, the endocytic process is governed in part by the tyrosine phosphorylation of endothelial cell integrins (11). Importantly, the endothelial cell receptor(s) that are bound by *C. albicans* and that mediate the endocytosis of the organism have not been identified previously. In the current study, we discovered that N-cadherin is an endothelial cell receptor for *C. albicans* hyphae.

**EXPERIMENTAL PROCEDURES**

**Fungal Strains and Culture—** *C. albicans* strains SC5314 and its *ura3Δ/ura3Δ* derivative, CAI-4 (12), were generous gifts from William Fonzi (Georgetown School of Medicine, Washington, D.C.). strain 36082 was from the American Type Culture Collection, and strain 15153 was obtained from the clinical laboratory at the Harbor-UCLA Medical Center. All of these strains are clinical isolates and have been shown previously to be endocytosed by and cause injury to vascular endothelial cells *in vitro* (9, 11, 13). The *tpk2Δ/tpk2Δ* mutant and a *TPK2*-reconstituted strain (*tpk2Δ/tpk2Δ*:*TPK2*), which were constructed from strain CAI-4 (14), were kindly provided by Joachim Ernst (Heinrich-Heine-Universität, Düsseldorf, Germany). *URA3* was re-

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stored to its native locus in CAI-4 and the tpk2Δ/tpk2Δ and tpk2Δ/tpk2Δ:TPK2 strains as described previously (15).

For all of the experiments, the organisms were grown overnight at room temperature on a rotating drum in yeast nitrogen base broth (Difco) supplemented with 2% glucose (w/v) (9). The resulting blastospores were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS)1 without Ca2+ and Mg2+, and enumerated with a hemacytometer. To obtain hyphal-phase organisms, C. albicans blastospores were suspended in RPMI 1640 medium (Irvine Scientific) at a final concentration of 3 × 106 cells/ml and incubated on a rotary shaker at 37 °C for 120 min. Because the hyphae of the tpk2Δ/tpk2Δ mutant elongated slightly more slowly than the hyphae of the other strains, this mutant was incubated in RPMI 1640 medium for 150 min. After this incubation, the hyphae of the tpk2Δ/tpk2Δ strain were similar in length to those of the other strains.

In some experiments, the hyphae were coated with serum proteins by incubation at 4 °C for 30 min at 37 °C in RPMI 1640 medium containing 10% pooled human serum (Gemini Bioproducts). To control for the stimulatory effects of the serum on hyphal growth, the hyphae were first killed by exposure to 100% methanol for 2 min (8) and then rinsed extensively in PBS before being incubated in serum. Control hyphae were killed and then incubated in RPMI 1640 medium without serum in parallel. The organisms were then rinsed in PBS and counted for use in the experiments. When there have found proteinase K treatment of hyphae are endocytosed by endothelial cells similarly to live hyphae (8).

Endothelial Cells and Chinese Hamster Ovary (CHO) Cells—Endothelial cells were isolated from the veins of human umbilical cords following the method of Jaffe et al. (16). They were maintained in M-199 medium (Invitrogen) containing 10% fetal bovine serum (Gemini Bio-Products, Inc.), 10% defined bovine calf serum (Gemini Bio-Products, Inc.), and 2 mML-glutamine with penicillin and streptomycin. CHO K-1 cells were obtained from the American Type Culture Collection and grown in Ham’s F-12K medium (American Type Culture Collection) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Both cell types were grown to confluency on fibronectin-coated glass coverslips in a 24-well tissue culture plate (BD Biosciences). The cells were rinsed once with warm Hank’s balanced salt solution (HBSS, Irvine Scientific) and infected with 106 cells of C. albicans in RPMI 1640 medium for 45 min at 37 °C in 5% CO2. Next, the cells were rinsed twice with 0.5 ml of HBSS in a standardized manner and then fixed with 3% parafomaldehyde. The adherent but non-endocytosed organisms were labeled with polyclonal rabbit anti-C. albicans antibodies (Biodiesgn International) that had been conjugated with green fluorescing Alexa 568 (Molecular Probes). The endothelial cells or CHO cells then were permeabilized with 0.5% Triton X-100 (Sigma) in PBS, after which both the endocytosed and non-endocytosed cells were rinsed twice with 0.5 ml of HBSS and infected with 105 cells of C. albicans SC5314 in RPMI 1640 medium. After a 45-min incubation at 37 °C in 5% CO2, the cells were washed once with HBSS to remove unbound organisms and then fixed with 3% paraformaldehyde. The cells were washed with PBS containing 0.1% bovine serum albumin and then blocked and permabilized for 5 min in 0.5% goat serum containing 1% Triton X-100. The cells were incubated with either an anti-N-cadherin monoclonal antibody (clone 32) or the anti-VE-cadherin monoclonal antibody, rinsed, and then incubated with a goat anti-mouse secondary antibody, and the bands were visualized using enhanced chemiluminescence (Pierce).

Preparation of Endothelial Cell Membrane Proteins—Endothelial cell membrane proteins were prepared using the method of Isberg and Leong (19). Confluent endothelial cells in 100-mm diameter tissue culture dishes were rinsed twice with warm PBS containing Ca2+ and Mg2+ (PBS+) and then incubated with Ez-Link Sulfo-NHS-LS Biotin (0.5 mg/ml, Pierce) in PBS+ for 12 min at 37 °C in 5% CO2. The cells then were rinsed extensively with cold PBS+ and scraped from the tissue culture dishes. The endothelial cells were collected by centrifugation at 500 × g for 5 min at 4 °C and then lysed by incubation for 20 min on ice in PBS+ containing 5.8% octyl-glucopyranoside (w/v) (Sigma) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The cell debris were removed by centrifugation at 5000 × g for 5 min at 4 °C. The supernatant was collected and centrifuged at 100,000 × g for 1 h at 4 °C. The concentration of the endothelial cell proteins in the resulting supernatant was determined by the Bradford assay (Bio-Rad).

Isolation of Endothelial Cell Surface Proteins that Bind to C. albicans—An affinity purification method using intact C. albicans cells was employed to isolate endothelial cell proteins that bind to C. albicans. Live C. albicans blastospores (8 × 106 cells) or an equivalent volume of hyphae (2 × 106 cells) were incubated for 1 h on ice with 50–250 μg of biotin-labeled endothelial cell surface proteins in buffer A (PBS+ contain-15.1% octyl-glucopyranoside and protease inhibitors). The unbound endothelial cell proteins were removed by three rinses with buffer A. The endothelial cell proteins that remained bound to the organisms were eluted with 10 mM EDTA, pH 8.0, in buffer A without Ca2+ and Mg2+. The proteins were separated on a 7.5% SDS-polyacryl- amide gel (Bio-Rad) and transferred to Immobilon-P nylon membranes (Millipore). After blocking the membranes overnight in Tris-buffered saline containing 10% casein, the membranes were immunoblotted with the biotin-labeled antibodies (clone MBN-14, rabbit pan-cadherin antibody (Sigma), an anti-N-cadherin monoclonal antibody (clone 32, Transduction Laboratories), or an anti-VE-cadherin monoclonal antibody (clone 75, Transduction Laboratories). The mem- branes then were incubated with an appropriate horseradish peroxi- dase-conjugated secondary antibody, and the bands were visualized using enhanced chemiluminescence (Pierce).

Measurement of Endocytosis of C. albicans by Endothelial Cells and CHO Cells—The number of organisms that were labeled with green fluorescing Alexa 488. The number of endocytosed organisms was determined by sub-traction of the number of organisms that were labeled with red fluorescing Alexa 688 from the number of cell-associated organisms. The results were expressed as the number of endocytosed or cell-associated organisms per high-powered field. An organism was considered to be endocytosed if an organism was internalized. All of the experiments were performed in triplicate at least three times.

Preparation of Endothelial Cell Membrane Proteins—Endothelial cell membrane proteins were prepared using the method of Isberg and Leong (19). Confluent endothelial cells in 100-mm diameter tissue culture dishes were rinsed twice with warm PBS containing Ca2+ and Mg2+ (PBS+) and then incubated with Ez-Link Sulfo-NHS-LS Biotin (0.5 mg/ml, Pierce) in PBS+ for 12 min at 37 °C in 5% CO2. The cells then were rinsed extensively with cold PBS+ and scraped from the

1 The abbreviations used are: PBS, phosphate-buffered saline without Ca2+ and Mg2+; PBS+, PBS containing Ca2+ and Mg2+; CHO, Chinese hamster ovary; HBSS, Hank’s balanced salt solution; siRNA, small interfering RNA.
FIG. 1. Effects of chelation of extracellular calcium on the endocytosis of C. albicans. Hyphae of C. albicans SC5314 were incubated for 45 min with endothelial cells in RPMI 1640 medium alone (Control) or in medium containing 1.9 mM EGTA (EGTA), 1.9 mM EGTA plus 1.9 mM CaCl$_2$ (EGTA + Ca$^{2+}$), or 1.9 mM EGTA plus 1.9 mM MgCl$_2$ (EGTA + Mg$^{2+}$). The number of endocytosed and cell-associated organisms was determined using a differential fluorescing assay. Results are expressed as a percentage of the number of organisms per high-powered field that were endocytosed by or cell-associated with control endothelial cells incubated without EGTA. Data are the mean ± S.D. of four independent experiments, each performed in triplicate. *$p < 0.0001$ compared with control. **$p = 0.03$ compared with control.

block ed on ice with 1% goat serum in HBSS. N-cadherin and VE-cadherin were detected using monoclonal antibodies directed against the extracellular domains of N-cadherin (clone GC34, Sigma) and VE-cadherin, respectively. The cells were labeled next with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma), rinsed, and then sorted using a FACSCaliber flow cytometer equipped with a cell sorter (BD Biosciences). The sorted cells were grown to confluency, and the percentage of cells expressing N-cadherin or VE-cadherin was determined by flow cytometry. The sorting procedure was repeated 3–4 times until at least 75% transfectants expressed either N-cadherin or VE-cadherin.

Down-regulation of Endothelial Cell N-cadherin Expression by Small Interfering RNA (siRNA)—Endothelial cells were grown to 60% confluency in six-well tissue culture plates in tissue culture medium containing 10% fetal bovine serum and 10% bovine calf serum without antibiotics. These cells were transfected with 2 µg of either N-cadherin siRNA duplex oligonucleotides (Santa Cruz Biotechnology) or random fluorescein isothiocyanate-labeled siRNA duplex control oligonucleotides (QiaGen) using Lipofectamine 2000 in 500 µl of serum-free medium accord ing to the manufacturer’s instructions. After 24 h, the cells were detached with trypsin-EDTA and seeded onto fibronectin-coated glass coverslips in a 24-well tissue culture plate. The next day, the endocytosis of C. albicans hyphae by the transfected endothelial cells was measured as described above. To verify that N-cadherin siRNA down-regulated endothelial cell N-cadherin expression, the transfected endothelial cells from one of the glass coverslips were lysed in 30 µl of 2× SDS sample buffer. The cellular proteins were resolved by SDS-PAGE, after which N-cadherin was detected by immunoblotting as outlined previously. Each immunoblot was then stripped and probed with a rabbit polyclonal anti-β-actin antisum (Cell Signaling Technology, Inc.) to confirm equality of protein loading.

Statistical Analysis—Differences were evaluated using analysis of variance, and $p$ values ≤ 0.05 were considered significant.

RESULTS

Endocytosis of C. albicans Requires Extracellular Calcium—Many host cell receptors that mediate the uptake of microorganisms by normally non-phagocytic host cells require calcium for their function (19, 21, 22). Therefore, we used the calcium chelator, EGTA, to investigate the role of extracellular calcium in the endocytosis of C. albicans by endothelial cells (Fig. 1). Adding EGTA alone to the medium reduced the endocytosis of C. albicans hyphae by 60% compared with control endothelial cells that were not exposed to this chelator. EGTA had no effect on the number of cell-associated organisms, indicating that the reduction in endocytosis was not due to inhibition of C. albicans adherence. The addition of calcium plus EGTA to the endothelial cells restored their capacity to endocytose hyphae to control levels and actually caused a slight but statistically significant increase in the number of cell-associated organisms. Magnesium did not reverse the inhibitory effect of EGTA on endocytosis, demonstrating that this effect was specifically due to the chelation of calcium. These results suggest that the interaction of C. albicans hyphae with its endothelial cell receptor is calcium-dependent.

Endocytosis of C. albicans by Endothelial Cells Does Not Require Serum Proteins—When C. albicans interacts with endothelial cells in vivo, the organisms are almost certainly coated with serum proteins. It is known that some microorganisms such as Staphylococcus aureus utilize serum components as bridging molecules to induce their own uptake by endothelial cells (22). To test whether serum proteins influence the endocytosis of C. albicans, we incubated C. albicans hyphae in the presence and absence of serum and then measured the uptake of the organisms by endothelial cells. Serum is a potent stimulator of C. albicans hyphal formation and elongation (23). Therefore, we killed the hyphae prior to incubating them in serum to control for the effects of serum on the organism. In the absence of serum, the number of killed organisms, which were endocytosed by and cell-associated with endothelial cells, was similar to that of live organisms (Fig. 2A), a finding we have reported previously (8). Incubation of killed hyphae in serum prior to adding them to the endothelial cells resulted in a 40% decrease in the number of endocytosed organisms, although it had no effect on the number of cell-associated organisms. These data indicate that serum proteins actually inhibit endocytosis rather than facilitate this process. Thus, they are unlikely to act as bridging molecules between the organism and the endothelial cell.

Detection of Endothelial Cell Surface Proteins that Bind to C. albicans Blastospores and Hyphae—Based on these above results, we hypothesized that the endocytosis of C. albicans is mediated by the direct interaction of an endothelial cell receptor with a ligand expressed on the surface of hyphae and that this interaction is calcium-dependent. Therefore, we used the affinity purification procedure developed by Isberg and Leong (19) to identify endothelial cell proteins that bound C. albicans hyphae in a calcium-dependent manner. We found that C. albicans blastospores bound only to an endothelial cell protein with a molecular mass of ~105 kDa (Fig. 2B). In contrast, C. albicans hyphae bound to a 135-kDa protein as well as the 105-kDa protein. Of note, the killed hyphae bound to the same endothelial cell proteins as did live hyphae (Fig. 2B). Furthermore, preincubating killed organisms in serum did not affect the binding of endothelial cell proteins to these cells. These results support our previous finding that serum proteins do not act as bridging molecules between the organism and the endothelial cell.

Endothelial Cell N-cadherin Binds to C. albicans—We scaled up the affinity purification procedure to obtain sufficient protein for sequencing. We found several potential matches with members of the cadherin family. Therefore, to verify that an endothelial cell cadherin bound to C. albicans hyphae, we probed blots containing endothelial cell proteins that bound to C. albicans with a pan-cadherin antiserum directed against the conserved C-terminal region of the classical cadherins. This antiserum recognized the 135-kDa endothelial cell protein that was bound specifically by hyphae (Fig. 3).

N-cadherin and VE-cadherin are the only cadherins with molecular masses in the range of 135 kDa that are known to be expressed by endothelial cells (24–26). Therefore, we investigated whether monoclonal antibodies directed against either of
these cadherins recognized an endothelial cell protein that was bound by \textit{C. albicans} hyphae. A monoclonal antibody directed against N-cadherin recognized the 135-kDa endothelial cell protein bound by hyphae. (Fig. 3). However, an anti-VE-cadherin monoclonal antibody did not recognize any endothelial cell protein that bound to \textit{C. albicans}, although this antibody did recognize VE-cadherin in the total membrane protein preparation (data not shown).

We next used indirect immunofluorescence to verify that N-cadherin on intact endothelial cells bound to \textit{C. albicans} hyphae that were being endocytosed. Parallel sets of endothelial cells were stained for VE-cadherin for comparison. As reported by others (25, 26), N-cadherin was expressed diffusely over the entire surface of uninfected endothelial cells (Fig. 4, A–C), whereas VE-cadherin was localized mainly at intercellular junctions (Fig. 4, H–J). When the cells were infected with \textit{C. albicans}, endothelial cell N-cadherin and microfilaments co-localized with \textit{C. albicans} hyphae that were being endocytosed (Fig. 4, D–G). Frequently, the N-cadherin could be seen to accumulate along the entire length of the hyphaus, even though there was only focal accumulation of actin around the organism. Although N-cadherin and actin usually co-localized around the same organism, a minority of organisms were surrounded by N-cadherin but not actin and others were surrounded by VE-cadherin but not N-cadherin (data not shown). In contrast to N-cadherin, VE-cadherin did not accumulate around the hyphae but remained located at the intercellular junctions (Fig. 4, K–N).

To confirm that N-cadherin bound to strains of \textit{C. albicans} other than strain SC5314, we investigated the endothelial cell proteins that were bound by two blood isolates of \textit{C. albicans} that are known to be endocytosed by endothelial cells in vitro (11). The hyphae of both of these strains bound to the same endothelial cell membrane proteins including N-cadherin as did strain SC5314 (Fig. 5). Collectively, the immunoblotting
and indirect immunofluorescent results indicate that endothelial cell N-cadherin binds to C. albicans hyphae.

A Mutant of C. albicans That Lacks Tpk2p Is Endocytosed Poorly by Endothelial Cells and Does Not Bind to N-cadherin—Many mutants of C. albicans that lack components of the signal transduction pathways that regulate hyphal formation are poorly endocytosed by endothelial cells (8). Tpk2p is a catalytic subunit of protein kinase A and is a member of the Ras protein kinase A pathway that regulates both hyphal formation and virulence in C. albicans (14, 23, 27, 28). We investigated the interactions of hyphae of a tpk2Δ::TPK2 mutant of C. albicans with endothelial cells. Approximately 50% fewer hyphae of the tpk2Δ::TPK2 mutant were endocytosed by endothelial cells as compared with wild-type parent strain and the TPK2-reconstituted strain (tpk2Δ::tpk2Δ::TPK2) (Fig. 6A). In contrast, the number of tpk2Δ::tpk2Δ hyphae that were cell-associated was similar to that of the other two strains (Fig. 6A). These results indicate that the tpk2Δ::tpk2Δ mutant was defective in its ability to induce endothelial cell endocytosis, although its adherence to endothelial cells was not impaired.

We next investigated the pattern of endothelial cell surface proteins that were bound by the tpk2Δ::tpk2Δ mutant. The hyphae of this strain bound very poorly to N-cadherin, although they bound normal amounts of the 105-kDa endothelial cell protein (Fig. 6, B and C). As expected, both the wild-type and TPK2-reconstituted strain bound to both N-cadherin and the 105-kDa protein. These results suggest that binding to N-cadherin is required for maximal endocytosis of C. albicans.

They also indicate that Tpk2p regulates the expression of the ligands on the surface of C. albicans that bind to N-cadherin.

N-cadherin Mediates Endocytosis of C. albicans Hyphae—To determine whether N-cadherin could mediate the endocytosis of C. albicans, we used a heterologous expression strategy. CHO cells, which normally express few or no cadherins (25, 26), were transfected with human N-cadherin, after which their ability to endocytose C. albicans hyphae was assessed. CHO cells expressing N-cadherin endocytosed almost 5-fold more organisms than did control cells transfected with the empty vector (Fig. 7A). CHO cells expressing human VE-cadherin endocytosed ~2-fold more organisms than did control cells but significantly fewer organisms than cells expressing N-cadherin. Expression of N-cadherin had no effect on the number of cell-associated organisms, indicating that the enhanced endocytosis was not the result of increased adherence.

We next examined the specificity of N-cadherin-mediated endocytosis by comparing the uptake of C. albicans hyphae and blastospores by CHO cells expressing N-cadherin. The N-cadherin transfectants endocytosed 7-fold more hyphae than blastospores (Fig. 7B). Endothelial cells also endocytosed significantly more hyphae than blastospores, a result we have reported previously (8). These results demonstrate that enhanced endocytosis of the N-cadherin transfectants is specific for C. albicans hyphae and is not just the result of a generalized increase in endocytic capacity of these transfectants.

Down-regulation of Endothelial Cell N-cadherin Expression Inhibits Endocytosis of C. albicans—To verify that N-cadherin mediates the endocytosis of C. albicans by endothelial cells, we used siRNA to down-regulate the expression of N-cadherin by these cells. Transfection of endothelial cells with the N-cadherin siRNA reduced the expressed of N-cadherin by at least 90% compared with cells transfected with a random control siRNA (Fig. 8A). Endothelial cells transfected with the N-cadherin siRNA endocytosed 34% fewer C. albicans hyphae than did endothelial cells transfected with the control siRNA (Fig. 8B). Down-regulation of N-cadherin expression did not have a significant effect on the number of cell-associated organisms (p = 0.14), indicating that the adherence of C. albicans to these cells was not altered.

**DISCUSSION**

Understanding the mechanisms by which pathogenic microorganisms induce their own uptake by normally non-phagocytic host cells is a critical step for developing therapeutic approaches to block this process. The fungi Cryptococcus neoformans and Aspergillus fumigatus as well as C. albicans are all known to induce their own endocytosis by human endothelial cells in vitro (9, 10, 29–31). However, the endothelial cell
Our data indicate that an additional function of N-cadherin is neuronal development and function (36–38) as well as the metastasis of breast cancer cells and vascular smooth muscle cells (32, 33). N-cadherin is also involved in the exchange of signals between endothelial cells. Although the normal function of N-cadherin in endothelial cells is not completely understood, this molecule is probably endocytosed by intact endothelial cells. Third, a strain of C. albicans that did not bind to N-cadherin was poorly endocytosed by endothelial cells. Fourth, CHO cells that expressed human N-cadherin endocytosed significantly more C. albicans hyphae than did control CHO cells that expressed either VE-cadherin or no human cadherins. Fifth, endothelial cells transfected with the indicated siRNAs were incubated with hyphae of C. albicans SC5314 for 45 min, after which the number of endocytosed and cell-associated organisms was determined by a differential fluorescence assay. The data are expressed as a percentage of the results obtained with hyphae incubated with either CHO cells expressing N-cadherin or endothelial cells. They are the mean ± S.D. of three experiments, each performed in triplicate. §, p < 0.001 compared with hyphae on the same cell type; Blasto., blastospores.

We found multiple lines of evidence indicating that N-cadherin is an endothelial cell receptor that mediates the endocytosis of C. albicans. First, the hyphae of three different strains of C. albicans, which are endocytosed avidly by endothelial cells, bound N-cadherin in endothelial cells plasma membrane extracts. In contrast, blastospores, which are poorly endocytosed by endothelial cells, did not bind to N-cadherin. Second, N-cadherin co-localized with hyphae of C. albicans that were being endocytosed by intact endothelial cells. Third, a tpk2Δ/tpk2Δ mutant of C. albicans that did not bind to N-cadherin was poorly endocytosed by endothelial cells. Fourth, CHO cells that expressed human N-cadherin endocytosed significantly more C. albicans hyphae than did control CHO cells that expressed either VE-cadherin or no human cadherins. Fifth, down-regulation of endothelial cell expression of N-cadherin with siRNA resulted in a significant reduction in the number of endocytosed organism.

Although the normal function of N-cadherin in endothelial cells is not completely understood, this molecule is probably involved in the exchange of signals between endothelial cells and vascular smooth muscle cells (32, 33). N-cadherin is also known to be important for the metastasis of breast cancer cells (34, 35) as well as neuronal development and function (36–38). Our data indicate that an additional function of N-cadherin is that it serves as a receptor that mediates the endocytosis of a microbial pathogen. Lambert et al. (39) found that beads coated with an N-cadherin-Fc fusion protein bound to and were internalized by N-cadherin-expressing cells. Thus, homotypic adhesion of N-cadherin on the beads to N-cadherin on the cells was sufficient to induce endocytosis. In addition, the binding of the beads to cells expressing N-cadherin resulted in the accumulation of host cell microfilaments and phosphotyrosine-containing proteins. This response is similar to the accumulation of microfilaments and phosphotyrosine that we have observed around C. albicans hyphae that are being endocytosed by endothelial cells (9, 11).

Another member of the cadherin family, E-cadherin, is known to be a receptor for internalin A of Listeria monocytogenes. The binding of internalin A to E-cadherin is sufficient to induce internalization of the organism by human epithelial cells (21). Interestingly, internalin A binds to human E-cadherin but not murine E-cadherin (40). Whether C. albicans displays a similar species-specific affinity for N-cadherin remains to be determined.

We also observed that chelating extracellular calcium with EGTA significantly inhibited the endocytosis of C. albicans hyphae. It is possible that this inhibition occurs because the interaction of C. albicans with N-cadherin is calcium-dependent. For example, the homotypic binding activity of N-cadherin requires extracellular calcium (41). Similarly, the binding of
the Yersinia pseudotuberculosis invasin to β1 integrins and the binding of the L. monocytogenes internalin A to E-cadherin are known to be calcium-dependent (19, 21, 40).

Our data indicate that adherence and endocytosis are distinct processes. We found that, even though the tkp2Δ/tkp2Δ mutant adhered normally to endothelial cells, it was poorly endocytosed. Also, the transfection of CHO cells with N-cadherin resulted in a significant increase in the endocytosis of C. albicans but had no effect on the adherence of C. albicans. In addition, the down-regulation of endothelial cell N-cadherin expression with siRNA inhibited the endocytosis but not the adherence of N-cadherin. Therefore, N-cadherin only mediates the endocytosis of C. albicans and plays little role in the adherence of the organism to endothelial cells.

Although the N-cadherin siRNA down-regulated the expression of N-cadherin by 90%, there was only partial inhibition of endothelial cell endocytosis of C. albicans. The most likely explanation for this result is that endothelial cell receptors other than N-cadherin also mediate the endocytosis of C. albicans. In support of this hypothesis, many microbial pathogens such as Neisseria gonorrhoeae and C. albicans also mediate the endocytosis of C. albicans and C. albicans C. albicans C. albicans C. albicans. Therefore, N-cadherin only mediates the endocytosis of C. albicans and plays little role in the adherence of the organism to endothelial cells.

In summary, the data presented in this report establish that N-cadherin is an endothelial cell receptor that mediates the endocytosis but not adherence of C. albicans hyphae in vitro. These data also indicate that the adherence of C. albicans to endothelial cells and its subsequent endocytosis are two distinct processes that are probably mediated by different endothelial cell surface proteins. Experiments to determine the C. albicans proteins that bind to N-cadherin and these additional endothelial cell surface proteins are in progress.

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