JOURNAL OF BACTERIOLOGY, Oct. 1999, p. 6339–6346 Vol. 181, No. 20
0021-9193/99/$04.00+0

Ras Signaling Is Required for Serum-Induced Hyphal Differentiation in *Candida albicans*

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Received 11 June 1999/Accepted 3 August 1999

Serum induces *Candida albicans* to make a rapid morphological change from the yeast cell form to hyphae. Contrary to the previous reports, we found that serum albumin does not play a critical role in this morphological change. Instead, a filtrate (molecular mass, <1 kDa) devoid of serum albumin induces hyphae. To study genes controlling this response, we have isolated the *RAS1* gene from *C. albicans* by complementation. The *Candida* Ras1 protein, like Ras1 and Ras2 of *Saccharomyces cerevisiae*, has a long C-terminal extension. Although *RAS1* appears to be the only *RAS* gene present in the *C. albicans* genome, strains homozygous for a deletion of *RAS1* (*ras1-2*/*ras1-3*) are viable. The Candida *ras1-2*/*ras1-3* mutant fails to form germ tubes and hyphae in response to serum or to a serum filtrate but does form pseudohyphae. Moreover, strains expressing the dominant active $RAS1^{V13}$ allele manifest enhanced hyphal growth, whereas those expressing a dominant negative $RAS1^{A16}$ allele show reduced hyphal growth. These data show that low-molecular-weight molecules in serum induce hyphal differentiation in *C. albicans* through a Ras-mediated signal transduction pathway.

The ability to switch from a cellular yeast form to a filamentous form is characteristic of many fungi. In *Saccharomyces cerevisiae*, the switch from yeast cells to pseudohyphae is signaled by nitrogen starvation and appears to be under the dual control of both the mitogen-activated protein (MAP) kinase and A kinase pathways (20, 23, 29, 30). There is considerable evidence that the *RAS2* gene acts upstream of both signaling pathways. Deletion of *RAS2* reduces filamentous growth, whereas the dominant active allele of *RAS2* enhances pseudohyphal growth (12, 30).

In *Candida albicans* (unlike *S. cerevisiae*) serum induces the switch from yeast to a variety of filamentous forms (germ tubes, pseudohyphae, and hyphae). This morphological change is thought to be essential for virulence (1). Serum appears to induce the morphologic change in *Candida* via several pathways (24). This conclusion is based on the response of mutants to serum. Single mutants in either *CPH1* (a *STE12* orthologue) or *EFG1* can still be induced to form hyphae by serum; only a strain containing two mutations, the double mutant *cph1 efg1*, fails to show vigorous serum induction of hyphae from yeast cells (21). This observation led to the proposal that serum triggers multiple signaling pathways in *Candida*, any one of which was capable of inducing the yeast to hyphal conversion.

The complexity of serum has hampered the identification of a single component that is responsible for its ability to induce the morphological change in *Candida*. Previous investigations had shown that the serum inducing factor(s) was heat stable and nondialyzable (5, 28) and that the peak of serum induction activity comigrated with the serum albumin fraction in purification by gel filtration (2). However, several observations argue against albumin itself as the inducing factor. Although some commercially purified serum albumins have the ability to induce hyphae, other preparations of albumin do not. Moreover, an active serum albumin fraction, when hydrolyzed to its constituent amino acids, retained its inductive activity (6, 7). These studies are consistent with the possibility that it is not albumin that evokes the morphologic change but rather components that copurify with it.

We provide here evidence that serum albumin does not play a key role in serum induction. Serum from a mutant rat strain that is completely devoid of albumin induces *Candida* to form germ tubes and hyphae as effectively as serum from a normal rat that has albumin. Furthermore, a low-molecular-weight serum filtrate (molecular mass, <1 kDa) induces hyphae with the same potency as does serum containing albumin. We show that the *Candida RAS1* gene is essential for hyphal differentiation in response to this low-molecular-weight serum factor. A dominant active *RAS1* allele can bypass the serum requirement for hyphal growth. Thus, the *RAS1* gene plays a key role in regulating the serum response in *C. albicans*.

MATERIALS AND METHODS

Screen for the *Candida RAS1* gene. The strain ASY240 (*ras1 ras2 ade2 ade3 trp1* [Table 1]) provided a convenient colony color system to screen for *Candida RAS* genes. Colonies of strains carrying the *ade2* mutation are red, whereas colonies of an *ade2 ade3* strain are white and require adenine and histidine because the *ade3* mutation blocks accumulation of the red pigment and results in both an adenine and histidine requirement (15). ASY240 contains the human *v-RAS* gene and the yeast *TRP1* and *ADE3* genes on an unstable ARS plasmid (pAS1076). The human *v-RAS* gene is required for the growth of ASY240 (yeast *ras1 ras2* strains are inviable), and the *ADE3* gene complements the *ade3* defect permitting the formation of the red pigment. Thus, ASY240 grows into homogeneous red colonies on plates containing low amounts of histidine and adenine.

The *Candida RAS* gene was cloned by transforming a *C. albicans* genomic library (19) on a plasmid lacking *ADE3* into ASY240. The resulting *Saccharomyces* transformants were screened for the appearance of white colonies. The presumption was that the white colonies were those that could lose the resident plasmid (pAS1076) containing the human *v-RAS* gene because they contained a *Candida RAS* gene capable of suppressing the *ras1 ras2* defect (16). The transformants were selected at 30°C for 4 days on a synthetic complete medium (SC) designed to highlight the color difference (it lacks uracil [32] and contains 0.1 mM adenine and 0.1 mM histidine). White colonies were single colony purified on standard SC-Ura plates and replicated to SC-Trp and SC-His plates. Plasmids were isolated from colonies that were white on low-adenine-histidine plates (and tested as Trp^- His^-, indicating loss of plasmid pAS1076) and transformed into *Escherichia coli*. Purified plasmids were retransformed into ASY240 to verify the phenotype. The *RAS1 Cla*I fragment was subcloned into pRS202 (Fink lab plasmid collection), resulting in plasmid R2cla, and sequenced.

Novel *hph-URA3-hph* disruption cassette. The creation of null mutations in *Candida* requires two sequential knockouts because *Candida* is diploid. To fa-

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TABLE 1. Strains, primers, and plasmids used in this study

Strain, primer, or plasmid	Genotype, sequence, or description
S. cerevisiae ASY240	*ura3-1 leu2-3 112 his3-11,15 trp1Δ63 ade2-1 ade3*::*hisG* *ras1*::*HIS3 ras2*::*LEU2* (pAS1076)
C. albicans	
RAS1/*ras1-1*	*RAS1*/*ras1*Δ::*hisG-URA3-hisG*
RAS1/*ras1-2*	*RAS1*/*ras1*Δ::*hisG*
ras1-2/*ras1-3*	*ras1*Δ::*hisG*/*ras1*Δ::*hph-URA3-hph*
ras1-2/*ras1-4*	*ras1*Δ::*hisG*/*ras1*Δ::*hph*
ras1-2/*ras1-4*/*RAS1*	*ras1*Δ::*hisG*/*ras1*Δ::*hph*/*ade2*::*RAS1-URA3*
RAS1/*RAS1*/*M-RAS1*	*RAS1*/*RAS1*/*ade2*::*Mal-RAS1-URA3*
RAS1/*RAS1*/*M-RAS1^{V13}*	*RAS1*/*RAS1*/*ade2*::*Mal-RAS1^{V13}-URA3*
RAS1/*RAS1*/*M-RAS1^{A16}*	*RAS1*/*RAS1*/*ade2*::*Mal-RAS1^{A16}-URA3*
Primers	
QF61 (*hph* gene)	5′-GGATCGATATGAAAAAGCCTGAACTCACCG-3′
QF62 (*hph* gene)	5′-GGATCGATCTATTCCTTTGCCCTCGG-3′
CaRAS1-NF (5′*RAS1*)	5′-GGTTAATGAATGAATGGCTTGC-3′
QF92 (5′*RAS1*)	5′-CGGGATCCGTATATGTGTGGATATGAATTTG-3′
QF93 (3′*RAS1*)	5′-CGGGATCCCCTATCCAATTGATTGTTTCC-3′
QF94 (3′*RAS1*)	5′-CTCCAGAAAGATCATTTGGTG-3′
QF91 (*RAS1*)	5′-TAAGCTTCATACCATGTTGAGAGAATAT-3′
QF87 (*RAS1^{V13}*)	5′-TTGTTGTTGGAGGTGTTGGTGTTGGTAAAT-3′
QF88 (*RAS1^{V13}*)	5′-ATTTACCAACACCAACACCTCCAACAACAA-3′
QF89 (*RAS1^{A16}*)	5′-GAGGTGGTGGTGTTGCTAAATCCGCTTTAAC-3′
QF90 (*RAS1^{A16}*)	5′-GTTAAAGCGGATTTAGCAACACCACCACCTC-3′
Plasmids	
pAS1076	Human *v-RAS* gene under Adh promoter in pACYC184 with *TRP1-ARS1* from YRP7
R2Cla	*RAS1 Cla*I fragment in pBluescript II KS(+)
pQF137	*RAS1* deletion construct with *hisG-URA3-hisG* cassette
pQF138	*RAS1* deletion construct with *hph-URA3-hph* cassette
BES116	*ADE2-URA3-ADE2 Asc*I fragment in pBluescript II KS(+)
BES119	*ADE2-URA3-pMAL2-ADE2 Asc*I fragment in pBluescript II KS(+)
pQF119	*RAS1* in BES116
pQF144.1	*RAS1* in BES119
pQF145.2	*RAS1^{V13}* in BES119
pQF146.1	*RAS1^{A16}* in BES119

cilitate this process, we created a new construct, the *hph-URA3-hph* disruption cassette (see Results). The *E. coli hph* gene was PCR amplified by using primers QF61, QF62, and B690 (Fink lab plasmid collection, *hph* gene clone) as the template, cloned into pCR2.1 (TA Cloning Kit; Invitrogen), resulting in pQF48.7 and pQF48.17, with the insert at opposite orientations in the vector respectively. The *Hin*dIII-*Xba*I fragment of *hph* from pQF48.7, the *Xba*I-*Sca*I fragment of *Candida URA3* from pQF65.2 and the *Eco*RV-*Bam*HI fragment of *hph* from pQF48.17 were cloned into the pBluescript II KS(+) *Hin*dIII-*Bam*HI site, yielding pQF86. The *hph-URA3-hph* cassette (3.4 kb) can be released by *Bam*HI digestion.

***C. albicans* growth and transformation.** The media for growth experiments was yeast extract-peptone-dextrose medium (YPD) with 2% glucose at room temperature and YPD with 4% glucose at 37°C. At 37°C *Candida* cells form hyphae that interfere with the measurement of growth by optical density (OD). The addition of 4% glucose at 37°C inhibits hyphal growth and permits an accurate measurement of the growth rate at this temperature.

Cells were grown in YPD at room temperature to late log phase (OD_{600} of ca. 5). The standard *S. cerevisiae* lithium acetate transformation procedure was followed for transformation of *C. albicans*.

Deletion of *RAS1*. *5′-RAS1* was PCR amplified by using QF92 and CaRAS-NF as primers and R2cla as the template. The product was cloned into pCR2.1, resulting in plasmid pQF121. *3′-RAS1* was PCR amplified with QF93 and QF94 as primers and R2cla as the template. The product was cloned into pCR2.1, resulting in plasmid pQF122. The *Spe*I-*Bam*HI fragment from pQF121, and the *Bam*HI-*Pst*I fragment from pQF122 were inserted at the *Spe*I/*Pst*I site in pBluescript II KS(+), resulting in pQF124. The *RAS1* deletion constructs, pQF137 and pQF138, were generated by inserting the *hisG-URA3-hisG* (10) cassette and the *Hph-URA3-Hph* cassette, respectively, at the *Bam*HI site in pQF124. The heterozygous deletion strain (*RAS1*/*ras1-1*) was obtained by transforming *C. albicans* CAI4 strain (10) with the *Pst*I/*Not*I fragment of pQF137. The homozygous deletion strain (*ras1-2*/*ras1-3*) was obtained by transforming the heterozygous deletion strain (*RAS1*/*ras1-2*) with the *Sac*I/*Cla*I fragment of pQF138.

Constructs for ectopic expression of *RAS1* in *Candida*. A *Pvu*II fragment containing a multiple cloning site and flanking sequences was removed from pBluescript II KS (Stratagene) and replaced by an *Asc*I site. A 2.2-kb *Candida ADE2* gene containing *Spe*I fragment was cloned into the new *Asc*I site, and then the *Candida URA3* gene on a 1.8 kb *Bam*HI/*Xba*I fragment was used to replace a 0.6-kb *Bam*HI/*Xba*I fragment within *ADE2*. Next, the multiple cloning site of pBluescript II KS resulting from *Bss*HII digestion was cloned into the *Xba*I site flanking the *URA3* gene. These constructions resulted in plasmid BES1116. Finally, a 0.5-kb fragment of the *Candida MAL2* gene promoter (up to but not including a start site) was cloned at *Sac*I/*Eco*RV within the multiple cloning site. This resulted in plasmid BES119.

The *Cla*I fragment of R2cla was cloned into the BES116 *Cla*I site, resulting in pQF119: the *RAS1* gene and *URA3* marker were flanked by the *ADE2* 5′- and 3′-end sequences. The *Asc*I fragment of pQF119 was transformed into the homozygous deletion strain (*ras1-2*/*ras1-4*), and Ura$^+$ colonies were selected.

Site-directed mutagenesis. The wild-type *RAS1* open reading frame (ORF) was PCR amplified with QF91 and T3 as primers and R2cla as the template. The product was cloned into pCR2.1 to form plasmid pQF120.1. The *RAS1^{V13}* mutant was generated by the *Dpn*I method (Stratagene QuickChange Site-Directed Mutagenesis Kit, catalog number 200518) by using primers QF87 and QF88 with pQF120.1 as the template to form plasmid pQF139.2. Similarly, the *RAS1^{A16}* plasmid was generated by using QF89 and QF90 as primers to form pQF140.1. The constructs were confirmed by sequencing. The *RAS1*, *RAS1^{V13}*, and *RAS1^{A16}* ORFs were released from the respective plasmids by *Hin*dIII/*Xho*I digestion and cloned into the plasmid BES119 to form pQF144.1, pQF145.2, and pQF146.1, respectively. The *Asc*I fragments of these plasmids were used to transform *C. albicans*. The transformants containing these constructs have *RAS1* alleles that are inducible when the strain is grown on maltose.

Induction of germ tubes and hyphae. *C. albicans* SC5314, the Ura$^+$ ancestor of CAI4 (10) was used to follow the formation of germ tubes and hyphae from yeast form cells. Portions (5 μl) of log-phase cells (OD_{600} of ca. 1) were mixed in 500 μl (10^5/ml) 50 mM potassium phosphate (pH 6)–10% serum or serum filtrate and incubated at 37°C. At various times after incubation the morphology of the cells was monitored under the microscope.

The serum filtrate was obtained by dialysis serum against 50 mM potassium phosphate at pH 6 by using a Spectra/Por regenerated cellulose dialysis membrane (CMS 265-015).

To determine colony and cell morphology on solid media, we streaked the cells on agar plates containing serum (5%) and incubated them at 37°C overnight. In other experiments, cells were streaked on SC-Ura sucrose (2%)–50 mM succinic acid (pH 5) plates and incubated at 30°C for a week.

Heat shock sensitivity and glycogen content. Cells growing in liquid SC-Ura sucrose medium to early stationary phase (OD_{600} of ca. 10 to 20) were incubated at 50°C for 10 min, diluted fivefold, spotted onto YPD plates, and incubated at 30°C for 2 days. Plates were photographed, and colonies from the last dilution were counted.

Different strains were patched either on synthetic complete medium without uracil plates with sucrose as the sole carbon source or on YPD plates, incubated at 30°C for 2 days, stained with iodine vapor, and photographed.

Nucleotide sequence accession number. Sequence data described in this report have been submitted to GenBank under accession no. AF177670.

RESULTS

Albumin is not the component of serum that induces hyphal growth. Serum is a potent and medically relevant inducer of *C. albicans* hyphal growth. Although *Candida* forms germ tubes and hyphae in response to diverse external treatments, some strains form germ tubes and hyphae only after exposure to serum (27), and some treatments will induce only stationary-phase cells (3), but not exponentially growing cells, to form germ tubes and hyphae. Previous studies indicated that serum albumin is the component of serum that mediates this induction (2). However, we and others have found that not all commercially available purified serum albumin induces germ tubes and hyphae in *Candida* (2).

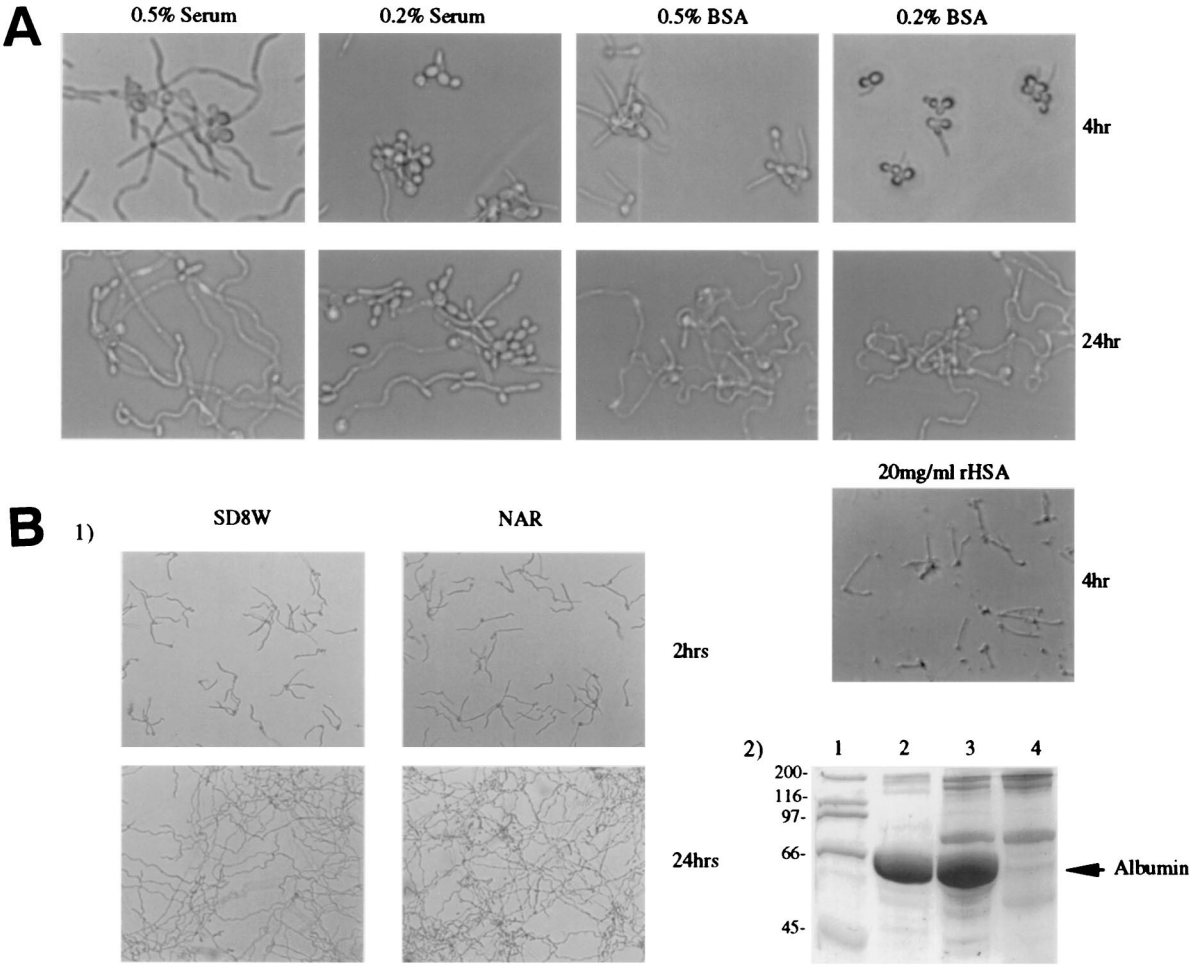

FIG. 1. Serum albumin is not the component of serum that induces germ tubes and hyphae. (A) Germ tubes and hyphae are induced by different concentrations of bovine serum and bovine serum albumin (BSA). Serum contains 40 mg of albumin per ml. Therefore, 0.5% serum contains 0.2 mg of albumin and 0.2% serum contains 0.08 mg of albumin. The recombinant human serum albumin (rHSA) only induced pseudohyphae at a high concentration. (B1) NAR serum induces germ tubes (2 h) and hyphae (24 h) as effectively as the wild-type rat (SD8W) serum. (B2) Sodium dodecyl sulfate-polyacrylamide gel. Lanes: 1, molecular weight marker; 2, 14.5 μg of purified bovine serum albumin; 3, 0.5 μl of SD8W serum; 4, 0.5 μl of NAR serum.

Four lines of evidence suggest that it is not the albumin in serum that causes the morphological induction. First, commercially purified serum albumin is not as effective as unfractionated serum in inducing the morphological change. At equal concentrations of protein (Fig. 1A), serum is at least 50-fold more effective than commercially purified serum albumin in inducing both germ tubes and hyphae. Second, filtration of serum through a molecular-weight cutoff membrane (of ca. 1 kDa) revealed that almost all of the inductive activity resides in the small molecules that flow through the membrane and not in the albumin, which is retained (data not shown). Third, purified recombinant human albumin from *Pichia pastoris* was not a potent inducer of filamentation in *Candida*. At concentrations where commercial purified serum albumin (purified from serum) produced complete conversion of yeast to filaments, the recombinant human albumin failed to induce any morphological change in *Candida*. Tenfold-higher concentrations of recombinant human albumin failed to induce hyphae but were capable of inducing pseudohyphae (Fig. 1A). Fourth, serum obtained from a rat mutant lacking albumin was as potent in inducing filaments as that containing albumin (Fig. 1B). The Nagase analbuminemic rat (NAR) reduces the albumin concentration in serum at least 7,000-fold compared to wild-type rat serum (SD8W) (14, 31). Serial dilution experiments show that 0.6% solutions of either wild-type or mutant sera induced 100% germ tubes and hyphae, 0.3% solutions of either induced 50% germ tubes and hyphae in 2 h, and 0.15% solutions of either failed to induce germ tubes and hyphae even after 24 h. Since serum albumin constitutes about 40% of serum protein, the protein concentration of NAR serum is about two-thirds that of the wild-type rat (SD8W) serum. These data show that serum from the mutant lacking albumin is as potent as serum from the wild type in inducing germ tubes and hyphae.

***C. albicans RAS1* gene.** The *C. albicans RAS1* gene was cloned from a genomic library (19) by its ability to suppress the viability defect of an *S. cerevisiae ras1 ras2* strain (see Materials and Methods). The ORF on the suppressing plasmid is homologous to *RAS* genes in *S. cerevisiae*, other fungi, and mammals. The predicted Ras protein encoded by the *C. albicans RAS1* gene was aligned with proteins encoded by the *S. cerevisiae RAS1* and *RAS2*, human *RAS* gene, and *RAS* genes from other fungi (Fig. 2A). The *C. albicans RAS1* protein sequence is most closely related to those predicted for the *S. cerevisiae RAS1* and *RAS2* genes (Fig. 2B). The *Candida* Ras1p has an extension in the C terminus typical of the *Saccharomyces* sequence but is

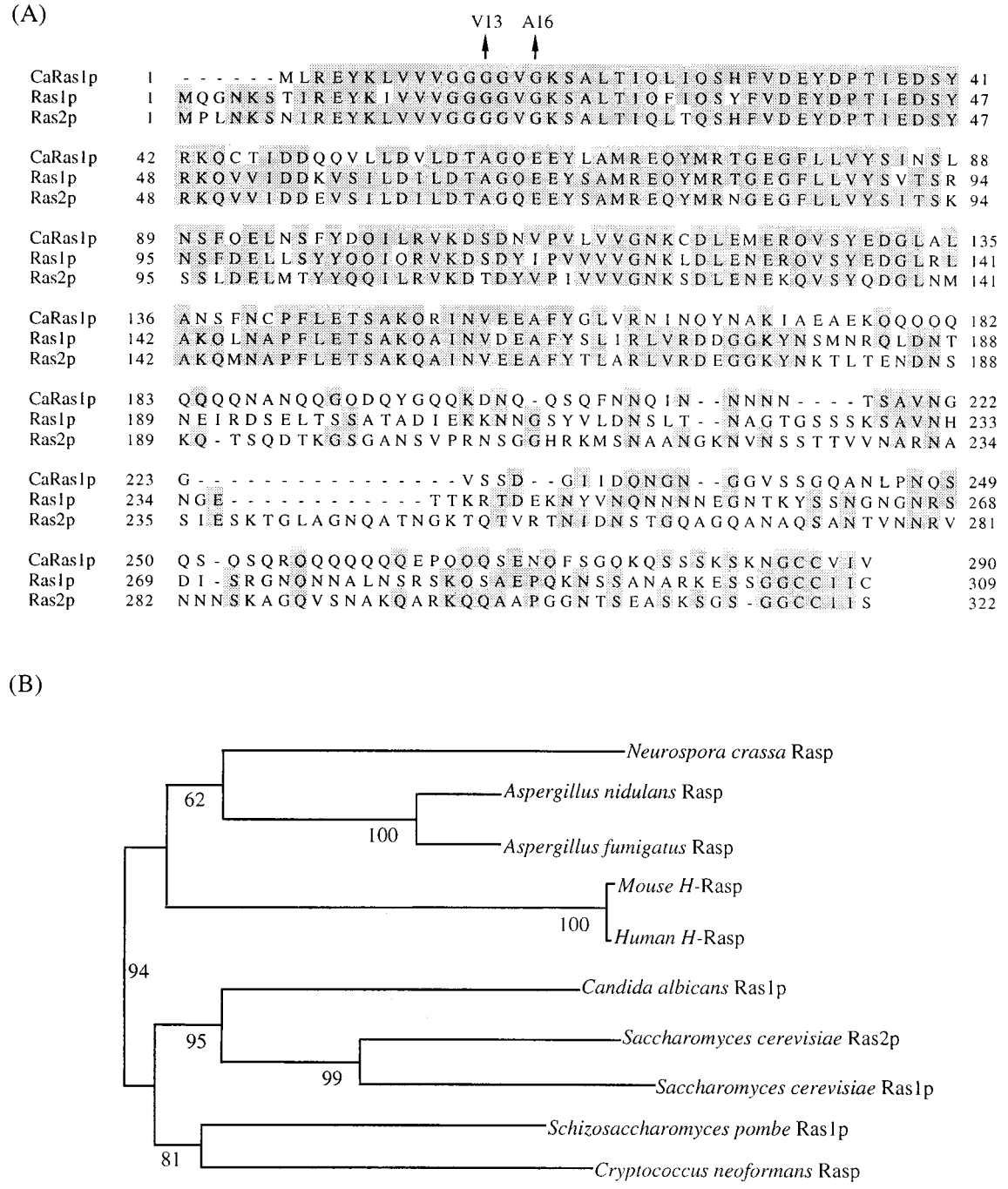

FIG. 2. The *C. albicans* Ras protein. (A) Sequence alignment of *C. albicans* Ras1, *S. cerevisiae* Ras1 and Ras2 proteins. Identical residues are shaded. Amino acids changed in different Ras proteins are indicated by arrows. (B) Phylogenetic tree of Ras proteins from different organisms. The 3′-end sequences of *S. cerevisiae* Ras1 and Ras2 and *C. albicans* Ras1 proteins were truncated to create the tree by using the CLUSTALX program. The phylogenetic tree was made with 1,000 bootstrap resamplings. The numbers are bootstrap percentages for each branch point.

6-amino-acid residues shorter at the N terminus than the *Saccharomyces* sequences. The C-terminal extension is glutamine-rich and has no significant homology to the *Saccharomyces* sequence or to any other sequence in the database. Low-stringency Southern analysis suggests that there is only one *RAS* gene in *Candida* genome (Fig. 3B).

Disruption of *C. albicans RAS1* gene. To test whether the *RAS1* gene plays a role in the response of *Candida* to serum, we deleted the *RAS1* gene in *C. albicans*. A strain heterozygous for the *RAS1* deletion was constructed by using a *hisG-URA3-hisG* cassette (*ras1-1*) (10). Segregants that had lost the *URA3* marker (*ras1-2*) were then isolated from this strain. The segregants were shown to be heterozygous for the *ras1-2* deletion both by PCR and by Southern analysis (Fig. 3C).

Disruption of the remaining *RAS1* gene in the heterozygote proved difficult when using transformation with a plasmid containing the standard *hisG-URA3-hisG* cassette. This plasmid integrates at high frequency into the *ras1-2* deletion allele ra-

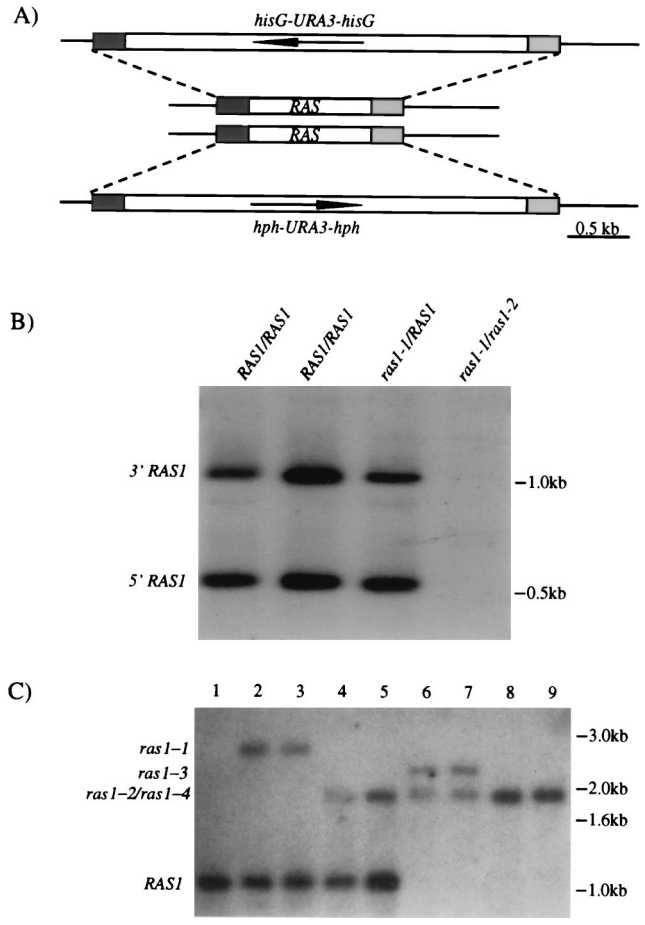

FIG. 3. Deletion of the *RAS1* gene in *C. albicans*. (A) The *RAS1* ORF (0.9-kb), *hisG-URA3-hisG* (4.0-kb), and *hph-URA3-hph* (3.4-kb) cassettes are represented by open boxes. The arrows in the boxes indicate the direction of the transcription of *URA3*. 5′- and 3′-end sequences of *RAS1* are represented by shaded boxes. (B) Low-stringency Southern blot analysis of *RAS* genes in *C. albicans*. *Candida* genomic DNAs from different strains were digested with *Ssp*I, run on 1% agarose gel, and transferred to the Hybond$^+$ membrane. The membrane was hybridized at 65°C overnight and washed at 60°C in 0.5 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The probe is *RAS1* ORF. (C) Southern blot analysis of *RAS1* deletion. Genomic DNAs were digested with *Ssp*I. The probe is the *RAS1* 3′ sequence. Lanes: 1, wild-type strain; 2 and 3, *RAS1*/*ras1-1*; 4 and 5, *RAS1*/*ras1-2*; 6 and 7, *ras1-2*/*ras1-3*; 8 and 9, *ras1-2*/*ras1-4*.

ther than the *RAS1* allele presumably because of the *hisG* homology between the *hisG* region on the plasmid and the *hisG* in the *ras1-2* gene on the chromosome. To avoid this problem, we constructed a novel disruption cassette *hph-URA3-hph* in which the *hisG* segment was replaced by the *E. coli* hygromycin gene (*hph*; see Materials and Methods). When this hygromycin construct (*ras1-3*) was used, approximately 10% of the transformants had the construct integrated in the intact *RAS1* allele in the heterozygote (as shown by PCR and Southern analysis [Fig. 3C]). Two independent strains homozygous for the *ras1* deletion (*ras1-2*/*ras1-3*) each derived from an independently constructed *RAS1*/*ras1* heterozygote were analyzed and tested for all the phenotypes described in the present study.

Strains homozygous for the deletion of *ras1* (*ras1-2*/*ras1-3*) are viable but grow more slowly than the progenitor *RAS1*/*RAS1* strain. The doubling times for *ras1-2*/*ras1-3*, *RAS1*/*ras1-1*, and *RAS1*/*RAS1* at 23°C are 3, 1.5, and 1 h, respectively, and at 37°C are 1.5, 1.16, and 1 h, respectively.

Strains lacking the *RAS1* gene have a severe defect in the response to serum. After 24 h, the homozygous *ras1-2*/*ras1-3* mutant failed to form either germ tubes or true hyphae in response to serum or the serum filtrate at pH 6 and 37°C. By contrast, strains heterozygous for the deletion *RAS1*/*ras1-1* form wild type-like germ tubes after 2 h and hyphae after 7 h (Fig. 4A). On an agar plate containing serum, the wild-type (*RAS1*/*RAS1*) strain and the heterozygous deletion (*RAS1*/*ras1-1*) strain form hyphae, whereas the homozygous deletion (*ras1-2*/*ras1-3*) strain forms round colonies without protruding hyphae (Fig. 4B). A *ras1-2*/*ras1-4* strain transformed with the *RAS1* gene (*ras1-2*/*ras1-4*/*RAS1*) appears to be identical to wild-type strains with respect to growth rate and the ability to form germ tubes and hyphae both on plates and in liquid serum-containing medium.

The *ras1-2*/*ras1-3* mutant responds slightly differently to unfractionated serum as compared with the serum filtrate. After 24 h of incubation, the *ras1-2*/*ras1-3* mutant formed some pseudohyphae when exposed to serum but remained as yeast forms when exposed to the serum filtrate. These data could be interpreted to mean that there is some large molecule in the prefractionated serum (and absent from the filtrate) that is capable of inducing pseudohyphae. However, this hypothetical molecule is not very potent and fails to promote conversion of the *ras1-2*/*ras1-3* mutant to hyphae.

Dominant *RAS1* mutations affect filamentous growth. Dominant-active (*RAS1*^{V13}) and dominant-negative (*RAS1*^{A16}) alleles (Fig. 2A) of the *Candida RAS1* gene were generated based on the amino acid substitutions observed for similar mutations in *S. cerevisiae*. Plasmids were constructed in which each of these alleles, *RAS1*, *RAS1*^{V13}, or *RAS1*^{A16}, was expressed from the inducible maltose 2 promoter (referred to as *M-RAS1*) (11), and each of these constructs was introduced into a *RAS1*/*RAS1* strain by transformation. On glucose medium, where the maltose promoter is repressed, all three strains formed hyphae after 1 week of incubation. On sucrose medium at 30°C, where the *MAL2* promoter is induced, the wild-type *RAS1*/*RAS1* strain began to form short hyphae after 7 days, whereas the *RAS1*/*RAS1*/*M-RAS1* strain formed longer and more abundant hyphae during the same time period. By contrast, the *RAS1*/*RAS1*/*M-RAS1*^{V13} strain produces abundant hyphae after only 3 days of incubation, and the *RAS1*/*RAS1*/*M-RAS*^{A16} strain failed to form hyphae even after 2 weeks of incubation (Fig. 5).

***Candida ras1* mutants have phenotypes similar to those of *ras2 Saccharomyces* mutants.** We examined *C. albicans* strains containing the *ras1* mutations to determine whether they had phenotypes in common with *Saccharomyces* containing comparable *ras2* mutations. Strains of *S. cerevisiae* containing the dominant active allele *RAS2*^{V19} are more sensitive to heat-shock than are wild-type strains and fail to accumulate glycogen. The *Candida* strains were heat shocked at 50°C for 10 min and then plated at 30°C for 2 days. The *Candida RAS1*/*RAS1*/*M-RAS*^{V13} strain was 10- to 20-fold more sensitive to heat shock than the wild type strain; the strain *RAS1*/*RAS1*/*M-RAS1* was twofold more sensitive. There was no difference in the heat shock sensitivity of the wild-type strain and the *RAS1*/*RAS1*/*M-RAS1*^{A16} strain (Fig. 6).

Colonies of the *Candida* wild-type strain stain brown with iodine vapor, whereas those of the *Candida RAS1*/*RAS1*/*M-RAS1*^{V13} strain stain yellow instead of brown (data not shown). This difference in staining suggests that strains containing the *Candida M-RAS1*^{V13} mutation do not accumulate as much glycogen as do the wild-type strains. No difference was observed between the intensity of staining of the wild-type strain

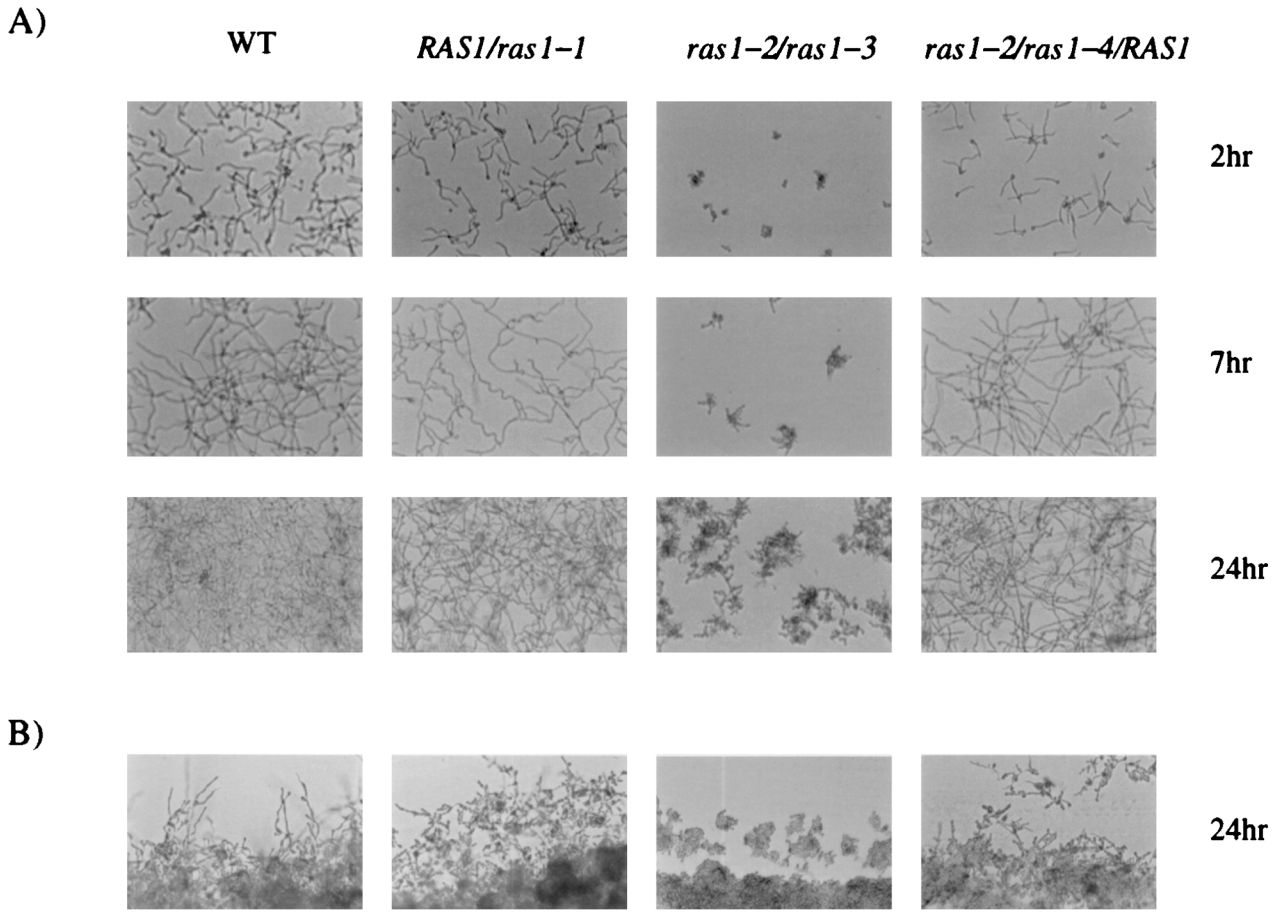

FIG. 4. The homozygous *ras1-2*/*ras1-3* deletion strain was defective for both germ tubes and hyphal formation. (A) Liquid medium assay. Log-phase cells (10^5/ml) were induced by 10% serum in 50 mM potassium phosphate (pH 6) at 37°C. (B) Solid medium assay. The agar plate contains 5% serum. Cells were streaked onto the plate and incubated at 37°C overnight.

(*RAS1*/*RAS1*) and either the *Candida RAS1*/*RAS1*/*M-RAS*^{A16} or the *ras1-2*/*ras1-3* strains.

DISCUSSION

The change in morphology in response to serum is a characteristic signature of *C. albicans*. When incubated with serum at 37°C and neutral pH, *C. albicans* undergoes a morphological change to form germ tubes and hyphae. Our data strongly suggest that serum albumin is not the key component in serum that induces this response. Serum from the NAR induces germ tubes and hyphae of *C. albicans* as effectively as the wild-type rat serum. Serum dilution experiments failed to detect a difference between these two sera in their ability to induce filamentation despite the fact that the quantitative difference in albumin concentration between these two rat sera is at least 7,000-fold (14, 31).

Although some samples of serum albumin induce germ tubes and hyphae, none of the samples is as potent as serum. It is likely that the inductive ability resides in other molecules that copurify with the albumin. Consistent with this idea, we found that small molecules filtered out of the serum induced filamentation in *Candida*. However, we were unable to purify a single small molecule that was responsible for the inductive activity, which raises the possibility that more than one component is required to induce this rapid morphological change. Previous studies have shown that proline (8) or a mixture of amino acids (6, 18) can induce germ tubes and hyphae in *C. albicans*. Under the conditions described here, an amino acid mixture (reconstituted to equal the known concentrations in serum) is not as effective as the serum filtrate in inducing germ tubes and hyphae.

Our finding that the *Candida RAS1* is essential for the morphological change induced by serum is consistent with observations on this morphological change in other fungi. For example, in *S. cerevisiae* the *RAS2*/cyclic AMP (cAMP) pathway is one component in the regulation of filamentous growth. Moreover, deletion of *RAS2* reduces filamentous growth (17), and elevated levels of cytoplasmic cAMP enhance filamentous growth (22). Both the phosphodiesterase (*pde2*) mutant and the dominant-active allele of *RAS2* (*RAS2*^{V19}) enhance filamentous growth (12). Recent studies show that of the three cAMP-dependent protein kinases (A kinase) in *S. cerevisiae* only one, Tpk2, is required for filamentous growth (29). The cAMP pathway has also been implicated in the morphogenesis of other fungi. In the corn pathogen *Ustilago maydis* and in the rice pathogen *Magnaporthe grisea*, the cAMP-dependent protein kinase catalytic subunit is required for both morphogenesis and virulence (9, 13, 25, 34). *RAS* activity also modulates many developmental decisions in *Aspergillus nidulans* (33).

The *C. albicans* Ras1 protein is most homologous to the *S. cerevisiae* Ras1p and Ras2p. The *Candida* Ras1p is the only

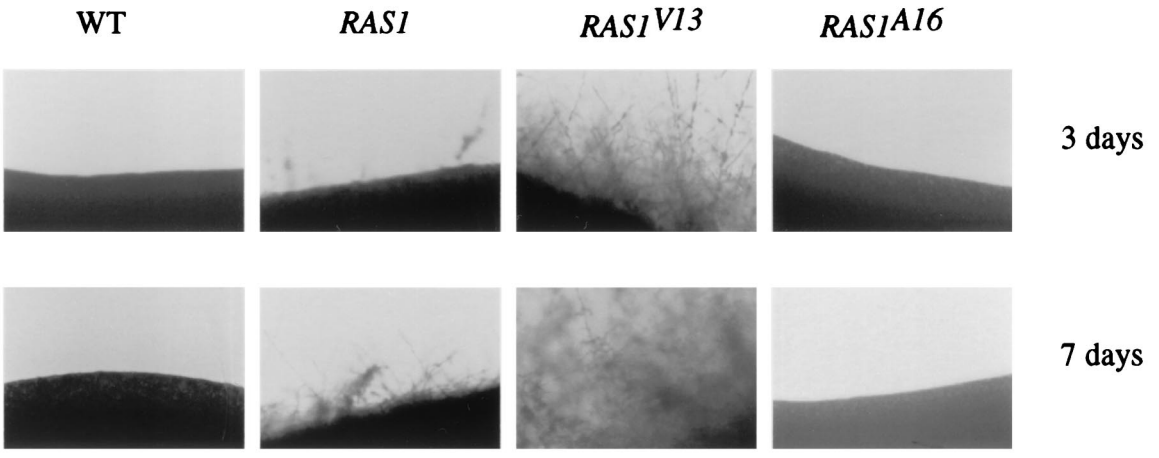

FIG. 5. The dominant-active allele *RAS1*^{V13} enhances filamentous growth, and the dominant-negative allele *RAS1*^{A16} suppresses filamentous growth. Cells were streaked onto a SC-Ura–sucrose (2%) plate containing 50 mM succinate at pH 5. The strain carrying the *RAS1*/*RAS1*/*M-RAS1*^{V13} (the *RAS1*^{V13} allele under control of the maltose 2 promoter) formed hyphae at the edge of the colonies after incubation at 30°C for 3 days, whereas *RAS1*/*RAS1*/*M-RAS1* (a strain containing wild-type *RAS1* allele under the maltose promoter) started to form hyphae after 7 days. The wild-type strain formed shorter and fewer hyphae after 7 days than did *RAS1*/*RAS1*/*M-RAS1*. The strain carrying the *RAS1*^{A16} allele did not form hyphae even after 2 weeks.

other known Ras protein that has a C-terminal extension like that found in the *S. cerevisiae* Ras proteins. This similarity is reflected in the comparable phenotypes of the *Candida* and *Saccharomyces ras* mutants. Strains overexpressing activated *RAS* are more sensitive to heat shock and accumulate less carbohydrates than Ras^{+} strains. In *Saccharomyces* the *ras2* deletion has reduced filamentation and in *Candida* the *ras1*/*ras1* strain is defective in the formation of germ tubes and hyphae.

Despite these similarities, the *ras* mutants in the two organisms show a number of differences. The *Saccharomyces ras2* deletion accumulates more carbohydrates than Ras^{+} strains, whereas the *Candida ras1-2*/*ras1-3* deletion strain does not. Moreover, deletion of *RAS1* in *C. albicans* has a more severe defect on filamentous growth than deletion of *RAS2* in *S. cerevisiae*. Ras function in *Saccharomyces* is essential, whereas it does not appear to be in *Candida*. Although we uncovered only a single *RAS* gene in *Candida*, it is possible that there is another *RAS* gene with insufficient homology to have yielded a signal in the Southern blot analysis and able to supply Ras function for viability.

Experiments in *S. cerevisiae* suggest that *RAS2* acts upstream of both the *STE12* MAP kinase pathway (26, 30) and *PHD1* pathways. The phenotype of the *ras1-2*/*ras1-3* mutant in *C. albicans* is also consistent with *RAS1* acting upstream of these pathways (*CPH1* = *STE12* and *EFG1* = *PHD1* pathways). Although the single *cph1* mutant and the *efg1* mutant are defective in filament formation on some media, each of these single mutant strains can be induced to form germ tubes and hyphae by serum. However, the *cph1 efg1* double mutant, like the *ras1-2*/*ras1-3* mutant, is defective in serum induction. Thus, the phenotypes of the mutants are compatible with a model in which *RAS* transmits a signal to two parallel pathways, the MAP kinase pathway and the *PHD1* pathway, each of which activates the downstream targets required for the formation of germ tubes and hyphae.

Several observations suggest that this linear model may not be an adequate representation of the network controlling the morphological change from yeast cells to filamentous cells. Although neither the *cph1 efg1* double mutant or the *ras1* mutant make hyphae and pseudohyphae in response to serum, both strains form filaments after growth on rich medium (YPD) at room temperature for a week. Since neither the *ras* mutant or the *cph1 efg1* double mutant block filament production under all environmental conditions, there must be additional routes to activate the downstream functions responsible for the morphological change. The multiple control of the switch from one cell type to another is not surprising because filament formation in *C. albicans* is induced by an enormous diversity of external cues (4, 8). The unraveling of this network of signaling pathways and their interactions may require not only traditional mutant analysis but also the application of novel technologies that await the completion of the *Candida* genome.

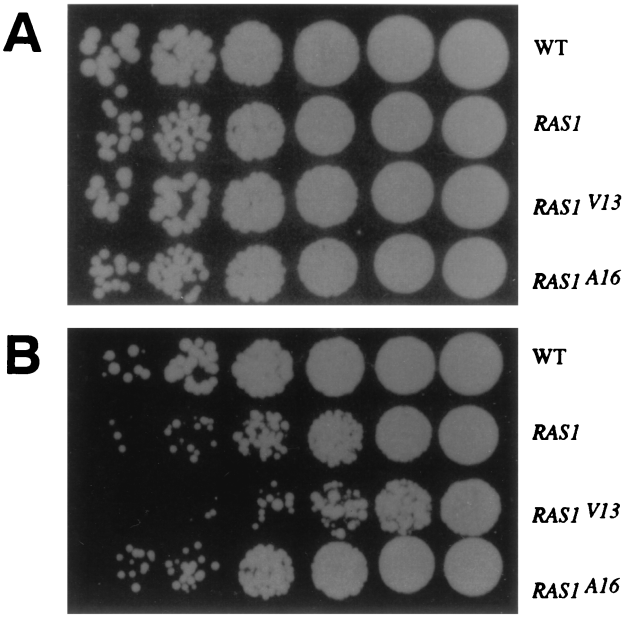

FIG. 6. The strain carrying the dominant active allele *RAS1*^{V13} was more sensitive to heat shock. The same number of cells were either left untreated (A) or heat shocked at 50°C for 10 min (B). Each spot represents the culture diluted successively by fivefold (from right to left) on a YPD plate. The plates were incubated at 30°C for 2 days.

ACKNOWLEDGMENTS

We thank Mike Lorenz, Hiten Madhani, and Julia Koehler for helpful comments on the manuscript; Amir Sherman for providing strains; and Seymour Packman and Hiroky Kodama for providing the NAR and wild-type rat sera. We also thank Fran Lewitter for helping create the phylogenetic relationships.

This work supported in part by a Damon Runyon Postdoctoral Fellowship DRG-1418 (Q.F.), National Institute of Health postdoctoral fellowship K08 AI01484-02 (E.S.), National Research Service Award F32GM19181-02 (B.G.), and National Institute of Health grant GM40266 (G.R.F.). G.R.F. is American Cancer Society Professor of Genetics.

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