

## A Rac Homolog Is Required for Induction of Hyphal Growth in the Dimorphic Yeast *Yarrowia lipolytica*

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Dimorphism in fungi is believed to constitute a mechanism of response to adverse conditions and represents an important attribute for the development of virulence by a number of pathogenic fungal species. We have isolated *YIRAC1*, a gene encoding a 192-amino-acid protein that is essential for hyphal growth in the dimorphic yeast *Yarrowia lipolytica* and which represents the first Rac homolog described for fungi. *YIRAC1* is not an essential gene, and its deletion does not affect the ability to mate or impair actin polarization in *Y. lipolytica*. However, strains lacking functional *YIRAC1* show alterations in cell morphology, suggesting that the function of *YIRAC1* may be related to some aspect of the polarization of cell growth. Northern blot analysis showed that transcription of *YIRAC1* increases steadily during the yeast-to-hypha transition, while Southern blot analysis of genomic DNA suggested the presence of several *RAC* family members in *Y. lipolytica*. Interestingly, strains lacking functional *YIRAC1* are still able to grow as the pseudohyphal form and to invade agar, thus pointing to a function for *YIRAC1* downstream of *MHY1*, a previously isolated gene encoding a C<sub>2</sub>H<sub>2</sub>-type zinc finger protein with the ability to bind putative stress response elements and whose activity is essential for both hyphal and pseudohyphal growth in *Y. lipolytica*.

The members of the Rho family of Ras-related small GTPases (Cdc42, Rac, and Rho proteins) are signaling molecules that, like other Ras proteins, act as molecular switches by transducing signals in the GTP-bound conformation, while being inactive in the GTP-bound state (12, 13). Although Rho family GTPases were originally thought to be involved solely in the organization of the actin cytoskeleton (22, 23, 56, 57, 66, 67, 70), recent evidence has implicated them in an increasing number of vital cellular processes, such as the activation of kinase cascades, regulation of gene expression and membrane trafficking, cell cycle control, and induction of apoptosis, and as critical regulators of oncogenic transformation in mammalian cells (10, 11, 16, 24, 31, 45, 55, 58, 59, 70, 74). Rac proteins have also been shown to regulate the NADPH oxidase system in phagocytes (7) and plants (30) and to induce cell death in rice

through a mechanism that shows biochemical and morphological features similar to those of apoptosis in mammalian cells (30).

In yeast, the Rho family has an important function in the budding process and is known to be involved in the control of actin cytoskeleton dynamics in response to extracellular signals (5, 14, 36). This family is represented in *Saccharomyces cerevisiae* by six proteins (Rho1p, Rho2p, Rho3p, Rho4p, Cdc42p, and Ynl180cp), which are thought to regulate partially overlapping pathways (19). No Rac homolog has yet been described for fungi.

The yeast *Yarrowia lipolytica* has received increasing attention as a model to study dimorphic transition because of its ability to alternate between a unicellular yeast form and distinct filamentous forms (hyphae and pseudohyphae). This fact,

TABLE 1. *Y. lipolytica* strains used in this study

Strain <sup>a</sup>	Genotype
E122.....	<i>MATA ura3-302 leu2-270 lys8-11</i>
22301-3.....	<i>MATB ura3-302 leu2-270 his1</i>
CHY1220.....	<i>MATA ura3-302 leu2-270 lys8-11 fi11</i>
CHY1220-A30.....	<i>MATA ura3-302 leu2-270 lys8-11 Δrac1::URA3</i>
CHY1220-B36.....	<i>MATB ura3-302 leu2-270 his1 Δrac1::URA3</i>
mhy1KO9.....	<i>MATA ura3-302 leu2-270 lys8-11 Δmhy1::URA3</i>
E122//22301-3.....	<i>MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ his1/+</i>
E122//CHY1220-B36.....	<i>MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ his1/+ Δrac1::URA3/+</i>
22301-3//CHY1220-A30.....	<i>MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ his1/+ Δrac1::URA3/+</i>
CHY1220-A30//CHY1220-B36.....	<i>MATA/MATB ura3-302/ura3-302 leu2-270/leu2-270 lys8-11/+ his1/+ Δrac1::URA3/Δrac1::URA3</i>

<sup>a</sup> Strains E122 and 22301-3 were from C. Gaillardin, Thiverval-Grignon, France. Strain mhy1KO9 was from work described previously (26). All other strains were from this study.

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TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence	Restriction site
KO1	5'-CTC TCC TGA TCT GCA TCT GAT CTG-3'	
KO2	5'-TAG CTG AAG ACT CAA TCT GGA GGG-3'	
T3	5'-CCA AGC TCG AAA TTA ACC CTC ACT AA-3'	
T7	5'-GTA ATA CGA CTC ACT ATA GGG CGA AT-3'	
CDC42U	5'-CCT AGC CCG TGC ACA GAC CCT CAA-3'	
CDC42M	5'-TCC GAG TGT GTA GGG CTC GTC TCC-3'	
PR1	5'-TTA GGG CCC AAT CTA AGA TAG ACA CAC GCT CAC CAC CCA-3'	<i>Apa</i> I
PR2	5'-CTG GTC GAC CAT TTT GGA ACC GGT AGC GAG AGT GGA TGT AGG-3'	<i>Sal</i> I
NT1	5'-ATG GTC GAC CAG AGT ATA AAA TGT GTC ACT GGC GAC GGG-3'	<i>Sac</i> I
NT2	5'-GGC CCG CGG TAT CCC AAA GTC CGA GGT TTA TCG GTT TGT-3'	<i>Sac</i> II
SE1	5'-ACT AGT GAG TCG ATG GGC AAC AAA CCA CAG-3'	<i>Spe</i> I
SE2	5'-AGA ATT CAG AGA GCT TAG TGC ACG GCT GGC TTG-3'	<i>Eco</i> RI
EH1	5'-TGA ATT CTT GTT GTG CTG AGT TTG TCT TTT TTT CAT CAA-3'	<i>Eco</i> RI
EH2	5'-AAA GCT TGT GGT TTG GGT GGT GAG CGT TGT TCT ATC-3'	<i>Hind</i> III
HB1	5'-CAA GCT TTC TTT TGC ACA CCA CCC CAC GAC CGA AAC-3'	<i>Hind</i> III
HB2	5'-CAG ATC TTG TAG TGA GTG ACG CAA AAA CTG AGA CCG-3'	<i>Bgl</i> II
BS1	5'-AAG ATC TGC ACA AGT CTC AAT CAA GAC ACT CGC AAG-3'	<i>Bgl</i> II
BS2	5'-GGT CGA CCA TTT TGG AAC CGG TAG CGA GAG TGG ATG TA-3'	<i>Sal</i> I

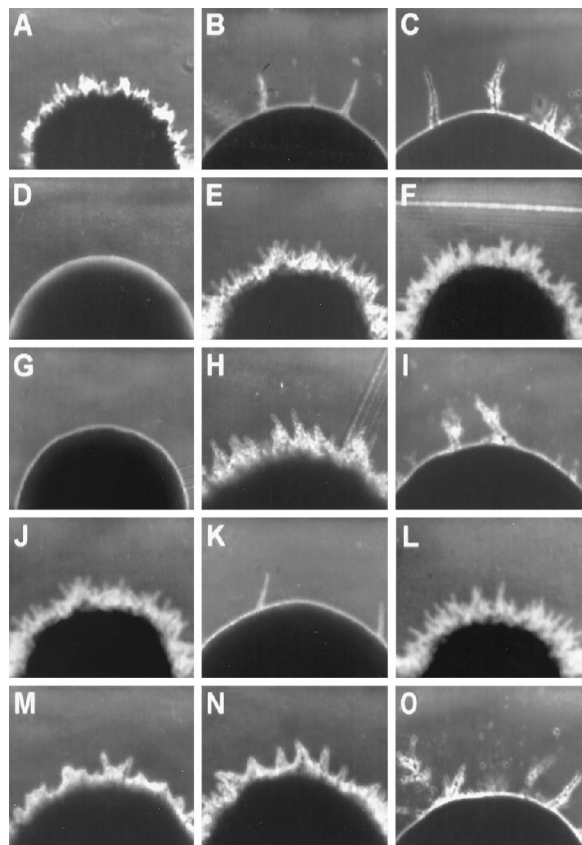


FIG. 1. Colony morphology of various *Y. lipolytica* strains. (A) Wild-type strain E122; (B) original mutant strain CHY1220; (C) *RAC1* disruptant strain CHY1220-A30; (D) *MHY1* disruptant strain mhy1KO9; (E) strain CHY1220 transformed with plasmid pRAC1; (F) strain CHY1220-A30 transformed with plasmid pRAC1; (G) strain mhy1KO9 transformed with plasmid pRAC1; (H) strain CHY1220 transformed with plasmid pMHY1; (I) strain CHY1220-A30 transformed with plasmid pMHY1; (J) wild-type strain 22301-3; (K) *RAC1* disruptant strain CHY1220-B36; (L) *RAC1/RAC1* diploid strain E122//22301-3; (M and N) *RAC1/rac1* diploid strains 22301-3//CHY1220-A30 and E122//CHY1220-B36, respectively; (O) *rac1/rac1* diploid strain CHY1220-A30//CHY1220-B36. Colonies were photographed after 3 days of incubation at 28°C on YNA agar plates. Magnification,  $\times 100$ .

combined with the availability of specific molecular and genetic tools, has provided *Y. lipolytica* with a number of advantages over *S. cerevisiae* and *Candida albicans* for investigation of the molecular mechanisms underlying dimorphic transition (26). The dimorphic switch is induced by environmental signals and is thought to be important for virulence in a number of pathogenic fungi (8, 38, 41, 52, 62).

In this paper, we report the isolation and initial characterization of *YIRAC1*, a gene encoding the first reported fungal Rac homolog, which is involved in the yeast-to-hypha transition in *Y. lipolytica*.

#### MATERIALS AND METHODS

**Yeast strains and microbial techniques.** The *Y. lipolytica* strains used in this study are listed in Table 1. The mutant strain CHY1220 was isolated after chemical mutagenesis of *Y. lipolytica* E122 cells with 1-methyl-3-nitro-1-nitrosoguanidine, as previously described (51). Media components were as follows: YEPD, 1% yeast extract, 2% peptone, and 2% glucose; YNA, 0.67% yeast nitrogen base without amino acids and 2% sodium acetate; YNBD, 0.67% yeast nitrogen base without amino acids and 2% glucose; YNBGlc, 1.34% yeast nitrogen base without amino acids and 1% glucose; and YNBGlcNAc, 1.34% yeast nitrogen base without amino acids, 1% *N*-acetylglucosamine, and 50 mM citric acid (pH 6.0). YNA and YNBD were supplemented with uracil, leucine, lysine, and histidine, each at 50  $\mu$ g/ml, as required. YNBGlc and YNBGlcNAc were supplemented with 2 $\times$  Complete Supplement Mixture (Bio101, Vista, Calif.) or 2 $\times$  Complete Supplement Mixture minus leucine, as required. Media, growth conditions, and procedures for mating, sporulation, and transformation of *Y. lipolytica* have been described previously (9, 51).

Standard techniques for DNA manipulation and growth of *Escherichia coli* were used as described previously (6).

**Mycelial induction.** Mycelial growth was induced as described previously (20). Cells were grown for 12 h in YNBGlc, harvested by centrifugation at room temperature, washed with sterile distilled water, kept on ice for 15 min in YNB without a carbon source, and inoculated at a final density of  $10^7$  cells/ml in prewarmed YNBGlcNAc (for induction of the yeast-to-hypha transition) or YNBGlc (for growth as the yeast form).

**Cloning and characterization of the *Y. lipolytica* *RAC1* gene.** The *Y. lipolytica* *RAC1* (*YIRAC1*) gene was isolated by functional complementation of strain CHY1220 using a *Y. lipolytica* genomic DNA library contained in the replicative *E. coli* shuttle vector pINA445 (51). Plasmid DNA was introduced into yeast cells by electroporation, and Leu<sup>+</sup> transformants were screened on YNA agar plates for their ability to give rise to filamentous colonies.

Complementing plasmids were recovered by transformation of *E. coli*, and the smallest fragment capable of restoring hyphal growth was determined. Restriction fragments prepared from the genomic insert of one of these constructs (pRAC1) were subcloned into the vector pGEM-5Zf(+) or pGEM-7Zf(+) (Promega, Madison, Wis.) or pBluescript II SK(+) (Stratagene, La Jolla, Calif.) for dideoxynucleotide sequencing of both strands. The deduced polypeptide sequence, *Y. lipolytica* Rac1p (*YIRac1p*), was compared to other known protein sequences using the BLAST Network Service of the National Center for Biotechnology Information (Bethesda, Md.).

-790 CCGCGACTAGTGAGTCGATGGGCAACAAACCACAGGCCGTGTCAACCAATCTATATGCTC  
 -730 CACACCCCATCTCTCTGATCTGCATCTGATCTGAAAAGTACTGCCGTGTGCTACTCGCA  
 -670 CTTGGGAGACTATCCGTAGAACAGGCCGCGAACAAACGGCGGACAAGCAAGCTGTGGCT  
 -610 GGTCACTGGGGGACTCTCTTTTTFIACCTAACCAACGTGCATACAGCCCCAGT  
 -550 AGCGGAATTCGCATATCGGGACCCCTGTGTACAAATACTACGATCACCCCCACGGATCT  
 -490 GCCACGGGAATATTTTATCTCTTGTGCTCACAGTCAAACAACCCCTTCAAGCCAGCCGT  
 -430 GCAC<sup>STRE1</sup>TAGCTCTCTGACCCCTTCTTGTGCTGAGTTTGTCTTTTTCATCAAATCTTTTCG  
 -370 CTTGGGACTTTAGGGCCAACTAAGATAGACACAGCTCACACCACAAACCACACCCCT  
 -310 TCTTTTGCACACCACCCACGACCGAAACACAATATCACAGAACGGCAAACATACTGTG  
 -250 ACGT<sup>mRNA</sup>TAA<sup>→</sup>CATACCGGACGAATACCAAGGACTCTTACAGGCTCTCACTCTTCATCTCCTCC  
 -190 ACTACCCAAAGTCGCGCTCCCTGTGAATACTCCGCTCATTACACGGTCTCAGTTTTCGCGT  
 -130 CACTCAC<sup>STRE3</sup>TACAACCCCTGCACAAGTCTCAATCAAGACTCGCAAGAGACCCCTTGA<sup>CTCT</sup>  
 -70 CTAGCGACCCATCCACACAGACTCGTTACACAAGCAGCTCCCTACATCCACTCTCGCTAC  
 -10 CGGTTCCAAATGCAGAGTATAAAATGTGTGCTCAC<sup>STRE2</sup>TGGCGACGGGTAAGT<sup>GA</sup>TGCTGAT  
 M Q S I K C V V T G D G 12  
 51 GCCATGAAGGTCATGGTTCAATTGACGCGACGTGTCCGGGATATGACGGCGGTCTTTAG  
 111 GCGAAACGATGGCCATCGTGTATTTGCTCCACGGGCGGCTTACACCCACGCAATACAC  
 171 GCTTGTCCCACCCCTCCGCCACCCCTACTAACACAGTCCCGTCCGTA<sup>AACT</sup>TGCATGCTA  
 A V G K T C M L 20  
 231 ATCTCATAACCCACAACCGCTTCCCAGGAGGTACATCCCCACCGTGTAAAGTAT<sup>TGACG</sup>  
 I S Y T T N A F P G E Y I P T V 36  
 291 CCCTCATTGATGCCCTCCACCTCTCTATCTAACTCAGCTTCGACA<sup>ACT</sup>TACTCTGCCAAT  
 F D N Y S A N 43  
 351 GTCATGGTGGATAACAAACCGATAAACCTCGGACTTTGGGATACCGCGGGCCAGGAAGAT  
 V M V D N K P I N L G L W D T A G Q E D 63  
 411 TAGACCGGCTGCGGCCACTGTCATACCCCGACCGCGCTGTTTCTTATCTGCTTCTCG  
 Y D R L R P L R P L S Y P Q T G V F L I C F S 83  
 471 CTCGTGTCGCCCCGCTCGTTCGAGAACGTC<sup>AAAGCT</sup>TAAGTGGCACCCCGAAATCTCGCAC  
 L V S P P S F E N V K A K W H P E I S H 103  
 531 CACGCCCCAATACGCCCATCATCCTCGTCCGACCAAGCTCGACCTGCGTAACGACAGT  
 H A P N T P I I L V G T K L D L R N D S 123  
 591 GAGACTCTGGCGCGCTCGCTGAAAAGCGACAAGCCCCATC<sup>ATAT</sup>GCAGAAGGCGCC  
 E T L A R L A E K R Q A P I T Y A E G A 143  
 651 AAGTGTGCTCGGGACATGGCGCGCTCAAATACTTTGAGTGC<sup>CGGC</sup>ATGACCCAAAAG  
 K C A R D I G A V K Y F E C S A L T Q K 163  
 711 GGACTCAAACAGTGT<sup>TTGACG</sup>AGGCCATTACGCGGTGCTGTGCGCTCCCAGCCAAAG  
 G L K T V F D E A I H A V L S P P Q P K 183  
 771 AAAAGAAGAAGAACTGTGTGATTC<sup>TCTAAAGT</sup>GGTGTGACTACGCGACAAACAAACCA  
 K K K K N C V I L - 192  
 831 CTGCTCCCCAGGAGTCAACCACGTGACGCTCGAATCTGGCGGGTCCA<sup>ACT</sup>TCCCTCCA  
 891 GAT<sup>TGAGT</sup>CTTCAGCTATGGCCAAAGACTTAAACGC<sup>TCTAG</sup>CACAAACACCAC<sup>TGTTTT</sup>  
 951 ATTGGCGGTCTTTCTCGGCTTCTTTGCCAGATCCCGCAATGACAGCTATGACTTGGTCC  
 1011 TCCACTAAAAGGAAC<sup>TGCTAGC</sup>TTAGGACTCCACTCAT<sup>TAGTTAA</sup>TGATGTG<sup>TTGG</sup>  
 1071 ATTG

FIG. 2. Nucleotide sequence of the *YRAC1* gene and deduced amino acid sequence of YRac1p. The transcriptional start site of the *YRAC1* gene is indicated by an arrow. Putative STREs are indicated. Consensus sequences for intron splicing are underlined. Putative transcription termination signals are doubly underlined.

**Nucleic acid manipulation.** Genomic DNA, plasmid DNA, and total RNA were prepared from *Y. lipolytica*, as described elsewhere (6). Southern and Northern blot analyses were performed with DNA probes prepared with the ECL direct nucleic acid labeling and detection system (Amersham Life Sciences, Oakville, Ontario, Canada). Electrophoresis and transfer to nitrocellulose membranes were carried out as described previously (6). Hybridization, stringency of washes, and signal generation and detection were as recommended by the manufacturer, except that for the analysis of *Y. lipolytica* genomic DNA, the temperature of hybridization and washes was reduced from 42 to 39°C.

**Immunofluorescence microscopy.** F-actin was detected by incubating cells with 1.3  $\mu$ M Oregon Green 488 phalloidin (Molecular Probes, Eugene, Oreg.), as previously described (2). Images were scanned using SPOT software 1.2.1 (Diagnostic Instruments, Sterling Heights, Mich.), processed in Photoshop 4.0.1 (Adobe Systems, San Jose, Calif.), and printed on a DS8650 PS color printer (Eastman-Kodak, Rochester, N.Y.).

**Mutagenesis of CCCCT elements in the *YRAC1* promoter region.** Three-base substitutions were introduced in the putative stress response elements (STREs) found in the promoter region of the *YRAC1* gene (CCCCT to ATTCT in STRE1, CCCCT to AGCTT in STRE2, and CCCCT to GATCT in STRE3) (see

Fig. 2) by PCR using the oligonucleotide pairs SE1 and SE2, EH1 and EH2, HB1 and HB2, and BS1 and BS2 (Table 2). The four PCR products (369-bp *SpeI-EcoRI*, 100-bp *EcoRI-HindIII*, 197-bp *HindIII-BglII*, and 122-bp *BglII-SalI* fragments) were then ligated to the 396-bp *SalI-SacII* fragment obtained by PCR using the oligonucleotides NT1 and NT2 (Table 2). The resulting *SpeI-SacII* fragment (~1.2 kbp) was used to replace its counterpart in the plasmid pRAC1. The integrity of the final construct was confirmed by sequencing.

**Nucleotide sequence accession number.** The sequence data reported here for *YRAC1* and *Y. lipolytica CDC42* (*YICDC42*) are available from EMBL/GenBank/DBJ under accession numbers AF176831 and AF209750, respectively.

## RESULTS

**Isolation of the *Y. lipolytica* mutant strain CHY1220.** The *Y. lipolytica* mutant strain CHY1220 (Fig. 1B) was initially isolated by its inability to form wild-type rough-surfaced colonies on YEPD agar plates after 3 days of incubation at 28°C (Fig.

1A and J). Further analysis revealed that, like *Δrac1* strains, strain CHY1220 was able to form colonies having a small number of peripheral extensions after prolonged periods of incubation on both rich and minimal media and that these extensions consisted of chains of elongated cells following a pseudohyphal pattern (Fig. 1B, C, K, and O; see Fig. 7).

**Isolation and characterization of the *YIRAC1* gene.** The *YIRAC1* gene was isolated from a *Y. lipolytica* genomic DNA library contained in the replicative *E. coli* shuttle vector pINA445 (51) by its ability to restore hyphal growth to CHY1220 cells. Of approximately 70,000 transformants screened, 5 showed a restored filamentous phenotype (Fig. 1E). Restriction enzyme analysis demonstrated that all complementing plasmids shared a 2.2-kbp *SpeI-ClaI* fragment capable of inducing hyphal growth in CHY1220 cells. Sequencing of this fragment revealed an open reading frame (ORF) of 576 bp interrupted by two introns, which are found at codons 12 and 36 (nucleotides +36 to +205 and +278 to +328 relative to the A residue of the potential initiating codon, respectively) (Fig. 2). The putative 5'-splice donor sequences of both introns (GTA AGTPu) diverge at the third and fourth positions from the GTGAGTPu and GTATGT consensus motifs found for *Y. lipolytica* (39, 65) and *S. cerevisiae* (69), respectively. As in the *Y. lipolytica* genes *SEC14* (39) and *PYK1* (65), a 3'-splice acceptor CAG sequence is found one nucleotide downstream of the consensus TACTAAC box (69) or its abbreviated form CTAAC (Fig. 2).

No obvious TATA box or CT/CA-rich region, which is believed to play a role in transcriptional regulation in *Y. lipolytica* (50, 71), is seen in the promoter region of *YIRAC1*. However, analysis of cDNA showed that transcription of the *YIRAC1* gene starts preferentially at position -286 relative to the A nucleotide of the first ATG codon and that polyadenylation occurs following the G nucleotide at position +1075. Other features of *YIRAC1* include the presence of conserved A nucleotides at positions -1 and -3 relative to the A nucleotide of the initiating ATG and three putative STRE (pentanucleotide CCCCT) (32) in its upstream region (Fig. 2).

The deduced protein product of *YIRAC1*, *YIRac1p*, is 192 amino acids in length and has a predicted molecular mass of 21,173 Da (Fig. 2). Comparison of the predicted amino acid sequence of *YIRac1p* with the sequences of Rac proteins from different sources suggests that its closest homolog is human Rac1 (Fig. 3). In addition, *YIRac1p* has a relatively high pI (8.47), which is an attribute that distinguishes Rac proteins from Rho and Ras proteins (whose pIs are in the range of 5.0 to 6.5) (17).

Consensus elements GXXXXGK (GDGAVGK, residues 10 to 16) and DXXG (DTAG, residues 57 to 60) (Fig. 3), which are involved in interactions with the phosphate portion of the GTP molecule, are found in *YIRac1p* at positions conserved among GTP-binding proteins (15, 18, 28, 46). Conserved motifs are also present at regions implicated in interaction with the GTPase-activating protein (TVFDNY, residues 35 to 40) (61) and in membrane association prior to biological activity (CVIL, residues 189 to 192) (23, 73) (Fig. 3). Notably, *YIRac1p* also contains the conserved motif TKXD (TKLD, residues 115 to 118), which is responsible for nucleotide specificity in Rac proteins and is involved in the determination of the unusually high intrinsic rate of GTP hydrolysis that distinguishes Rac proteins from other Rho family members (17).

**Isolation and characterization of the *YICDC42* gene.** Since no *RAC* gene had previously been reported for fungi and since the *CDC42* gene, which encodes a protein that belongs to the Rac subfamily of Rho GTPases (19), had been shown to be involved in the regulation of filamentous growth in *S. cerevisiae*

<i>YIRac1</i>	MQS IKCVVTDGAVGKTCMLISYTTNAPFGEY IPTVFDNY SANVMVDNKP	50
<i>HsRac1</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
<i>HsRac2</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGSKP	50
<i>HsRac3</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
<i>MmRac1</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
<i>MmRac2</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGSKP	50
<i>DmRac1</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
<i>DmRac2</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
<i>CeRac1</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY STNVMVDGSKP	50
<i>CfRac1</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
<i>XIRac</i>	MQA IKCVVVDGAVGKTCCLISYTTNAPFGEY IPTVFDNY SANVMVDGKP	50
	* * * * * . * * * * * . * * * * * . * * * * * . * * * * * . *	50
<i>YIRac1</i>	INLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>HsRac1</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>HsRac2</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>HsRac3</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>MmRac1</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>MmRac2</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>DmRac1</i>	INLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>DmRac2</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>CeRac1</i>	INLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>CeRac2</i>	INLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>CfRac1</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
<i>XIRac</i>	VNLGLWDTAGQEDYDRLRPLSYPTQDVFVLCFSLVSPSPSEFENVRKAWFEE	100
	. * * * * * . * * * * * . * * * * * . * * * * * . * * * * * . *	100
<i>YIRac1</i>	ISHHAPNPTPI I LVGFKLIDL RNDSETLRLA LKQRAPITVAEGAKCARDIG	150
<i>HsRac1</i>	VRHHCNPPTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>HsRac2</i>	VRHHCNPSTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>HsRac3</i>	VRHHCNPPTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>MmRac1</i>	VRHHCNPPTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>DmRac1</i>	VRHHCNPSTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>DmRac2</i>	VRHHCNPSTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>CeRac1</i>	VSHHCNPPTPI I LVGFKADLRDRDTEIKLRERRIQPVSTQGYVMAKEIK	150
<i>CeRac2</i>	VSHHCNPPTPI I LVGFKADLRDRDTEIKLRERRIQPVSTQGYVMAKEIK	150
<i>CfRac1</i>	VRHHCNPPTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
<i>XIRac</i>	VRHHCNPPTPI I LVGFKLIDL RDKDTEIKLKEKLTPTI TPYQGLAMAKEIG	150
	. * * * * * . * * * * * . * * * * * . * * * * * . * * * * * . *	150
<i>YIRac1</i>	AVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--KKRKRKCVLL	192
<i>HsRac1</i>	AVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--KKRKRKCVLL	191 (79.58%)
<i>HsRac2</i>	SVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--RQQRKRCSLL	192 (76.04%)
<i>HsRac3</i>	SVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--KKPKKCTVF	192 (78.13%)
<i>MmRac1</i>	AVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--KKRKRKCVLL	192 (79.17%)
<i>MmRac2</i>	SVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--RQQRKRCSLL	192 (76.56%)
<i>DmRac1</i>	AVKYLECSALTOGKGLKTVFDEAIRSVLCPVQL--PKSKRRCALL	192 (77.60%)
<i>DmRac2</i>	AVKYLECSALTOGKGLKTVFDEAIRSVLCPVQL--GPKRKRRCALL	192 (75.52%)
<i>CeRac1</i>	AVKYLECSALTOGKGLKTVFDEAIRAVVTPPQ--RAKRSKCTVL	191 (74.87%)
<i>CeRac2</i>	AVKYLECSALTOGKGLKTVFDEAIRAVVTPPQ--RAKRSKCTVL	195 (69.27%)
<i>CfRac1</i>	AVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--KKRKRKCVLL	192 (79.17%)
<i>XIRac</i>	AVKYLECSALTOGKGLKTVFDEAIRAVLCPFPV--KKRKRKCVLL	192 (78.65%)
	. * * * * * . * * * * * . * * * * * . * * * * * . * * * * * . *	192

FIG. 3. Amino acid sequence alignment of Rac1p of *Y. lipolytica* (*YIRac1p*) and Rac proteins from *Homo sapiens* (*HsRac1*, *HsRac2*, and *HsRac3*), *Mus musculus* (*MmRac1* and *MmRac2*), *Drosophila melanogaster* (*DmRac1* and *DmRac2*), *Caenorhabditis elegans* (*CeRac1* and *CeRac2*), *Canis familiaris* (*CfRac1*), and *Xenopus laevis* (*XIRac*). GenBank accession numbers are M29870 (*HsRac1*), CAB45265 (*HsRac2*), AAC51667 (*HsRac3*), CAA40545 (*MmRac1*), Q05144 (*MmRac2*), AAA62870 (*DmRac1*), P48554 (*DmRac2*), AAA28141 (*CeRac1*), AAB40386 (*CeRac2*), P15154 (*CfRac1*), and AAD50299 (*XIRac*).

and *C. albicans* (46, 47), we decided to provide further evidence that we had identified a fungal *RAC* gene by isolating the *CDC42* gene of *Y. lipolytica* (*YICDC42*).

We combined the sequence of a partial *YICDC42* clone previously obtained by probing a *Y. lipolytica* genomic DNA library with an oligonucleotide derived from a highly conserved sequence in the Rab family of proteins (53) with the sequence of a *YICDC42* cDNA obtained by PCR of a *Y. lipolytica* cDNA library constructed in the ZAP Express vector (Stratagene) with oligonucleotides T3, T7, CDC42U, and CDC42M (Table 2) to obtain the sequence of the *YICDC42* gene. The *YICDC42* gene contains an ORF of 573 bp, which is interrupted by two introns, as is the *YIRAC1* gene (Fig. 4). The introns are found between codons 16 and 17 and at codon 45 (nucleotides +49 to +157 and +244 to +327 relative to the A nucleotide of the potential initiating codon, respectively). The putative 5'-splice donor sequences of both introns are identical to the consensus motif GTGAGTPu found in other *Y. lipolytica* genes (39, 65),

```

-123 CATATCACACTCCACACATACACACCCACACAACACCCACATTCTCACACTCTCACGG
-63 CCCCACCACGCTCCTTGAACAAGCTCCCGCCCTAGCCCGTGCACAGACCCTCAAAAAGAT
-3 AAAATGCAGACCATAAAATGTGTTGTTGTGCGGCGATGGTGCCGTCGGAAAGGTGAGTATA
  M Q T I K C V V V G D G A V G K 16
58 AAGACAAAACGCCAATTATCAAGGTTCTCGTCTGCGGCCACGATCAGGATGTCGTTCTGG
118 ACGATGGATTAAATGGCCCTGGAGAGCTCCTACTAACGCAGACTTGTCTCCTCATCTCATA
  T C L L I S Y 23
178 CACAACAAAACAAGTTCGCCCTCTGAATACGTTCCACCAGTTTTTGACAACATATGCCGTGAC
  T T N K F P S E Y V P T V F D N Y A V T 43
238 TGTCAATGTGAGTATAACGCGTTCGCATTCTGCAAGAAACCAAGCCCCCCCCCCCCCCCC
  V M 45
298 CCAAGCAACGAATCGAGATACTAACCCAGGATTGGAGACGAGCCCTACACACTCGGACT
  I G D E P Y T L G L 55
358 GTTCGACACCCCGGTCAGGAGGATTACGACCGACTGCGACCTCTTTGTTACCCCTCAGAC
  F D T A G Q E D Y D R L R P L C Y P Q T 75
418 CGATGTTTTTCCTCGTCTGCTTTTTCCGTCACCTCTCCCGCCTCCTTTGAGAACGTCAGGA
  D V L L V C F S V T S P A S F E N V K E 95
478 GAAGTGGTCCCTGAGGTCCACCACCCTGCCCGGCGTGCCTTGCCTCATTGTTGGTAC
  K W F P E V H H H C P G V P C L I V G T 115
538 CCAGGTTGATCCCGGAAGTGACAGGATGATTCTTGACAAGCTTTCCGACACAAGTGGCG
  Q V D P R S D R M I L D K L S R H K L R 135
598 ACCCATGACCACTGAGCAAGGCTACCAGCTCGCCCGAGAAGTCCGGTGCCTCAAGTACGT
  P M T T E Q G Y Q L A R E L G A V K Y V 155
658 CGAGTGTCTGCCCTTACTCAGAAGGCTCAAGGACGTTTTTCGACGAGGCCATCGTGGC
  E C S A L T Q K G L K D V F D E A I V A 175
718 AGCTCTTGAGCCTCCAGTGGTCAAGAAGAACAAGTGCATTGTCTAGTTTTTGCA
  A L E P P V V K K N K K C I V L - 191
778 TGCTAATGGGTTAACACAACGAAAATGAATCAAGCAAGAAAAAATGTTTC

```

FIG. 4. Nucleotide sequence of the *Y1CD42* gene and deduced amino acid sequence of Y1Cdc42p. Consensus sequences for intron splicing are underlined.

and a 3'-splice acceptor CAG sequence is found one or two nucleotides downstream of the consensus TACTAAC box (69) (Fig. 4).

The deduced protein of *Y1CD42*, Y1Cdc42p, is 191 amino acids long and has a predicted molecular mass of 21,336 Da (Fig. 4) and an estimated pI of 6.09, which is characteristic of Cdc42 proteins (17). In addition, Y1Cdc42p contains all motifs required for the biological function of small GTPases of the Rho family (GDGAVGK, residues 10 to 16; TVFDNY, residues 35 to 40; DTAG, residues 57 to 60; and CIVL, residues 188 to 191) (Fig. 4 and 5). Comparison of Y1Cdc42p with Cdc42 proteins from a number of different organisms suggests that its closest relative is *Schizosaccharomyces pombe* Cdc42 (Fig. 5). Importantly, Y1Cdc42p contains the signature sequence of Cdc42 proteins (27), the motif TQXD (TQVD, residues 115 to 118) in the region responsible for nucleotide specificity.

**Strains with the *Y1RAC1* gene disrupted are viable and unaffected in mating ability.** A 1.0-kbp *ApaI-NdeI* fragment of *Y1RAC1* was replaced by a 1.6-kbp *ApaI-NdeI* fragment containing the *Y. lipolytica* *URA3* gene (Fig. 6). This construct was digested with *DraI* and *XbaI* to liberate a 2.4-kbp fragment containing the entire *URA3* gene flanked by 0.5 and 0.3 kbp of the 5' and 3' regions of the *Y1RAC1* gene, respectively. This linear fragment was used to transform the wild-type *Y. lipolytica* strain E122 to uracil prototrophy.

Of 303 Ura<sup>+</sup> transformants obtained, 3 showed a smooth phenotype after 3 days on YEPD agar plates. Two of these transformants were confirmed by Southern blot analysis and PCR to have had the *Y1RAC1* gene correctly replaced by the *URA3* gene (Fig. 6), and one of them, CHY1220-A30 (Fig. 1C and Table 1), was selected for further analysis.

Because mating has been found to be intimately connected

to dimorphism and, like dimorphism, is a phenomenon that involves dramatic changes in cell morphology in response to environmental conditions (40), we investigated whether disruption of the *Y1RAC1* gene had any effect on the mating ability of *Y. lipolytica*. Crossing of the A mating type strain CHY1220-A30 ( $\Delta$ *rac1*) with the B mating type wild-type strain 22301-3 was readily attained (Fig. 1M), indicating that no mating defect was associated with disruption of *Y1RAC1* in these strains. To determine whether the lack of effect on mating by disruption of the *Y1RAC1* gene was confined to A mating type cells, a B mating type strain, CHY1220-B36 (Fig. 1K and Table 1), with its *Y1RAC1* gene deleted, was obtained by sporulation of the diploid strain 22301-3//CHY1220-A30 (Fig. 1M and Table 1) and selection of haploids for their inability to form hyphal cells. The  $\Delta$ *rac1::URA3* genotype of the CHY1220-B36 strain was confirmed by cosegregation of the Ura<sup>+</sup> and Fil<sup>-</sup> phenotypes in random spore analysis (data not shown), and this strain was found to be able to mate to both wild-type E122 and CHY1220-A30 strains (Fig. 1N and O), demonstrating that *Y1RAC1* is not essential for mating. One copy of the *Y1RAC1* gene was sufficient to support dimorphic transition in diploid strains of *Y. lipolytica*, although a slight reduction in the proportion of hyphal cells could be observed in these strains (Fig. 1M and N).

**Disruption of the *Y1RAC1* gene affects cell morphology but does not impair actin polarization or cell invasiveness.** Since the organization of the actin cytoskeleton is directly involved in the determination of cell shape and because Rac proteins play a fundamental role in this process (21, 22, 57, 67), disruption of *Y1RAC1* was anticipated to result in morphological defects in *Y. lipolytica* cells. Indeed, exponentially growing  $\Delta$ *rac1* mutant cells were found to be round in shape, clearly contrasting with

YLcd42	MQTIKCVVVDGAVGKTCLLISYTTNKFPESEYVPTVFDNYAVTVMI GDEP	50
SpCdc42	MPTIKCVVVDGAVGKTCLLISYTTNKFPSDYVPTVFDNYAVTVMI GDEP	50
ScCdc42	MQTIKCVVVDGAVGKTCLLISYTTNKFPPADYVPTVFDNYAVTVMI GDEP	50
CaCdc42	MQTIKCVVVDGAVGKTCLLISYTTNKFPPADYVPTVFDNYAVTVMI GDEP	50
CeCdc42	M---KCVVVDGAVGKTCLLISYTTNKFPESEYVPTVFDNYAVTVMI GDEP	47
MmCdc42	MQTIKCVVVDGAVGKTCLLISYTTNKFPESEYVPTVFDNYAVTVMI GDEP	50
HsCdc42	MQTIKCVVVDGAVGKTCLLISYTTNKFPESEYVPTVFDNYAVTVMI GDEP	50
	* ****	
YLcd42	YTLGLEDTAGQEDYDRLRPLCYPQTDVFLVCFVSPTSPASFENVKEKWFEE	100
SpCdc42	YTLGLEDTAGQEDYDRLRPLSYPTDVLVCFVSPTSPASFENVKEKWFEE	100
ScCdc42	YTLGLEDTAGQEDYDRLRPLSYPTDVLVCFSVISPPSFENVKEKWFEE	100
CaCdc42	FTLGLEDTAGQEDYDRLRPLSYPTDVLVCFSVISPPSFENVKEKWFEE	100
CeCdc42	YTLGLEDTAGQEDYDRLRPLSYPTDVLVCFSVVAPASFENVKEKWFEE	97
MmCdc42	YTLGLEDTAGQEDYDRLRPLSYPTDVLVCFSVVSPSSFENVKEKWFEE	100
HsCdc42	YTLGLEDTAGQEDYDRLRPLSYPTDVLVCFSVVSPSSFENVKEKWFEE	100
	. ****	
YLcd42	VHHHCPCVPECLIVGTQVDRSDRMILDKLSRHKLKRPMTTEQGYQLARELG	150
SpCdc42	VHHHCPCVPECLIVGTQIDLRDDPSVQOKLARQHQHPLTHEQGERLARELG	150
ScCdc42	VHHHCPCVPECLIVGTQIDLRDDKVIIEKLRQRLRPIITSEQGSRLARELG	150
CaCdc42	VHHHCPCVPECLIVGTQIDLRDDVILQRLHROKLSPIITQEQGKLAKELR	150
CeCdc42	ISHHCSKTFPFLVGTQVDRDDPGMLKLEKLNKQKPVSTVDGKLAKELR	147
MmCdc42	ITHHCFKTFPFLVGTQIDLRDDPSTIEKLNKQKPIITPETAEKLARDLK	150
HsCdc42	ITHHCFKTFPFLVGTQIDLRDDPSTIEKLNKQKPIITPETAEKLARDLK	150
	. ***	
YLcd42	AVKYVECSALTQKGLKDVFEAIVAALEPPVVKKNK-KCIVL	191
SpCdc42	AVKYVECSALTQKGLKNVFEAIVAAALDPVPHKKSKKCLVL	192 (86.39%)
ScCdc42	AVKYVECSALTQKGLKNVFEAIVAALEPPVIKSK-KCAIL	191 (84.29%)
CaCdc42	AVKYVECSALTQKGLKTVFEAIVAALEPPVIKSK-KCTIL	191 (83.77%)
CeCdc42	AVKYVECSALTQKGLKNVFEAIVAAALDPVQKQK-KCNIL	188 (78.19%)
MmCdc42	AVKYVECSALTQKGLKNVFEAIVAALEPPETQPKR-KCCIF	191 (76.96%)
HsCdc42	AVKYVECSALTQKGLKNVFEAIVAALEPPEPKSR-RCVLL	191 (78.53%)
	*****	

FIG. 5. Amino acid sequence alignment of Cdc42p of *Y. lipolytica* (YLcd42p) and its homologs in *S. cerevisiae* (ScCdc42p) (28), *C. albicans* (CaCdc42p) (46), *S. pombe* (SpCdc42p) (44), *Caenorhabditis elegans* (CeCdc42p) (15), *Mus musculus* (MmCdc42p) (43), and *Homo sapiens* (HsCdc42p) (48).

the typically ovoid cells observed for wild-type strains (Fig. 7, top panels). Continued incubation of yeast cells, pseudohyphae, and a few germ tubes, while  $\Delta rac1$  cultures contained only a small proportion of pseudohyphal cells and no germ tubes (Fig. 7, middle panels). In general, pseudohyphal  $\Delta rac1$  cells were found to be shorter than their wild-type counterparts (Fig. 7, middle panels). As stationary phase was reached, hyphal growth became predominant in wild-type cultures, while only a limited number of chains composed of pseudohyphal cells were seen in the  $\Delta rac1$  cultures (Fig. 7, bottom panels). Germ tubes arising from pseudohyphal cells were sometimes seen in the wild-type strain (Fig. 7, bottom right panel, inset). Interestingly, invasive pseudohyphal growth was found to be substantially induced in the  $\Delta rac1$  strain by incubation on minimal medium containing glucose as the sole carbon source (YNBd agar), whereas this effect was not observed in  $\Delta rac1$  cells grown on minimal medium containing acetate as the sole carbon source (YNA agar) or in  $mhy1KO9$  ( $\Delta mhy1$ ) cells incubated under either condition (Fig. 8). No difference in invasiveness was observed between the original mutant strain CHY1220 and the  $\Delta rac1$  strain CHY1220-A30 when they were subjected to growth under the same conditions (data not shown).

As described for a number of fungi (1, 3, 4, 25, 34, 42, 60, 72), actin-rich zones at the sites of growth (apices of germ tubes, hyphae, pseudohyphae, and yeast cells), combined with a background of diffuse actin staining with punctate actin patches, were observed for wild-type cells of *Y. lipolytica* (Fig. 9A to G). Interestingly, despite alterations in cell morphology,  $\Delta rac1$  mutant cells appeared to retain the ability to concentrate actin granules at the apices of pseudohyphal cells and emerging buds (Fig. 9H and I).

**Transcription of the *YIRAC1* gene is increased during the yeast-to-hypha transition.** The dimorphic switch was induced in exponentially growing E122 cells by a 15-min carbon source

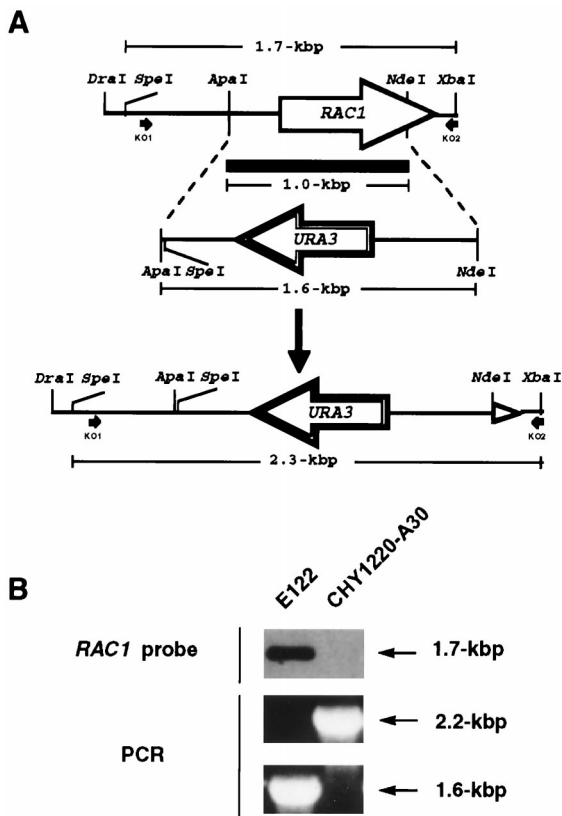


FIG. 6. Integrative disruption of the *YIRAC1* gene. (A) Diagram illustrating the replacement of a 1.0-kbp *ApaI-NdeI* fragment of *YIRAC1* by a 1.6-kbp *ApaI-NdeI* fragment containing the *Y. lipolytica* *URA3* gene. (B) Southern blot analysis of *SpeI-HpaI*-digested genomic DNA, and PCR analysis of total genomic DNA, from wild-type strain E122 and strain CHY1220-A30, confirming the correct replacement of the *YIRAC1* gene with the *URA3*-containing linear molecule in strain CHY1220-A30. Primers KO1 and KO2 (Table 2) are indicated by black arrows in panel A.

starvation period at 4°C, followed by transfer to prewarmed (28°C) YNBGlcNAc. Under these conditions, more than 80% of the cell population gave rise to germ tubes after 10 h of incubation, while cells transferred to fresh YNBGlc grew almost exclusively as the budding form, as described previously (20, 26). Northern blot analysis performed with total RNA extracted from cells harvested at 0, 1, 3, and 10 h of incubation in YNBGlcNAc showed that *YIRAC1* mRNA levels increased steadily during the yeast-to-hypha transition, but they remained virtually constant during incubation in YNBGlc (Fig. 10).

**Putative STREs in the *YIRAC1* gene are not necessary for the induction of hyphal growth.** Genetic interaction between *YIRAC1* and *MHY1* was initially suggested by the observation that a pINA445-based multicopy plasmid bearing the *MHY1* gene (pMHY1) (26) was able to restore hyphal growth upon introduction into CHY1220, but not CHY1220-A30 ( $\Delta rac1$ ), cells (Fig. 1H and I). The inability of pRAC1 to induce dimorphic transition in  $\Delta mhy1$  cells (Fig. 1D and G); the presence of three copies of the pentanucleotide STRE sequence CCCCT in the promoter region of *YIRAC1* (Fig. 2), one of which has been shown to specifically bind in vitro-synthesized Mhy1p (26); and the finding that  $\Delta rac1$  mutant cells can form pseudohyphae, while  $\Delta mhy1$  mutants are unable to grow as either the hyphal or pseudohyphal form (Fig. 7 and 8) sug-

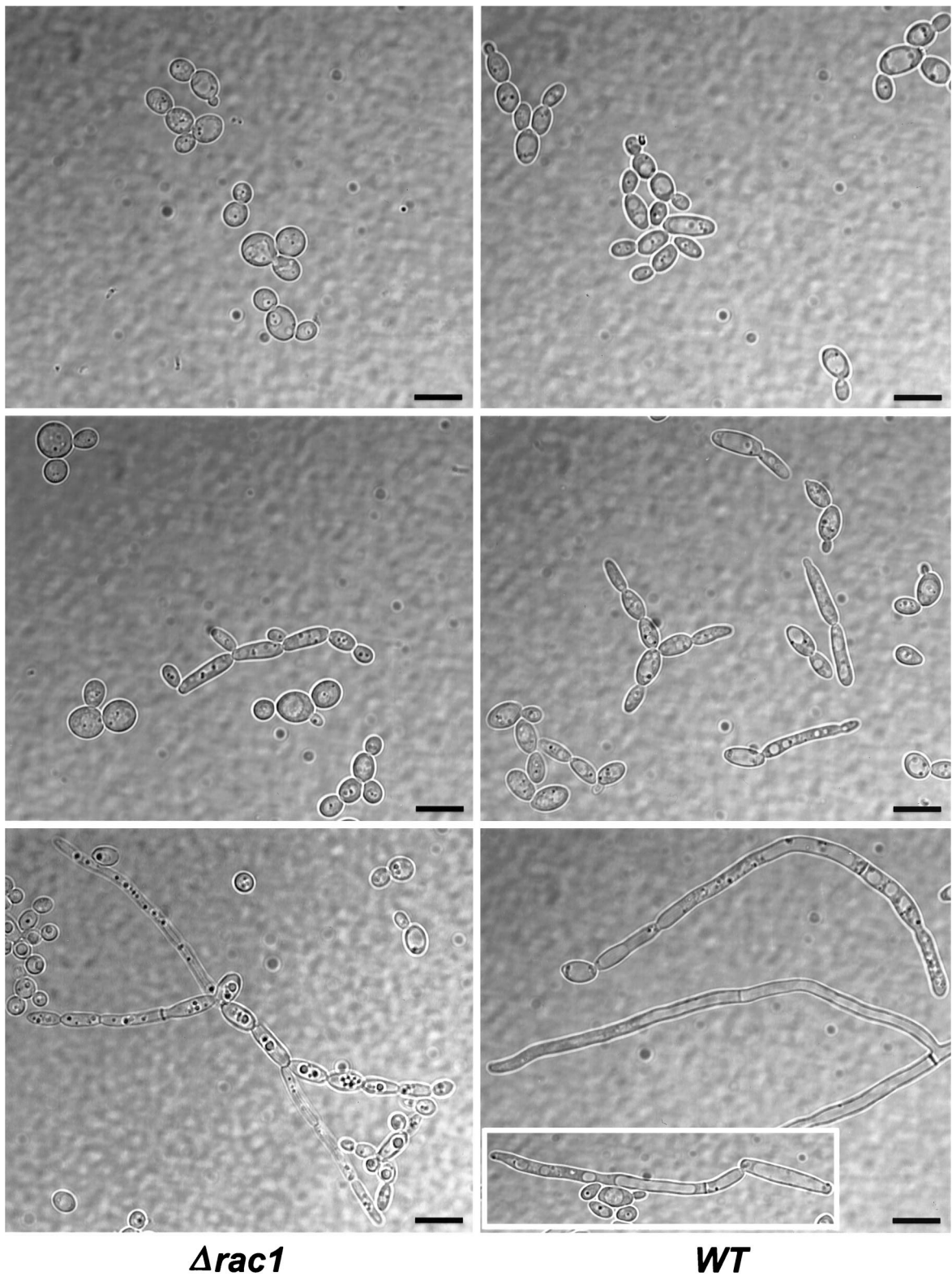


FIG. 7. Disruption of *YIRAC1* affects cell morphology and impairs hyphal growth, but not pseudohyphal growth, in *Y. lipolytica*. Strains were grown in YEPD. Top panels, exponential growth phase (optical density at 600 nm [OD<sub>600</sub>] = 1). Middle panels, late exponential growth phase (OD<sub>600</sub> = 4). Bottom panels and inset, stationary phase (OD<sub>600</sub> = 10). WT, wild-type strain E122. *Δrac1*, strain CHY1220-A30. Bars, 5 μm.

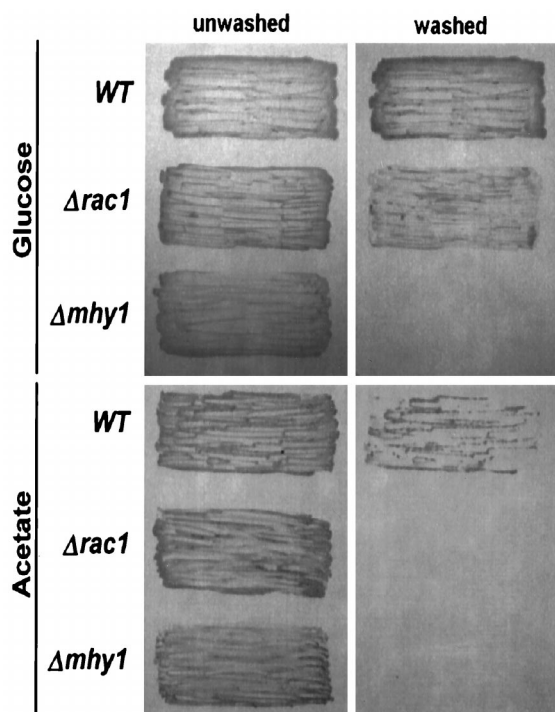


FIG. 8. Invasive filamentous growth by different *Y. lipolytica* strains. Following 5 days of incubation at 28°C on minimal agar medium containing glucose or acetate as the sole carbon source, plates were washed with running water to remove cells from the agar surface. Pictures were taken before and after washes. WT, wild-type strain E122.  $\Delta rac1$ , strain CHY1220-A30.  $\Delta mhy1$ , strain mhy1KO9.

gested that Mhy1p might act to promote hyphal growth through these regulatory elements via *YIRAC1*. In order to investigate the role of these putative STRE sequences in the induction of hyphal growth, mutagenesis of these elements in pRAC1 was performed. No defect was observed in the ability to induce the dimorphic transition upon introduction of the plasmid pRAC1-Mut (which contains mutations in all three STREs) into a  $\Delta rac1$  strain (CHY1220-A30), suggesting that these elements are not necessary for the induction of hyphal growth via *YIRAC1* (data not shown).

**Genomic DNA analysis of RAC genes in *Y. lipolytica*.** As most organisms have multiple Rac and Rho homologs, we looked for evidence of other RAC genes in *Y. lipolytica*. Genomic DNA from the E122 strain was digested with various combinations of restriction endonucleases and analyzed by Southern blotting under low-stringency conditions with a labeled 240-bp *SacII-NdeI* fragment of the *YIRAC1* gene. A complex pattern of bands was observed, suggestive of the presence of several RAC-related superfamily members in *Y. lipolytica* (Fig. 11).

## DISCUSSION

Rac proteins have been implicated in the control of a diverse and extensive set of cellular processes (74), including notably the regulation of the assembly and organization of the actin cytoskeleton (21, 22, 49, 56, 57, 66, 67, 70). As a consequence, Rac-mediated changes in actin organization or in gene expression are believed to play a pivotal role in the control of cell shape, cell attachment, cell motility and invasion, cell-cell interaction, and cell proliferation and differentiation (74). In plants, Rac homologs are thought to be involved in the regulation of growth of the pollen tube, a process that shares

several characteristics with filamentous growth in fungi. Pollen tube elongation is based on a process known as tip growth, where polarized secretion is restricted to the apex, and cell membrane and cell wall material are delivered exclusively to this location (33, 37, 64, 68). Significantly, plant Rac homologs have been shown to be localized to the plasma membrane of the pollen tube in the region of the tip (37). The localization of *YIRac1p* in *Y. lipolytica* has so far remained elusive. Although an epitope-tagged *YIRac1p* could be detected at the growing tip region of filamentous *Y. lipolytica* cells (data not shown), this fusion protein was unable to induce hyphal growth in  $\Delta rac1$  cells, making it impossible to ascertain whether the distribution of the tagged protein was truly representative of that of the natural protein. The availability of antibodies specific for *YIRac1p* will be extremely useful in addressing this question.

Although no Rac homolog had previously been described for fungi, a newly identified *S. cerevisiae* ORF, Ynl180c, codes for a protein with a number of features typical of Rac proteins, including the presence of all conserved motifs required for biological function and an estimated pI of 8.76. However, this putative protein, which is only 52.1% identical to *YIRac1p*, is at 331 amino acids considerably larger than all known Rac proteins, and it is not known at this time whether it is functional.

Disruption of the *YIRAC1* gene is not lethal and does not abolish the ability of cells to polarize actin at the site of growth. These findings might be explained by the presence of other genes in *Y. lipolytica* that are closely related to *YIRAC1*, as suggested by the complex banding pattern revealed by Southern analysis of *Y. lipolytica* genomic DNA under conditions of low stringency. Nevertheless, alterations in the cell morphology of  $\Delta rac1$  mutants and the inability of these strains to grow as the hyphal form strongly suggest that *YIRAC1* functions in some aspect of the polarization of cell growth.

We have previously reported the isolation and characterization of the gene *MHY1*, which codes for a putative transcription factor that binds in vitro to an oligonucleotide containing STRE1 from *YIRAC1* (Fig. 2) and is essential for filamentous growth in *Y. lipolytica* (26). Here we report that while deletion of *MHY1* completely abolishes the ability of *Y. lipolytica* to grow as both the hyphal and pseudohyphal forms on solid minimal medium containing either glucose or acetate as the sole carbon source, strains lacking a functional *YIRAC1* gene are still able to form pseudohyphae and invade agar on glucose-based minimal medium, suggesting, as has been suggested for *C. albicans* (38), that these two morphologies in *Y. lipolytica* are controlled, at least in part, by two parallel signaling pathways, each with a different and additive input, or that they represent a sequence of events in a single pathway of filamentous growth requiring a quantitatively stronger regulatory input to produce hyphae rather than pseudohyphae. Likewise, the analysis of a Ras homolog in *Aspergillus nidulans* has suggested a scenario in which several thresholds of Ras concentration exist, each of which allows development to proceed to a certain point, producing the proper cell type while inhibiting further development (63). Our findings that overexpression of *MHY1* can restore hyphal growth in CHY1220 but not in CHY1220-A30 ( $\Delta rac1$ ) cells, that disruption of *YIRAC1* affects only hyphal growth while disruption of *MHY1* blocks both hyphal and pseudohyphal growth, and that pseudohyphal cells can give rise to hyphae (Fig. 7, bottom right panel, inset) support such a scenario and suggest that *MHY1* acts upstream of *YIRAC1* in the filamentous pathway(s).

It is important to point out that regardless of the fact that mutagenesis of all three putative STREs in the promoter of *YIRAC1* did not affect its ability to induce hyphal growth in *Y. lipolytica* (data not shown), a role for these elements in the



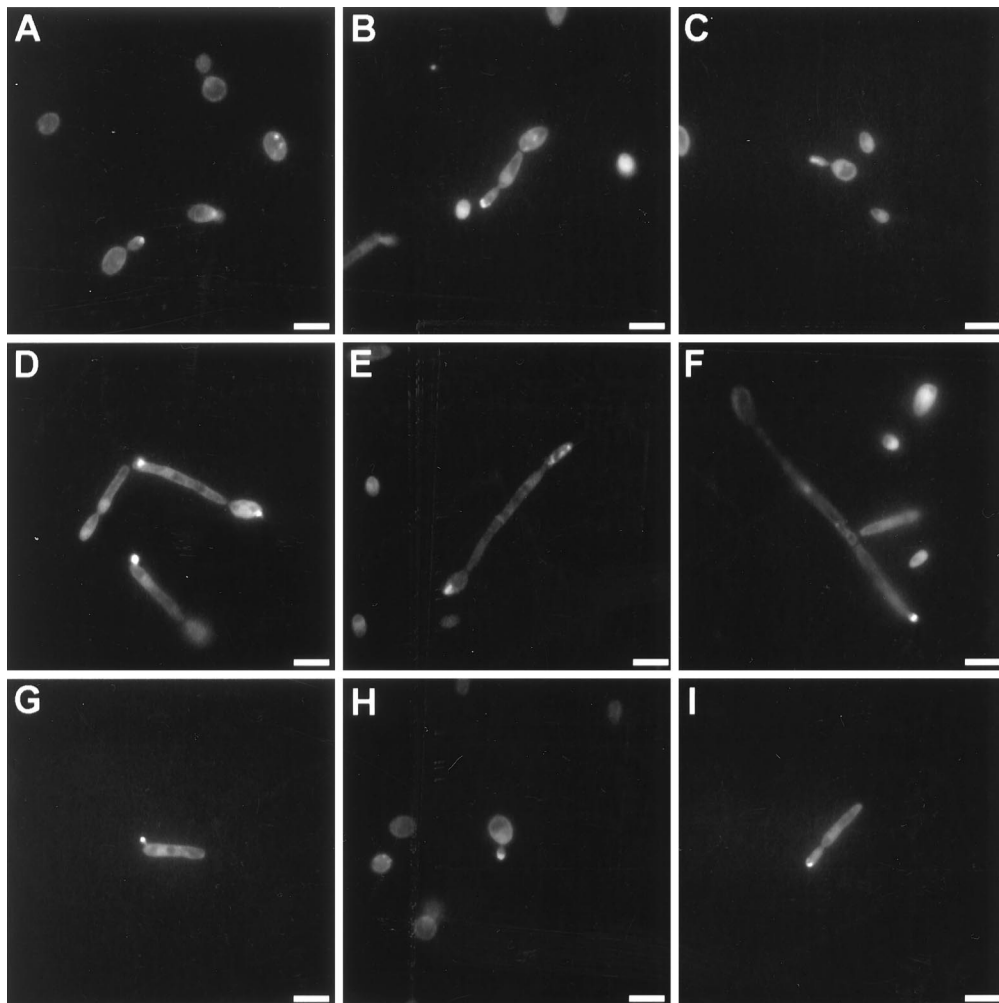


FIG. 9. Actin localization during different stages of development of wild-type and  $\Delta rac1$  cells. Actin was detected by staining of cells with Oregon Green 488 phalloidin followed by fluorescence microscopy. (A to G) Wild-type strain E122. (H and I)  $\Delta rac1$  strain CHY1220-A30. (A and H) Yeast-like cells; (B, G, and I) pseudohyphal growth; (C) early germ tube formation; (D) late germ tube formation; (E and F) hyphal growth. Bars, 5  $\mu\text{m}$ .

induction of dimorphism cannot be ruled out. The activity of other unidentified regulatory elements in the *YIRAC1* gene or compensation for the loss of transcriptional induction of *YIRAC1* by the activation of other related GTPases may explain this negative result. We are currently investigating these possibilities.

Here we also report the isolation and initial characterization of the *YICDC42* gene. In *C. albicans*, a transient increase in the *CDC42* mRNA levels was observed during the switch to hyphal growth (46), but it still remains to be determined whether Cdc42p also plays a role in pseudohyphal formation in this organism. Although no variation in the abundance of Cdc42p has been observed during the cell cycle of *S. cerevisiae* (73), *CDC42* has been shown to be a potent regulator of filamentous growth in this yeast, acting downstream of *RAS2* and activating pseudohyphal growth of diploid cells and invasive growth of haploid cells in response to nitrogen starvation via *STE20* (35, 47, 54). We are currently investigating whether *YICDC42* also plays a role in the induction of filamentous growth in *Y. lipolytica*.

In conclusion, we report the isolation and initial characterization of the first fungal Rac homolog and provide evidence that *YIRac1p* plays an important role in the regulation of hy-

phal growth in *Y. lipolytica*. Greater knowledge of the function(s) of *YIRAC1* and of its interactions with other *Y. lipolytica* genes will be important for a better understanding of the mechanisms by which environmental conditions induce

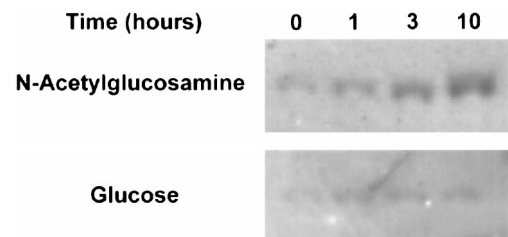


FIG. 10. *YIRAC1* mRNA levels are increased during the dimorphic transition. Total RNA was isolated from E122 cells incubated at 28°C in YNBGlucNAc (induction of filamentous growth) or YNBGluc (control culture, yeast-like cells) for the times indicated and subjected to Northern blot analysis. Ten micrograms of RNA from each time point was separated on a formaldehyde agarose gel and transferred to nitrocellulose. Blots were hybridized with a probe specific for the *YIRAC1* gene (1.0-kbp *ApaI-NdeI* fragment [see Fig. 6]). Equal loading of RNA was ensured by ethidium bromide staining (data not shown).

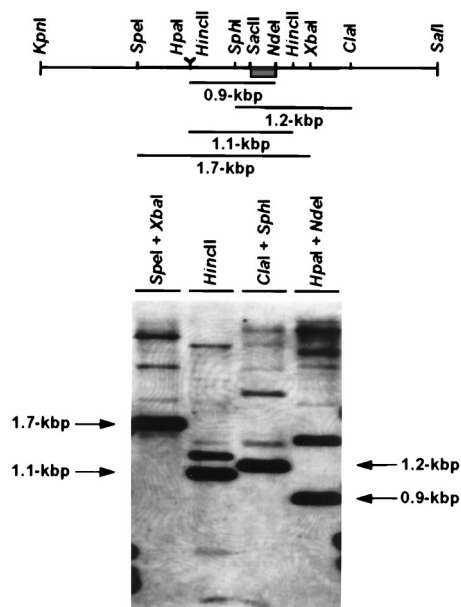


FIG. 11. Southern blot analysis of E122 genomic DNA. Ten micrograms of DNA per lane was digested with the indicated restriction enzymes, separated by electrophoresis, transferred to nitrocellulose, and probed with a 240-bp *SacII-NdeI*-labeled fragment from *YIRAC1* (boxed), as described in Materials and Methods.

changes in the pattern of cell growth, a phenomenon with strong implications for the development of virulence by fungal pathogens (8, 38, 41, 52, 62) and for the elucidation of the molecular mechanisms controlling differentiation in higher eukaryotes. Furthermore, as one of the mechanisms of Ras transformation relies on signaling cascades controlled by Rac and Rho GTPases, the dimorphic yeast *Y. lipolytica* may represent a more suitable model for the understanding of such complex events in mammalian cells, with important implications for the search for potential drug targets for Ras-mediated malignancies (29, 66, 74).

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