Crosstalk between the Ras2p-controlled Mitogen-activated Protein Kinase and cAMP Pathways during Invasive Growth of *Saccharomyces cerevisiae*

Hans-Ulrich Mösch,* Eric Kübler,* Sven Krappmann,* Gerald R. Fink,[†] and Gerhard H. Braus*[‡]

*Institute for Microbiology and Genetics, Georg-August-University, D-37077 Göttingen, Germany; and [†]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142

The two highly conserved *RAS* genes of the budding yeast *Saccharomyces cerevisiae* are redundant for viability. Here we show that haploid invasive growth development depends on *RAS2* but not *RAS1*. Ras1p is not sufficiently expressed to induce invasive growth. Ras2p activates invasive growth using either of two downstream signaling pathways, the filamentation MAPK (Cdc42p/Ste20p/MAPK) cascade or the cAMP-dependent protein kinase (Cyr1p/cAMP/PKA) pathway. This signal branch point can be uncoupled in cells expressing Ras2p mutant proteins that carry amino acid substitutions in the adenylyl cyclase interaction domain and therefore activate invasive growth solely dependent on the MAPK cascade. Both Ras2p-controlled signaling pathways stimulate expression of the filamentation response element-driven reporter gene depending on the transcription factors Ste12p and Tec1p, indicating a crosstalk between the MAPK and the cAMP signaling pathways in haploid cells during invasive growth.

INTRODUCTION

When starved for nitrogen, diploid strains of *Saccharomyces cerevisiae* undergo a developmental transition from growth as single yeast form cells to a multicellular form consisting of filaments of pseudohyphal cells. Filamentous growth, also referred to as pseudohyphal growth, is a composite of several distinct and genetically dissectable processes: cell elongation, filament formation, and agar penetration (Gimeno *et al.*, 1992; Mösch and Fink, 1997). A related phenomenon, invasive growth, occurs in haploid cells (Roberts and Fink, 1994). Haploid invasiveness is distinct from diploid filamentous growth, because haploids are able to invade agar substrate rich in nitrogen, whereas diploids are not. In addition, haploid invasion does not require *BUD* gene functions, which regulate the budding patterns of haploid and diploid cells.

Haploid invasive growth requires some of the signaling components that also regulate diploid filamen-tous growth. These include the p65[PAK] kinase homologue Ste20p, Ste11p (MAPK kinase kinase), and Ste7p (MAPK kinase) protein kinases and the transcription factor Ste12p proteins that constitute part of the MAPK module, which is also required for mating (Liu *et al.*, 1993). In diploids, the transcription factor Tec1p, a regulator originally identified to control expression of yeast transposons (Laloux *et al.*, 1990), is also required for filamentous growth (Gavrias *et al.*, 1996; Mösch and Fink, 1997). Tec1p contains the TEA/ATTS DNA-binding domain, which is sheared by several eukaryotic transcription factors, including the human TEF-1 and *Aspergillus* abaA (Andrianopoulos and Timberlake, 1991; Burglin, 1991). One of the functions of Tec1p is to target Ste12p to filamentation response elements (FREs) through cooperative binding (Madhani and Fink, 1997). FREs are necessary and sufficient to direct filamentation-specific gene expression and are present in the promoter regions of at least two genes required for pseudohyphal development, *TEC1* (Madhani and Fink, 1997) and *FLO11* (Lo and Dranginis, 1998). The role of Tec1p in haploid invasive growth has not been studied.

[‡] Corresponding author: E-mail address: gbraus@gwdg.de.

Table 1. Strains used in this study

Strain	Genotype	Source
YHUM86	*MATα, ras2Δ::ura3::HIS3, FG(Ty1)::lacZ::URA3, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM108	*MATα, ras1Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM114	*MATα, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM120	*MATα, ras2Δ::ura3::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM143	*MATα, ste12Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM145	*MATα, tec1Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM158	*MATα, ras2Δ::ura3::HIS3, ste20Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM167	*MAT**a**, ras2Δ::ura3::HIS3, tec1Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM175	*MATα, ras2Δ::ura3::HIS3, ste12Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study
YHUM184	*MAT**a**, ste20Δ::HIS3, FRE(Ty1)::lacZ::LEU2, ura3-52, leu2::hisG, his3::hisG, trp1::hisG*	This study

Activated alleles of *CDC42* (*CDC42^Val12^* and *CDC42^Leu61^*) encoding a small G protein, induce diploid filamentous growth, and also increase transcription of the FRE reporter gene (Mösch *et al.*, 1996). Therefore, they are presumably located upstream of the MAPK module in the filamentous growth signal transduction pathway. The function of Cdc42p in regulation of haploid invasive growth has not yet been studied.

Recently it was found that the dominant active form of *RAS2*, *RAS2^Val19^*, stimulates expression of the transcriptional reporter that contains the FRE filamentous growth response element (Mösch *et al.*, 1996). Full induction of the reporter and of filamentous growth of diploid yeast requires *STE20, STE11, STE7* and *STE12*. Genetic studies suggest that *RAS2* is located upstream of *CDC42* in the signal transduction pathway. It is unknown whether *RAS2* is also involved in haploid invasive growth. The Ras guanine nucleotide–binding proteins play a key role in signal processes that regulate proliferation and differentiation in eukaryotes (McCormick, 1995). The budding yeast *S. cerevisiae* possesses two Ras protein homologues encoded be the two genes *RAS1* and *RAS2*. The best characterized target of Ras proteins in *S. cerevisiae* is adenylyl cyclase encoded by the *CYR1* gene (for review, see Broach, 1991). Activation of Ras in *S. cerevisiae* results in elevated intracellular cAMP levels that in turn activate the cAMP-dependent protein kinase (PKA or A kinase). This enzyme is composed of an inhibitory subunit Bcy1p and a catalytic subunit encoded by the three redundant genes, *TPK1, TPK2,* and *TPK3*. Activation of the A kinase by expression of the dominant *RAS2^Val19^* allele also confers sensitivity to heat shock and nutrient starvation, loss of carbohydrate reserves, and a block to sporulation. In addition, Ras2 protein seems to be involved in the control of cell division (Morishita *et al.*, 1995) and the yeast lifespan (Sun *et al.*, 1994).

Several recent studies indicate that filamentous growth is also regulated by the cAMP/A kinase pathway (Ward *et al.*, 1995; Kübler *et al.*, 1997; Lorenz and Heitman, 1997). This suggests that Ras2p regulates filamentous growth by two separate pathways, one involving the MAPK cascade and another mediated by cAMP. The function of *RAS1*, the second *RAS* gene in *S. cerevisiae*, in regulating pseudohyphal development or haploid invasive growth is not known.

Elements of the invasive growth signaling network are highly conserved in evolution and may provide an interesting paradigm for the understanding of signal transduction in all eukaryotes. Here we studied the role of Ras proteins in haploid invasive growth of yeast. We present evidence that Ras proteins perform distinct functions in regulating invasive growth development. We show that 1) only *RAS2* but not *RAS1* is sufficiently expressed to regulate invasive growth development; 2) invasive growth signaling by Ras2p is mediated by both effector pathways, the Cdc42p/Ste20/MAPK cascade and the A kinase pathway; 3) expression of specific *RAS2* alleles can uncouple these two effector pathways; and 4) the A kinase stimulates expression of the MAPK cascade–controlled FRE reporter gene, indicating a crosstalk between both signaling pathways.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

All yeast strains used in this study are congenic to the Σ1278b genetic background (Table 1). The *ras1Δ::HIS3, ste20Δ::HIS3, ste12Δ::HIS3, tec1Δ::HIS3* deletion mutations were introduced using deletion plasmids pras1Δ::HIS3, pste20Δ::HIS3, pste12Δ::HIS3, and ptec1Δ::HIS3 (Table 2). The *ras2Δ::ura3::HIS3* deletion mutation was obtained by 1) using the deletion plasmid pras2Δ::URA3 (Kataoka *et al.*, 1984) and 2) subsequent disruption of the *ras2Δ::URA3* locus using the linear ura3::HIS3 *URA3* disruption cassette (from Yona Kassir, Technion, Haifa, Israel). The *bcy1* mutation was introduced using the disruption plasmid pbcy1::URA3 (Toda *et al.*, 1987a). The *FG(Ty1)::lacZ* reporter cassette was integrated at the *URA3* locus using plasmid YIp356R-FG(Ty1)::lacZ (from Steve Kron, University of Chicago, Chicago IL). The *FRE(Ty1)::lacZ* reporter was integrated at the *LEU2* locus using YIpFRE(Ty1)::lacZ.

Standard methods for genetic crosses and transformation were used, and standard yeast culture medium was prepared essentially as described (Guthrie and Fink, 1991). Synthetic complete (SC) medium lacking appropriate supplements was used for scoring invasive growth or β-galactosidase assays with either 2% glucose or

Table 2. Plasmids used in this study

Plasmid	Description	Source
pras1Δ::HIS3	Cassette for full deletion of *RAS1*-open reading frame	This study
pras2Δ::URA3	Cassette for full deletion of *RAS2*-open reading frame	M. Wigler
pste20Δ::HIS3	Cassette for full deletion of *STE20*-open reading frame	This study
pste12Δ::HIS3	Cassette for full deletion of *STE12*-open reading frame	This study
ptec1Δ::HIS3	Cassette for full deletion of *TEC1*-open reading frame	This study
pRS316-RAS2	3-kb fragment containing *RAS2* in pRS316	This study
YCplac22-RAS2	3-kb fragment containing *RAS2* in YCplac22	This study
YEplac112-RAS2	3-kb fragment containing *RAS2* in YEplac112	M. Wigler
YCp50-RAS2^{Val19}	3-kb fragment containing *RAS2^{Val19}* in YCp50	M. Wigler
YCplac22-RAS2^{Val19}	3-kb fragment containing *RAS2^{Val19}* in YCplac22	This study
YCplac22-RAS2^{Gly41}	3-kb fragment containing *RAS2^{Gly41}* in YCplac22	This study
YCplac22-RAS2^{Asn45}	3-kb fragment containing *RAS2^{Asn45}* in YCplac22	This study
YCplac22-RAS2^{Val19Gly41}	3-kb fragment containing *RAS2^{Val19Gly41}* in YCplac22	This study
YCplac22-RAS2^{Val19Asn45}	3-kb fragment containing *RAS2^{Val19Asn45}* in YCplac22	This study
pRS314-RAS2ΔC	3-kb fragment containing *RAS2* with C-terminal deletion in pRS314	This study
pRS314-R2N-R1C	3.5-kb fragment containing *RAS2-RAS1* chimeric gene in pRS314	This study
YCplac22-R2p-R1orf	3.6-kb fragment containing *RAS2*-promoter fused to *RAS1*-ORF in YCplac22	This study
YEplac112-RAS1	3-kb fragment containing *RAS1* in YEplac112	This study
pRS316-GAL-CDC42^{Val12}	2.5-kb fragment containing *CDC42^{Val12}* fused to *GAL1-10*-promoter in pRS316	This study
pRS316-GAL-CDC42^{Leu61}	2.5-kb fragment containing *CDC42^{Leu61}* fused to *GAL1-10*-promoter in pRS316	This study
pRS426-STE20	4.9-kb fragment containing *STE20* in pRS426	Mösch *et al.*, 1996
pYGEX-STE20	*GST-STE20* fusion gene under control of the *GAL1-10*-promoter	Roberts *et al.*, 1997
B2616	5.6-kb fragment containing *STE11-4* in YCp50	G. Sprague
pRS426-STE12	5-kb fragment containing *STE12* in pRS426	This study
B2065	*GAL1-10::STE12* fragment in *URA3* marked 2-micron plasmid	B. Errede
pRS202-TEC1	6.5-kb fragment containing *TEC1* in pRS202	Mösch and Fink, 1997
pGAL-TEC1	*TEC1*-cDNA fused to *GAL1-10*-promoter in pRS316	This study
YEplac195-TPK1	2.5-kb fragment containing *TPK1* in YEplac195	This study
pRS426-TPK2	5.3-kb fragment containing *TPK2* in pRS426	This study
pRS426-TPK3	3.9-kb fragment containing *TPK3* in pRS426	This study
YIpFRE(Ty1)::lacZ	Integrative *FRE(Ty1)::lacZ* reporter construct	This study
YIp356R-FG(Ty1)::lacZ	Integrative *FG(Ty1)::lacZ* reporter construct	S. Kron

a mixture of 3% raffinose and 0.1% galactose as carbon sources. Invasive growth tests were performed as described previously using solid SC medium lacking appropriate supplements (Roberts and Fink, 1994).

Plasmids

Plasmids are listed in Table 2. Deletion alleles for *RAS1*, *STE20*, *STE12*, and *TEC1* were created by replacement of coding sequences by either the *HIS3*- or the *TRP1*-selectable marker. Plasmids pRS316-RAS2 and YCplac22-RAS2 were constructed by insertion of a 3-kb *RAS2 Eco*RI–*Hin*dIII fragment into vectors pRS316 (Sikorski and Hieter, 1989) and YCplac22 (Gietz and Sugino, 1988), respectively. YCplac22-RAS2^{Val19} was obtained by subcloning of a 3-kb *RAS2^{Val19} Eco*RI–*Hin*dIII fragment from YCp50-RAS2^{Val19} (Michael Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) into YCplac22. The *RAS2^{Gly41}*, *RAS2^{Asn45}*, *RAS2^{Val19Gly41}*, and *RAS2^{Val19Asn45}* alleles were constructed by oligonucleotide-directed mutagenesis using a two-step PCR technique. pRS314-RAS2ΔC and pRS314-R2N-R1C were obtained by subcloning of a 3-kb *Sal*I–*Not*I fragment from p413-RAS2Δ or a 3.5-kb *Sal*I–*Not*I fragment from p413-r2r1 (Hurwitz *et al.*, 1995) into pRS314 (Sikorski and Hieter, 1989). Plasmid YCplac22-R2p-R1orf, carrying the *RAS1* ORF under the control of the *RAS2* promoter, was constructed by 1) separate amplification of both the *RAS1* ORF and the *RAS2* promoter region using PCR and 2) insertion of both PCR products into YCplac22 (Gietz and Sugino, 1988). YEplac112-RAS1 was obtained by subcloning of a 3-kb *Bam*HI–*Eco*RI fragment carrying *RAS1* from p413-RAS1 (Hurwitz *et al.*, 1995) into YEplac112 (Gietz and Sugino, 1988).

pRS316-CDC42^{Val12} and pRS316-CDC42^{Leu61} were constructed by subcloning of 2.4-kb fragments containing either *CDC42^{Val12}* or *CDC42^{Leu61}* under control of the *GAL1-10* promoter from plasmids pGAL-CDC42^{Val12} and pGAL-CDC42^{Leu61} (Ziman *et al.*, 1991) into pRS316. pRS426-STE12 was obtained by insertion of a 5-kb *Sal*I–*Hin*dIII fragment carrying the *STE12* locus into pRS426 (Christianson *et al.*, 1992). YEplac195-TPK1 was constructed by insertion of a 2.5-kb *Sph*I–*Hin*dIII fragment carrying *TPK1* from plasmid YEp-TPK1 (Toda *et al.*, 1987b) into YEplac195 (Gietz and Sugino, 1988). pRS426-TPK2 and pRS426-TPK3 were obtained by subcloning of a 5.3-kb *Sal*I–*Eco*RI fragment containing *TPK2* from pMB05 (Maria Mazón, Universidad Autonoma de Madrid, Madrid, Spain) or a 3.9-kb *Sal*I–*Sac*I fragment carrying *TPK3* from plasmid pSA103 (Kelly Tatchell, Louisiana State University, Shreveport, LA) into pRS426. pGAL-TEC1 was isolated from the yeast cDNA library constructed by (Liu *et al.*, 1992) using colony hybridizations with a *TEC1*-specific ^{32}P-radiolabeled probe. YIpFRE(Ty1)::lacZ was constructed by replacing a 5-kb *Sal*I–*Hin*dIII fragment carrying both the yeast 2μ DNA region and *URA3* for a 2.5-kb *LEU2* fragment in plasmid FRE(Ty1)::lacZ (Madhani and Fink, 1997).

β-Galactosidase Assay

Assays were performed on extracts of cultures grown on solid media for 30 h as described previously (Mösch *et al.*, 1996). Specific β-galactosidase activity was normalized to the total protein in each extract and equals $(OD_{420} \times 1.7)/(0.0045 \times$ protein concentration $\times$ extract volume $\times$ time). Assays were performed on at least three

independent transformants, and the mean value is presented. SD did not exceed 15%.

Cell Extracts and Western Blot Analysis

Protein extracts were prepared from 10^7 cells of cultures grown on solid SC medium lacking the appropriate supplements for 30 h according to a method described earlier (Yaffe and Schatz, 1984). Proteins were then separated using SDS-PAGE and transferred onto nitrocellulose membranes. Ras proteins were detected using ECL technology (Amersham, Buckinghamshire, United Kingdom) after incubation of membranes with the rat monoclonal anti-H-Ras antibody (259) (Santa Cruz Biotechnology, Santa Cruz, CA) and a peroxidase-coupled goat anti-rat IgG secondary antibody (Dianova, Hamburg, Germany).

Northern Blot Analysis

Total RNA was prepared from cultures grown on solid media for 30 h according to the method described by Cross and Tinkelenberg (1991). Total RNA was separated on a 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes as described earlier (Mösch *et al.*, 1992). *RAS1*, *RAS2*, and *ACT1* transcripts were detected using gene-specific ^{32}P-radiolabeled DNA probes.

RESULTS

RAS2 Is Required for Invasive Growth Development in Haploid Yeast

The requirement of both *RAS* genes, *RAS1* and *RAS2*, for haploid invasive growth was determined, because Ras2p is a potent regulator of pseudohyphal differentiation of diploid cells under nitrogen starvation conditions (Gimeno *et al.*, 1992; Mösch *et al.*, 1996). Haploid strains carrying complete deletions of *RAS1* or *RAS2* were constructed and tested for invasive growth on rich medium. Yeast strains with full deletions of either *STE20*, *STE12*, or *TEC1* were used as controls. Figure 1A shows that deletion of *RAS2* prevents invasive growth to the same extent as inactivation of *STE20*, *STE12*, or *TEC1*. However, deletion of *RAS1* does not affect invasive growth, because a *ras1* strain still penetrates agar indistinguishable from a control strain carrying both *RAS* genes. Thus, haploid yeast strains require *RAS2* but not *RAS1* for their invasive growth phase.

Ras2 mutants are phenotypically identical to *ste20*, *ste12*, and *tec1* strains. Therefore, we tested whether *RAS2* is required for FRE-dependent reporter gene expression. A single copy of the *FRE(Ty1)::lacZ* reporter gene (Madhani and Fink, 1997) was integrated at the *LEU2* locus in *ras1*, *ras2*, *ste20*, *ste12*, and *tec1* mutants, and expression was measured on rich medium. FRE-dependent reporter gene expression is reduced twofold in the absence of *RAS2*, threefold without *STE20*, and ~50-fold when either *STE12* or *TEC1* is deleted (Figure 1B). The same twofold decrease in FRE-dependent reporter gene expression in the absence of *RAS2* is found when the *FG(Ty1)::lacZ* reporter gene (Mösch *et al.*, 1996) is used (Figure 2D).
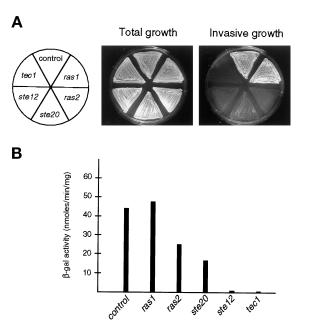
Figure 1. *RAS2* is required for haploid invasive growth and FRE-dependent transcription. (A) Yeast strains YHUM114 (control), YHUM108 (*ras1*), YHUM120 (*ras2*), YHUM184 (*ste20*), YHUM143 (*ste12*), and YHUM145 (*tec1*) were patched on SC-Leu medium. After incubation the plate was photographed before (Total growth) and after (Invasive growth) washing the cells of the plate. (B) *FRE(Ty1)::lacZ* expression levels. β-Galactosidase activity was measured on the strains shown in A. Units are nanomoles per minute per milligram. Bars depict means of three independent measurements.

Furthermore, expression of the dominant active *RAS2^Val19* allele induces transcription of an FRE reporter gene sevenfold when compared with a strain lacking *RAS2* (Figure 3B). These data show that *RAS2* is essential for invasive growth and suggest a function of Ras2p in the haploid invasive growth signaling pathway described earlier (Roberts and Fink, 1994; Madhani *et al.*, 1997).

Expression of the Conserved N Terminus of Ras2p Is Sufficient to Activate Haploid Invasive Growth

In contrast to *RAS2*, *RAS1* is not required for invasive growth. We determined whether this distinct requirement of both *RAS* genes in invasive growth regulation is due to structural differences between the two Ras proteins. Although the N-terminal 180 amino acids of Ras1p and Ras2p are 91% homologous, no similarity is found between the hypervariable C-terminal regions. Thus, different *RAS* gene constructs were transformed into a *ras2* mutant carrying a copy of the *FG(Ty1)::lacZ* reporter gene (Mösch *et al.*, 1996). Resulting strains were assayed for invasive growth and expression of the FRE-driven *FG(Ty1)::lacZ* reporter gene. Expression levels of the different *RAS* gene constructs were monitored using Western blot analysis (Figure 2).
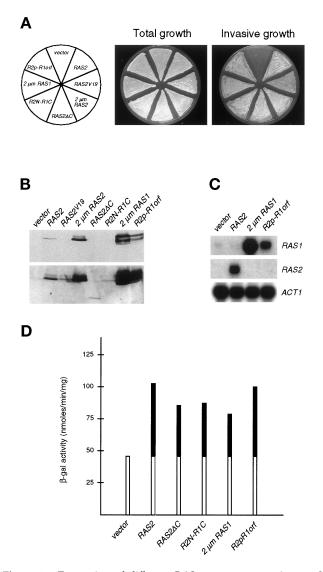
A

Total growth   Invasive growth

B

C

D

Figure 2. Expression of different *RAS* gene constructs in a *ras2* mutant strain. (A) Shown is invasive growth of yeast strain YHUM86 (*ras2*) containing YCplac22 (vector), YCplac22-RAS2 (*RAS2*), YCplac22-RAS2[Val19] (*RAS2V19*), YEplac112-RAS2 (2 μm *RAS2*), pRS314-RAS2ΔC (*RAS2ΔC*), pRS314-R2N-R1C (*R2N-R1C*), YEplac112-RAS1 (*RAS1* 2 μm) or YCplac22-R2p-R1orf (*R2p-R1orf*). Plates were incubated for 4 d and photographed before (Total growth) and after (Invasive growth) washing cells of the agar surface. (B) Expression levels of Ras proteins was determined in strains described in A by Western blot analysis using monoclonal anti-H-Ras antibody (259) that cross-reacts equally well with either Ras1p or Ras2p (upper panel, short exposure; lower panel, long exposure). (C) Autoradiogram showing steady-state levels of *RAS1* and *RAS2* mRNA in strains described in A. *ACT1* gene expression was used as internal standard. (D) *FG(Ty1)::lacZ* expression levels. β-Galactosidase activity was measured on strains described in A. Units are nanomoles per minute per milligram. Bars depict means of three independent measurements. White bars indicate *RAS2*-independent expression of *FG(Ty1)::lacZ*.

We find that expression of *RAS2ΔC*, a construct lacking the sequences encoding the hypervariable re-

gion of Ras2p (amino acids 175–300) (Hurwitz *et al.*, 1995) is sufficient to complement a *ras2* strain for both invasive growth and expression of the *FG(Ty1)::lacZ* reporter gene. This result suggests that the conserved N terminus alone conveys activation of haploid invasive growth and that for this function the hypervariable C terminus of Ras2p is dispensable. We further tested whether the hypervariable C terminus of Ras1p has an inhibiting function on invasive growth. Therefore, a Ras2p–Ras1p chimera consisting of the Ras2p N-terminal 151 residues followed by amino acids 152–309 of Ras1p (Hurwitz *et al.*, 1995) was expressed in a *ras2* strain. Invasive growth of the resulting strain is indistinguishable from a strain expressing the full Ras2p or the Ras2ΔCp deletion form. In summary, our findings indicate that the hypervariable C terminus of yeast Ras proteins is not required for regulating invasive growth.

High Expression of RAS1 Restores Invasive Growth in ras2 Mutants

Whereas *RAS2* is expressed under invasive growth conditions resulting in clearly detectable amounts of Ras2p, only very low levels of Ras1p are detectable (Figure 2B). In addition, only low levels of *RAS1* transcripts are detectable under these conditions (Figure 2C), suggesting that *RAS1* is poorly expressed. When assayed in a wild-type strain, expression of *RAS2* is ~10 times higher that expression of *RAS1* (our unpublished results). To test whether Ras1p can substitute for Ras2p in invasive growth regulation when expressed at appropriate levels, *RAS1* was expressed from a high-copy plasmid or under the control of the *RAS2* promoter (Figure 2). We find that strains lacking *RAS2* but overexpressing *RAS1* were restored for invasive growth as well as FRE-dependent transcription (Figure 2, A and D). This result shows that both Ras proteins Ras1p and Ras2p are functionally redundant for invasive growth signaling, but that only *RAS2* is sufficiently expressed under these conditions.

Activation of the Cdc42p/Ste20p/MAPK Cascade Can Suppress Defective Invasive Growth of a ras2 Mutant

In diploids Ras2p induces filamentous growth by activating the Cdc42p/Ste20p/MAPK signaling pathway (Mösch *et al.*, 1996). Because we found that *RAS2* is required for regulation of haploid invasive growth and FRE-dependent transcription, we examined whether in haploids Ras2p activates invasive growth development also via the MAPK pathway. Therefore, we tested whether activation of the MAPK pathway is sufficient to suppress the invasive growth defect of a *ras2* mutant strain. A strain lacking *RAS2* was transformed with an array of different plasmids that either express activated alleles of *CDC42* or *STE11* or that
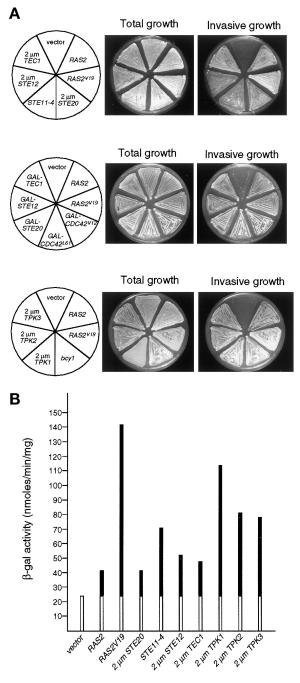
Figure 3. Activation of either the Cdc42p/Ste20p/MAPK pathway or the PKA is sufficient to induce invasive growth in strains lacking *RAS2*. (A) Invasive growth of yeast strain YHUM120 (*ras2*) containing pRS426 (vector), pRS316-RAS2 (*RAS2*), YCp50-RAS2^{Val19} (*RAS2^{V19}*), pRS426-STE20 (2 μm *STE20*), B2616 (*STE11-4*), pRS426-STE12 (2 μm *STE12*), pRS202-TEC1 (2 μm *TEC1*), pRS316-GAL-CDC42^{Val12} (*GAL-CDC42^{V12}*), pRS316-GAL-CDC42^{Leu61} (*GAL-CDC42^{L61}*), pYGEX-STE20 (*GAL-STE20*), B2065 (*GAL-STE12*), pGAL-TEC1 (*GAL-TEC1*), YEplac195-TPK1 (2 μm *TPK1*), pRS426-TPK2 (2 μm *TPK2*), pRS426-TPK3 (2 μm *TPK3*), or a *bcy1* disruption mutation (*bcy1*), respectively. Strains were grown on solid SC-Ura medium containing either 2% glucose (upper and lower panels) or a mixture of 3% raffinose and 0.1% galactose (middle panel). After

overexpress *STE20*, *STE12*, or *TEC1*, respectively. Invasive growth of the resulting strains was assayed on rich medium. Expression of the different *CDC42* alleles was controlled by growing strains with pGAL::CDC42 constructs on raffinose plus 0.1% galactose to avoid growth inhibition caused by high levels of these mutant proteins. Under all conditions tested, expression of either the dominant active *CDC42* alleles *CDC42^{Val12}* and *CDC42^{Leu61}* or the hyperactive *STE11* allele *STE11-4* or overexpression of *STE20*, *STE12*, or *TEC1* was sufficient to suppress defective invasive growth caused by a deletion of *RAS2* (Figure 3A). In addition, activation of the MAPK pathway in *ras2* mutant strains induces FRE-dependent transcription at least to the levels found in strains harboring a functional *RAS2* gene (Figure 3B). These results further corroborate that in haploids Ras2p regulates invasive growth by activating the Cdc42p/Ste20p/MAPK pathway.

Hyperactive PKA Induces Invasive Growth in Strains Lacking RAS2

Recent studies indicate that filamentous growth of diploid yeast is regulated by cAMP (Ward *et al.*, 1995; Kübler *et al.*, 1997; Lorenz and Heitman, 1997). Thus, we determined whether regulation of haploid invasive growth by Ras2p can also be achieved by activation of PKA. A *ras2* mutant strain was either disrupted at the *BCY1* locus, encoding the inhibitory subunit of PKA, or was transformed with plasmids overexpressing any of the three *TPK* genes. All constructions result in high A kinase activity in the cell (Toda *et al.*, 1987a,b). Resulting strains were tested for agar penetration and expression of the *FRE(Ty1)::lacZ* reporter (Figure 3). We find that overexpression of any of the catalytic A kinase subunits encoding *TPK1*, *TPK2*, or *TPK3* genes induces invasive growth in the absence of Ras2p.

Surprisingly, in haploid cells high A kinase activity not only induces invasive growth in the absence of *RAS2* but also stimulates expression of the *FRE(Ty1)::lacZ* reporter gene to levels comparable to strains with an activated MAPK pathway (Figure 3B). This suggests that the MAPK cascade and the A kinase pathway are interconnected. Therefore, we examined whether the A kinase induces expression of the FRE reporter gene depending on elements of the MAPK cascade. All three *TPK* genes, *TPK1*, *TPK2*, and *TPK3*, were overexpressed in either

Figure 3 (cont). incubation for 4 d, plates were photographed before (Total growth) or after (Invasive growth) washing cells from the agar surface. (B) *FRE(Ty1)::lacZ* expression levels. β-Galactosidase activity was measured on strains described in A. Units are nanomoles per minute per milligram. Bars depict means of three independent measurements. White bars indicate *RAS2*-independent expression of *FG(Ty1)::lacZ*.
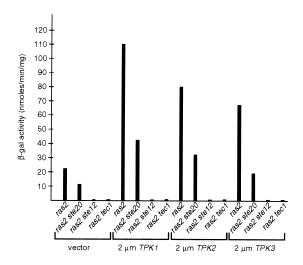
Figure 4. Activation of FRE-dependent transcription by the A kinase depends on Ste12p and Tec1p. Yeast strains YHUM120 (*ras2*), YHUM158 (*ras2 ste20*), YHUM175 (*ras2 ste12*), and YHUM167 (*ras2 tec1*) containing pRS426 (vector), YEplac195-TPK1 (2 μm *TPK1*), pRS426-TPK2 (2 μm *TPK2*), or pRS426-TPK3 (2 μm *TPK3*) were assayed for *FRE(Ty1)::lacZ* expression levels by measuring β-galactosidase activity. Units are nanomoles per minute per milligram. Bars depict means of three independent measurements.

ras2, ras2 ste20, ras2 ste12, or *ras2 tec1* mutants, and resulting strains were assayed for expression of the *FRE(Ty1)::lacZ* reporter gene (Figure 4). The *ras2* mutation was introduced to prevent crosstalk between the A kinase and the MAPK pathway via Ras2p. Stimulation of FRE-dependent transcription by all three Tpk subunits completely depends on the presence of both Ste12p and Tec1p but is only partially attenuated by deletion of *STE20*. These findings suggest that regulation of FRE-dependent gene expression by the A kinase and the Cdc42p/Ste20/MAPK cascade might be interconnected at a level downstream of Ste20p.

Alleles of Activated RAS2^Val19 Carrying Second Site Mutations in the Adenylyl Cyclase-interacting Domain Require STE20, STE12, or TEC1 for Activation of Haploid Invasive Growth

Ras2p regulates invasive growth by activating both the Cdc42p/Ste20p/MAPK and the A kinase pathways. Therefore, we determined whether these two functions of Ras2p are separable. We created *RAS2* mutant alleles *RAS2^Gly41^*, *RAS2^Asn45^*, *RAS2^Val19Gly41^*, and *RAS2^Val19Asn45^*, encoding single amino acid substitutions at positions 41 and 45 either alone or in combination with the activating mutation of codons for valine instead of glycine at position 19 (Figure 5A). Analogous mutant proteins of the highly conserved human Ha-Ras, Ha-Ras^Val12Gly34^ and Ha-Ras^Val12Asn38^, are specifically blocked for binding and activating yeast adenylyl cyclase Cyr1p (Akasaka *et al.*, 1996).
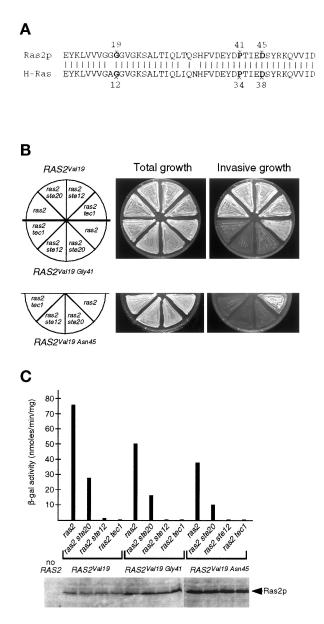

Figure 5. Properties of *RAS2* mutants. (A) Sequence alignment between N-terminal regions of yeast Ras2p (amino acids 10–75) and human H-Ras (amino acids 3–68). Identical residues are indicated by vertical lines. Substitution of Gly19 in yeast Ras2p or Gly12 in H-Ras for Val leads to activated mutant proteins. Amino acids Pro34 and Asp38 of H-Ras and corresponding residues Pro41 and Asp45 of Ras2p are indicated. (B) Invasive growth of yeast strains YHUM120 (*ras2*), YHUM158 (*ras2 ste20*), YHUM175 (*ras2 ste12*), and YHUM167 (*ras2 tec1*) containing the plasmid YCplac22-RAS2^Val19 (*RAS2^Val19^*), YCplac22-RAS2^Val19Gly41^ (*RAS2^Val19Gly41^*) or YCplac22-RAS2^Val19Asn45^ (*RAS2^Val19Asn45^*) on solid SC-Trp medium. After incubation, plates were photographed before (Total growth) and after (Invasive growth) washing under a stream of water. (C) Expression levels of *FRE(Ty1)::lacZ* and Ras2 proteins. β-Galactosidase activity was measured on strains described in B, and expression of Ras2 mutant proteins was analyzed by Western blot analysis.

Yeast Ras2p and human Ha-Ras share 90% identity in their N terminus, and both amino acids Pro34 and Asp38 of Ha-Ras required for Cyr1p binding are conserved in yeast Ras2p (Figure 5A). In addition, yeast *RAS2* effector mutants *RAS2^{Ser42}* and *RAS2^{Ala42}*, coding for single amino acid substitutions at position 42 in Ras2p, are specifically attenuated for adenylyl cyclase-stimulating activity (Marshall *et al.*, 1988; Sun *et al.*, 1994).

The two single mutant alleles *RAS2^{Gly41}* and *RAS2^{Asn45}* were expressed in a *ras2* mutant strain and tested for induction of invasive growth. We find that expression of either *RAS2^{Gly41}* or *RAS2^{Asn45}* leads to strains exhibiting reduced invasive growth, although the amount of invasively growing cells is clearly above levels of strains lacking *RAS2* (our unpublished results). Thus, amino acid residues Pro41 and Asp45 of Ras2p are required for full induction of invasive growth. We concluded that part of the invasive growth–stimulating activity of Ras2p is achieved by interaction with Cyr1p, because these residues have been shown to be required for interaction of H-Ras with adenylyl cyclase. Therefore, the remaining invasive growth activation potential of Ras2p mutant proteins carrying the P41G or D45N substitutions should depend on elements of the Ste20p/MAPK pathway. However, testing this prediction by expression of the *RAS2^{Gly41}* and *RAS2^{Asn45}* single mutant alleles in strains with a defective MAPK pathway is not conclusive, because, e.g., *ste20* and *tec1* mutants are defective for invasive growth, although they carry an intact *RAS2*. Expression of the dominant *RAS2^{Val19}* allele, however, activates invasive growth in strains that lack *STE20*, *STE12*, or *TEC1* (Figure 5B). Therefore, we asked whether an activated Ras^{Val19} protein with an additional defect in the adenylyl cyclase interaction domain is still able to overcome the defects in invasive growth caused by inactivation of the MAPK pathway. For this purpose, we expressed the double mutant alleles *RAS2^{Val19Gly41}* and *RAS2^{Val19Asn45}* in *ras2*, *ras2 ste20*, *ras2 ste12*, and *ras2 tec1* mutant strains. Invasive growth behavior, FRE-dependent reporter gene expression, and intracellular Ras2 mutant protein levels were measured in the resulting strains (Figure 5, B and C). We find that expression of either *RAS2^{Val19Gly41}* or *RAS2^{Val19Asn45}* in the *ras2* single mutant leads to invasive growth induction comparable to that achieved by wild-type *RAS2* but below that induced by *RAS2^{Val19}*. The same result is found for stimulation of the FRE-dependent reporter gene expression (Figure 5C). This suggests that the Ras2^{Val19Gly41} and Ras2^{Val19Asn45} effector mutant proteins activate invasive growth independent of Cyr1p interaction by stimulating other downstream effector pathways, e.g., the MAPK pathway. Indeed, expression of the Ras2^{Val19Gly41} and Ras2^{Val19Asn45} effector mutants is not sufficient to restore the invasive growth defect of the *ras2 ste20*, *ras2*

ste12, and *ras2 tec1* double mutant strains with a defective MAPK pathway, whereas it is restored by expression of Ras2^{Val19} (Figure 5B). The failure of Ras2^{Val19Gly41} or Ras2^{Val19Asn45} to induce invasive growth in MAPK mutant strains cannot be due to reduced expression of the different Ras2 proteins, because expression of Ras2p is independent of *STE20*, *STE12*, or *TEC1* (Figure 5C). Thus, both Ras2^{Val19Gly41} and Ras2^{Val19Asn45} depend on an intact Ste20p/MAPK cascade to induce invasive growth, whereas Ras2^{Val19} does not. This indicates that amino acid substitutions P41G or D45N disrupt the ability of Ras2p to activate the cAMP/PKA pathway by interaction with Cyr1p, but not the effector domain of Ras2p responsible for activation of the MAPK cascade.

In summary, results in this section indicate that the two functions by which Ras2p activates invasive growth can be separated. Amino acid substitutions within Ras2p uncouple activation of the Cdc42p/Ste20p/MAPK cascade from the PKA pathway.

DISCUSSION

Differential Transcriptional Expression of RAS Genes in Yeast Growth and Development

We have investigated the role of Ras proteins in cellular growth and development using the developmental switch of *S. cerevisiae* from growth as single haploid yeast cells to invasive filaments as a paradigm. Our studies show that the two *RAS* homologues of *S. cerevisiae*, *RAS1* and *RAS2*, fulfill divergent roles in regulating yeast growth and development. Both *ras1* and *ras2* single mutant strains exhibit normal growth, whereas a *ras1 ras2* double mutant is not viable. Therefore, at least one of the *RAS* genes must be expressed to ensure normal growth. Already very low levels of Ras1p in a *ras2* mutant are sufficient to allow growth but not invasive growth development. High levels of either Ras1p or Ras2p are required to induce invasive growth development. In vivo, yeast cells are only able to make use of *RAS2* but not *RAS1* to regulate invasive growth. The two Ras proteins are differentially expressed at the level of transcription resulting in *RAS1* mRNA levels that are too low during invasive growth. When the *RAS1* ORF is under the control of the *RAS2* promoter, both *RAS1* mRNA and Ras1 protein levels are sufficient to induce invasive growth. Thus, expressional divergence of the two Ras proteins is achieved by transcriptional regulators that differentially control the *RAS1* and *RAS2* promoters.

Ras2p Stimulates Distinct Pathways to Induce Haploid Invasive Growth

In diploid yeast, Ras2p signals via the filamentous growth MAPK cascade to induce pseudohyphal development under starvation conditions (Mösch *et al.*,
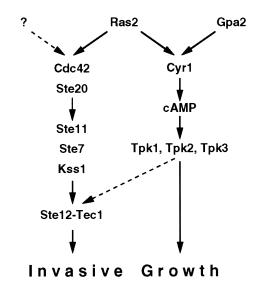
Figure 6. Model for the regulatory network controlling invasive growth in *S. cerevisiae* (see DISCUSSION for details).

1996). Here we extend this finding by demonstrating that in haploid yeast Ras2p is required for invasive growth differentiation under nonstarvation conditions. Loss of Ras2p function can be overcome by activation of either of two signaling units, the Cdc42p/Ste20p/MAPK cascade or the PKA pathway. Therefore, Ras2p constitutes a junction that affects both of these signaling units as effector pathways (Figure 6). This is supported by our finding that expression of activated *RAS2* alleles, $RAS2^{Val19Gly41}$ and $RAS2^{Val19Asn45}$ carrying effector mutations in the adenylyl cyclase–stimulating domain activate invasive growth solely depending on elements of the MAPK cascade. Thus, similar to higher eukaryotes, yeast Ras proteins control cell growth and development by inducing distinct effector pathways. In mammalian cells, ectopic expression of activated *H-RAS*, $H\text{-}RAS^{Val12}$, induces at least two distinct RAS effector pathways, a MAPK cascade and a signal pathway inducing membrane ruffling. Stimulation can be separated by expression of specific effector mutants of *H-RAS*, $H\text{-}RAS^{Val12Ser35}$ and $H\text{-}RAS^{Val12Cys40}$ (Joneson *et al.*, 1996). $H\text{-}RAS^{Val12Cys40}$ is defective for MAPK activation but has retained the ability to induce membrane ruffling, whereas $H\text{-}RAS^{Val12Ser35}$ still activates the MAPK but is defective for stimulation of membrane ruffling (Joneson *et al.*, 1996). The effector protein for stimulation of the MAPK cascade, which directly binds to activated H-RAS, is the RAF protein kinase (McCormick, 1994). Stimulation of membrane ruffling by H-RAS involves the RAC pathway, but the effector protein directly binding H-RAS remains to be determined. In yeast, only the adenylyl cyclase protein Cyr1p has been identified as an effector protein di-

rectly binding to activated forms of Ras2p. No direct effector protein of Ras2p has been identified for stimulation of filamentous invasive growth via the Cdc42p/Ste20p/MAPK pathway. Our results indicate that Ras2p must target effectors distinct from adenylyl cyclase to activate the MAPK cascade.

Crosstalk between the cAMP-dependent Kinase and the Invasive MAPK Pathway

We have demonstrated that in haploid yeast the A kinase can stimulate expression of the FRE-dependent reporter gene depending on the transcription factors Ste12p and Tec1p. This appears to be in contrast to earlier findings showing that hyperactive A kinase only slightly induces expression of an FRE-dependent reporter gene (Mösch *et al.*, 1996) or that very high A kinase activity inhibits FRE-dependent transcription (Lorenz and Heitman, 1997). However, these earlier measurements were all performed in diploid cells under conditions of nitrogen starvation and not in haploid cells under conditions in which supply with ammonia is sufficient. In addition, one major difference between haploid and diploid cells is that expression of both the FRE-dependent reporter gene and the FRE-controlled *FLO11* gene is ~10 times higher in haploids than in diploids (Mösch *et al.*, 1996; Lo and Dranginis, 1998). Thus, although the signaling pathways for diploid filamentous growth and haploid invasive growth share identical components, signaling by these pathways in response to the different nutritional stimuli must not be the same. The mating type or starvation for nitrogen could control the activity of the A kinase toward the MAPK pathway, switching from activating to repressing by a specificity factor.

The finding that in haploid cells the A kinase stimulates expression of an FRE-dependent reporter gene implies the existence of a crosstalk between PKA and the invasive MAPK cascade. How does the A kinase act on FRE-controlled transcription? Activated A kinase could target any of the protein kinases of the invasive MAPK cascade, thereby stimulating their activity toward the transcriptional activators Ste12p and Tec1p. However, deletion of the Ste20p protein kinase only partially attenuates transcriptional induction of the FRE-dependent reporter gene by PKA, suggesting a link at a level downstream of Ste20p. At the moment, we favor a model in which Ste12–Tec1p forms a junction connecting the A kinase pathway to the invasive MAPK cascade (Figure 6). In this model PKA stimulates FRE-dependent transcription by acting on the Ste12p–Tec1p transcription factor, thereby inducing target genes of the invasive growth MAPK cascade. The exact molecular mechanism by which PKA acts on the MAPK pathway to induce FRE-dependent gene expression remains to be elucidated.

Induction of FRE-dependent transcription does not appear be the only way by which PKA induces invasive growth, because expression of *RAS2^Val19^* induces invasion in strains that lack *STE12* (Figure 4). This suggests that activation of the cAMP pathway by Ras2p stimulates invasive growth by targets distinct from Ste12p. The transcription factor Sfl1p is a good candidate for such a target, because genetic evidence indicates that Tpk2p acts upstream of Sfl1p in the regulation of Flo11p (Robertson and Fink, 1998). However, the exact interplay between PKA and the different transcriptional factors that form putative downstream targets regulating invasive growth is not clear.

A Highly Regulated Signaling Network for Invasive Growth Development

Our study shows that invasive growth development is under the control of a highly regulated signaling network (Figure 6). Two central signal transduction pathways, the Cdc42p/Ste20p/MAPK cascade and the Cyr1p/cAMP/PKA pathway, control invasive growth by targeting a number of downstream transcription factors. Thus, any regulator affecting the activity of these two pathways is able to control invasive growth. This study defines Ras2p as a central regulator for stimulating both the Cdc42p/Ste20p/MAPK cascade and the Cyr1p/cAMP/PKA pathway. However, a number of recent studies show that apart from Ras2p additional proteins are able to control both growth and filamentous development via the cAMP/PKA pathway. These proteins include Gpa2p, a G-protein alpha subunit (Kübler *et al.*, 1997; Lorenz and Heitman, 1997), Gpr1p, a putative G protein–coupled receptor that associates with Gpa2p (Xue *et al.*, 1998), and Mep2p, a high-affinity ammonium permease with a putative ammonium sensor function (Lorenz and Heitman, 1998). How these genes regulate invasive growth of haploids under conditions of high ammonia, however, has not been carefully studied.

What are the factors acting upstream of Ras2p to regulate invasive growth development? A number of proteins are known that regulate Ras2p activity, including Cdc25p, which functions as guanine nucleotide exchange factor for Ras2p (Engelberg *et al.*, 1990; Jones *et al.*, 1991), and Ira1p and Ira2p, which are thought to act as GTPase-activating proteins (Tanaka *et al.*, 1990). Thus, changes in Cdc25p or Ira1p/Ira2p intracellular levels or activity may be mechanisms to regulate invasive growth. Interestingly, the cellular content of Cdc25p is regulated by destabilization through a cyclin destruction box (Kaplon and Jacquet, 1995), opening the possibility that modulation of Cdc25p stability is an important mechanism to control Ras2p-mediated yeast development. Alternatively, invasive growth may be regulated by modulation of intracellular Ras2p content rather than variation in

activity or localization control. Indeed, we have found that expression of both Ras proteins is strictly regulated during invasive growth. Therefore, factors that control development-specific expression of both Ras1p and Ras2p might be important regulators of yeast development.

In summary, our study shows that the control of yeast growth and development by Ras is mediated by a highly regulated signaling network. Because elements of this network are conserved in evolution, the molecular mechanisms underlying their function provide a paradigm for signal transduction in all eukaryotes.

REFERENCES

Akasaka, K., Tamada, M., Wang, F., Kariya, K., Shima, F., Kikuchi, A., Yamamoto, M., Shirouzu, M., Yokoyama, S., and Kataoka, T. (1996). Differential structural requirements for interaction of Ras protein with its distinct downstream effectors. J. Biol. Chem. *271*, 5353–5360.

Andrianopoulos, A., and Timberlake, W.E. (1991). ATTS, a new and conserved DNA binding domain. Plant Cell *3*, 747–748.

Broach, J.R. (1991). *RAS* genes in *Saccharomyces cerevisiae*: signal transduction in search of a pathway. Trends Genet. *7*, 28–33.

Burglin, T.R. (1991). The TEA domain: a novel, highly conserved DNA-binding motif. Cell *66*, 11–12.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene *110*, 119–122.

Cross, F.R., and Tinkelenberg, A.H. (1991). A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. Cell *65*, 875–883.

Engelberg, D., Simchen, G., and Levitzki, A. (1990). In vitro reconstitution of cdc25 regulated *S. cerevisiae* adenylyl cyclase and its kinetic properties. EMBO J. *9*, 641–651.

Gavrias, V., Andrianopoulos, A., Gimeno, C.J., and Timberlake, W.E. (1996). *Saccharomyces cerevisiae TEC1* is required for pseudohyphal growth. Mol. Microbiol. *19*, 1255–1263.

Gietz, R.D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-bp restriction sites. Gene *74*, 527–534.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., and Fink, G.R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and. RAS. Cell *68*, 1077–1090.

Guthrie, C., and Fink, G.R. (1991). Guide to Yeast Genetics and Molecular Biology, San Diego: Academic Press.

Hurwitz, N., Segal, M., Marbach, I., and Levitzki, A. (1995). Differential activation of yeast adenylyl cyclase by Ras1 and Ras2 depends

on the conserved N terminus. Proc. Natl. Acad. Sci. USA *92*, 11009–11013.

Jones, S., Vignais, M.L., and Broach, J.R. (1991). The CDC25 protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotides bound to ras. Mol. Cell. Biol. *11*, 2641–2646.

Joneson, T., White, M.A., Wigler, M.H., and Bar-Sagi, D. (1996). Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. Science *271*, 810–812.

Kaplon, T., and Jacquet, M. (1995). The cellular content of Cdc25p, the Ras exchange factor in *Saccharomyces cerevisiae*, is regulated by destabilization through a cyclin destruction box. J. Biol. Chem. *270*, 20742–20747.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J., and Wigler, M. (1984). Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell *37*, 437–445.

Kübler, E., Mösch, H.U., Rupp, S., and Lisanti, M.P. (1997). Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J. Biol. Chem. *272*, 20321–20323.

Laloux, I., Dubois, E., Dewerchin, M., and Jacobs, E. (1990). *TEC1*, a gene involved in the activation of Ty1 and Ty1-mediated gene expression in *Saccharomyces cerevisiae*: cloning and molecular analysis. Mol. Cell. Biol. *10*, 3541–3550.

Liu, H., Krizek, J., and Bretscher, A. (1992). Construction of a *GAL1*-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics *132*, 665–673.

Liu, H., Styles, C.A., and Fink, G.R. (1993). Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science *262*, 1741–1744.

Lo, W.S., and Dranginis, A.M. (1998). The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. Mol. Biol. Cell *9*, 161–171.

Lorenz, M.C., and Heitman, J. (1997). Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog. EMBO J. *16*, 7008–7018.

Lorenz, M.C., and Heitman, J. (1998). The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J. *17*, 1236–1247.

Madhani, H.D., and Fink, G.R. (1997). Combinatorial control required for the specificity of yeast MAPK signaling. Science *275*, 1314–1317.

Madhani, H.D., Styles, C.A., and Fink, G.R. (1997). MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell *91*, 673–684.

Marshall, M.S., Gibbs, J.B., Scolnick, E.M., and Sigal, I.S. (1988). An adenylate cyclase from *Saccharomyces cerevisiae* that is stimulated by RAS proteins with effector mutations. Mol. Cell. Biol. *8*, 52–61.

McCormick, F. (1994). Raf: the holy grail of Ras biology? Trends Cell Biol. *4*, 347–350.

McCormick, F. (1995). Ras-related proteins in signal transduction and growth control. Mol. Reprod. Dev. *42*, 500–506.

Morishita, T., Mitsuzawa, H., Nakafuku, M., Nakamura, S., Hattori, S., and Anraku, Y. (1995). Requirement of *Saccharomyces cerevisiae* Ras for completion of mitosis. Science *270*, 1213–1215.

Mösch, H.U., and Fink, G.R. (1997). Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. Genetics *145*, 671–684.

Mösch, H.U., Graf, R., and Braus, G.H. (1992). Sequence-specific initiator elements focus initiation of transcription to distinct sites in the yeast TRP4 promoter. EMBO J. *11*, 4583–90.

Mösch, H.-U., Roberts, R.L., and Fink, G.R. (1996). Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *93*, 5352–5356.

Roberts, R.L., and Fink, G.R. (1994). Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. Genes & Dev. *8*, 2974–2985.

Robertson, L.S., and Fink, G.R. (1998). The three yeast A kinases have specific signaling functions in pseudohyphal growth. Proc. Natl. Acad. Sci. USA *95*, 13783–13787.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19–27.

Sun, J., Kale, S.P., Childress, A.M., Pinswasdi, C., and Jazwinski, S.M. (1994). Divergent roles of *RAS1* and *RAS2* in yeast longevity. J. Biol. Chem. *269*, 18638–18645.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M.S., Gibbs, J.B., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990). *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. Cell *60*, 803–807.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., McMullen, B., Hurwitz, M., Krebs, E.G., and Wigler, M. (1987a). Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *7*, 1371–1377.

Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987b). Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50*, 277–287.

Ward, M.P., Gimeno, C.J., Fink, G.R., and Garrett, S. (1995). *SOK2* may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. Mol. Cell. Biol. *15*, 6854–6863.

Xue, Y., Batlle, M., and Hirsch, J.P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. EMBO J. *17*, 1996–2007.

Yaffe, M.P., and Schatz, G. (1984). Two nuclear mutations that block mitochondrial protein import in yeast. Proc. Natl. Acad. Sci. USA *81*, 4819–4823.

Ziman, M., O'Brien, J.M., Ouellette, L.A., Church, W.R., and Johnson, D.I. (1991). Mutational analysis of *CDC42Sc*, a *Saccharomyces cerevisiae* gene that encodes a putative GTP-binding protein involved in the control of cell polarity. Mol. Cell. Biol. *11*, 3537–3544.