The NDR Kinase Cbk1 Downregulates the Transcriptional Repressor Nrg1 through the mRNA-Binding Protein Ssd1 in Candida albicans

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NDR (nuclear Dbf2-related) kinases are essential components for polarized morphogenesis, cytokinesis, cell proliferation, and apoptosis. The NDR kinase Cbk1 is required for the hyphal growth of Candida albicans; however, the molecular functions of Cbk1 in hyphal morphogenesis are largely unknown. Here, we report that Cbk1 downregulates the transcriptional repressor Nrg1 through the mRNA-binding protein Ssd1, which has nine Cbk1 phosphorylation consensus motifs. We found that deletion of SSD1 partially suppressed the defective hyphal growth of the C. albicans cbk1ΔΔ mutant and that Ssd1 physically interacts with Cbk1. Cbk1 was required for Ssd1 localization to polarized growth sites. The phosphomimetic SSD1 allele (ssd1-9E) allowed the cbk1ΔΔ mutant to form short hyphae, and the phosphodeficient SSD1 allele (ssd1-9A) resulted in shorter hyphae than did the wild-type SSD1 allele, indicating that Ssd1 phosphorylation by Cbk1 is important for hyphal morphogenesis. Furthermore, we show that the transcriptional repressor Nrg1 does not disappear during hyphal initiation in the cbk1ΔΔ mutant but is completely absent in the cbk1ΔΔ::ssd1ΔΔ double mutant. Deletion of SSD1 also increased Als3 expression and internalization of the cbk1ΔΔ mutant in the human embryonic kidney cell line HEK293T. Collectively, our results suggest that one of the functions of Cbk1 in the hyphal morphogenesis of C. albicans is to downregulate Nrg1 through Ssd1.

Cell polarity, a structural and functional asymmetry of cellular organization, is observed in diverse animal cell types, such as neurons and epithelial cells, and a variety of unicellular organisms, including yeast, fungi, protozoa, and prokaryotes (1–3). Cell polarity is established and maintained by directional movement and an unbalanced distribution of proteins, mRNAs, and subcellular organelles (4–7). Directional movement of cellular components requires delicately coordinated spatial and temporal regulation of polarity proteins, actin and tubulin cytoskeletons, and endomembranes (3, 4). In yeast, polarized cell growth occurs during budding, mating, and filamentous growth. Although the polarization events differ in spatial cues and spatiotemporal controls, the events are generally initiated and established from local accumulation of active GTP-bound Cdc42 and scaffold proteins, as well as the directed orientation of cytoskeletal elements (8–10).

Candida albicans is an important opportunistic fungal pathogen that causes not only superficial infection but also systemic or life-threatening infections in immunocompromised hosts (11, 12). C. albicans switches between yeast and hyphal forms to rapidly disseminate inside the host and escape from host defense systems, thus resulting in fatal infection (13, 14). The morphological shape of C. albicans is determined by multiple signaling pathways that respond to the numerous environmental challenges C. albicans encounters in the host. The known major signaling pathways include the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway via Efg1, a mitogen-activated protein kinase pathway through Cph1, a pH-responsive pathway through Rim101, Tup1-mediated repression through Rfg1 and Nrg1, and pathways represented by the transcription factors Cph2, Tec1, and Czf1 (15). Additionally, the nutrient-sensing Brg1/Hda1 chromatin remodeling pathway and the hypoxia plus high CO2 sensing pathway control the levels of the hypha-specific transcription factor Ume6 to maintain hyphal development and virulence (16–18).

In addition to these pathways, regulation of the Ace2 transcription factor and polarized morphogenesis (RAM) network, which consists of the two kinases Cbk1 and Kic1 and four associated proteins (Mob2, Hym1, Pag1, and Sog2), has been reported to be essential for the hyphal growth of C. albicans (19). In the RAM network, the terminal kinase Cbk1, which is a serine/threonine kinase belonging to the LATS/NDR (nuclear Dbf2-related) protein family, functions to maintain polarisome components at the hyphal tips and, thus, maintains hyphal growth (20). Moreover, Cbk1 regulates the transcription factor Ace2 to control mother-daughter cell separation, agar invasion, and biofilm formation (19, 21). Furthermore, the findings that the sensitivity of cbk1 null mutants to cell wall-disturbing agents is suppressed by SSD1 deletion and that Ssd1 sequences contain the consensus Cbk1 phosphorylation motif suggest that Cbk1 may regulate Ssd1 activity to control cell wall integrity (19, 22, 23). However, this has not been experimentally demonstrated.

Ssd1 was identified as an mRNA-binding protein in Saccharomyces cerevisiae (24). Although Ssd1 was reported to be genetically linked to various cellular functions, including stress signaling, cellular aging, and virulence (25–28), the primary function of Ssd1 in S. cerevisiae is likely to modulate the expression and localization of mRNAs for cell wall proteins (29–31). Although Ssd1 phosphorylated by Cbk1 actively translates bound mRNAs, nonphosphorylated Ssd1 interacts with processing bodies (P bodies) and thus...
represses mRNA translation in *S. cerevisiae* (30–33). These studies indicate that Cbk1 regulates Ssd1 activity to precisely coordinate cell wall remodeling during isotropic and polarized *S. cerevisiae* growth.

In *C. albicans*, it is also probable that Cbk1 negatively regulates Ssd1 to control cell wall integrity (19, 22, 23). However, deletion of *SSD1* did not result in noticeable phenotypes related to hyphal growth of *C. albicans* (19), which seemingly defies the involvement of Ssd1 in the yeast-to-hypha transition. Although an independent study demonstrated that *ssd1* null mutants were less virulent in a mouse model of hematogenously disseminated candidiasis, the attenuated virulence was ascribed to decreased antimicrobial peptide resistance that is conferred by Ssd1 in *C. albicans* (34). Thus, it is not yet known whether Ssd1 is required for the hyphal growth of *C. albicans* and, if so, how Ssd1 controls the morphogenesis. In this study, we demonstrate that Cbk1 regulates Ssd1 to control the hyphal growth of *C. albicans*.

We further show that Ssd1 regulation by Cbk1 is responsible for Nrg1 downregulation during hyphal initiation.

**MATERIALS AND METHODS**

**Strains, plasmids, media, and growth conditions.** All strains, plasmids, and primers used in this work are described in Tables S1 to S3 in the supplemental material. *C. albicans* cells were grown at 30°C in YPG medium (1% yeast extract, 2% Bacto peptone, 2% glucose). To select transformants, synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, amino acid dropout mixture, and 2% glucose) was used with appropriate auxotrophic requirements. For hyphal growth, cells cultured overnight in YPG were diluted 1:100 in YPG medium containing 10% newborn calf serum (Gibco) and incubated at 37°C. To induce the MET3 promoter, cells grown overnight in YPG were washed twice in phosphate-buffered saline (PBS) and resuspended in SC-Met-Cys medium. The *Escherichia coli* strain DH5α was used for DNA manipulation and grown at 37°C in Luria-Bertani (LB; 0.5% yeast extract, 1% tryptone, 1% NaCl) medium supplemented with 100 μg/ml ampicillin.
To construct plasmids for yeast two-hybrid analyses, the complete coding regions and various domains of the CBK1 and SSD1 genes were generated from *C. albicans* genomic DNA by PCR. Amplified DNA fragments were cloned between EcoRI and XhoI restriction sites and fused to the DNA binding domain in the pLexA vector and the activating domain in the pB42AD vector. The EGY48[p8op-lacZ] strain was cotransformed with the two hybrid plasmids. Transformants were tested for β-galactosidase activity on selective medium (SD/Gal/Raf).

**Two-hybrid analysis.** To construct plasmids for yeast two-hybrid analyses, the complete coding regions and various domains of the CBK1 and SSD1 genes were generated from *C. albicans* genomic DNA by PCR. Amplified DNA fragments were cloned between EcoRI and XhoI restriction sites and fused to the DNA binding domain in the pLexA vector and the activating domain in the pB42AD vector. The EGY48[p8op-lacZ] strain was cotransformed with the two hybrid plasmids. Transformants were tested for β-galactosidase activity on selective medium (SD/Gal/Raf).

**FIG 2**  
SSD1 deletion partially suppresses the defective morphogenesis of the cbk1ΔΔ mutant in *C. albicans*. (A) The cbk1ΔΔ ssd1ΔΔ mutant exhibits reduced cell lysis, smaller cell size, and less aggregation than the cbk1ΔΔ mutant in YPG medium at 30°C. Dark arrows indicate lysed cells. (B) SSD1 deletion partially restores hyphal growth of cbk1ΔΔ mutants in YPG medium plus 10% serum at 37°C for 3 h. Scale bar, 5 μm. (C) Unlike cbk1ΔΔ mutant cells, cbk1ΔΔ ssd1ΔΔ mutant cells form normal vacuoles. Cells grown in YPG at 30°C were stained with 1.6 M FM4-64. (D) cbk1ΔΔ ssd1ΔΔ mutant cells polarize lipid rafts. Cells grown in YPG at 30°C or YPG plus 10% serum at 37°C were stained with 10 g/ml filipin for 5 min.
His/−Trp/−Ura/BU salts [Na₂HPO₄·7H₂O and NaH₂PO₄, pH 7.0]/X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]).

Immunoprecipitation. C. albicans cells were harvested, washed, and resuspended in lysis buffer (10 mM Tris-Cl [pH 8.0], 250 mM NaCl, 0.1% NP-40, 0.5 mM dithiothreitol [DTT], 1 mM phenyl methane sulfonic acid [PMSF]) and an equal volume of glass beads (Biospec). Cells were lysed using a bead beater (Biospec), and lysates were centrifuged at 13,000 rpm for 10 min at 4°C. To test for interactions between green fluorescent pro-
tein-labeled Cbk1 (Cbk1-3×GFP) (35) and Ssd1-6myc, protein extracts (500 μl) containing 1 mg of protein were immunoprecipitated using the anti-GFP or anti-myc antibodies (Santa Cruz Biotechnology) and 20 μl protein A/G plus agarose beads (Santa Cruz Biotechnology). After incubation overnight at 4°C, protein-bound beads were washed four times with cold PBS and eluted in SDS sample loading buffer by incubation for 5 min at 95°C.

**Mass spectrometry identification of phosphorylation.** Ssd1-6myc proteins were immunoprecipitated using anti-myc antibodies from SSD1-6MYC-expressing wild-type cells grown in YPG medium at 30°C. The proteins were resolved on a 6% SDS-PAGE gel and stained with Coomassie blue. Bands corresponding to Ssd1 were excised, digested in gel with trypsin (Promega), and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a reversed-phase capillary high-performance liquid chromatograph directly coupled to a Finnigan LCQ ion trap mass spectrometer and TurboSEQUEST software (Thermo Quest).

**Staining and microscopic observations.** Vacuole morphology was visualized using the lipophilic fluorescent dye FM4-64 (Molecular Probes). Cells suspended in 50 μl YPG plus 1 μl of 1.6 μM FM4-64 (in dimethyl sulfoxide [DMSO]) were incubated for 20 min at 30°C. After a chase in fresh YPG for 30 to 120 min, cells were washed with sterile water and resuspended in liquid SC-Ura medium. For the visualization of sterol-rich domains, cells grown in YPG or YPG-plus-serum medium for 3 h at 30°C or 37°C, respectively, were stained with filipin (10 μg/ml prepared in DMSO; Sigma) for 5 min at room temperature. To analyze Als3 expression during hyphal growth, cells cultured in YPG medium plus 10% serum were fixed with 3% formaldehyde (Sigma) in PBS for 30 min and added to poly-L-lysine (Sigma)-coated glass coverslips. After 30 min, cells blocked with 3% bovine serum albumin were stained with an Als3 monoclonal antibody (36) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Molecular Probes). Photographs were taken using an Olympus BX61 microscope equipped with differential interference contrast (DIC) optics, appropriate filters, and a camera (Olympus DP71). Images were adjusted using ImageJ software (National Institutes of Health).

**Internalization assays.** HEK293T cells grown on glass coverslips for 3 days in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) were infected with 10⁵ cells of each C. albicans strain for 120 min at 37°C. The culture medium was aspirated, and HEK293T cells were rinsed three times with PBS to remove free fungal cells. Cells were fixed with 3% formaldehyde (Sigma) in PBS for 10 min. Adherent but noninternalized C. albicans cells were stained with anti-C. albicans antibody (Meridian Life Science) and Alexa Fluor 350-conjugated secondary antibody (Molecular Probes). HEK293T cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS, and HEK293T- associated C. albicans cells (both internalized and noninternalized cells) were stained with anti-C. albicans antibody and Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Actin was stained using rhodamine-phalloidin (Sigma) to visualize HEK293T cells. The percentage of internalized C. albicans cells was determined by dividing the number of internalized cells by the total number of HEK293T-associated C. albicans cells. At least 100 C. albicans cells were counted on each coverslip, and the values presented represent the average results from at least three independent experiments.

**Virulence test.** Wild-type, cbk1ΔΔ mutant, and cbk1ΔΔ ssd1ΔΔ mutant cultures were grown overnight at 30°C in YPG, harvested, and washed twice with sterile saline. Cells were counted using a hemocytometer, and 5 × 10⁶ cells (in 0.1 ml) were injected into the lateral tail veins of mice. Ten female BALB/c mice (aged 8 weeks and weighing 18 to 20 g) were used to test each C. albicans strain. The protocol was approved by the Institutional Animal Care and Use Committee of Chungnam National University.

**RESULTS**

SSD1 deletion partially restores the defective hyphal growth of the cbk1 mutant. We previously reported that a C. albicans ssd1ΔΔ mutant was more sensitive than the wild type to Calcofluor white and Congo red staining (19), which suggests that the molecular functions of Ssd1 may involve the regulation of cell wall-remodeling protein expression. To investigate whether Ssd1 is also required for C. albicans hyphal growth, we analyzed the
morphological phenotypes of the ssd1Δ/Δ mutant. As shown by the images in Fig. 1A, however, ssd1Δ/Δ mutant cells formed normal true hypha in medium containing serum. Next, we examined the morphology of a gain-of-function mutant overexpressing Ssd1 from the MET3 promoter (Fig. 1B). The yeast cell size of Ssd1-overexpressing cells was larger than that of wild-type cells, and the hyphae of the Ssd1-overexpressing cells were approximately 30% shorter than those of the wild type and appeared to have irregular widths (Fig. 1C and D). These results suggest that Ssd1 is involved in the morphogenesis of C. albicans.

Because Ssd1 overexpression inhibited hyphal growth in wild-type cells, we hypothesized that deletion of SSD1 in the cbk1Δ/Δ mutant might suppress, at least partially, the defective morphogenesis of the C. albicans cbk1Δ/Δ mutant. Interestingly, cbk1Δ/Δ ssd1Δ/Δ double mutant cells were less fragile, smaller in cell size, and less aggregated than cbk1Δ/Δ mutant cells in yeast growth medium (Fig. 2A). Moreover, they formed hyphae in serum-containing medium (Fig. 2B). This result was confirmed with the mob2Δ/Δ ssd1Δ/Δ double mutant (Fig. S1B). However, the hyphal length of the double mutant strain was much shorter than that of the wild type, and the hyphal width was irregular. The ability to form normal vacuoles is related to the hyphal morphogenesis of C. albicans (37). Therefore, we stained the vacuoles in cbk1Δ/Δ ssd1Δ/Δ mutants using the fluorescent lipophilic dye FM4-64. In contrast to the aberrant, fragmented vacuoles of cbk1Δ/Δ mutants, cbk1Δ/Δ ssd1Δ/Δ mutants formed large, round vacuoles (Fig. 2C). We also examined the polarization of sterol- and sphingolipid-enriched microdomains (lipid rafts), which is linked to morphogenesis (19, 38), in cbk1Δ/Δ ssd1Δ/Δ mutant cells. Whereas cbk1Δ/Δ mutant cells were uniformly stained with filipin, a fluorescent sterol-binding polyene, cbk1Δ/Δ ssd1Δ/Δ mutant cells were stained distinctly at the budding site and bud neck in yeast cells and at the growing tips in hyphae (Fig. 2D). Collectively, these results support the role of Ssd1 in the hyphal growth of C. albicans.

Phosphorylation of Ssd1 by Cbk1 is required for hyphal morphogenesis of C. albicans. It is known that S. cerevisiae Cbk1 phosphorylates Ser or Thr residues within the conserved motif HX(K/R)/(K/R)X(S/T), with His at the −5 position and Lys or Arg at either the −3 or −2 position (39). In C. albicans, there are nine potential phosphorylation sites for Cbk1 in the N terminus of Ssd1 (Fig. 3C). Yeast two-hybrid analyses revealed that Cbk1 and Ssd1 physically interact and that the kinase domain of Cbk1 targets Ssd1 (see Fig. S2 in the supplemental material). Therefore, we examined Ssd1 phosphorylation using mass spectrometry, which revealed that three Cbk1 consensus motifs are phosphorylated (Fig. 3C). Thus, we propose that Cbk1 phosphorylates Ssd1 to control C. albicans morphogenesis.

To examine whether Ssd1 phosphorylation at the conserved Cbk1 target sites is necessary for regulating Ssd1 activity to control the hyphal morphogenesis of C. albicans, the wild-type SSD1 gene was replaced with the phosphomimetic SSD1 allele (ssd1-9E) in the cbk1Δ/Δ mutant and with the phosphodeficient SSD1 allele (ssd1-9A) in the wild-type strain. We found that the cbk1Δ/Δ ssd1Δ/Δ mutant was less fragile than the cbk1Δ/Δ mutant in yeast growth medium and appeared to form hyphae better than the cbk1Δ/Δ Δ mutant in hypha-inducing medium (Fig. 3D). In contrast, the expression of the ssd1-9A allele in wild-type cells increased cell size and caused a round cell shape in yeast growth medium and decreased hypha length in hypha-inducing serum medium (Fig. 3E and F). In addition, the hyphae of Ssd1-9A-expressing cells appeared to bulge at the tip (Fig. 3E). Collectively, these results indicate that Cbk1 phosphorylates Ssd1 at conserved motifs for Cbk1 and that this phosphorylation is associated with the role of Ssd1 in C. albicans hyphal morphogenesis.

Phosphorylation of Ssd1 by Cbk1 is important for polarized localization of Ssd1 in C. albicans. Our data showing that Ssd1 is involved in cell wall remodeling and hyphal morphogenesis in C. albicans imply that Ssd1 may localize at growing sites of yeast cells and hyphae. We found that Ssd1 localizes as puncta largely at the emerging-bud site of mother cells, in the small buds attached to the mother cell, and in the bud neck, as well as at the growing tips of hyphae. The protein is also dispersed within the cytoplasm (Fig. 4A and C), which is similar to the localization pattern of Ssd1 in S. cerevisiae (31). Next, we examined whether Ssd1 localization is controlled by Cbk1. Although Ssd1 was present in small buds and bud necks in the wild-type strain, Ssd1 was randomly spread into daughter and mother cells in the cbk1Δ/Δ mutant strain, suggesting that the Ssd1 localization is affected by Cbk1 function (Fig. 4A). However, Cbk1 localization was not altered by the absence of Ssd1 (Fig. 4B). To investigate whether the phosphorylation of Ssd1 by Cbk1 is required for Ssd1 localization to growing sites during hyphal growth, we analyzed the localization of the phosphomimetic Ssd1-9E in the cbk1Δ/Δ mutant strain. We found that Ssd1-9E partially restored polarized localization in the cbk1Δ/Δ mutant strain, although not to the extent of the Ssd1 protein in the wild-type strain (Fig. 4C and D). These results indicate that Ssd1 phosphorylation by Cbk1 directs the localization of Ssd1 to growing sites in C. albicans.

Nrg1 and Ssd1 function downstream from Cbk1 for hyphal growth. Microarray data from our previous study indicated that regulation of the Ace2 transcription factor and polarized morphogenesis (RAM)-dependent hypha-specific genes (including ECE1, ALS3, RBFI, RBFT, KIP4, IHD1, CDC10, PHR1, and UME6) were repressed by Tup1 and Nrg1 (19, 40). These data suggest that the RAM network may be involved in regulating the expression or activity of Nrg1. If Nrg1 functions downstream from the RAM network, disruption of NRG1 in RAM mutants may, at least partially, rescue the defective hyphal growth of the RAM mutant. To test the possibility, a cbk1Δ/Δ nrg1Δ/Δ double mutant strain was constructed and its phenotype examined. In yeast growth medium, cbk1Δ/Δ nrg1Δ/Δ mutant cells exhibited a polar budding pattern that was in contrast to the random budding pattern of cbk1Δ/Δ mutant cells; however, they remained as aggregated yeast form cells, unlike the nrg1Δ/Δ mutant cells, which form hyphae regardless of the culture conditions (Fig. 5A). This result was confirmed with the mob2Δ/Δ nrg1Δ/Δ mutant (see Fig. S2 in the supplemental material). Moreover, the cbk1Δ/Δ nrg1Δ/Δ mutant formed short, curved hyphae in hypha-inducing medium (Fig. 5A). However, cbk1Δ/Δ nrg1Δ/Δ mutant showed aberrant vacuole morphology (Fig. 5B). Thus, it is likely that Nrg1 functions downstream from Cbk1 but that elimination of Nrg1 is not sufficient for normal hyphal morphogenesis in the cbk1Δ/Δ mutant.

Next, we tested whether deletion of SSD1 would enable the cbk1Δ/Δ nrg1Δ/Δ mutant to form longer hyphae. The cbk1Δ/Δ nrg1Δ/Δ ssd1Δ/ΔΔ triple mutant developed pseudo-yeast in yeast growth medium and decreased hypha length in hypha-inducing serum medium (Fig. 3E and F). In addition, the hyphae of Ssd1-9A-expressing cells appeared to bulge at the tip (Fig. 3E). Collectively, these results indicate that Cbk1 phosphorylates Ssd1 at conserved motifs for Cbk1 and that this phosphorylation is associated with the role of Ssd1 in C. albicans hyphal morphogenesis.
Cbk1 is required for Ssd1 localization to polarized growing sites in *C. albicans*. (A) Ssd1-3×GFP localizes as puncta in the emerging bud tip and bud neck during yeast growth and at the hyphal tip during hyphal growth in wild-type cells. In *cbk1Δ/Δ* cells, Ssd1-3×GFP puncta are distributed in the cytoplasm. (B) Cbk1 normally localizes to sites of polarized growth in *ssd1Δ/Δ* cells. (C) Phosphorylation of Ssd1 by Cbk1 is important for polarized localization of Ssd1 in *C. albicans*. (D) Measurement of Ssd1 localization. Each strain was grown in YPG medium plus 10% serum at 37°C and Ssd1 localization was evaluated. Polar, Ssd1 at the hyphal tip or the end of the growing bud; Nonpolar, Ssd1 throughout cytoplasm. Values are the average results ± SD from three independent cultures (>100 cells for each culture).
growth medium and made longer hyphae than the \( cbk1/Δ \) \( nrg1/Δ \) mutant in hypha-inducing medium (Fig. 5A and B). Moreover, the aberrant vacuole morphology of the \( cbk1/Δ \) mutant, which remained abnormal in the \( cbk1/Δ \) \( nrg1/Δ \) mutant, was almost completely recovered in the \( cbk1/Δ \) \( nrg1/Δ \) \( ssd1/Δ \) mutant (Fig. 5B). Nonetheless, hyphae of \( cbk1/Δ \) \( nrg1/Δ \) \( ssd1/Δ \) triple mutant cells remained atypical in shape and shorter in size compared to those of the wild type. These results suggest that other downstream effectors of Cbk1 might be necessary for establishing and maintaining normal long hyphae in \( C. albicans \).

**Cbk1 is required for Nrg1 downregulation during hyphal initiation.** Nrg1 is downregulated very rapidly, but temporarily, when hyphal development is initiated (Fig. 6A) (17, 41). Based on our finding that \( NRG1 \) deletion recovered the polarized budding pattern and germ tube formation of the \( cbk1/Δ \) mutant, we reasoned that Cbk1 might be associated with Nrg1 downregulation at the early stages of hyphal morphogenesis in \( C. albicans \). Surprisingly, in contrast with the rapid disappearance of Nrg1 in the wild-type and \( ssd1/Δ \) mutant strains, Nrg1 remained relatively stable in the \( cbk1/Δ \) mutant (Fig. 6A). In addition, we found that Nrg1 rapidly disappeared in the \( cbk1/Δ \) \( ssd1/Δ \) mutant, which agrees with the observation that \( SSD1 \) deletion partially restored the ability of \( cbk1/Δ \) \( ssd1/Δ \) mutant cells to form germ tubes in hypha-inducing medium (Fig. 2B, Fig. 6A). Moreover, we observed that Nrg1 disappeared in the \( cbk1/Δ \) \( ssd1/Δ \) \( 9E \) mutant, suggesting that the phosphorylation of Ssd1 by Cbk1 is involved in Nrg1 downregulation (Fig. 6A). We also noticed that ALS3, whose transcription is repressed by Nrg1, was rarely expressed in the \( cbk1/Δ \) mutant but highly expressed in the \( cbk1/Δ \) \( ssd1/Δ \) mutant (Fig. 6B). These results suggest that Cbk1 is involved in Nrg1 downregulation during hyphal initiation in \( C. albicans \).

**Deletion of SSD1 enables the cbk1/Δ mutant to invade HEK293T cells.** Host cell invasion is likely a critical virulence attribute of \( C. albicans \). One of the host invasion processes is endocytosis by the host, which requires invasin-like proteins to be expressed on the surface of \( C. albicans \) hyphae (42, 43). The \( cbk1/Δ \) mutant strain does not express Als3, which is a fungal invasin that induces endocytosis by epithelial cells (44). However, the \( cbk1/Δ \) \( ssd1/Δ \) mutant strain produces Als3 on the surface of its short hyphae (Fig. 6B). We observed that \( cbk1/Δ \) cells were rarely internalized by HEK293T cells, a human embryonic kidney cell line, likely because they lack Als3 (Fig. 7A). Finally, we examined whether \( SSD1 \) deletion restores the ability of the \( cbk1/Δ \) strain to invade HEK293T cells. In contrast to the 10% internalization of \( cbk1/Δ \) cells, approximately 50% of \( cbk1/Δ \) \( ssd1/Δ \) cells were internalized by HEK293T cells (Fig. 7A and B). Thus, we conclude that Cbk1 controls Ssd1 for host cell invasion as well as hyphal morphogenesis in \( C. albicans \).

**DISCUSSION**

The molecular functions of the substrates being considered, NDR kinases, are thought to coordinate the transcription, translation, and vesicle transport of cell polarity proteins in order to establish and maintain cell polarity and morphogenesis (30, 31, 45–48). In this study, we demonstrated that Cbk1 regulates the activity and localization of the mRNA-binding protein Ssd1 to control the hyphal morphogenesis of \( C. albicans \). In particular, it is surprising that Cbk1 is required for downregulation of the transcriptional repressor Nrg1 during hyphal initiation, and the inability of the

![Figure 5](http://ec.asm.org/41B/July2015Volume14Number7/FIG5.png)
The cbk1ΔΔ mutant to eliminate Nrg1 can be rescued by SSD1 deletion. Thus, we propose that Cbk1 coordinates the expression of many genes for hyphal morphogenesis through Nrg1 and for cell wall remodeling at sites of polarized growth in C. albicans.

This study revealed that Cbk1 is essential for Nrg1 downregulation and that Ssd1 is involved in this process. We found that cbk1ΔΔ mutants levels are not downregulated in the cbk1ΔΔ mutant and Als3 is not expressed in the absence of Cbk1 (Fig. 6), which suggests that the molecular functions of Cbk1 may be associated with transcriptional repression and/or degradation of Nrg1. Genes such as ALS3, CDC10, ECE1, FTR1, IHD1, KIP4, PHR1, RBT1, RBT5, and UME6, which are repressed by Nrg1 (40, 49–51), were not expressed in the cbk1ΔΔ mutant strain (19), supporting the possibility that Cbk1 may control at least one of the

FIG 6 Cbk1 is required for Nrg1 degradation during hyphal initiation. (A) Western blot analyses of Nrg1 expression during hyphal induction in wild type, cbk1ΔΔ, ssd1ΔΔ, and cbk1ΔΔssd1ΔΔ strains. Lysates of the strains expressing Nrg1-myc, grown in YPG medium plus 10% serum at 37°C, were analyzed by Western blotting using an anti-myc antibody. (B) Immunofluorescence micrographs of hyphal cells expressing Als3. Wild-type, cbk1ΔΔ, ssd1ΔΔ, and cbk1ΔΔssd1ΔΔ strains grown in YPG medium plus 10% serum at 37°C for 3 h were fixed in 3% formaldehyde and stained with anti-Als3 antiserum and FITC-conjugated anti-mouse antibody.
two distinct regulatory pathways for Nrg1 downregulation. Recently, haploinsufficiency-based genetic interaction screens revealed that the Cbk1 and PKA signaling pathways coregulate a common set of genes during hyphal growth, implying a potential interaction between the Cbk1 and PKA pathways (47). Because Nrg1 expression is repressed by active cAMP-PKA signaling (17), it is conceivable that one of the Cbk1 downstream effectors may be involved in PKA signaling. In addition, there are three potential Cbk1 phosphorylation sites (S200, T251, and T281) in the Nrg1 protein (see Fig. S3 in the supplemental material). Thus, it would be interesting to examine whether these sites are important for regulation of Nrg1 levels.

FIG 7 SSD1 deletion induces invasion of HEK293T cells by *cbk1Δ/Δ* mutants. (A) Invasion of HEK293T cells by wild-type, *cbk1Δ/Δ, ssd1Δ/Δ,* and *cbk1Δ/Δ ssd1Δ/Δ* strains. Extracellular *C. albicans* cells were stained with anti-*C. albicans* antibody and Alexa Fluor 350-conjugated secondary antibody. After HEK293T cells were permeabilized with 0.1% Triton X-100, HEK293T-associated *C. albicans* cells were stained with anti-*C. albicans* antibody and Alexa Fluor 488-conjugated secondary antibody. Actin was stained using rhodamine-phalloidin to visualize HEK293T cells. (B) The percentage of invasion was determined by dividing the number of partially intracellular *C. albicans* cells by the total number of HEK293T-associated *C. albicans* cells. Values are the average results ± SD from three independent experiments (>100 cells for each experiment).
Cbk1 Downregulates Nrg1 in C. albicans

Because the cbk1ΔΔ ssd1ΔΔ mutant strain could form hyphae and invade HEK293T cells (Fig. 2 and 7), we examined whether the strain was virulent. The cbk1ΔΔ and cbk1ΔΔ ssd1ΔΔ mutants exist as chains or clumps of cells; therefore, we injected mice with a large number of cells (5 × 10⁶) into the tail vein. Whereas mice injected with the wild-type strain all died within 3 days, mice injected with the cbk1ΔΔ ssd1ΔΔ mutant strain survived longer than 3 weeks (see Fig. S4 in the supplemental material), indicating that other Cbk1 substrates are required for the recovery of virulence traits in the cbk1ΔΔ ssd1ΔΔ mutant. The transcriptional regulator Bcr1, which is the only known Cbk1 substrate in C. albicans, regulates biofilm development (22, 53). Ace2, which is presumed to be a Cbk1 substrate in C. albicans (23), is important for virulence (21). We are currently investigating whether the expression of constitutively active BCR1 and/or ACE2 alleles restores virulence in the cbk1ΔΔ ssd1ΔΔ mutant.

In summary, this study provides new insight into the molecular mechanisms by which Cbk1 controls hyphal growth of C. albicans. Cbk1 is essential for downregulating Nrg1 levels during hyphal initiation, and the mRNA-binding Ssd1 protein is involved in Nrg1 downregulation. Thus, this study suggests that Cbk1 coordinates processes for cell wall remodeling and polarized growth by temporally and spatially controlling the translation of Ssd1-bound mRNAs. Future work to identify Ssd1-bound mRNAs and their molecular functions is expected to reveal a novel mechanism regarding how Ssd1 plays a central role in coordinating multiple processes for hyphal morphogenesis, including cell wall remodeling and polarized growth, in C. albicans.

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