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Crk1, a Novel Cdc2-Related Protein Kinase, Is Required for Hyphal Development and Virulence in *Candida albicans*

JIANGYE CHEN,[1,2] SONG ZHOU,[1] QIN WANG,[1] XI CHEN,[1] TING PAN,[2] AND HAOPING LIU[2]*

State Key Laboratory of Molecular Biology, Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai 200031, China,[1] and Department of Biological Chemistry, College of Medicine, University of California, Irvine, Irvine, California 92697-1700[2]

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Both mitogen-activated protein kinases and cyclin-dependent kinases play a role in hyphal development in *Candida albicans*. Using an oligonucleotide probe-based screen, we have isolated a new member of the Cdc2 kinase subfamily, designated Crk1 (Cdc2-related kinase). The protein sequence of Crk1 is most similar to those of *Saccharomyces cerevisiae* Sgv1 and human Pkl1/Cdk9. In *S. cerevisiae*, *CRK1* suppresses some, but not all, of the defects associated with an *sgv1* mutant. Deleting both copies of *CRK1* in *C. albicans* slows growth slightly but leads to a profound defect in hyphal development under all conditions examined. *crk1*/*crk1* mutants are impaired in the induction of hypha-specific genes and are avirulent in mice. Consistent with this, ectopic expression of the Crk1 kinase domain (*CRK1N*) promotes filamentous or invasive growth in *S. cerevisiae* and hyphal development in *C. albicans*. The activity of Crk1 in *S. cerevisiae* requires Flo8 but is independent of Ste12 and Phd1. Similarly, Crk1 promotes filamentation through a route independent of Cph1 and Efg1 in *C. albicans*. *RAS1*^{V13} can also activate filamentation in a *cph1*/*cph1 efg1*/*efg1* double mutant. Interestingly, *CRK1N* produces florid hyphae in *ras1*/*ras1* strains, while *RAS1*^{V13} generates feeble hyphae in *crk1*/*crk1* strains.

Candida albicans is the fungus most frequently identified from clinical isolates. It can cause a variety of opportunistic infections, including deadly systemic candidiasis in immunocompromised patients (for a review, see reference 52). *C. albicans* is capable of dramatic morphological switching between budding yeast growth and filamentous hyphal growth. Both growth forms coexist in infected tissues. Because mutant strains defective in morphological switching are much less virulent than wild-type strains (14, 22, 35, 39), competence to perform the switch has been linked with pathogenicity in *C. albicans*. Hyphal cells have been suggested to aid in adhesion and penetration of epithelial or endothelial cell layers to facilitate the infection (24).

C. albicans cells are able to respond to and integrate a large variety of environmental signals during their morphological development. Serum, nitrogen starvation, high temperature, and neutral pH, for example, promote hyphal development (51). Hyphal development is also accompanied by transcriptional induction of many hypha-specific genes, such as *ECE1*, *HWP1*, and *HYR1* (5, 7, 59). A conserved mitogen-activated protein (MAP) kinase pathway has been shown to regulate hyphal development; mutations in Cst20 (PAK), Hst7 (MEK), Cek1 (MAP kinase), and Cph1 (a transcription factor) partially block hyphal colony formation on certain hypha-inducing media (15, 31, 34, 36). The Cek1 MAP kinase pathway functions in parallel with Efg1, a member of a family of basic-helix-loop-helix proteins important for developmental processes in several fungi (39, 60). Efg1 may function downstream of Tpk2, the catalytic subunit of protein kinase A (PKA), in hyphal development (57). Furthermore, a *C. albicans* Ras protein has been shown to be required for serum-induced hyphal differentiation (20). The complicated nature of dimorphic regulation is underscored by the discovery of more signaling pathways necessary for proper hyphal development. A two-component histidine kinase, Cos1/Nik1, and a Hog1 MAP kinase are involved in hyphal morphogenesis (2, 15, 48, 49, 58). More recently, we have found a G_1 cyclin-dependent kinase (Cdk) to be important for hyphal development under specific hypha-inducing conditions and for transcription of hypha-specific genes (42). Negative regulators of hyphal development have also been identified. The deletion of *TUP1*, which encodes a global transcriptional corepressor, causes hyperfilamentation under yeast growth conditions (9). Considering that *C. albicans* cells can respond to a large number of extracellular signals and growth conditions in monitoring hyphal development, they are likely to utilize many parallel signal transduction pathways to integrate these signals.

Many of the regulatory components for dimorphic switching are conserved in filamentous fungi despite their enormous diversity in size and shape and their genetic distance. For example, elements of the same conserved MAP kinase pathway involved in hyphal development in *C. albicans* are also required for filamentous growth in other fungi (45). In *Saccharomyces cerevisiae*, the switch from a unicellular yeast growth to a pseudohyphal growth upon nitrogen starvation depends on this MAP kinase pathway. Four protein kinases, Ste20, Ste11, Ste7, and Kss1, function in sequence to activate the transcriptional factors Ste12 and Tec1 (12, 37, 44, 46). Similarly, the same MAP kinase pathway is necessary for filamentous growth and virulence in the corn smut *Ustilago maydis* (6) and for appressorium formation and virulence in the rice fungus *Magnaporthe grisea* (65). Cyclic AMP (cAMP)/PKA is another conserved signal transduction pathway important for filamentous growth in several fungi (45). In *S. cerevisiae*, changing the level of intracellular cAMP either by an activated allele of *RAS2* GTPase or by the G protein α subunit homologue Gpa2 affects the amount of filamentation (23, 32, 43). The cAMP-mediated signal transduction in filamentous growth is independent of the Kss1 MAP kinase pathway; instead it requires Flo8, a transcriptional regulatory necessary for pseudohyphal growth (38, 53, 55). The cAMP/PKA pathway is also important for fila-

* Corresponding author. Mailing address: Department of Biological Chemistry, College of Medicine, University of California, Irvine, Irvine, CA 92697-1700. Phone: (949) 824-1137. Fax: (949) 824-2688. E-mail: H4LIU@UCI.EDU.

TABLE 1. *C. albicans* strains used

Strain	Genotype	Reference
SC5314	*CRK1/CRK1 URA3/URA3*	21
CAI4	*ura3::1 imm434/ura3::1 imm434*	21
CAW1	*crk1::hisG-URA3-hisG/CRK1 ura3::1 imm434/ura3::1 imm434*	This study
CAW2	*crk1::hisG/crk1 ura3::1 imm434/ura3::1 imm434*	This study
CAW3	*crk1::hisG/crk::hisG-URA3-hisG ura3::1 imm434/ura3::1 imm434*	This study
CAW4	*crk1::hisG/crk1::hisG ura3::1 imm434/ura3::1 imm434*	This study
CAW5	*crk1::hisG/crk1::hisG ura3::1 imm434/ura3::1 imm434* (pYPB1-ADHpt)	This study
CAW6	*crk1::hisG/crk1::hisG ura3::1 imm434/ura3::1 imm434* (pYPBCRK1)	This study
CAW7	*crk1::hisG/crk1::hisG ura3::1 imm434/ura3::1 imm434* (pYPB*CRK1N*)	This study
JKC97	*cst20::hisG/cst20::hisG-URA3-hisG ura3::1 imm434/ura3::1 imm434*	31
JKC129	*hst7::hisG/hst7::hisG-URA3-hisG ura3::1 imm434/ura3::1 imm434*	31
JKC19	*cph1::hisG/cph1::hisG-URA3-hisG ura3::1 imm434/ura3::1 imm434*	36
HLC52	*efg1::hisG/efg1::hisG-URA3-hisG ura3::1 imm434/ura3::1 imm434*	39
HLC54	*cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG ura3::1 imm434/ura3::1 imm434*	39
Ras1-2/ras1-3	*ras1Δ::hisG/ras1Δ::hph-URA3-hph ura3::1 imm434/ura3::1 imm434*	20

mentous growth, virulence, and mating in the human pathogen *Cryptococcus neoformans* (3). In addition to the MAP kinase and cAMP/PKA pathways, the Cdk Cdc28 has been shown to regulate filamentous growth in *S. cerevisiae* (1, 19, 41). Depending on its associated cyclins, Cdc28 plays different roles in filamentous growth (1, 19, 41).

Here we report the identification of another protein kinase in the same Cdc2 subfamily as MAP kinases and Cdks. We have designated it Crk1, for Cdc2-related kinase. Disruption of both copies of *CRK1* in *C. albicans* leads to defective hyphal formation under all conditions examined. Furthermore, *crk1/crk1* mutants fail to induce hypha-specific genes and are avirulent in mice. Consistent with mutant phenotypes, the ectopic expression of a *CRK1* catalytic domain promotes the formation of hyphal colonies under conditions suited for yeast growth. Expression of the Crk1 catalytic domain in *S. cerevisiae* and *C. albicans* mutants defective in components of known filamentation signaling pathways suggested that Crk1 can activate filamentous growth via a route independent of the filamentation MAP kinase pathway and that of Phd1/Efg1. The relationship between Ras1 and Crk1 in hyphal development is also discussed.

MATERIALS AND METHODS

Strains and culture conditions. The *C. albicans* and *S. cerevisiae* strains used in this study are listed in Tables 1 and Table 2, respectively. Yeast strains were routinely grown on YPD medium or on SD medium for selection of prototropic strains (56). Synthetic low-ammonia medium (SLAD) was used for observing pseudohyphal colony formation of *S. cerevisiae* (23). Invasive growth of *S. cerevisiae* was examined as described by Roberts and Fink (54) except that uracil-deficient synthetic complete medium (SC−Ura) was used instead of YPD medium. Transformation of *S. cerevisiae* was performed as described by Ito et al. (29). *C. albicans* strains were cultured as described previously (42). Ura$^-$ *C. albicans* strains were selected on 5-fluoro-orotic acid (FOA)-containing medium (8). The protoplasting method of Kurtz et al. (33) was used for *C. albicans* transformation. Cell and colony morphologies were photographed as described by Loeb et al. (42).

Cloning and sequencing of *CRK1*. Two oligonucleotides, 5′AAAATTTGTG AC(or T)TTTGGTTTA and 5′TCTTGCTAAACCA, were synthesized according to a nucleotide sequence that encodes KICDFGLAR, a conserved region in the subdomain VII of Cdc2-related protein kinases. The two oligonucleotides were annealed to each other, and the two ends were labeled by blunt-end filling in with Klenow enzyme in the presence of [α-^{32}P]dATP (Amersham). The oligonucleotide was used as a hybridization probe to screen a *C. albicans* genomic library inserted in λGEM12 phage (Promega) (10). The hybridization was performed at 40°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhart solution–100 μg of yeast tRNA/ml–0.05% sodium pyrophosphate. The membrane was washed with 6× SSC at room temperature. Sixty-five positive λ plaques were isolated. The recombinant λ DNA was digested with restriction enzymes and analyzed by Southern hybridization using the same oligonucleotide probe. The 65 λ phage clones were classified into 13 groups. Two groups had restriction patterns of the known MAP kinase genes *CEK1* and *MKC1* (50, 63). The inserts from the other putative λ phage clones were released by digestion with *Bam*HI and cloned into the *Bam*HI site of pBSK (Stratagene). Two clones, pBSZS1 and pBSZS2, had much stronger hybridization signals than the others and were analyzed in detail. pBSZS2 was found to contain a gene for a new MAP kinase (J. Chen et al., unpublished data). A 9-kb *Bam*HI fragment

TABLE 2. *S. cerevisiae* strains used

Strain	Genotype	Reference or source
KMG58-7A[a]	*MAT***a** *sgv1 ura3 leu2 trp1*	28
YPH499[a]	*MAT***a** *ura3-53 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 cir*$^+$	64
L5528	*MAT***a** *ura3-52 his3::hisG*	37
HLY367	*MAT***a** *ste7::LEU2 ura3-52 leu2::hisG*	37
HLY362	*MAT***a** *ste12::LEU2 ura3-52 leu2::hisG*	37
HLY2000	*MAT***a** *tec1::HIS3 ura3-52*	Derived from L6149 44
HLY850	*MAT***a** *flo8::hisG ura3-52*	38
CG31	*MAT***a**/α *ura3-52/ura3-52*	23
HLY351	*MAT***a**/α *ste7::LEU2/ste7::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG*	37
HLY352	*MAT***a**/α *ste12::LEU2/ste12::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::hisG*	37
HLY2002	*MAT***a**/α *tec1::HIS3/tec1::HIS3 ura3-52/ura3-52*	Derived from L6149 44
HLY852	*MAT***a**/α *flo8::hisG/flo8::hisG ura3-52/ura3-52*	38
L6235	*MAT***a**/α *his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52/ura3-52 phd1::hisG-URA3-hisG/ phd1::hisG-URA3-hisG ste12::LEU2/ste12::LEU2* (pRS314, B2552)	39
L6235b	*MAT***a**/α *phd1::hisG/phd1::hisG ste12::LEU2/ste12::LEU2 ura3-52/ura3-52*	39

[a] Not Σ strain.

TABLE 3. Plasmids used

Plasmid	Description	Reference
pYPB1-ADHpt	*C. albicans ADH1* promoter and *ADH1* terminator on a *C. albicans URA3/ARS/2*μm vector	13
pYPB*CRK1*	2.25-kb full-length *CRK1* in pYPB1-ADHpt	This study
pYPB*CRK1N*	1.1-kb Crk1 kinase domains *CRK1N* in pYPB1-ADHpt	This study
BES119*CRK1N*	1.1-kb CRK1N in BES119	20, this study
PQF145.2	$RAS1^{V13}$ in BES119	20
pVT102U	*S. cerevisiae ADH1* promoter and *S. cerevisiae ADH1* terminator in *S. cerevisiae URA3/2*μm vector	62
pVTU*CRK1*	Full-length *CRK1* in pVT102U	This study
pVTU*CRK1N*	Crk1 kinase domains *CRK1N* in pVT102U	This study
pVTUHA*CRK1*	*CRK1-HA* in pVT102U	This study
pVTUHA*CRK1N*	*CRK1N-HA* in pVT102U	This study
pVTU*SGV1*	Full-length *SGV1* in pVT102U	This study
pVTU*SGV1N*	Sgv1 kinase domains *SGV1N* in pVT102U	This study
pUC19*CRK1*	4-kb *Sac*I *CRK1* genomic fragment in pUC19	This study
pUC19*CRK1URA3*	*crk1::hisG-URA3-hisG* in pUC19	This study
pBSKZS1	9-kb *Bam*HI *CRK1* genomic fragment in pBSK	This study

in pBSZS1 was digested with *Eco*RI, *Pst*I, and *Bal*31 and then subcloned into pBSK for sequence analysis. Nucleotide sequences of the DNA fragment that hybridized to the probe were determined by the dideoxy-chain termination method using Sequenase (U.S. Biochemical) and [α-^{35}S]dATP (Amersham). Protein sequence comparisons were conducted by using the BLAST algorithm of Altschul et al. (4). Plasmid pBSZS1 contained a DNA sequence with a 2,241-bp open reading frame, corresponding to a protein of 746 amino acids, designated Crk1.

Plasmid and *C. albicans* strain construction. A 4-kb *Sac*I fragment containing the entire coding region of *CRK1* was subcloned from pBSZS1 into the *Sac*I site of a pUC19 vector, generating plasmid pUC19*CRK1* (Table 3). The internal 2-kb *Eco*RV-*Xho*I fragment in plasmid pUC19*CRK1* was replaced with a 4.8-kb *Sal*I-*Sca*I *hisG-URA3-hisG* fragment from plasmid pCUB6 (21) (see Fig. 3A), generating plasmid pUC19*CRK1URA3*. *Sac*I-digested pUC19*CRK1URA3* DNA (see Fig. 3A) was used to transform *Candida ura3/ura3* strain CAI4 (21) to produce *CRK1/crk1* and *crk1/crk1* strains (Table 1).

For complementation and ectopic expression in *S. cerevisiae* and *C. albicans*, several plasmids carrying the *CRK1* or *SGV1* gene under regulation of the *ADH1* promoter were constructed (Table 3). Full-length *CRK1* and *CRK1N* (truncated *CRK1*, encoding just the 11 kinase domains of Crk1) were generated by PCR. The primers used for synthesis of *CRK1* and *CRK1N* were 5′GTCGGATCCATGTCTGTTATTGCTGGCCAT, 5′GCTAAGCTTACATAGATTTGTGTCC, and 5′GCTAAGCTTTATCAATTTCGTGAC. *CRK1* and *CRK1N* PCR products were digested with *Bam*HI and *Hin*dIII and cloned into the *Bam*HI-*Hin*dIII site of pVT102U (*URA3*, 2μm) (62), generating *S. cerevisiae* expression plasmids pVTU*CRK1* and pVTU*CRK1N*, respectively. *CRK1* and *CRK1N* PCR products were also cloned into the *Eco*RV site of plasmid pYPB1-ADHpt (*C. albicans URA3* and *ARS*) (13), generating *C. albicans* expression plasmids pYPB*CRK1* and pYPB*CRK1N*.

For the kinase assay, a synthetic linker containing a hemagglutinin (HA) coding sequence was fused in frame to the N terminus of *CRK1* and *CRK1N* in plasmids pVTU*CRK1* and pVTU*CRK1N*, generating plasmids pVTUHA*CRK1* and pVTUHA*CRK1N*, respectively. The linker sequence was 5′GAGCTCATGGCTTACCCATACGATGTTCCAGATTACGCTAGCGGATCCATG. Full-length *SGV1* and *SGV1N*, encoding just the kinase domain, were generated by PCR. The primers used for synthesis of *SGV1* and *SGV1N* were 5′GTCGGATCCATGAGTGATAATGGTTCCCCC, 5′CTGGAGCTCTTAATATCAGCTTCA, and 5′CTGGAGCTCCGTAATTAGCCACGAGGC. *SGV1* and *SGV1N* PCR products were digested with *Bam*HI and *Sac*I and then cloned into the *Bam*HI-*Sac*I site of pVT102U, generating expression plasmids pVTU*SGV1* and pVTU*SGV1N*, respectively. The *Bam*HI-*Hin*dIII *CRK1N* fragment from pVTU*CRK1N* was inserted into BES119 (20) to generate plasmid BES119 *CRK1N*.

Southern and Northern analyses. Methods for DNA isolation and Southern blot hybridization were as previously described (11). Total RNA extraction and Northern blot hybridization were performed as described in *Current Protocols in Molecular Biology* (26). DNA probes were labeled with the Bethesda Research Laboratories random-primer labeling kit and [α-^{32}P]dATP (Amersham); 4-kb *Sac*I and 2-kb *Eco*RV-*Xho*I *CRK1* fragments from pUC19*CRK1*, a 1.4-kb *Xba*I-*Sca*I *URA3* fragment from pUR3 (30), and a *Cla*I-*Sal*I *ACT1* fragment from plasmid p1595/3 (18) were used as probes. *C. albicans ECE1* and *HWP1* PCR products were used for probing Northern blots. The primers used were 5′GCCATCCACCATGCTCC and 5′GTGCTACTGAGCCGGCATCTC for *ECE1* and 5′TGCTCCAGGTACTGAATCCGC and 5′GGCAGATGGTTGCATGAGTGG for *HWP1*. The 2-kb *CRK1* fragment (see Fig. 3) was used as a probe in Northern hybridization. The sizes of mRNAs on Northern blots correlated with the expected lengths based on information from the *C. albicans* genome database.

Kinase assays. For kinase assays, plasmids pVTUHA*CRK1* and pVTUHA*CRK1N* were introduced into YPH499. Extract preparation, immunoprecipitation, and kinase assays of immune complexes were performed as described elsewhere (61, 64). For each immunoprecipitation, 2.5 mg of protein total extract was used, with 1 μg of myelin basic protein (MBP; Sigma) or histone H1 protein (Sigma) as a substrate.

Bioassay for response to α-factor. A halo bioassay was performed as described by Irie et al. (28). In short, 0.1 ml of overnight culture (10^7 cells) was mixed with 5 ml of 0.7% soft agar and spread onto a YPD plate. Whatman paper disks (6 mm in diameter) were placed on the nascent lawn. Different quantities (0, 0.5, and 5 μg) of synthetic α-factor (Sigma) were dotted onto each disk in 5-μl aliquots. Photographs were taken after 48 h.

Virulence studies. The virulence of *C. albicans* strains was tested as described by De Bernardis et al. (16). *C. albicans* strains were grown on SD−Ura plates for 48 h at 30°C. The cells were suspended in physiological saline solution and counted in a hemacytometer. Following quantitation, cells were adjusted to densities of 5×10^7 and 5×10^6 cells/ml. Each *C. albicans* strain was tested for virulence by injecting 0.1 ml of cells (5×10^6 and 5×10^5 cells) into the lateral tail veins of ICR male mice (18 to 21 g each; Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China). Eight mice were injected for each strain. Surviving mice were observed daily after infection with *C. albicans*.

Nucleotide sequence accession number. The GenBank accession number for the *CRK1* nucleotide sequence is U92261.

RESULTS

Cloning of protein kinase gene *CRK1*. We used an oligonucleotide probe-based screen to clone putative protein kinases in the Cdc28/Cdc2 subfamily from a *C. albicans* genomic library. The oligonucleotide sequence was designed from a region in the kinase subdomain VII, which is conserved among all MAP kinases and Cdks (Fig. 1B; Materials and Methods). Four putative kinase genes were cloned: one for a new MAP kinase (Chen et al., unpublished); two known MAP kinase genes, *CEK1* and *MKC1* (50, 63); and a novel gene that encodes a 746-amino-acid predicted protein (Fig. 1). The amino-terminal half of the coding sequence contains all 11 kinase catalytic domains that are highly conserved among members of the Cdc2 subfamily (Fig. 1B) (27). It shares the highest similarity with *S. cerevisiae* Sgv1 (28) (47% identical and 63% similar). A *Schizosaccharomyces pombe* Cdc2-like gene ranked second in our BLAST search, with 46% identity and 61% similarity. Two human kinases, a sequence of cDNA isolated from brain tissue and a PITALRE kinase (Pk11/Cdk9) (17, 25), also gave comparable scores in the search. In addition, the *C. albicans* kinases have an insertion common to all members of the Cdc2 branch of the kinase family (27) (Fig. 1B, underlined region between X and XI). Thus, we designated the *C. albicans* protein Crk1, for Cdc2-related kinase. However, Crk1 lacks some of the conserved residues that are known to be important for Cdk functions (47), including the highly conserved regulatory residue Tyr15 (Fig. 1B, Tyr19 for Cdc28), whose phosphorylation state modulates the kinase activity, and

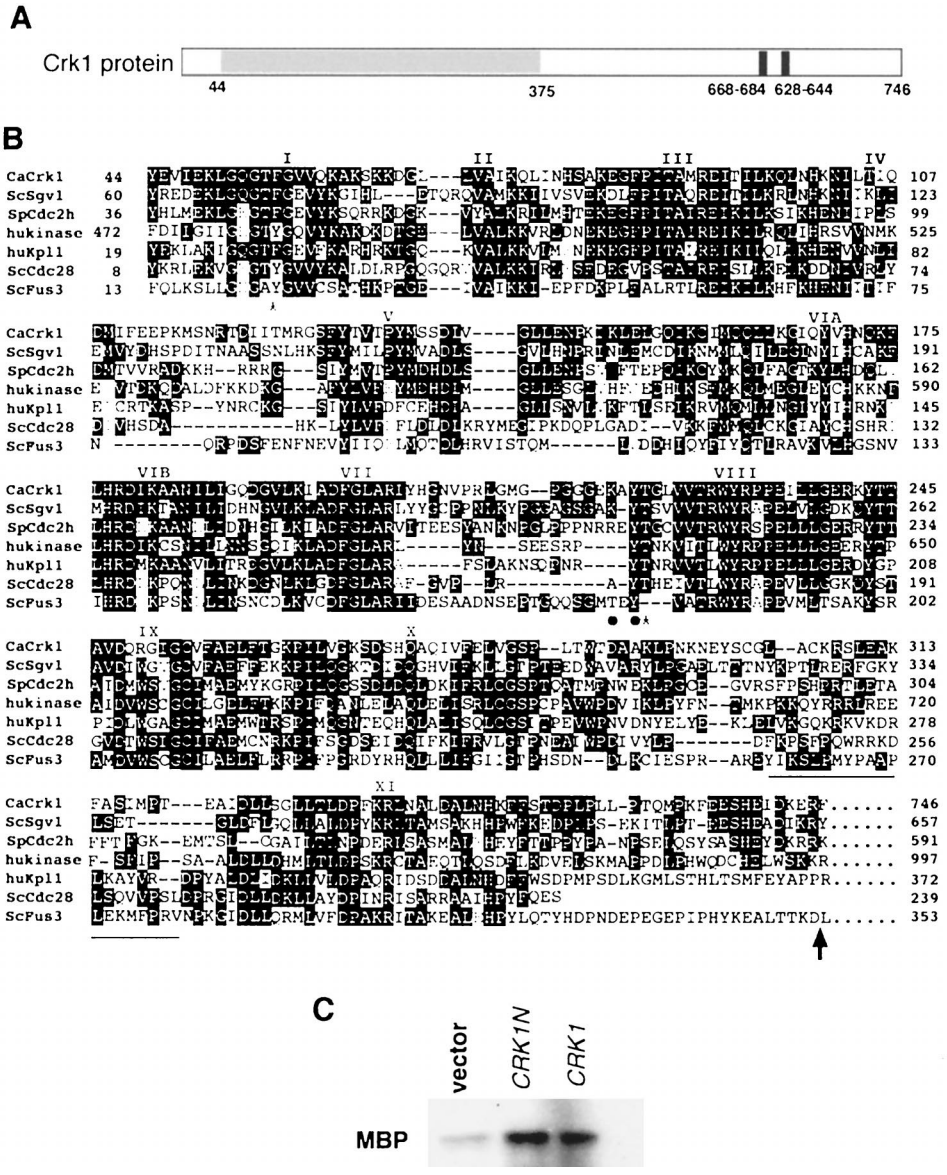

FIG. 1. Comparison of the Crk1 sequence to sequences of other Cdc2-related kinases. (A) Diagram of predicted functional domains in Crk1. The shaded region contains the conserved kinase domain, as shown in panel B. Potential nuclear localization sequences (based on the PSORT program) near the carboxyl terminus are also indicated. (B) Sequence alignment of *C. albicans* Crk1 (CaCrk1) kinase domains with those of *S. cerevisiae* Sgv1 (ScSgv1), an *S. pombe* Cdc2 homologue (SpCdc2h; accession no. AB004534), a human Ser/Thr kinase (hukinase; accession no. AB020711), the human PITALRE kinase HuKp11/Cdk9, *S. cerevisiae* Cdc28, and *S. cerevisiae* Fus3. Subdomains are labeled according to Hanks et al. (27). Shaded residues represent identities among these kinases. Conserved phosphorylation sites in Cdc28 and Fus3 are indicated with asterisks and dots, respectively. Underlined sequences denote the insertion unique for the Cdc2 branch of the kinases. The arrow indicates the ending position of Crk1N and Sgv1N. (C) Kinase activity associated with Crk1 and Crk1N immunocomplexes. Yeast cell extracts were immunoprecipitated with anti-HA antibodies. Immunocomplexes were assayed for the ability to phosphorylate MBP (2-h exposure).

the conserved PSTAIRE sequence (Fig. 1B, domain III) important for interacting with the cell cycle-regulated cyclins. It also lacks the conserved MEK phosphorylation site TXY at the L12 region between subdomains VII and VIII of all MAP kinases (66). Very few similarities outside the kinase domains exist between Crk1 and the other four kinases, except for a short sequence immediately following subdomain XI which shows significant similarity between Crk1 and Sgv1 (Fig. 1B).

We tagged full-length Crk1 and the Crk1 catalytic domain (designated Crk1N) with the HA epitope and expressed both in *S. cerevisiae* Crk1 and Crk1N proteins were then precipitated with anti-HA antibodies and protein A-conjugated agarose beads. Protein kinase activity was assayed with either histone H1 or MBP as the substrate. MBP was phosphorylated by both Crk1 and Crk1N immunocomplexes, whereas the control showed a significantly reduced level of MBP phosphorylation (Fig. 1C). Histone H1, however, was not phosphorylated by either Crk1 or Crk1N immunocomplexes (data not shown). The human PITAIRE kinase Kpl1/Cdk9 also prefers MBP to histone H1 as the substrate in in vitro assays (25). The result of our immunoprecipitation kinase assay is consistent with the deduced protein sequence of Crk1 being a protein kinase.

***CRK1* suppresses the hypersensitive pheromone-induced growth arrest phenotype of *S. cerevisiae sgv1* mutants.** As shown in Fig. 1, Crk1 is most similar to *S. cerevisiae* Sgv1 in protein sequence. *SGV1* was isolated in a mutant screen for suppres-

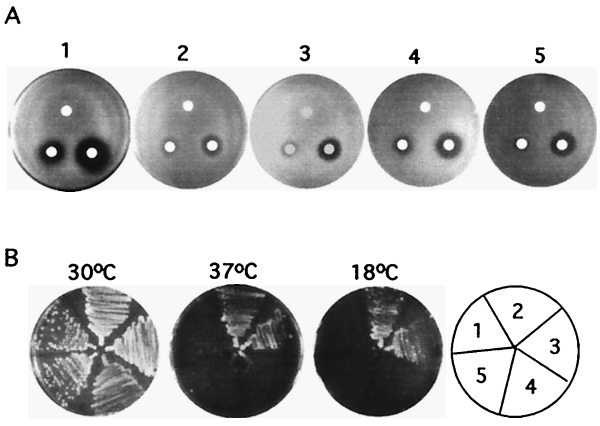

FIG. 2. Suppression of *S. cerevisiae sgv1* mutants by *C. albicans* Crk1. (A) Pheromone-induced growth arrest assay. Haploid *S. cerevisiae sgv1* mutants were transformed with vector (pVTU) (1), *SGV1* (pVTU*SGV1*) (2), *SGV1N* (pVTU*SGV1N*) (3), *CRK1* (pVTU*CRK1*) (4), and *CRK1N* (pVYU*CRK1N*) (5). Approximately 10^7 cells were plated on each YPD plate. Sterile filter disks were placed on the nascent cell lawns; α-factor in the amounts of 0 ng (top), 50 ng (left), and 500 ng (right) was added to the disks. Plates were incubated for 2 days at 30°C. (B) Effect of temperature on growth. The strains used for panel B were used to test growth properties at 37 and 18°C. Cells were streaked onto SC−Ura plates, which were incubated for 5 days at 30°C, 7 days at 37°C, and 7 days at 18°C.

sors that could repress hyperadaptation from pheromone-induced growth arrest in $GPA1^{Val50}$ cells (28). *GPA1* encodes the α subunit of the G protein for pheromone receptors in *S. cerevisiae*. It also plays a positive role in promoting recovery from pheromone-induced growth arrest. The effect of *sgv1* on recovery from pheromone treatment is not specific to the $GPA1^{Val50}$ mutation, as *sgv1* mutants in otherwise wild-type strains are more sensitive to pheromone-induced growth arrest (28). The same *sgv1* mutation also causes temperature-sensitive and cold-sensitive growth phenotypes, consistent with the fact that *SGV1* is essential for vegetative growth (28).

To test whether *CRK1* can complement *S. cerevisiae sgv1* mutants, *CRK1* and *CRK1N* were cloned into an *S. cerevisiae* expression vector under the regulation of a constitutive *ADH1* promoter. The constructs were transformed into a *MAT***a** *sgv1* strain to test whether *CRK1* could complement *sgv1*. *sgv1* mutants were hypersensitive to pheromone and produced a large halo ring around the disks containing α-factor as observed previously (28). The hypersensitive growth arrest by pheromone in *sgv1* mutants was complemented by expression of either *SGV1* or *SGV1N* (Fig. 2A). Similarly, both *CRK1* and *CRK1N* were able to partially suppress the hypersensitive pheromone-induced growth arrest phenotype in *sgv1* mutants, based on results of the halo assay (Fig. 2A). However, neither *CRK1* nor *CRK1N* suppressed the temperature-sensitive or cold-sensitive growth defect of *sgv1* (Fig. 2B).

Chromosomal deletion of *CRK1* in *C. albicans*. To elucidate cellular functions of *CRK1*, we deleted *CRK1* in *C. albicans*. Part of the *CRK1* coding region was replaced by *URA3* with two flanking sequences of *hisG* for gene deletion by homologous recombination (Fig. 3A). A sequential gene disruption strategy was used to delete both copies of *CRK1* in *C. albicans* as described by Fonzi and Irwin (21). Of 185 transformants from the first round of transformation, 90% had the *hisG-URA3-hisG* insertion at the *CRK1* locus, based on Southern hybridizations (Fig. 3B, lane 2). The pattern of Southern hybridization with the 4-kb *CRK1* probe is consistent with integration of the *crk1::hisG-URA3-hisG* construct at the *CRK1* locus. After growth selection on FOA to remove the *URA3* marker, the second copy of *CRK1* was deleted by another round of transformation with the same *crk1::hisG-URA3-hisG* construct; 16 out of 78 transformants displayed the homologous recombination at the second copy of the *CRK1*. This was determined by the loss of the wild-type *CRK1* gene shown by Southern hybridization (Fig. 3B, middle, lane 3).

The ability to obtain homozygous *crk1*/*crk1* mutants suggests that Crk1 is not essential for cell viability. However, we observed that all *crk1*/*crk1* homozygous mutants grew slightly slower than the wild-type parental strain. The wild-type strain had a doubling time of 1.5 h in YPD at 30°C, while the *crk1*/*crk1* strains required 2.2 h for each doubling under the same conditions. On solid YPD medium, *crk1*/*crk1* mutant strains produced slightly smaller wild-type colonies, than and the difference in colony size was more evident at 22°C (Fig. 4A). The low growth rate was caused by the *CRK1* deletion, as the phenotype was reversed by reintroducing wild-type *CRK1* on an autonomous replicating plasmid into the *crk1*/*crk1* strain (Fig. 4A).

Deletion of both copies of *CRK1* also had a subtle effect on cell morphology. *crk1*/*crk1* cells are larger than wild-type cells (Fig. 4B), a phenotype similar to that of the *S. cerevisiae sgv1* mutant at the restrictive temperature (28). In addition, *crk1*/*crk1* cells tend to form chains whereas wild-type cells detach after cytokinesis (Fig. 4B). The phenotypes of cell morphology and incomplete cell-cell separation were enhanced at 22°C. About 4% of *crk1*/*crk1* cells showed an abnormally elongated morphology (Fig. 4B). The morphological defect of *crk1*/*crk1* was reversed by retransformation with the wild-type *CRK1* gene (data not shown).

Crk1 is necessary for hyphal development. Deletion of *CRK1* caused a profound defect in hyphal development on all solid hypha-inducing media tested (Fig. 5A). On serum-containing agar medium, *crk1*/*crk1* strains produced mostly round cells, with a very low percentage of stunted hyphal cells in the initial hours, whereas wild-type strains produced long hyphae. After 3 days of incubation, the wild-type strains generated florid hyphal colonies, whereas *crk1*/*crk1* strains produced round colonies (Fig. 5A, top row). The colonies remained round even after a longer incubation time (Fig. 5A). The heterozygous strain *CRK1*/*crk1* produced intermediate hyphal colonies (Fig. 5A). The defective hyphal growth was most likely caused by the *CRK1* deletion, since reintroducing a wild-type *CRK1* gene on an autonomous replicating plasmid rescued the mutant phenotype (Fig. 5A). On solid Lee's medium, *crk1*/*crk1* strains also failed to develop hyphal colonies after 5 days (Fig. 5A, third row). On Lee's medium, the wild-type strain produced highly filamentous hyphal colonies and the heterozygous strain made intermediate hyphal colonies. We consistently observed that the defect in hyphal development persisted after 7 days. *crk1*/*crk1* strains were also defective in developing hyphal colonies on all other solid media that we tried, including Spider medium and SLAD medium (data not shown).

The defect in hyphal filament formation associated with *crk1*/*crk1* mutants was also observed in all liquid media examined. Serum, in combination with a temperature shift to 37°C, is one of the most effective hypha-inducing conditions and therefore tends to be a more stringent test for cell elongation than growth on solid medium. Wild-type cells form germ tubes within 1 h of incubation (not shown). Longer hyphae are usually observed after 3.5 h (Fig. 5B). *crk1*/*crk1* strains, on the other hand, generated a mixture of mostly round yeast cells and some short pseudohypha-like cells, as well as a limited number of hyphal cells (about 5%) (Fig. 5B, first row). The homozygous strain was impaired in serum-induced hyphal development regardless of the duration of induction. *crk1*/*crk1* mutants were also defective in hyphal formation in Lee's me-

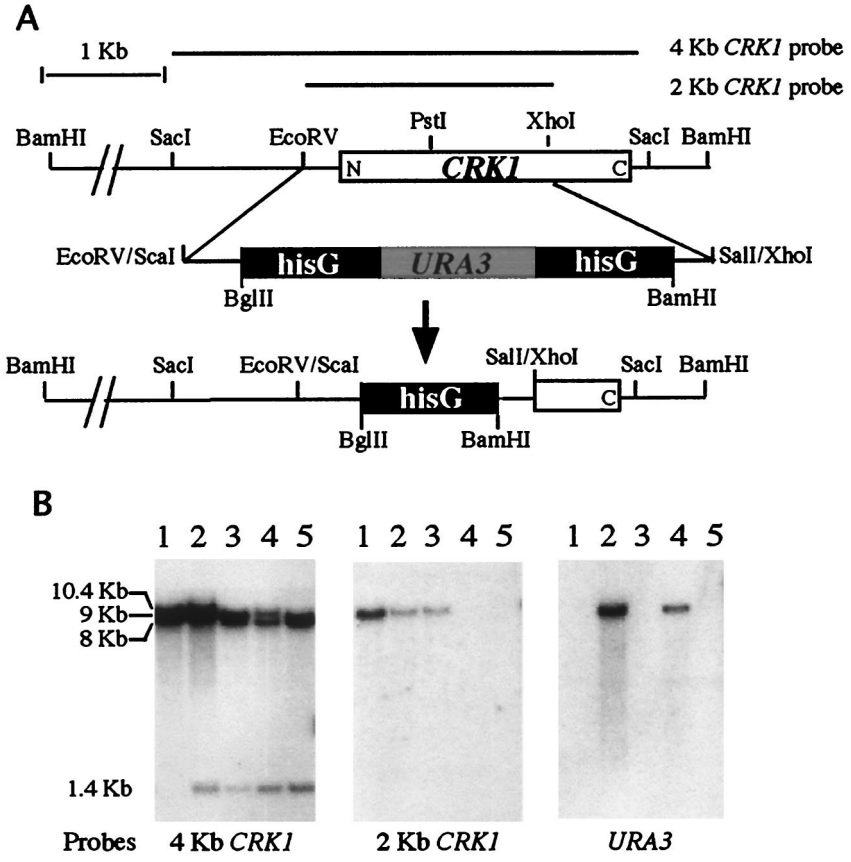

FIG. 3. Disruption of the *C. albicans CRK1* gene. (A) Restriction map and disruption strategy for *CRK1*. (B) Southern analysis of transformants with the *CRK1* disruption construct. Genomic DNA from the recipient strain (lane 1, CAI4), a heterozygote transformant (lane 2, CAW1), an FOA^r/*ura3* derivative of CAW1 (lane 3, CAW2), a homozygote transformant (lane 4, CAW3), and an FOA^r/*ura3* derivative of CAW3 (lane 5, CAW4) were digested with *Bam*HI. The Southern blot on the left was probed with the 4-kb *Sac*I fragment shown in panel A. The *Bam*HI site in the *hisG-URA3-hisG* sequence generated two new hybridization fragments of 10.4 and 1.4 kb from the original 9-kb wild-type *Bam*HI fragment. The 10.4-kb *crk1::hisG-URA3-hisG* fragment became an 8-kb *crk1::hisG* fragment after selection on an FOA plate to loop out the *URA3* and one copy of *hisG*. This size difference between *crk1::hisG* and *crk1::hisG-URA3-hisG* is evident in lane 4, where the doublet represents fragments of 10.4 and 8 kb, respectively. The Southern blot in the middle was probed with the 2-kb *Eco*RV-*Xho*I fragment shown in panel A. The *Eco*RV-*Xho*I region was replaced with the *hisG-URA3-hisG* sequence in the deletion construct. Therefore, the 2-kb probe is expected to hybridize only to the 9-kb *Bam*HI fragment from the wild-type *CRK1* locus. Homozygous *crk1*/*crk1* mutants do not contain the 9-kb *Bam*HI fragment. The Southern blot on the right was probed with *C. albicans URA3*.

dium at pH 7. After 6 h of incubation in Lee's medium, the wild type made long hyphal cells whereas the *crk1*/*crk1* mutants made mostly round cells mixed with occasional long cells (about 5%) (Fig. 5B, second row). Wild-type strains produced mycelia after 15 h of growth, whereas the *crk1*/*crk1* strain generated clusters of mostly round cells, with some hyphal cells surrounding the clusters (Fig. 5B, third row). These hyphal cells produced in Lee's medium were different from the long *crk1*/*crk1* cells seen in YPD at 22°C (Fig. 4B) in that hyphal cells were longer and thinner. Furthermore, hypha-specific transcripts were undetectable by Northern blotting in *crk1*/*crk1* cells under yeast growth conditions (not shown).

The existence of occasional long hyphal cells in *crk1*/*crk1* strains suggested that Crk1 might not be directly responsible for the polarization of actin cytoskeleton. Rather, it might be involved in the transcriptional regulation of hypha-specific genes necessary for filamentation and cell elongation. Therefore, we examined the ability of Crk1 to induce transcription of the hypha-specific genes *ECE1* (extent of cell elongation) and *HWP1* (hyphal wall protein) by Northern analysis. Expression of the *ECE1* transcript has been found to directly correlate with the extent of cell elongation regardless of the conditions or media used for hyphal induction (5, 7, 59), making it a suitable marker for this study. Overnight cultures were diluted into YPD plus 10% serum at 37°C or Lee's medium at 37°C for hyphal induction. *ECE1* expression was dramatically induced in wild-type cells after 3 h in serum or 6 h in Lee's medium (Fig. 5C). The *ECE1* transcript was equally induced in the heterozygous mutant and the wild-type strain (Fig. 5C). However, *ECE1* expression in both hyphal induction conditions was severely reduced in the *crk1*/*crk1* strains. Compared to the wild type, the level of *ECE1* expression in the *crk1*/*crk1* mutant was 7-fold lower in YPD-serum medium and 10-fold lower in Lee's medium (Fig. 5C). The defect in transcriptional induction of hypha-specific genes was not limited to *ECE1*. The expression of *HWP1*, which encodes a hyphal wall protein (5, 7, 59), was similarly affected in the *crk1*/*crk1* strain. The level of *HWP1* in the *crk1*/*crk1* strain was 8-fold lower than that in wild-type cells in YPD serum medium and 12-fold lower than wild-type transcription in Lee's medium. Therefore, Crk1 is required for normal induction of the hypha-specific transcriptional program.

The defect in hyphal development observed in the *crk1*/*crk1* mutant was caused by deleting *CRK1*. Introducing a wild-type *CRK1* into the *crk1*/*crk1* mutant restored its competence in producing hyphal colonies (Fig. 5A) and hyphal filaments in liquid hypha-inducing media (Fig. 5B). The *CRK1* gene also

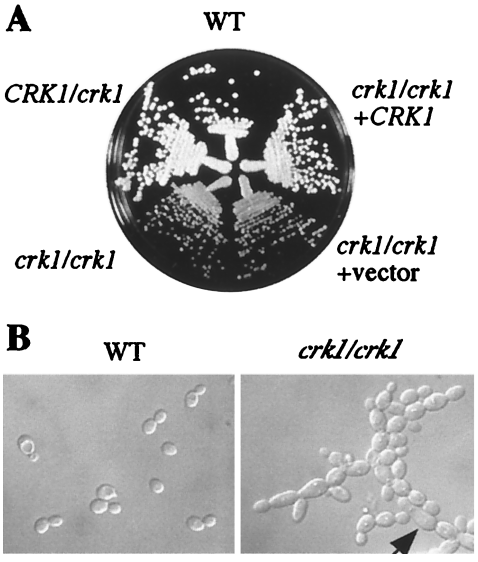

FIG. 4. Effects of *CRK1* disruption on cell growth. (A) *crk1*/*crk1* strains grow slower than wild type. Wild-type (WT; SC5314), *CRK1*/*crk1* (CAW1), *crk1*/*crk1* (CAW3), *crk1*/*crk1* carrying a vector (CAW5), and *crk1*/*crk1* carrying *CRK1* (CAW6) were grown on a YPD plate for 5 days at 22°C. (B) Comparison of cell morphologies. Wild-type (SC5314) and *crk1*/*crk1* (CAW3) cells were grown in YPD medium at 22°C for 15 h and photographed.

complemented the defect in the hypha-specific transcriptional program, as both *ECE1* and *HWP* were expressed in the *CRK1*-transformed *crk1*/*crk1* strains (Fig. 5C).

***crk1*/*crk1* is avirulent in mice.** The dimorphic transition ability of *C. albicans* has been linked with its pathogenicity in mice (22, 35, 39). Here we assessed the virulence of *crk1*/*crk1* mutant strains by the intravenous injection of mice (Materials and Methods). Injection of mice with wild-type *C. albicans* cells is fatal. Injection with an inoculum of 5×10^6 cells caused all mice to die in 6 days, and a smaller inoculum of 5×10^5 cells killed all mice in 13 days (Fig. 6A). The heterozygotic *CRK1*/*crk1* mutant strain was less virulent than the wild-type strain despite being capable of hyphal development (Fig. 5A and B). All mice survived for over 20 days after injection with 5×10^5 cells, and 60% survived after 10 days with an inoculum of 5×10^6 cells (Fig. 6B). The *crk1*/*crk1* cells were avirulent at both inoculum sizes: all mice survived for more than 20 days after injection with 5×10^6 or 5×10^5 *crk1*/*crk1* cells (Fig. 6C). This is comparable to the virulence level of the *cph1*/*cph1 efg1*/*efg1* double mutant (Fig. 6F), which has been previously shown to be avirulent with a similar inoculum size (39). We also used *cph1*/*cph1* and *hst7*/*hst7* single mutants as controls in our experiments. The two strains showed similar survival curves and were slightly less virulent than the wild type (Fig. 6E and D) (39). In comparison, the heterozygotic *CRK1*/*crk1* mutant was less virulent than either *cph1*/*cph1* or *hst7*/*hst7* strains.

***CRK1N* promotes invasive or filamentous growth in *S. cerevisiae* through Flo8 but not through the filamentation MAP kinase pathway or Phd1.** Many of the regulatory components of dimorphic transition are conserved between *C. albicans* and *S. cerevisiae*. Furthermore, several *C. albicans* hyphal regulatory proteins were identified by their ability to promote pseudohyphal growth in *S. cerevisiae* (36, 60). Therefore, we decided to use *S. cerevisiae* as an initial step to investigate potential regulatory targets of Crk1.

Ectopic expression of the Crk1 catalytic domain (*CRK1N*) in *S. cerevisiae* promoted pseudohyphal growth in diploids (Fig. 7A and Table 3). In addition, it enhanced invasive growth in haploid *S. cerevisiae* (Fig. 7B), a phenomenon that shares many features and regulatory components with pseudohyphal growth. Ectopic expression of full-length Crk1 did not alter the level of filamentation (Table 3) or invasive growth (not shown), suggesting that the noncatalytic domain is potentially inhibitory to Crk1 activity, at least in *S. cerevisiae*. Nevertheless, ectopic expression of *CRK1N* in *S. cerevisiae* mutations in components of known signaling pathways could be used to dissect the pathway with which Crk1 is associated.

One of the pathways for invasive or filamentous growth in *S. cerevisiae* is the Kss1-mediated MAP kinase pathway (37, 44). Stimulation of the filamentation MAP kinase pathway is achieved through Ste7, which activates the MAP kinase Kss1, thereby eliminating the inhibitory activity of Kss1 on the transcriptional factor Ste12, leading to the activation of Ste12 (44), which in turn is necessary for the pseudohyphal transcriptional program. Ectopic expression of *CRK1N* bypassed this requirement for the MAP kinase pathway in both filamentous and invasive growth (Fig. 7). *CRK1N* promoted filamentation in diploid *ste7*/*ste7*, *ste12*/*ste12*, and *tec1*/*tec1* strains under nitrogen starvation conditions (Fig. 7A). Quantification of the percentage of pseudohyphal colonies in various strains showed that *CRK1N* increased the magnitude of filamentation as well as the percentage of pseudohyphal colonies (Table 4). *CRK1N* also suppressed the defect in invasive growth in haploid *ste7*, *ste12*, and *tec1* mutants (Fig. 7B). *S. cerevisiae PHD1* has been suggested to function in a pathway parallel to *Ste12* during pseudohyphal growth (39). We found that *CRK1N* bypassed the requirement for filamentous growth in a *ste12*/*ste12 phd1*/*phd1* double mutant. Our result suggests that Crk1 activates filamentous growth via a third pathway, independent of the Phd1 and the MAP kinase pathways.

The cAMP PKA pathway is another pathway implicated in filamentous or invasive growth. Increasing the level of intracellular cAMP promotes filamentous growth (53, 55). Function of the cAMP pathway in filamentous or invasive growth requires the transcriptional regulator Flo8 (53, 55), which is necessary for both invasive growth and filamentous growth (38). We found that mutations in *FLO8* blocked *CRK1N*-promoted filamentous growth (Fig. 7A and Table 4). *flo8* also blocked *CRK1N*-promoted haploid invasive growth (Fig. 7B). Therefore, Crk1-stimulated filamentous or invasive growth in *S. cerevisiae* requires Flo8.

Ectopic expression of *CRK1N* promotes hyphal growth under conditions favorable for yeast growth. While complementing the hypha-defective phenotype in *crk1*/*crk1* mutants, we observed that the ectopic expression of *CRK1* enhanced hyphal growth under conditions that are otherwise favorable for the yeast form of growth. The ectopic expression of *CRK1* allowed formation of visible hyphal colonies after 3 days of growth on solid YPD medium (Fig. 8A). No filaments were observed in the wild-type control strain grown under the same conditions until day 5 (Fig. 8A). We also observed that the expression of the catalytic domain of Crk1 promoted more filamentous growth than Crk1 (Fig. 8A), indicating that Crk1N might be more active than Crk1.

The ectopic expression of *CRK1* or *CRK1N* also promoted the expression of hypha-specific genes under yeast growth conditions. Northern analysis demonstrated that the levels of *CRK1* and *CRK1N* expression from plasmids were higher than from the endogenous chromosomal copies (Fig. 8B). While the *ECE1* transcript was not detectable at 30°C in YPD in a wild-type strain, it was detected in strains overexpressing either *CRK1* or *CRK1N*. However, the level of *ECE1* transcript in *CRK1*- and *CRK1N*-expressing strains was about 20- to 30-fold lower than that of wild-type hyphal cells (compare Fig. 8B to

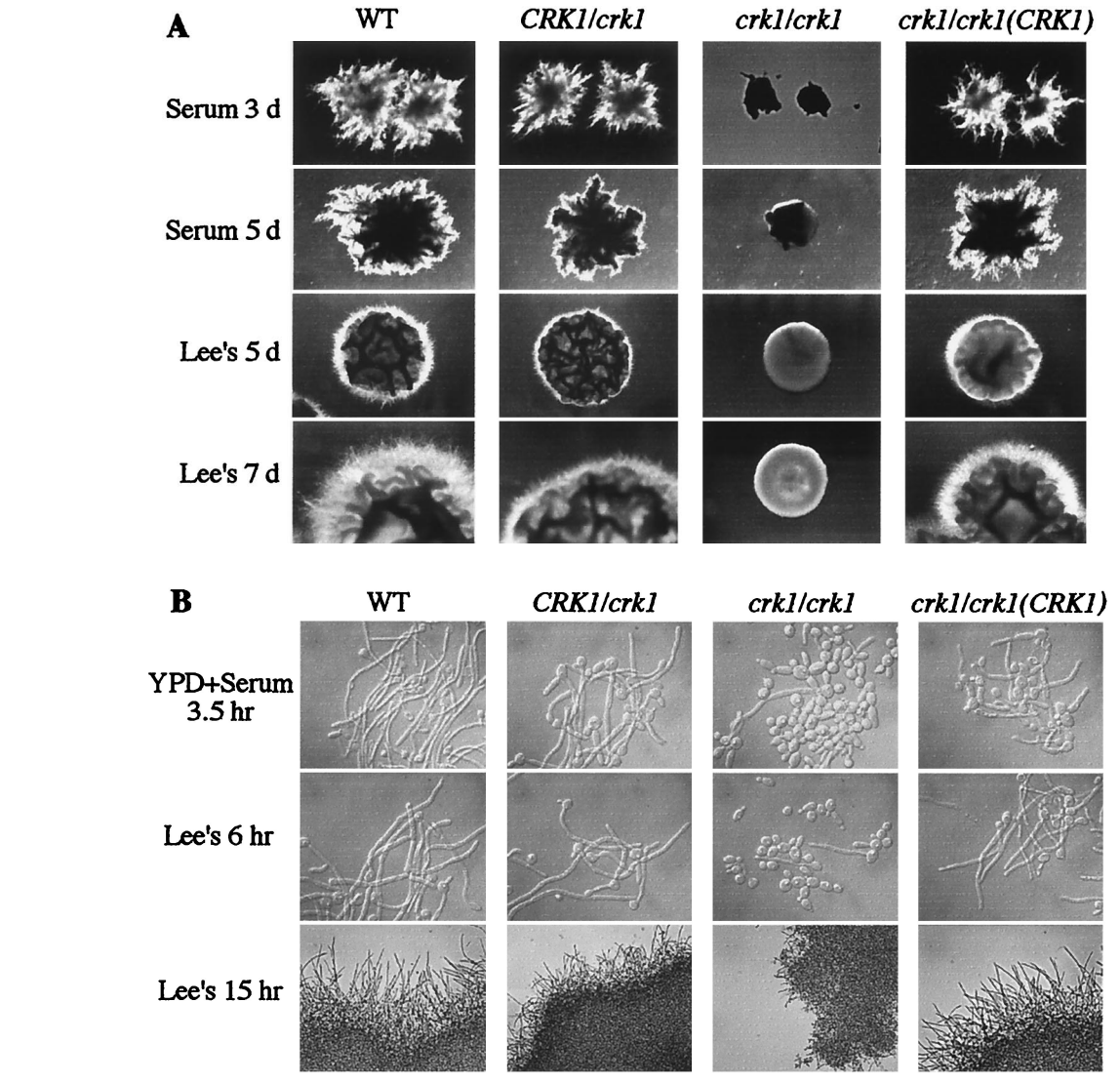

FIG. 5. *crk1*/*crk1* strains are defective in hyphal development. (A) *crk1*/*crk1* strains cannot develop hyphal colonies. Ura$^+$ strains, including wild type (WT; SC5314), *CRK1*/*crk1* (CAW1), *crk1*/*crk1* (CAW3), and *crk1*/*crk1* carrying ectopically expressed *CRK1* (CAW6), were plated at a density of about 50 colonies per plate on solid serum-containing medium and solid Lee's medium. Colonies were photographed after incubation at 37°C for the days (d) indicated. (B) *crk1*/*crk1* strains are impaired in hyphal filament formation in liquid media. Overnight cultures of the four Ura$^+$ strains used for panel A were diluted in YPD medium containing 10% serum or modified Lee's medium for hyphal induction. Cells were photographed after incubation at 37°C for the time indicated. (C) *crk1*/*crk1* mutants are defective in induction of hypha-specific genes. RNA from wild-type cells grown in YPD at 30°C for 6 h was used as a control for gene expression in the yeast growth form (left). RNA from cells of the same Ura$^+$ strains induced for 3.5 h by serum (panel B, top row) and induced for 6 h in Lee's medium (panel B, middle row) were subjected to Northern analysis, as shown in panel C, middle and right gels, respectively. Northern probes are as indicated. The image for the *CRK1* Northern blot was obtained after 3 days of exposure. Images for *ECE1*-, *HWP1*-, and *ACT1*-probed filters were obtained after 3 h of exposure. Transcript levels were quantified with a PhosphorImager.

Fig. 5C). This may explain why the ectopic expression of *CRK1N* did not generate hyphal cells in liquid YPD at 30°C (not shown).

***CRK1* can promote hyphal development through a pathway that is independent of Cph1 and Efg1 in *C. albicans*.** To address the function of Crk1 in the context of known *C. albicans* signaling components, we expressed *CRK1N* in various *C. albicans* strains defective in the filamentation MAP kinase pathway and the parallel pathway Efg1. *CRK1N* suppressed the hyphal formation defect in *cst20*/*cst20* strains (Fig. 9). The effect of *CRK1N* was evident after 3 days but more obvious after a longer incubation. Although Cst20 is supposedly in the same pathway as Hst7 and Cph1, the filament-promoting activity of Crk1N was much weaker in *hst7*/*hst7* and *cph1*/*cph1*

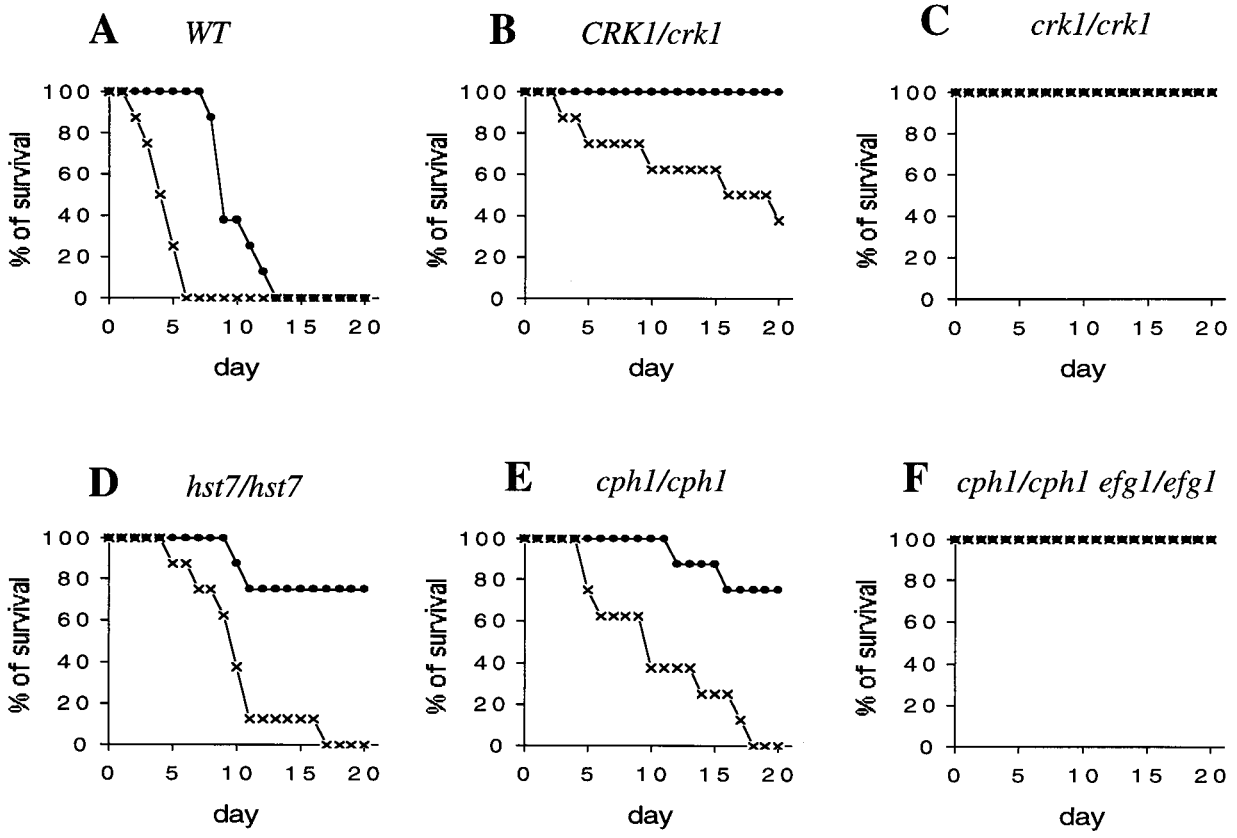

FIG. 6. Virulence assay. ICR male mice were injected with wild-type (*WT*; SC5314; A), *CRK1*/*crk1* (CAW1; B), *crk1*/*crk1* (CAW3; C), *hst7*/*hst7* (JKC129; D), *cph1*/*cph1* (JKC19; E), and *cph1*/*cph1 efg1*/*efg1* (HLC54; F) strains. The mice were injected with 5×10^5 (●) and 5×10^6 (×) *C. albicans* cells. Mice injected with either *crk1*/*crk1* cells or *cph1*/*cph1 efg1*/*efg1* cells all survived for more than 20 days.

strains than in the *cst20*/*cst20* strain. As shown in Fig. 9, *hst7*/*hst7* and *cph1*/*cph1* colonies were more filamentous than *cst20*/*cst20* colonies, but with the expression of *CRK1N*, they were less filamentous than the *cst20*/*cst20* strain. This indicated that the signaling pathway from Cst20 to Hst7 might not be linear. We also showed that *CRK1N* could partially suppress the defect of hyphal development in the *efg1*/*efg1* mutants (Fig. 9). Surprisingly, when transformed with *CRK1N*, *efg1*/*efg1 cph1*/*cph1* double mutants generated more hyphal filaments than either *efg1*/*efg1* or *cph1*/*cph1* single mutants with *CRK1N* (Fig. 9). Comparable to this observation, $RAS1^{V13}$ promoted dramatic hyphal formation in *efg1*/*efg1 cph1*/*cph1* double mutant. Subtle differences existed between *CRK1N*- and $RAS1^{V13}$-activated filamentation. *CRK1N*-promoted hyphal filamentation was most evident around the initial streaks and well-separated single colonies, whereas $RAS1^{V13}$-activated filamentation was seen throughout the streak regardless of the colony density. Interestingly, the expression of $RAS1^{V13}$ in either MAP kinase pathway-defective strains or *efg1*/*efg1* strains led to the formation of large wrinkled sheet-like colonies (Fig. 9). The fact that both *CRK1N* and $RAS1^{V13}$ generated more filaments in the double mutant than in each of the single mutants indicated that there might be complicated negative regulation by Cph1 and Efg1 on filamentation. The phenotypes of *CRK1N* and $RAS1^{V13}$ in the *efg1*/*efg1 cph1*/*cph1* strain suggested that they both could activate hyphal development through pathways independent of Efg1 and Cph1.

To further examine the relationship between Ras1 and Crk1, *CRK1N* and $RAS1^{V13}$ were expressed in *C. albicans ras1*/*ras1* and *crk1*/*crk1* mutants, respectively. The *ras1*/*ras1* mutant produced fewer hyphae than the wild type (not shown), and *CRK1N* dramatically enhanced filament formation in the *ras1*/*ras1* strain (Fig. 9). On the other hand, although $RAS1^{V13}$ suppressed the hyphal formation defect in *crk1*/*crk1* strains, the level of filamentation produced by $RAS1^{V13}$ in the *crk1*/*crk1* strain was much lower than that of *CRK1N* in the *ras1*/*ras1* strain (Fig. 9).

DISCUSSION

A new member of the Cdc2-related protein kinase family with a regulatory carboxyl terminus. We have cloned a novel gene, *CRK1*, which encodes a Ser/Thr kinase with a catalytic domain highly conserved among kinases of the Cdk subfamily. The kinase domain of Crk1 is most similar to those of the *S. cerevisiae* protein Sgv1 and three other Cdc2-related protein kinases. In addition to being similar in sequence, Crk1 may also share overlapping functions with Sgv1, because *CRK1* partially suppresses the hypersensitive pheromone-induced growth arrest phenotype in *sgv1* mutants. This suppression is specific to *sgv1*, as neither Crk1 nor Crk1N promotes adaptation to pheromone induction in wild-type strains. However, Crk1 and Sgv1 also have nonoverlapping functions. First, Crk1 cannot complement the conditional growth defect of *sgv1*. Second, overexpression of full-length Sgv1 or its catalytic domain does not promote invasive or filamentous growth in *S. cerevisiae* (Chen, unpublished observation). Third, Sgv1 is essential for viability in *S. cerevisiae* whereas Crk1 is dispensable in *C. albicans*. Since the deletion of *SGV1* leads to lethality in *S. cerevisiae*, it is impossible to address whether *sgv1*/*sgv1* mu-

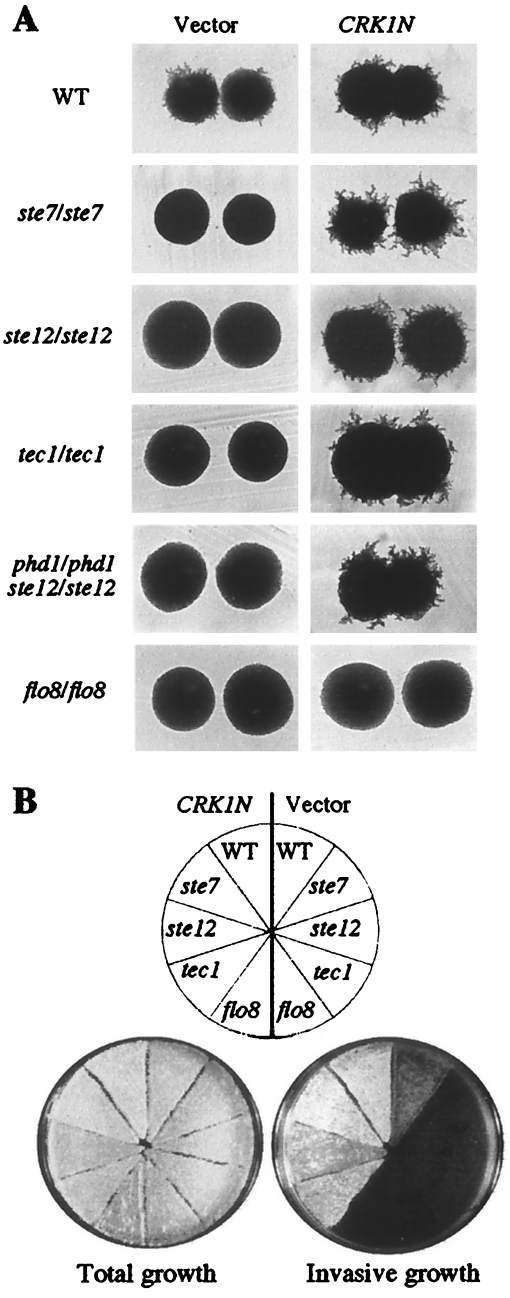

FIG. 7. *CRK1N* stimulated filamentous and invasive growth in *S. cerevisiae*. (A) Colony morphologies of isogenic wild-type (WT; CG31), *ste7*/*ste7* (HLY351), *ste12*/*ste12* (HLY352), *tec1*/*tec1* (HLY2002), *phd1*/*phd1 ste12*/*ste12* (L6235), and *flo8*/*flo8* (HLY852) strains carrying vector (left) or *CRK1N* (right) grown on SLAD at 30°C for 4 days. (B) Total and invasive growth of wild-type (L5528), *ste7* (HLY367), *ste12* (HLY362), *tec1* (HLY2000), and *flo8* (HLY850) strains carrying a vector (left) or *CRK1N* (right) after 5 days of growth on SC−Ura.

tants will block pseudohyphal growth. All of these findings indicate that functional differences exist between Crk1 and Sgv1. Similar to Crk1, the Ras proteins from *S. cerevisiae* and *C. albicans* also have functional differences; *S. cerevisiae* Ras proteins are essential, whereas the *C. albicans* Ras1 protein is not. The functional differences between Crk1 and Sgv1 may reflect variation in substrate specificity. The substrate specificity is likely to be defined by a region in the catalytic domain of Crk1 and Sgv1 since the activity to promote invasive/filamentous growth and the ability to complement the conditional growth in *sgv1* are supported by the catalytic domains of Crk1 and Sgv1, respectively.

All four kinases, Crk1, Sgv1, the uncharacterized *S. pombe* Cdc2-like protein, and the predicted protein from a human brain cDNA, have a long noncatalytic carboxyl domain. For Crk1, the catalytic domain alone seems to be more active than the complete protein. Therefore, the noncatalytic domain may function as an inhibitor to the kinase activity. One possible mechanism of Crk1 activation is to unfold the inhibitory domain and thus expose the catalytic domain during the dimorphic switch. Although the noncatalytic domains of these four kinases are not similar, it is still possible that the mechanisms for their regulation are similar, but the regulators of the noncatalytic domain are different in each organism.

Role of Crk1 in hyphal development in *C. albicans*. Crk1 is required for hyphal development under all hypha-inducing conditions investigated. Deleting *CRK1* dramatically impaired hyphal formation under various hypha-inducing conditions, whereas the ectopic expression of its catalytic domain promoted hyphal colony formation even under conditions favorable for yeast form growth. Crk1 is probably not directly responsible for changes in cytoskeleton that are necessary for the polarized growth during hyphal development. Rather, several lines of evidence support its role in regulating the transcriptional program of hypha-specific genes. First, *crk1*/*crk1* mutants are severely impaired in the induction of two hypha-specific genes. Second, the catalytic domain of Crk1 can induce the expression of hyphal genes under yeast growth conditions when hyphal genes are normally undetectable. Third, the ectopic expression of the Crk1 catalytic domain in *S. cerevisiae* promoted invasive growth, a phenomenon caused by the expression of a cell wall protein, Flo11 (40). Flo11 is necessary for invasive growth, and its expression is regulated by transcription factors required for invasive/pseudohyphal growth. Finally, the Crk1 sequence predicts two conserved basic bipartite nuclear localization sequences at the carboxyl terminus (Fig. 1), suggesting a nuclear function. Taken together, these findings indicate that Crk1 plays a role in regulating the hyphal transcriptional program. This could be achieved by its phosphorylation of some transcription factor(s) or regulator(s) important for hyphal development.

The substrate directly phosphorylated and controlled by Crk1 during *C. albicans* hyphal development is not known. Our studies of *S. cerevisiae* suggest that Crk1 acts independently of Ste12 and Phd1, which correspond to Cph1 and Efg1 in *C. albicans*. Consistent with this, *CRK1N* can suppress the hyphal development defect of *cph1*/*cph1 efg1*/*efg1* double mutants in *C. albicans*. Thus, Crk1 promotes filamentation through a pathway independent of Cph1 and Efg1. The invasive/pseudohyphal growth-promoting activity of Crk1 in *S. cerevisiae* is blocked by Flo8, which is necessary for the cAMP-mediated signaling (53, 55). The sequence and functional conservation between the Ras proteins from *C. albicans* and *S. cerevisiae* suggests that *C. albicans* Ras1 may act in the cAMP pathway (20). Based on experiments in *S. cerevisiae*, *C. albicans Ras1* has been suggested to function upstream of the Cph1 and Phd1 pathways (20). The phenotypes of $RAS1^{V13}$ in mutant strains defective in the MAP kinase pathway or in *EFG1* are supportive of this view; mutations in either pathway dramatically reduce the activity of $RAS1^{V13}$ in filamentation. However, Ras1 can also activate hyphal filament formation through additional routes, as $RAS1^{V13}$ generates florid hyphal filaments in *efg1*/*efg1 cph1*/*cph1* double mutants. It is interesting that *CRK1N* and $RAS1^{V13}$ have similar patterns of suppression in mutants of these two pathways. Further epistasis studies show that *CRK1N* can promote dramatic filamentation in *ras1*/*ras1*

TABLE 4. Filament formation on SLAD medium

Plasmid	Filament formation[a]					
	CG31 (WT)	HLY351 (*ste7*/*ste7*)	HLY352 (*ste12*/*ste12*)	HLY2002 (*tec1*/*tec1*)	HLY852 (*flo8*/*flo8*)	HLY1873b (*phd1*/*phd1 ste12*/*ste12*)
Vector	+++ (80)	+ (10)		+ (10)	++ (10)	+ (50)
	− (20)	− (90)	− (100)	− (90)	− (90)	− (50)
CRK1	+++ (80)	+ (20)		+ (10)	++ (10)	++ (50)
	− (20)	− (80)	− (100)	− (90)	− (90)	+ (50)
CRK1N	+++++ (80)	+++++ (50)	++++ (40)	+++++ (50)	++ (10)	+++++ (50)
	− (20)	+ (50)	+ (60)	+ (50)	− (90)	+ (50)

[a] −, smooth colonies; +, filamentous colonies. The level of cell elongation is indicated by the number of +'s. The percentage of filamentous or smooth colonies in total colonies counted is given in parentheses.

strains, whereas *RAS1*^{V13} shows weaker suppression in *crk1*/*crk1* strains. It is tempting to suggest that Crk1 might be one of the downstream targets of Ras1 in hyphal development. However, a linear model of signal transduction may not be adequate to explain the Ras1/cAMP-mediated regulation of hyphal development. Therefore, epistasis experiments alone might not be able to dissect the complicated network involved in hyphal development.

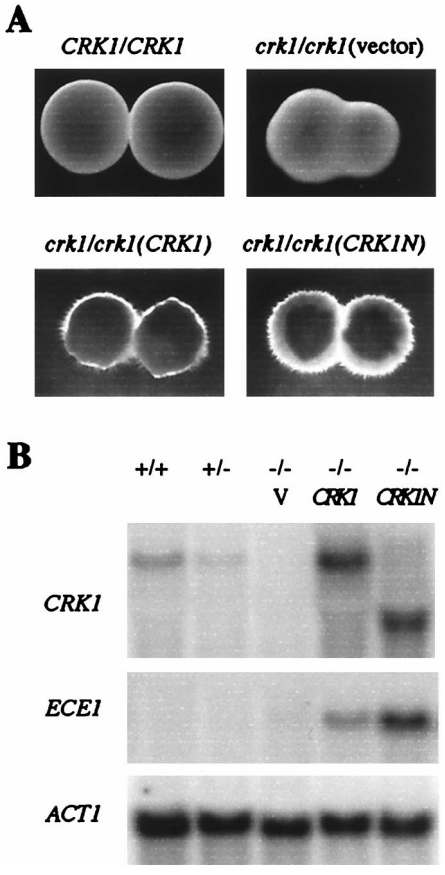

FIG. 8. Ectopic expression of *CRK1*- or *CRK1N*-promoted filamentation under yeast growth conditions. (A) Colony phenotypes of wild-type (SC5314), *crk1*/*crk1* with vector (CAW5), *crk1*/*crk1* with ectopic expression of *CRK1* (CAW6), and *CRK1N* (CAW7) strains grown on YPD medium at 30°C for 3 days. (B) Induction of *ECE1* transcription by ectopic expression of *CRK1* and *CRK1N*. Wild-type (SC5314), *CRK1*/*crk1* (CAW1), *crk1*/*crk1* carrying a vector (V; CAW5), *crk1*/*crk1* carrying *CRK1* (CAW6), and *crk1*/*crk1* carrying *CRK1N* (CAW7) strains were grown in YPD at 30°C for 6 h, and total RNA was extracted for Northern analysis. The Northern blot was probed with *CRK1*, *ECE1*, and *ACT1* and exposed for 3 days (for the *CRK1* and *ECE1* transcripts) and 3 h (for *ACT1*).

Integrative regulation of multiple pathways for hyphal development. Our data suggest that Ras1 and Crk1 regulate additional pathways independent of Cph1 and Efg1, and all of these pathways converge to regulate a common set of hypha-specific genes. This is reminiscent of the pseudohyphal development program in *S. cerevisiae*, where the Kss1 MAP kinase pathway and the cAMP-regulated pathway converge on the promoter of *FLO11* (53, 55). Since both *crk1*/*crk1* and *efg1*/*efg1* mutants are unable to induce hyphal development under similar in vitro hyphal growth conditions, it is unlikely that each pathway responds to a particular hypha-inducing condition; rather, each pathway may respond to a specific signal in a

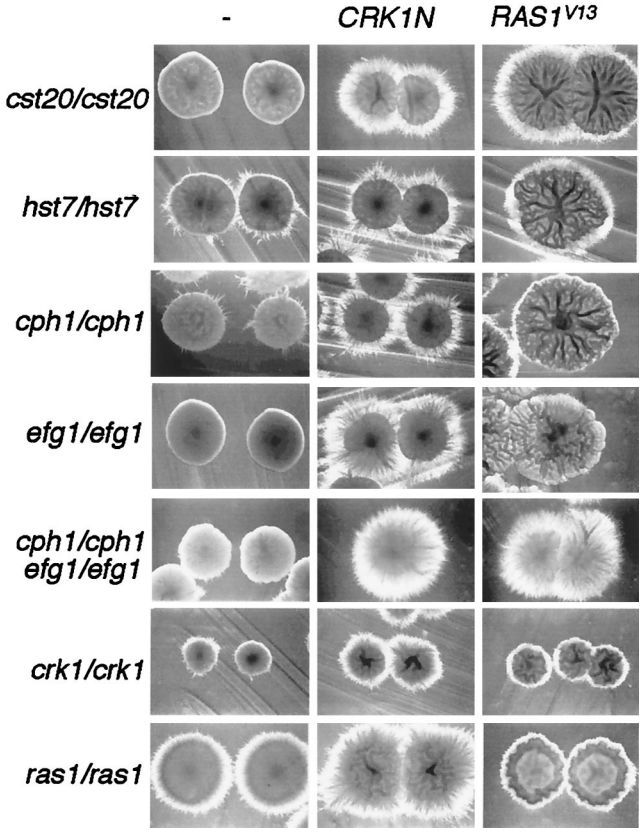

FIG. 9. Functional relationship of Crk1 with the filamentation MAP kinase pathway, Efg1, and Ras1 in *C. albicans* hyphal development. The *C. albicans* mutant strains indicated on the left (described in Table 1) were transformed with *CRK1N* (BES119CRK1N) and *RAS1*^{V13} (pQF145.2). Both genes are under the control of the *MAL2* promoter. The *C. albicans* transformants were grown on an SC−Ura+sucrose (2%) plate containing 50 mM succinate at pH 5 for 4 days at 30°C (20). Well-separated colonies near the edge of each steak were photographed.

hypha-inducing condition. The strong activation for any one pathway may be enough to reach a threshold required for hyphal development, but in many cases, integrated inputs from more than one pathway may be required to reach the threshold required for hyphal development. A defect in any one of the signaling pathways will reduce the total integrated inputs and hamper hyphal development. This integrative regulation may be necessary for fine-tuning of the signaling system, such as in rapid response versus sustained activation. Alternatively, multiple pathways could be used by *C. albicans* cells to sense subtle differences in the growth conditions of its native host environment. Our defined laboratory media, which have been chosen for its all or no hyphal growth property, may fail to mimic the subtle differences in growth conditions in the host.

Virulence. The *crk1*/*crk1* is avirulent under the conditions used in our investigation. The reduced growth rate of *crk1*/*crk1* may contribute to the reduced virulence. The reduced virulence could also be due to the impaired ability of *crk1*/*crk1* strains to undergo hyphal formation, as many mutants defective in hyphal formation have been shown to be less virulent or avirulent compared to the wild type (14, 22, 35, 39). Alternatively, there may be other targets of Crk1 that are not involved in hyphal growth but are required for virulence. Given its role in virulence, uncovering the proteins regulated by Crk1 should reveal new targets for antifungal drug development.

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