Krh1p and Krh2p act downstream of the Gpa2p G$_\alpha$ subunit to negatively regulate haploid invasive growth

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Summary

The yeast G$_\alpha$ subunit Gpa2p and its coupled receptor Gpr1p function in a signaling pathway that is required for the transition to pseudohyphal and invasive growth. A two-hybrid screen using a constitutively active allele of *GPA2* identified the *KRH1* gene as encoding a potential binding partner of Gpa2p. Strains containing deletions of *KRH1* and its homolog *KRH2* were hyper-invasive and displayed a high level of expression of *FLO11*, a gene involved in pseudohyphal and invasive growth. Therefore, *KRH1* and *KRH2* encode negative regulators of the invasive growth pathway. Cells containing *krh1*Δ *krh2*Δ mutations also displayed increased sensitivity to heat shock and decreased sporulation efficiency, indicating that Krh1p and Krh2p regulate multiple processes controlled by the cAMP/PKA pathway. The *krh1*Δ *krh2*Δ mutations suppressed the effect of a *gpa2*Δ mutation on *FLO11* expression and eliminated the effect of a constitutively active *GPA2* allele on induction of *FLO11* and heat shock sensitivity, suggesting that Krh1p and Krh2p act downstream of Gpa2p. The Sch9p kinase was not required for the signal generated by deletion of *KRH1* and *KRH2*; however, the cAMP-dependent kinase Tpk2p was required for generation of this signal. These results support a model in which activation of Gpa2p relieves the inhibition exerted by Krh1p and Krh2p on components of the cAMP/PKA signaling pathway.

Key words: GPA2, KRH1, KRH2, Kelch repeat

Introduction

The *GPA2* gene of the yeast *Saccharomyces cerevisiae* encodes a protein that is homologous to heterotrimeric G protein α-subunits (Nakafuku et al., 1988). The function of *GPA2* is required for pseudohyphal and invasive growth in response to changes in nutrient conditions (Kübler et al., 1997; Lorenz and Heitman, 1997; Lorenz et al., 2000). Nutrient limitation causes both haploid and diploid cells to undergo morhphological changes, although the nutritional condition that induces the transition is different in each case. Whereas nitrogen limitation causes diploid cells to form pseudohyphae, glucose limitation causes haploid cells to undergo invasive growth (Cullen and Sprague, 2000; Gimeno et al., 1992). Pseudohyphae formation and invasive growth are regulated by both the MAPK and cAMP/PKA signaling pathways (Gancedo, 2001; Levin and Errede, 1995; Madhani and Fink, 1998). However, little is known about the proteins involved in signal transduction through Gpa2p.

One component of the Gpa2p pathway that has been identified is the G protein-coupled receptor that interacts with the Gpa2p α-subunit. This receptor, called Gpr1p, was identified by a two-hybrid screen using Gpa2p as the bait (Kraakman et al., 1999; Xue et al., 1998; Yun et al., 1997). The presence of this receptor on the cell surface suggests that it recognizes an extracellular ligand (Xue et al., 1998). However, the ligand that activates Gpr1p has not been identified as yet. Like *GPA2, GPR1* is required for the switch to pseudohyphal and invasive forms of growth (Lorenz et al., 2000; Tamaki et al., 2000). A constitutively active allele of *GPA2* suppresses the filamentous growth defect conferred by a *gpr1*Δ mutation, in agreement with the idea that Gpr1p couples to Gpa2p and initiates signaling through the Gpa2p pathway (Lorenz et al., 2000).

The signaling pathway that functions downstream of Gpr1p and Gpa2p is not well understood. One possibility that has been proposed is that Gpa2p acts in an analogous manner to the mammalian α-subunit G$_{\alpha s}$, which directly activates adenylyl cyclase. This potential mechanism would constitute a second way of activating adenylyl cyclase in yeast, in addition to the known mechanism of direct activation of adenylyl cyclase by Ras proteins (Broach, 1991; Thevelein and de Winde, 1999). Production of cAMP by adenylyl cyclase activates the cAMP-dependent kinases Tpk1p, Tpk2p and Tpk3p. These kinases phosphorylate substrates that regulate metabolism, growth and filament formation (Borges-Walmsley and Walmsley, 2000; Madhani and Fink, 1998).

cAMP-dependent kinase is involved in many cellular processes, and the three different forms of this kinase that are present in yeast are not equivalent for all functions. Although *TPK1*, *TPK2* or *TPK3* can provide the essential function that is revealed when all three genes are mutated (Toda et al., 1987b), they appear to play different roles with respect to filamentous growth. Whereas Tpk2p is required for filamentous growth, Tpk1p and Tpk3p have either no effect or a small inhibitory effect on this process (Pan and Heitman, 1999; Robertson and Fink, 1998). Activation of the cAMP-

dependent kinases occurs by binding of cAMP to a regulatory subunit, Bcy1p, which is presumed to bind to all three forms of the kinase. It is therefore likely that the different cAMP-dependent kinases are subject to additional types of regulation that affect their involvement in different cellular processes.

Several observations suggest that there is a relationship between Gpa2p-mediated signaling and cAMP-dependent signaling. First, overexpression of *GPA2* augments the rapid increase in cAMP levels that occurs when glucose is added to glucose-starved cells (Nakafuku et al., 1988). Second, the defect in pseudohyphal growth conferred by a *gpa2*Δ or *gpr1*Δ mutation is reversed by the addition of cAMP (Kübler et al., 1997; Lorenz and Heitman, 1997; Lorenz et al., 2000; Tamaki et al., 2000). Finally, a *gpa2*Δ or *gpr1*Δ mutation eliminates the glucose-induced increase in cAMP levels under certain conditions (Colombo et al., 1998; Kraakman et al., 1999; Lorenz et al., 2000; Yun et al., 1998). Gpa2p has been shown to function independently of Ras, which would be consistent with the possibility that Gpa2p directly activates adenylyl cyclase (Colombo et al., 1998; Xue et al., 1998). However, direct activation of adenylyl cyclase by Gpa2p has not been demonstrated experimentally as yet.

Another candidate for a downstream component of the Gpa2p pathway is the kinase Sch9p. The *SCH9* gene was isolated based on the fact that its overexpression suppresses the growth defect conferred by mutations in genes that encode components of the cAMP signaling pathway (Toda et al., 1988). Sch9p appears to function in a separate pathway from Ras, adenylyl cyclase and the cAMP-dependent kinases (Hartley et al., 1994; Toda et al., 1988). The possibility that Sch9p acts downstream of Gpa2p was raised by the finding that an *sch9*Δ mutation eliminates the sensitivity to heat shock conferred by a constitutively active version of Gpa2p (Xue et al., 1998). However, Sch9p and Gpa2p do not appear to function in a linear signaling pathway because *sch9*Δ mutant cells have a different phenotype from *gpa2*Δ mutant cells. Cells containing an *sch9*Δ mutation display a significant growth defect (Toda et al., 1988) and are defective for induction of trehalase activity in response to nitrogen addition (Crauwels et al., 1997). Cells containing a *gpa2*Δ mutation do not have a growth defect (Nakafuku et al., 1988) and respond normally to nitrogen addition (Kraakman et al., 1999). Moreover, double mutant *sch9*Δ *gpa2*Δ cells display a much more severe growth defect than single mutant *sch9*Δ cells, indicating that the functions of these two genes cannot be completely overlapping (Kraakman et al., 1999; Lorenz et al., 2000).

In summary, the Gpa2p α-subunit cannot be placed in the context of a simple, linear signaling pathway because all potential downstream components of the Gpa2p pathway respond to multiple inputs. Here, we report the identification of two novel genes, *KRH1* and *KRH2*, encoding proteins that play a role in the Gpa2p pathway. The *KRH1* and *KRH2* gene products act downstream of Gpa2p and function by exerting a negative regulatory effect on haploid invasive growth.

Materials and Methods

Plasmid construction

Construction of plasmids pGBT9-GPA2 and pG2CT-112.2 was described previously (Xue et al., 1998). Plasmid pG2CT-T9.2 was constructed by changing the arginine at position 273 in the *GPA2* sequence of pGBT9-GPA2 to an alanine, as described for pG2CT-112.2.

Construction of plasmids YEpSCH9 and YEpADH-SCH9 was described previously (Toda et al., 1988). Plasmid pS9CLON.111 was made by cloning a 7.6 kb *Bam*HI fragment from YEpSCH9 into the *Bam*HI site of YEplac111. Plasmid pS9-7K.Bs was made by cloning a 7.0 kb *Bam*HI-*Kpn*I fragment from pS9CLON.111 into the *Bam*HI-*Kpn*I sites of pBluescript. Plasmid pURA3.Bs was made by cloning a 1.2 kb *Xba*I fragment containing the *URA3* gene from pAC100-2 into the *Spe*I site of pBluescript. To construct a *URA3* disruption of *SCH9*, a 1.0 kb *Eco*RI-*Nsi*I fragment from pURA3.Bs was cloned into the *Eco*RI-*Pst*I sites of pS9-7K.Bs to produce pS9-7K::URA3.Bs.

Construction of plasmid YEpTPK2 was described previously (Toda et al., 1987b). Plasmid YEpTPK2.2 was made by cloning a 2.0 kb *Bgl*II fragment from YEpTPK2 into the *Bam*HI site of YEp351.

The *KRH1* gene was cloned by amplifying a 1.2 kb fragment from yeast genomic DNA by polymerase chain reaction (PCR) using primers 5TH14-1 [5′-CG**CTGCAG**<u>TGATTCATTGGCAGGTCC</u>-3′ (genomic sequences are underlined in all primers)], which contains a flanking *Pst*I site (shown in bold), and 3TH14-1 (5′-<u>CGGTCCGTTAATTT**GGATCC**</u>-3′), which contains an internal *Bam*HI site present in the genomic DNA site (shown in bold). This fragment was cloned into the *Pst*I/*Bam*HI sites of pUC19 to create pUC19.Th14. To construct a *HIS3* disruption of *KRH1*, a 1.8 kb *Hinc*II-*Sma*I fragment from pUC18-HIS3 was cloned into the *Hinc*II sites of *KRH1* to produce Th14::HIS3. Plasmid YEp181-FLKRH1 was cloned by PCR using yeast genomic DNA as a template and contains sequences from the *Eco*RI site at nucleotide –755 (where +1 is the A of the start codon) to nucleotide +2778.

The *KRH2* gene was cloned by amplifying a 0.8 kb fragment from yeast genomic DNA by PCR using primers oYOR1.5 (5′-CC**GAGCTC**<u>GTATGGTATGGTGCCCATCAC</u>-3′), which contains a flanking *Sac*I site (shown in bold), and oYOR4 (5′-<u>CCTTAGGTCTACCGTCAAAAGC</u>-3′). This fragment was cloned into the *Sac*I/*Hin*dIII sites of pUC19, using the introduced *Sac*I site and an internal *Hin*dIII site, to produce pYOR37.1. A 1.1 kb fragment was amplified from yeast genomic DNA by PCR using primers oYOR5 (5′-<u>CGAGTGTAATGCCAAGTGCCA</u>-3′) and oYOR6 (5′-CC**AAGCTT**<u>AATTGCATCATCCTCTAAATA</u>-3′), which contains a flanking *Hin*dIII site (shown in bold). This fragment was cloned into the *Hin*dIII site of pYOR37.1, using the introduced *Hin*dIII site and an internal *Hin*dIII site, to produce pYOR37.12. To construct a *URA3* disruption of *KRH2*, a 1.2 kb *Xba*I fragment from pAC100-2 was cloned into the *Xba*I sites of pYOR37.12 to produce pYOR37.12U. Plasmid YEp112-KRH2 was cloned by PCR using yeast genomic DNA as a template and contains sequences from the *Eco*RI site at nucleotide –766 (where +1 is the A of the start codon) to nucleotide +2701.

The plasmid used for isolating the *FLO11* probe was made by amplifying a 0.5 kb fragment from pYSL12 (Lo and Dranginis, 1998) by PCR using primers oFLO3, 5′-GG**GGATCC**<u>GTAACT-CCTGCCACTAATGC</u>-3′, which contains a flanking *Bam*HI site (shown in bold), and oFLO4, 5′-<u>CCACATAAAGTTTCCAAGA-ACCTTG</u>-3′. The fragment was cloned into the *Bam*HI/*Xho*I sites of pBluescript, using the introduced *Bam*HI site and an internal *Xho*I site, to produce pFLO11CT.Bs. The plasmid used for isolating the *ACT1* probe was made by amplifying a 0.9 kb fragment from yeast genomic DNA by PCR using primers GMS288 (5′-<u>CCTCG-TGCTGTCTTCCCATCTATC</u>-3′) and GMS289 (5′-<u>GCATTCTT-TCGGCAATACCTGG</u>-3′). The fragment was cloned into plasmid PCR2.1-TOPO to produce pActin.21.

Strain construction and media

Strains used in this study are listed in Table 1. The *gpa2::TRP1* allele was made by transformation of cells with the 1.4 kb *Bam*HI fragment from pgpa2-1::TRP1 (Xue et al., 1998). The *sch9::URA3* allele was

Table 1. Strains used in this study

Strain	Genotype	Source
W3031B	*MAT*α *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein (Columbia University, New York, NY)
*B14-53B	*MAT*α *sch9::URA3*	This study
*G102-5C	*MAT*α *krh1::HIS3*	This study
*G103-4A	*MAT*α *krh2::URA3*	This study
*H137-22A	*MAT*α *gpa2::TRP1 sch9::URA3*	This study
*H147-3C	*MAT*α *krh1::HIS3 krh2::URA3*	This study
*H148-6A	*MAT*α *krh1::HIS3 krh2::URA3 sch9::URA3*	This study
*H152-28A	*MAT*α *gpa2::TRP1 krh1::HIS3 krh2::URA3 sch9::URA3*	This study
*H174	*MAT***a**/α *krh1::HIS3 krh2::URA3 CYH2/cyh2*	This study
SKY763	*MAT*α *ura3-52 trp1::hisG leu2::hisG his3::hisG*	S. Palecek and S. Kron (University of Chicago, Chicago, IL)
†BS1B	*MAT*α	This study
†BS3B	*MAT*α *tpk2::HIS3*	This study
†BS13B	*MAT*α *gpa2::TRP1*	This study
†HS154-3D	*MAT***a** *krh1::HIS3 krh2::URA3*	This study
†HS161-4C	*MAT*α *krh1::HIS3 krh2::URA3 tpk2::TRP1*	This study
†HS182-1D	*MAT*α *krh1::HIS3 krh2::URA3*	This study
†HS183-3D	*MAT*α *krh1::HIS3 krh2::URA3 gpa2::TRP1*	This study
†HS184-5A	*MAT*α *flo11::lacZ-HIS3*	This study
†HS197-13D	*MAT*α *krh1::HIS3 krh2::URA3 flo11::lacZ-HIS3*	This study
†SKY.k1-2B	*MAT*α *krh1::HIS3*	This study
†SKY.k2-4C	*MAT*α *krh2::URA3*	This study

*W3031B background.
†SKY763 background; SKY763 is derived from Σ1278b (Liu et al., 1993).

made by transformation of cells with a 3.8 kb *Hin*dIII fragment from plasmid pS9-7K::URA3.Bs. The *tpk2::HIS3* allele was made by transformation of cells with a 3.6 kb *Eco*RI fragment from plasmid ptpk2::HIS3 (Toda et al., 1987b). The *tpk2::TRP1* allele was made by transformation of a *tpk2::HIS3* strain with a 3.8 kb *Sma*I/*Xho*I fragment from marker swap plasmid pHT6 (Cross, 1997). The *krh2::URA3* allele was made by transformation of cells with the 1.6 kb *Sac*I/*Hin*dIII fragment from pYOR37.12U. The *krh2::TRP1* allele was made by transformation of a *krh2::URA3* strain with a 3.6 kb *Sma*I fragment from marker swap plasmid pUT11 (Cross, 1997). The *krh1::HIS3* allele was made by transformation of cells with the 2.3 kb *Sac*I fragment from Th14::HIS3. The *flo11::lacZ-HIS3* allele was made by transformation of cells with *Bst*EII-digested plasmid pMUC1-lacZ. All strain constructions involving transformations were confirmed by Southern blot.

Strains were grown on YEPD (2% glucose), and strains under

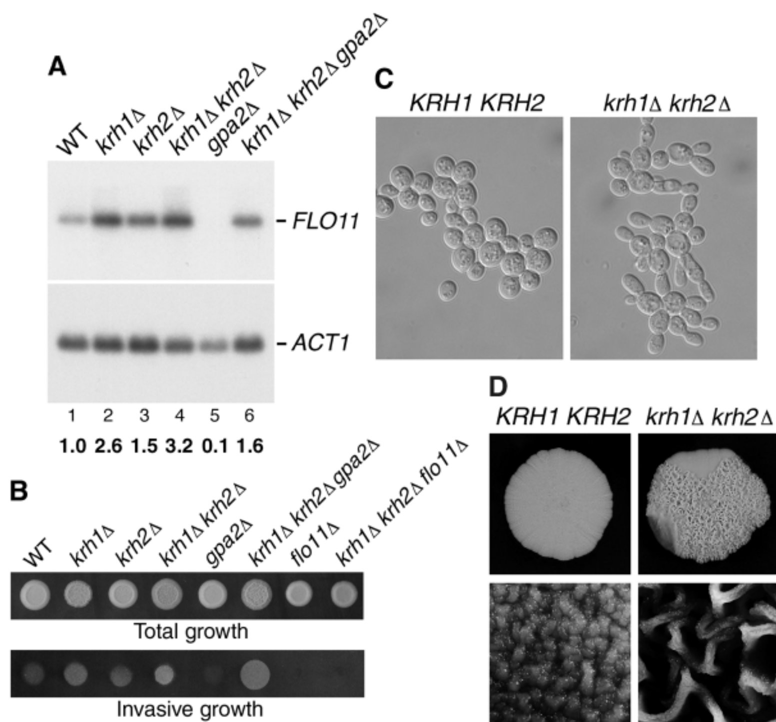

Fig. 1. Effect of *krh1*Δ *krh2*Δ mutations on *FLO11* induction, invasive growth and cell morphology. (A) RNA was isolated from wild-type strain SKY763 (lane 1), *krh1*Δ strain SKY.k1-2B (lane 2), *krh2*Δ strain SKY.k2-4C (lane 3), *krh1*Δ *krh2*Δ strain HS182-1D (lane 4), *gpa2*Δ strain BS13B (lane 5) and *krh1*Δ *krh2*Δ *gpa2*Δ strain HS183-3D (lane 6). The RNA was transferred to nylon membrane, hybridized with a *FLO11* probe, and rehybridized with an *ACT1* probe. The relative amount of *FLO11* RNA, normalized to *ACT1* RNA, is shown below each lane. (B) Wild-type strain SKY763 (WT), *krh1*Δ strain SKY.k1-2B (*krh1*Δ), *krh2*Δ strain SKY.k2-4C (*krh2*Δ), *krh1*Δ *krh2*Δ strain HS182-1D (*krh1*Δ *krh2*Δ), *gpa2*Δ strain BS13B (*gpa2*Δ), *krh1*Δ *krh2*Δ *gpa2*Δ strain HS183-3D (*krh1*Δ *krh2*Δ *gpa2*Δ), *flo11*Δ strain HS184-5A (*flo11*Δ), and *krh1*Δ *krh2*Δ *flo11*Δ strain HS197-13D (*krh1*Δ *krh2*Δ *flo11*Δ) were patched onto YEPD/2.5% agar medium, incubated for 4 days at 25°C, and photographed before (Total growth) and after (Invasive growth) rubbing the surface of the plate with a glass rod under a stream of water. (C) Wild-type strain BS1B (*KRH1 KRH2*) and double mutant strain HS154-3D (*krh1*Δ *krh2*Δ) were diluted 1:20 from an overnight saturated culture into fresh YEPD medium and incubated at 30°C with shaking for 2 days. (D) Wild-type strain BS1B (*KRH1 KRH2*) and double mutant strain HS154-3D (*krh1*Δ *krh2*Δ) were patched onto YEPD plates and incubated at 30°C for 5 days. Magnification: upper panel, 1×; lower panel, 25×.

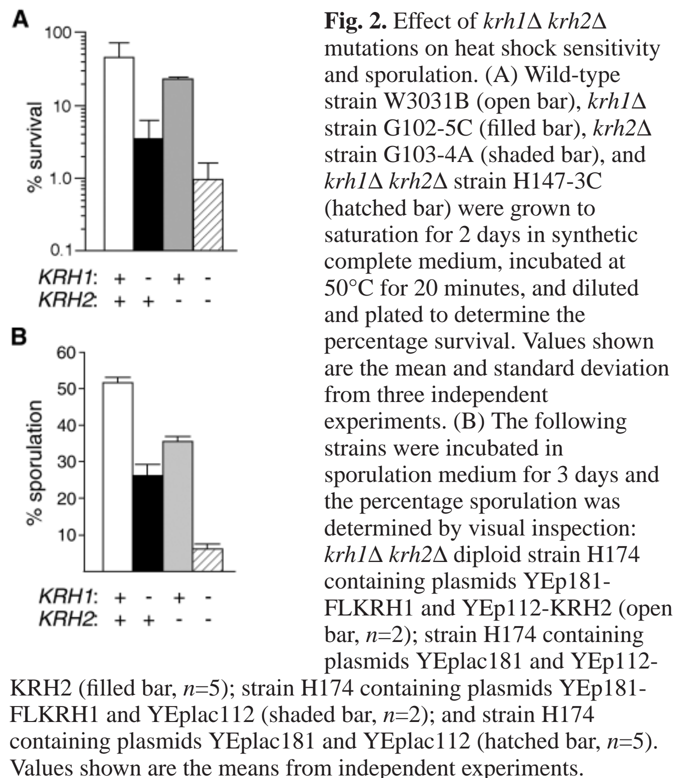

Fig. 2. Effect of *krh1*Δ *krh2*Δ mutations on heat shock sensitivity and sporulation. (A) Wild-type strain W3031B (open bar), *krh1*Δ strain G102-5C (filled bar), *krh2*Δ strain G103-4A (shaded bar), and *krh1*Δ *krh2*Δ strain H147-3C (hatched bar) were grown to saturation for 2 days in synthetic complete medium, incubated at 50°C for 20 minutes, and diluted and plated to determine the percentage survival. Values shown are the mean and standard deviation from three independent experiments. (B) The following strains were incubated in sporulation medium for 3 days and the percentage sporulation was determined by visual inspection: *krh1*Δ *krh2*Δ diploid strain H174 containing plasmids YEp181-FLKRH1 and YEp112-KRH2 (open bar, *n*=2); strain H174 containing plasmids YEplac181 and YEp112-KRH2 (filled bar, *n*=5); strain H174 containing plasmids YEp181-FLKRH1 and YEplac112 (shaded bar, *n*=2); and strain H174 containing plasmids YEplac181 and YEplac112 (hatched bar, *n*=5). Values shown are the means from independent experiments.

selection were grown on synthetic dropout media, as described (Guthrie and Fink, 1991).

Two-hybrid screen and yeast methods

pG2CT-T9.2 was transformed into reporter strain HF7c (Clontech) and the resulting strain was individually transformed with each of three yeast genomic DNA fusion libraries, Y2HL-C1, Y2HL-C2 and Y2HL-C3 (James et al., 1996). Transformation mixtures were plated on medium lacking histidine, and positive transformants were retested for β-galactosidase expression by incubation in the presence of 0.3 mg/ml X-gal. Plasmid TH14, which encodes a fusion to *KRH1* at codon 531, was isolated in this screen.

Heat shock assays, sporulation assays, and yeast RNA extraction were performed as described previously (Xue et al., 1998). Invasive growth assays were performed according to Kuchin et al. (Kuchin et al., 2002). Yeast transformations were performed by the lithium acetate method using standard procedures (Guthrie and Fink, 1991).

Northern blots

Total RNA was isolated from cells grown to logarithmic phase. RNA was electrophoresed in a 0.9% agarose formaldehyde-containing gel. Following electrophoresis, the gel was incubated in 0.05 M NaOH, 0.15 M NaCl for 20 minutes and neutralized in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl for 30 minutes. RNA was transferred to a nylon GeneScreen membrane (NEN) by applying a pressure of 80 mmHG with the Posiblot Pressure Blotter (Stratagene) for 2.5 hours using 1.5 M NaCl, 0.15 M sodium citrate as the transfer buffer. The RNA was UV crosslinked to the membrane using a Stratalinker UV box. Prehybridization was carried out at 42°C in a buffer containing 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate (pH 6.5), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% SDS, and 0.1 mg/ml of denatured salmon testes DNA (Sigma). Hybridization was carried out at 42°C in a buffer containing 50% formamide, 0.75 M NaCl, 75 mM sodium citrate,

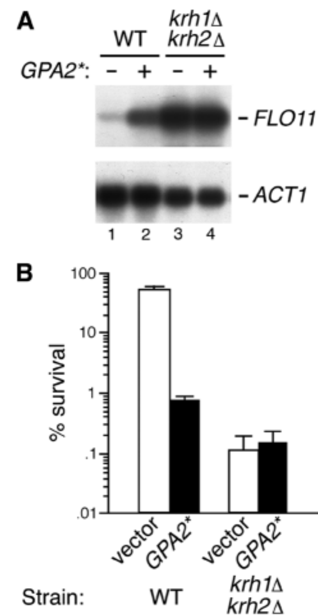

Fig. 3. Relationship between *KRH1* and *KRH2* and *GPA2*. Wild-type strain BS1B (lanes 1,2) and *krh1*Δ *krh2*Δ strain HS154-3D (lanes 3,4) transformed with either plasmid pG2CT-112.2 (lanes 2,4) or vector YEplac112 (lanes 1,3) were grown to log phase and RNA was isolated and hybridized as described in the legend to Fig. 1A. (B) Wild-type haploid strain W3031B (WT) and double mutant strain H147-3C (*krh1*Δ *krh2*Δ) transformed with either plasmid pG2CT-112.2 (*GPA2**) or YEplac112 (vector) were grown to saturation for 2 days in synthetic dropout medium lacking tryptophan, incubated at 50°C for 20 minutes, and diluted and plated to determine the percent survival. Values for YEplac112 are represented by the open bars; values for pG2CT-112.2 are represented by the filled bars. Values shown are the mean and standard deviation from three independent experiments. Note that the *krh1*Δ *krh2*Δ strain is more heat-shock sensitive when grown in medium lacking tryptophan than when grown in complete medium (Fig. 2A).

50 mM sodium phosphate (pH 6.5), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 12.5% dextran sulfate, and 0.1 mg/ml of denatured salmon testes DNA. Blots were washed four times for 15 minutes at 65°C with 0.3 M NaCl, 30 mM sodium citrate, 0.5% SDS and exposed to a phosphor storage screen and scanned with a PhosphorImager (Molecular Dynamics) or exposed to film. The probes used were gel-purified DNA restriction fragments ^{32}P-labeled by random primer labeling using a Prime-It kit (Stratagene). The fragments used were a 0.5 kb *Bam*HI/*Xho*I fragment from plasmid pFLO11CT.Bs for the *FLO11* probe and a 0.9 kb *Eco*RI fragment from plasmid pActin.21 for the *ACT1* probe.

Microscopy

Cells were viewed using Nomarski optics for differential interference contrast microscopy on a Zeiss Axiophot microscope. They were photographed with a 100× objective. Plates were viewed using a Zeiss Axioplan 2 microscope and photographed with a 2.5× objective.

Results

Identification of *KRH1*

To identify proteins that interact with the active form of the Gpa2p α-subunit, a two-hybrid screen was performed using as

Table 2. Two-hybrid assays

Plasmids	Genes	β-galactosidase activity
Th14*/pGBT9	*KRH1* C-term/vector	0.12
Th14/pGBT9-GPA2	*KRH1* C-term/*GPA2*	0.67
Th14/pG2CT-T9.2	*KRH1* C-term/*GPA2*^{R273A}	1.61
pGPE2-424.2†/pGBT9	*KRH1*/vector	0.12
pGPE2-424.2/pGBT9-GPA2	*KRH1*/*GPA2*	0.07
pGPE2-424.2/pG2CT-T9.2	*KRH1*/*GPA2*^{R273A}	0.12
pGAD424/pGBT9-GPA2	vector/*GPA2*	0.07
pGAD424/pG2CT-T9.2	vector/*GPA2*^{R273A}	0.13

Assays were performed in strain HF7c. Units were calculated as 1000 × OD_{420}/time(minutes) × mg protein. The experiment was repeated four times with essentially identical results. A representative experiment is shown.

*Plasmid Th14 contains a region of *KRH1* that encodes amino acids 531-740.

†Plasmid pGPE2-424.2 contains the entire coding region of *KRH1*.

bait the constitutively active form of Gpa2p encoded by the *GPA2*^{R273A} allele. This allele encodes a protein that is predicted to exist predominantly in the GTP-bound state as a result of its impaired GTPase activity (Freissmuth and Gilman, 1989). The two-hybrid screen resulted in the isolation of a plasmid encoding amino acids 531-740 of open reading frame YAL056W, which encodes a protein of 880 amino acids. This gene had been assigned the name *KRH1* (for kelch repeat homologue; see Discussion) in the *Saccharomyces* Genome Database. Expression of the C-terminal region of Krh1p fused to the Gal4p activation domain produced a positive signal for β-galactosidase activity when present in cells expressing either wild-type or constitutively active Gpa2p fused to the Gal4p DNA binding domain (Table 2, plasmids Th14 and pGBT9-GPA2 or pG2CT-T9.2). Moreover, the *GPA2*^{R273A} construct gave a two- to threefold higher level of β-galactosidase activity than the wild-type *GPA2* construct. This result suggests that there is a slight preference for interaction of Krh1p with the GTP-bound form of Gpa2p. In contrast, a construct containing the entire open reading frame of *KRH1* fused to the *GAL4* activation domain did not generate a positive signal for β-galactosidase activity when expressed with either the *GPA2* or *GPA2*^{R273A} constructs (Table 2, plasmids pGPE2-424.2 and pGBT9-GPA2 or pG2CT-T9.2). Therefore, if Krh1p binds to Gpa2p under physiological conditions, this interaction may be subject to additional regulatory mechanisms that expose the C-terminal binding site on Krh1p.

KRH1 and *KRH2* encode negative regulators of *FLO11* expression and invasive growth

A search of the *Saccharomyces* Genome Database revealed that a gene with high homology to *KRH1* is present in the yeast genome. The protein encoded by this gene, which is called *KRH2*, is 35% identical to Krh1p. If Krh1p and Krh2p function in the Gpa2p signaling pathway, then deletion of the *KRH1* and *KRH2* genes would be expected to affect cellular processes that require *GPA2*. Such processes include the ability of cells to undergo the transition to pseudohyphal or invasive growth (Kübler et al., 1997; Lorenz and Heitman, 1997). The involvement of the *KRH1* and *KRH2* gene products in invasive growth was investigated in a strain of the Σ1278b background, which contains an intact signaling pathway for this process (Kron, 1997).

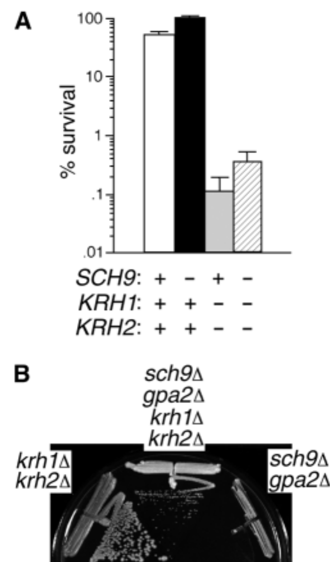

Fig. 4. Relationship between *KRH1* and *KRH2* and *SCH9*. (A) Wild-type strain W3031B, *sch9*Δ strain B14-53B, *krh1*Δ *krh2*Δ strain H147-3C, and *krh1*Δ *krh2*Δ *sch9*Δ strain H148-6A, all transformed with vector YEplac112, were grown to saturation for 2 days in synthetic dropout medium lacking tryptophan, incubated at 50°C for 20 minutes, and diluted and plated to determine the percentage survival. Value for W3031B is represented by the open bar; value for B14-53B is represented by the filled bar; value for H147-3C is represented by the shaded bar; value for H148-6A is represented by the hatched bar. Values shown are the mean and standard deviation from three independent experiments. (B) Strains with the following genotypes were streaked out for single colonies onto YEPD medium and grown for 2 days at 30°C: H147-3C (*krh1*Δ *krh2*Δ), H152-28A (*gpa2*Δ *krh1*Δ *krh2*Δ *sch9*Δ) and H137-22A (*gpa2*Δ *sch9*Δ).

Haploid invasive growth correlates with induction of the *FLO11* gene, which encodes a flocculin that is required for both pseudohyphal and invasive growth (Lo and Dranginis, 1998). To test whether the *KRH1* and *KRH2* genes are involved in the signaling pathway that results in invasive growth, the expression of *FLO11* was determined in strains containing deletions of these genes. Under conditions of log phase growth, the abundance of *FLO11* RNA was low but detectable in wild-type cells (Fig. 1A, lane 1). In cells containing a *gpa2*Δ mutation, the level of *FLO11* RNA was greatly decreased relative to wild-type cells (lane 5), as described previously (Lorenz et al., 2000; Tamaki et al., 2000). In contrast, individual deletions of *KRH1* and *KRH2*, or double deletion of both *KRH1* and *KRH2* resulted in an increase in *FLO11* RNA abundance over the level seen in wild-type cells (lanes 2-4). Quantification of the normalized results from several experiments showed that the level of *FLO11* RNA in *krh1*Δ *krh2*Δ cells is three- to fourfold higher than in wild-type cells. Because deleting the genes causes activation of the signaling pathway, these results indicate that *KRH1* and *KRH2* act as negative regulators of signaling. One possible interpretation of these findings is that Krh1p and Krh2p inhibit signaling of a component that acts downstream of Gpa2p under conditions that promote turning the pathway off. Alternatively, Krh1p and Krh2p could be required for modification or localization of the Gpa2p protein. To distinguish between these possibilities, the

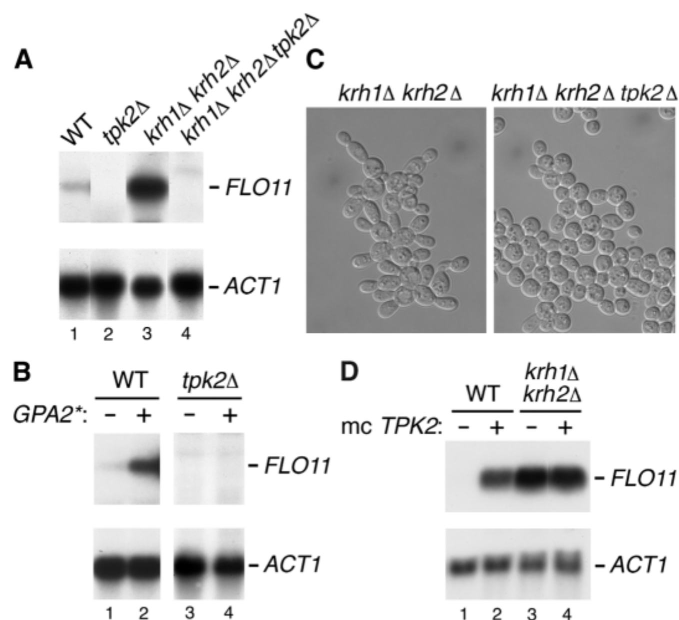

Fig. 5. Relationship between *KRH1* and *KRH2* and *TPK2*. (A) RNA from wild-type strain BS1B transformed with YEplac112 (lane 1), *tpk2*Δ strain BS3B transformed with YEplac112 (lane 2), *krh1*Δ *krh2*Δ strain HS154-3D (lane 3), and *krh1*Δ *krh2*Δ *tpk2*Δ strain HS161-4C (lane 4) was isolated and hybridized as described in the legend to Fig. 1A. (B) RNA from wild-type strain BS1B (lanes 1,2) and *tpk2*Δ strain BS3B (lanes 3,4) transformed with either plasmid pG2CT-112.2 (lanes 2,4) or vector YEplac112 (lanes 1,3) was isolated and hybridized as described in the legend to Fig. 1A. (C) *krh1*Δ *krh2*Δ strain HS154-3D and *krh1*Δ *krh2*Δ *tpk2*Δ strain HS161-4C were diluted 1:20 from an overnight saturated culture into fresh YEPD medium and incubated at 30°C with shaking for 2 days. (D) RNA from wild-type strain BS1B (lanes 1,2) and *krh1*Δ *krh2*Δ strain HS154-3D (lanes 3,4) transformed with either plasmid YEpTPK2.2 (lanes 2,4) or vector YEp351 (lanes 1,3) was isolated and hybridized as described in the legend to Fig. 1A.

effect of the *krh1*Δ and *krh2*Δ mutations was determined in a strain lacking Gpa2p. In triple mutant *krh1*Δ *krh2*Δ *gpa2*Δ cells, the level of *FLO11* RNA was substantially higher than that seen in *gpa2*Δ cells (lanes 5 and 6). A comparison of *krh1*Δ *krh2*Δ cells with *krh1*Δ *krh2*Δ *gpa2*Δ cells revealed that the presence of the *gpa2*Δ mutation in a *krh1*Δ *krh2*Δ background causes an approximately two- to threefold decrease in *FLO11* RNA abundance (lanes 4 and 6). However, the level of *FLO11* RNA was more than tenfold higher in *krh1*Δ *krh2*Δ *gpa2*Δ cells than in *gpa2*Δ cells, indicating that the predominant effect of the *krh1*Δ *krh2*Δ mutations is to suppress the phenotype of the *gpa2*Δ mutation.

Induction of the *FLO11* gene generally correlates with an increase in invasive growth in haploid cells and pseudohyphal growth in diploid cells. To test whether the increased level of *FLO11* expression in *krh1*Δ *krh2*Δ cells has physiological consequences, the ability of wild-type and *krh1*Δ *krh2*Δ strains to undergo haploid invasive growth was determined. *krh1*Δ, *krh2*Δ and *krh1*Δ *krh2*Δ cells showed a significant increase in their ability to invade solid medium when compared with wild-type cells (Fig. 1B). The most pronounced effect was seen with *krh1*Δ *krh2*Δ double mutant cells. Whereas *gpa2*Δ cells did not invade the medium to a significant degree, *krh1*Δ *krh2*Δ *gpa2*Δ cells displayed substantial invasive growth. Therefore, the *krh1*Δ *krh2*Δ mutations suppress the defect in invasive growth conferred by a *gpa2*Δ mutation. Deletion of the *FLO11* gene eliminated the increase in invasiveness conferred by the *krh1*Δ *krh2*Δ mutations, consistent with previously identified characteristics of invasive growth (Fig. 1B).

In addition to the phenotype displayed by *krh1*Δ *krh2*Δ cells on solid medium, *krh1*Δ *krh2*Δ cells grown in rich liquid medium for two days displayed an altered morphology. In liquid medium, *krh1*Δ *krh2*Δ cells formed chains of elongated cells that appear similar to cells undergoing the early stages of pseudohyphal growth (Fig. 1C). Depletion of glucose from the medium, which occurs under these conditions, is known to promote invasive growth in haploid cells (Cullen and Sprague, 2000). Introduction of a *gpa2*Δ mutation into a *krh1*Δ *krh2*Δ strain had no effect on the altered morphology of cells grown to saturation in liquid medium (data not shown). Given that pseudohyphal formation normally requires growth on solid medium, and that *krh1*Δ *krh2*Δ cells overcome this requirement, it can be concluded that the *KRH1* and *KRH2* gene products exert a strong negative effect on filamentous forms of growth in wild-type cells.

Another morphological phenotype conferred by the *krh1*Δ *krh2*Δ mutations involves the macroscopic appearance of patches of cells. Deletion of the *KRH1* and *KRH2* genes causes cell cultures incubated on solid medium to grow up off the plate in extended sheets (Fig. 1D). This macroscopic phenotype is reminiscent of that seen when *S. cerevisiae* is grown under conditions of fungal biofilm formation (Reynolds and Fink, 2001). Biofilm formation in *S. cerevisiae* requires *FLO11* (Reynolds and Fink, 2001), suggesting that the phenotype of *krh1*Δ *krh2*Δ mutants may be the result of increased cell adhesion due to higher levels of *FLO11* expression.

KRH1 and *KRH2* negatively regulate processes controlled by the cAMP/PKA pathway

Gpa2p activity also has effects in strains that are not capable of undergoing pseudohyphal or invasive growth. For example, cells of a non-filamentous strain that contain a constitutively active allele of *GPA2* display a decrease in sporulation efficiency and an increase in heat shock sensitivity (Xue et al., 1998). Increased sensitivity to heat shock is a phenotype associated with cells that have increased activity of growth control pathways, including both the cAMP/PKA pathway and other redundant pathways (Cameron et al., 1988; Toda et al., 1987a). To determine whether the *KRH1* and *KRH2* genes play a role in heat shock sensitivity, survival following a heat shock was measured for wild-type, *krh1*Δ, *krh2*Δ and *krh1*Δ *krh2*Δ cells in a W303 background. Whereas 48% of wild-type cells in stationary phase survived after a 50°C heat shock, only 1.0% of *krh1*Δ *krh2*Δ cells survived after this treatment (Fig. 2A). Single *krh1*Δ and *krh2*Δ mutants displayed intermediate survival levels of 2.8% and 22.4%, respectively. This finding confirms that the *KRH1* and *KRH2* gene products are negative regulators of the signaling pathway because an increase in heat shock sensitivity occurs either when constitutively active *GPA2* is present (Xue et al., 1998) or when the *KRH1* and *KRH2* genes are deleted.

The effect of *krh1*Δ *krh2*Δ mutations on the ability of cells to sporulate was also examined. Cells containing intact *KRH1*

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Krh1          KLPALTYHCSVELNGNIYIFGGLMPCYSYEEDAPMLNDFFVDGIKNLPP 324
Krh2          KLPSLSYHCSVELNDQLFIVGGLMACHRYDEEAPDLKDFYVDGIKNLPP 337

Krh1          PPPLLCVQGSKLTDRHIFFYGGFEIR--TETRGDENGKYHLKKRLYVNN 408
Krh2          PPPLVCTQGCKLTERHIFLYGGFEIK--SETQVDDKGRYFIRKRAFLNN 421

Krh1          AVLSSLPH---NTVHTVIIFGGYRQT--GDDRYEAMNDLWKIEIPVIRR 532
Krh2          KHCSTATHIC-SSVNTILIFGGYSQT--GDDKYEAMNDMWKINIPVVSR 574

Krh1          GRQRLILSQEKPVGKTVVLHGGSNGLNVLDDMWLMDLECETWTPIETFA 724
Krh2          TPPLNPSKKCASIGRTIAFHGGSDGYDVCSDMWWFDFDSETWTKIDLYA 748

Krh1          VNVGLVGHRMESIGRICVCIGGMVQE--DVDQFYSENDDESPRKRKVDT 785
Krh2          INLCMVGHSMTTVGHKVVLIGGLRQG--DVDRIYR---DETLPEEVISG 803

Krh1          NIVVGVGGTSLQCDKSIILIGGLISR--RSNVKEIYLHGTITKSIFPSV 876
Krh2          HQVLTVAGTIELVKGTMTLIGGVVAG--REDISSLYLRGAVLQFILPSM 893

                          I H III                           I
consensus:             S  L K LVLIGG       EE      E        L
                       C  V R VFFF         DD      D        V

                                IY I
Kelch repeat:                 G LLIVGG              Y      W
                              D VFVF
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Fig. 6. Repeat sequences in Krh1p and Krh2p. The six repeated sequences from Krh1p and Krh2p were aligned based on the double glycine present in each repeat. The invariant double glycine is highlighted in black. Other conserved residues that are present in at least four repeat sequences are highlighted in gray. The kelch repeat consensus sequence from Adams et al. (Adams et al., 2000) is shown below. The Krh1p and Krh2p protein sequences were derived from the nucleotide sequence of the genes from the Σ1278b strain background, which differs from that in the *Saccharomyces* Genome Database by about 1% at the nucleotide level.

and *KRH2* genes displayed 51% sporulation efficiency (Fig. 2B). *krh1*Δ, *krh2*Δ and *krh1*Δ *krh2*Δ cells all showed a decrease in sporulation efficiency. The largest effect was seen in *krh1*Δ *krh2*Δ cells, which had a sporulation efficiency of 6%. Single *krh1*Δ and *krh2*Δ mutants displayed intermediate levels of sporulation of 26% and 35%, respectively. Therefore, deletion of the *KRH1* and *KRH2* genes results in multiple physiological changes that are associated with increased activation of the cAMP/PKA pathway.

The interaction between Krh1p and Gpa2p revealed by the two-hybrid assay raised the possibility that Krh1p and Krh2p are proteins that interact with G_α subunits in general. Therefore, it was possible that these proteins also interact with Gpa1p, the G_α subunit that mediates the pheromone response pathway. *krh1*Δ, *krh2*Δ and *krh1*Δ *krh2*Δ cells were tested for their ability to undergo cell cycle arrest in response to pheromone by measuring the density of cell growth in an area surrounding a filter disk containing α-factor (halo assay). No differences were detected in this assay among wild-type, *krh1*Δ, *krh2*Δ or *krh1*Δ *krh2*Δ cells (data not shown). Therefore, the function of *KRH1* and *KRH2* appears to be specific to the Gpa2p pathway.

KRH1 and *KRH2* act downstream of *GPA2*

If Krh1p and Krh2p couple Gpa2p to downstream components, then deletion of the *KRH1* and *KRH2* genes would be expected to eliminate the effects of a constitutively active form of Gpa2p. To test this idea, the effect of Gpa2p activation on *FLO11* RNA abundance was determined by isolating RNA from strains containing either vector or the constitutively active $GPA2^{R273A}$ allele. In wild-type cells, *FLO11* RNA levels were significantly higher in cells containing the $GPA2^{R273A}$ allele than in cells containing vector alone (Fig. 3A, lanes 1,2). These findings are in agreement with previous results that indicate that *GPA2* is involved in the transition to filamentous growth (Kübler et al., 1997; Lorenz and Heitman, 1997). The presence of the $GPA2^{R273A}$ allele had no effect on *FLO11* RNA abundance in the *krh1*Δ *krh2*Δ strain (lanes 3 and 4). One interpretation of this result is that transmission of the signal generated by activated Gpa2p is largely prevented by deletion of *KRH1* and *KRH2*.

To determine whether the *KRH1* and *KRH2* genes are required for the effect of Gpa2p activation on heat shock sensitivity, survival following a heat shock was measured in wild-type and *krh1*Δ *krh2*Δ strains. Heat shock treatment of wild-type cells containing the constitutively active $GPA2^{R273A}$ allele caused a decrease in survival of approximately 70-fold compared with vector-containing cells (Fig. 3B), confirming previous results (Xue et al., 1998). However, *krh1*Δ *krh2*Δ cells containing either vector or the $GPA2R^{273A}$ plasmid displayed the same survival levels (Fig. 3B). Elimination of the effects of the $GPA2R^{273A}$ allele by the *krh1*Δ *krh2*Δ mutations is consistent with a model in which Krh1p and Krh2p act downstream of the Gpa2p α-subunit.

Signal generated by lack of *KRH1* and *KRH2* does not require *SCH9*

Previous results from our laboratory suggested that the Sch9p kinase acts downstream of Gpa2p (Xue et al., 1998). It was therefore of interest to test whether deletion of the *SCH9* gene blocks the signal resulting from deletion of the *KRH1* and *KRH2* genes. If Krh1p and Krh2p act immediately downstream of Gpa2p, then components that act further downstream in the same pathway would be expected to eliminate the signal generated by the absence of the negative regulators. To determine the effect of an *sch9*Δ mutation on the phenotype conferred by the *krh1*Δ and *krh2*Δ mutations, the heat shock sensitivity of strains containing different mutations was measured. Cells containing an *sch9*Δ mutation did not display a heat shock sensitive phenotype (Fig. 4A), in agreement with previous results (Xue et al., 1998). Heat shock treatment of the triple mutant *krh1*Δ *krh2*Δ *sch9*Δ strain caused a large decrease in survival, similar to the effect seen in a *krh1*Δ *krh2*Δ strain (Fig. 4A). Therefore, the *sch9*Δ mutation does not eliminate the phenotype caused by deletion of the *KRH1* and *KRH2* genes. This finding suggests that Sch9p does not act downstream in a pathway that is negatively regulated by Krh1p and Krh2p.

The data presented above are consistent with the idea that Krh1p and Krh2p act downstream of Gpa2p to negatively regulate a signaling pathway that does not require Sch9p. If this were the case, then *krh1*Δ *krh2*Δ mutations would be expected to suppress phenotypes present in *gpa2*Δ *sch9*Δ double mutants by activating the downstream pathway. To test this possibility, strains were constructed that contained either deletions of *KRH1* and *KRH2*, deletions of *GPA2* and *SCH9*, or all four mutations together. *krh1*Δ *krh2*Δ mutant strains did not display an apparent growth defect (Fig. 4B). *gpa2*Δ *sch9*Δ mutant strains displayed a severe growth defect and did not produce visible colonies after 2 days of growth, as described previously (Kraakman et al., 1999; Lorenz et al., 2000). Strains containing all four mutations showed substantial suppression of the growth defect seen in the *gpa2*Δ *sch9*Δ strain. These results indicate that loss of *KRH1* and *KRH2* results in activation of a pathway that partially compensates for the lack of *SCH9*.

Signal generated by lack of *KRH1* and *KRH2* or activation of Gpa2p requires *TPK2*

Activation of the *RAS*/cAMP pathway suppresses the growth defect conferred by an *sch9*Δ mutation (Hartley et al., 1994; Toda et al., 1988). Although the Ras proteins function independently of Gpa2p (Colombo et al., 1998; Xue et al., 1998), suppression of the *sch9*Δ growth defect by *krh1*Δ *krh2*Δ mutations could be explained by activation of downstream components of the *RAS*/cAMP pathway under these conditions. One candidate for a downstream component that is activated in *krh1*Δ *krh2*Δ cells is the cAMP-dependent protein kinase Tpk2p. In yeast, cAMP-dependent protein kinase is encoded by three genes, *TPK1*, *TPK2* and *TPK3* (Toda et al., 1987b). However, only the *TPK2* gene product positively regulates *FLO11* transcription (Pan and Heitman, 1999; Robertson and Fink, 1998). To determine whether *TPK2* is required for the signal generated by *krh1*Δ *krh2*Δ mutations, the basal level of *FLO11* RNA was measured in cells containing different combinations of mutations. *FLO11* RNA was present at a basal level in wild-type cells, but was undetectable in cells containing a *tpk2*Δ mutation (Fig. 5A, lanes 1,2), as described previously (Pan and Heitman, 1999; Robertson and Fink, 1998). *FLO11* RNA was also undetectable in *krh1*Δ *krh2*Δ *tpk2*Δ triple mutant cells (lane 4), demonstrating that *TPK2* is required for the signal generated in *krh1*Δ *krh2*Δ cells. These results imply that the signal transmitted through Krh1p and Krh2p inhibits the activity of the Tpk2p kinase, either directly or indirectly.

Krh1p and Krh2p appear to act downstream of Gpa2p and upstream of the cAMP-dependent protein kinase Tpk2p. To investigate further the ordering of the signaling pathway, the requirement for *TPK2* in signaling through activated Gpa2p was determined. Wild-type cells carrying the $GPA2^{R273A}$ allele contained significantly more *FLO11* RNA than cells carrying vector alone (Fig. 5B, lanes 1,2), as described above. However, no detectable *FLO11* RNA was present in *tpk2*Δ mutant cells carrying either vector or the $GPA2^{R273A}$ allele (lanes 3,4), indicating that *TPK2* acts downstream of *GPA2*.

The effect of a *tpk2*Δ mutation on the altered cell morphology conferred by the *krh1*Δ *krh2*Δ mutations was also examined. Whereas *krh1*Δ *krh2*Δ mutants grown to saturation in rich medium formed chains of elongated cells, *krh1*Δ *krh2*Δ *tpk2*Δ mutants formed clusters of more rounded cells (Fig. 5C). Therefore, the *tpk2*Δ mutation largely suppresses the cell morphology phenotype of *krh1*Δ *krh2*Δ cells. In addition, *krh1*Δ *krh2*Δ *tpk2*Δ cells displayed significantly less haploid invasive growth than *krh1*Δ *krh2*Δ cells when the plate washing was done after 3 days of growth (data not shown).

The effect of *TPK2* overexpression was determined in both wild-type and *krh1*Δ *krh2*Δ strains. In wild-type cells, *TPK2* overexpression resulted in a large increase in the abundance of *FLO11* RNA (Fig. 5D, lanes 1,2). However, *TPK2* overexpression had no effect on the high level of *FLO11* RNA present in cells containing deletions of the *KRH1* and *KRH2* genes (lanes 3,4).

Discussion

Cells containing *krh1*Δ *krh2*Δ mutations display phenotypes also seen in cells containing a constitutively active allele of *GPA2*. These phenotypes include increased expression of *FLO11*, increased invasive growth, decreased survival following heat shock, and decreased efficiency of sporulation. Moreover, deletion of the *TPK2* gene eliminates the effects of both *krh1*Δ *krh2*Δ mutations and constitutively active *GPA2*. These observations, coupled with the finding that *KRH1* was identified in a two-hybrid screen with *GPA2*, strongly suggest that Krh1p, Krh2p and the Gpa2p α-subunit are involved in the same signaling process. To investigate the relationship between *KRH1*, *KRH2* and *GPA2*, the phenotype of cells containing mutations in all three genes were studied. Whereas *gpa2*Δ mutant cells did not invade the agar and expressed a very low level of *FLO11* RNA, *krh1*Δ *krh2*Δ *gpa2*Δ mutant cells displayed increased invasive growth and a high level of *FLO11* RNA. Similarly, *krh1*Δ *krh2*Δ mutations suppressed the growth defect of *gpa2*Δ *sch9*Δ mutant cells. The finding that *krh1*Δ *krh2*Δ mutations suppress a *gpa2*Δ mutation indicates that the negative regulatory function of Krh1p and Krh2p is likely to act on a downstream component of the Gpa2p signaling pathway rather than on Gpa2p itself. One model to explain these results is that binding of Krh1p and Krh2p to Gpa2p prevents them from inhibiting the activity of a downstream component. This idea is supported by results showing that *krh1*Δ *krh2*Δ mutations eliminate the effects of a constitutively active allele of *GPA2*. In this case, the link between Gpa2p and downstream components of the pathway would be missing due to the absence of the *KRH1* and *KRH2* gene products. An observation not explained by this model is the finding that *krh1*Δ *krh2*Δ *gpa2*Δ cells express two- to threefold less *FLO11* RNA than *krh1*Δ *krh2*Δ cells. This situation could occur if signaling through Gpa2p is partially independent of Krh1p and Krh2p under some circumstances. Alternatively, Krh1p and Krh2p could have multiple negative regulatory functions and could exert their effects by inhibiting Gpa2p signaling as well as inhibiting another component in the pathway that acts upstream of Tpk2p. Nevertheless, several experiments show that deletion of *KRH1* and *KRH2* activates signaling in cells that lack Gpa2p and prevents signaling by constitutively active Gpa2p. These observations are most consistent with a model in which Krh1p and Krh2p mediate signaling from Gpa2p to downstream components.

The association between *GPA2* mutant phenotypes and cAMP signaling has led to the suggestion that yeast adenylyl cyclase is directly activated by the G_α subunit Gpa2p. This situation would be analogous to the direct activation of mammalian adenylyl cyclase by the G_α subunit $G_{\alpha s}$. In contrast to this idea, we have shown that the novel proteins Krh1p and Krh2p appear to act immediately downstream of Gpa2p. Moreover, Krh1p and Krh2p require the cAMP-dependent kinase Tpk2p for their signaling function, in agreement with previous observations that show an association between *GPA2* and cAMP signaling. Therefore, it seems likely that the effect of Gpa2p on cAMP signaling is mediated by Krh1p and Krh2p. However, overexpression of *TPK2* had no effect in cells containing *krh1*Δ *krh2*Δ mutations. Therefore, further experiments will be needed to determine whether this result is due to maximal activation of the pathway in *krh1*Δ *krh2*Δ cells or to the existence of a complex relationship between Krh1p, Krh2p and Tpk2p.

Krh1p and Krh2p do not display any obvious sequence identity to other known proteins, but they do contain six repeats that display some similarity to kelch repeats (Fig. 6). Kelch

repeats are segments of about 50 amino acids that contain a characteristic double glycine motif (Adams et al., 2000). The double glycine is situated C-terminal to four hydrophobic residues and N-terminal to conserved tyrosine and tryptophan residues that are separated from it by spacer regions. The repeats in Krh1p and Krh2p also contain a double glycine motif situated C-terminal to four hydrophobic residues. However, only one of these repeats (Fig. 6, repeat 3) contains the conserved tyrosine and tryptophan residues, and the spacer region between these residues and the double glycine repeat is smaller than that seen in the consensus for kelch repeats. The other repeats are missing either the tyrosine or tryptophan at the appropriate position. Therefore, they can be thought of as variant kelch repeats. The crystal structure of a protein containing seven kelch repeats has revealed that each repeat forms a four-stranded β-sheet, resulting in a protein that consists of a seven-bladed β-propeller (Ito et al., 1994). This 3D structure is similar to that of G protein β-subunits, which contain seven WD domains that form a seven-bladed β-propeller (Sondek et al., 1996; Wall et al., 1995). These observations raise the interesting possibility that Krh1p and Krh2p bind to Gpa2p in a manner similar to that by which G_β subunits bind to G_α subunits. Given that Gpa2p is a positive regulator of the signaling pathway and that Krh1p and Krh2p are negative regulators, it would be predicted that binding of Gpa2p to Krh1p and Krh2p blocks their ability to inhibit downstream steps in the pathway.

The *KRH1* gene was identified previously by a mutation that causes cells to continue to grow when incubated in alkaline sporulation medium (Ohkuni and Yamashita, 2000). This medium contains acetate as a carbon source and is limiting for nitrogen, a nutritional condition that induces sporulation in wild-type cells. Previous results have shown that mutational activation of Gpa2p inhibits sporulation to a significant degree (Xue et al., 1998). The finding that a loss-of-function mutation in the *KRH1* gene causes cells to continue to divide under conditions that induce sporulation is consistent with our observation that the sporulation efficiency of *krh1*Δ *krh2*Δ mutants is very low.

The *KRH2* gene was isolated previously as one of 29 genes that encode proteins that interact with the nuclear export factor Crm1p in a two-hybrid assay (Jensen et al., 2000). Although *KRH2* was not characterized further in that study, interaction with Crm1p could indicate that Krh2p is capable of nucleocytoplasmic shuttling. It is therefore of interest to note that other proteins involved in cAMP signaling, such as the catalytic and regulatory subunits of cAMP-dependent kinase, localize differentially to the nucleus or cytoplasm in response to different physiological conditions (Griffioen et al., 2000). Differential localization of Krh2p could limit inhibition of the signaling pathway to a particular cellular compartment, providing an additional level of regulation to the pathway.

Although it is likely that Krh1p and Krh2p function by transmitting a signal from Gpa2p to downstream components, the pathway that relays this signal to its ultimate physiological targets is not clearly understood. For example, work presented here demonstrates that the Sch9p kinase is not required for the increase in *FLO11* RNA that is observed in cells lacking *KRH1* and *KRH2*. However, we have shown previously that *SCH9* is required for the increase in heat shock sensitivity conferred by a constitutively active allele of *GPA2* (Xue et al., 1998). Other experiments exploring the relationship between *SCH9* and *GPA2* showed that *SCH9* is required for the increase in trehalase activity observed when a nitrogen source is added to cells starved for nitrogen, but that *GPA2* is not required for this process (Crauwels et al., 1997; Kraakman et al., 1999). Similarly, *GPA2* is required for the increase in cAMP observed when glucose is added to cells starved for glucose, but *SCH9* is not required for this process (Colombo et al., 1998; Crauwels et al., 1997). Moreover, double mutant *sch9*Δ *gpa2*Δ cells have a much more severe growth defect than either of the single mutants, suggesting that *GPA2* and *SCH9* function, at least in part, in different pathways (Kraakman et al., 1999; Lorenz et al., 2000). One interpretation of the relationship between *SCH9* and *GPA2* is that the Sch9p kinase is required only for a subset of the phenotypes associated with activation of Gpa2p. In that case, it would suggest that there are branchpoints in the signaling pathway downstream of Gpa2p. Alternatively, it is possible that Sch9p is required only for signaling through the Gpa2p pathway under particular growth conditions. Such a result would suggest that Gpa2p couples to different downstream components depending on growth conditions.

The uncovering of a novel class of signaling molecules that act downstream of a G_α subunit could have implications for G protein signaling in a wide variety of eukaryotic organisms. A homologue of *KRH2* is present in the genome of the yeast *Kluyveromyces lactis* (Ozier-Kalogeropoulos et al., 1998), and potential homologues also exist in multicellular organisms. It is likely that these *KRH1* and *KRH2* homologues couple G proteins to downstream signaling components in other organisms. Therefore, further studies of the function of Krh1p and Krh2p in signaling through the Gpa2p pathway have the potential to contribute to the understanding of general mechanisms of G protein signaling.

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References

Adams, J., Kelso, R. and Cooley, L. (2000). The kelch repeat superfamily of proteins: propellers of cell function. *Trends Cell Biol.* **10**, 17-24.

Borges-Walmsley, M. I. and Walmsley, A. R. (2000). cAMP signalling in pathogenic fungi: control of dimorphic switching and pathogenicity. *Trends Microbiol.* **8**, 133-141.

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

Cameron, S., Levin, L., Zoller, M. and Wigler, M. (1988). cAMP-independent control of sporulation, glycogen metabolism, and heat shock resistance in S. cerevisiae. *Cell* **53**, 555-566.

Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J. H., Gorwa, M.-F., Colavizza, D. et al. (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **17**, 3326-3341.

Crauwels, M., Donaton, M. C. V., Pernambuco, M. B., Winderickx, J., de Winde, J. H. and Thevelein, J. M. (1997). The Sch9 protein kinase in the

yeast *Saccharomyces cerevisiae* controls cAPK activity and is required for nitrogen activation of the fermentable-growth-medium-induced (FGM) pathway. *Microbiology* **143**, 2627-2637.

Cross, F. R. (1997). 'Marker swap' plasmids: convenient tools for budding yeast molecular genetics. *Yeast* **13**, 647-653.

Cullen, P. J. and Sprague, G. F., Jr (2000). Glucose depletion causes haploid invasive growth in yeast. *Proc. Natl. Acad. Sci. USA* **97**, 13619-13624.

Freissmuth, M. and Gilman, A. G. (1989). Mutations of $G_{s\alpha}$ designed to alter the reactivity of the protein with bacterial toxins. *J. Biol. Chem.* **264**, 21907-21914.

Gancedo, J. M. (2001). Control of pseudohyphae formation in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **25**, 107-123.

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

Griffioen, G., Anghileri, P., Imre, E., Baroni, M. D. and Ruis, H. (2000). Nutritional control of nucleocytoplasmic localizaiton of cAMP-dependent protein kinase catalytic and regulatory subunits in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 1449-1456.

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

Hartley, A. D., Ward, M. P. and Garrett, S. (1994). The Yak1 protein kinase of *Saccharomyces cerevisiae* moderates thermotolerance and inhibits growth by an Sch9 protein kinase-independent mechanism. *Genetics* **136**, 465-474.

Ito, N., Phillips, S. E., Yadav, K. D. and Knowles, P. F. (1994). Crystal structure of a free radical enzyme, galactose oxidase. *J. Mol. Biol.* **238**, 794-814.

James, P., Halladay, J. and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425-1436.

Jensen, T. H., Neville, M., Rain, J. C., McCarthy, T., Legrain, P. and Rosbash, M. (2000). Identification of novel *Saccharomyces cerevisiae* proteins with nuclear export activity: cell cycle-regulated transcription factor Ace2p shows cell cycle-independent nucleocytoplasmic shuttling. *Mol. Cell. Biol.* **20**, 8047-8058.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W. R. H., Donaton, M. C. V., Van Dijck, P., Winderickx, J., de Winde, J. H. and Thevelein, J. M. (1999). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* **32**, 1002-1012.

Kron, S. J. (1997). Filamentous growth in budding yeast. *Trends Microbiol.* **5**, 450-454.

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

Kuchin, S., Vyas, V. K. and Carlson, M. (2002). Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate *FLO11*, haploid invasive growth, and diploid pseudohyphal differentiation. *Mol. Cell. Biol.* **22**, 3994-4000.

Levin, D. E. and Errede, B. (1995). The proliferation of MAP kinase signaling pathways in yeast. *Curr. Opin. Cell Biol.* **7**, 197-202.

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 α homolog. *EMBO J.* **16**, 7008-7018.

Lorenz, M. C., Pan, X., Harashima, T., Cardenas, M. E., Xue, Y., Hirsch, J. P. and Heitman, J. (2000). The G protein-coupled receptor Gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Genetics* **154**, 609-622.

Madhani, H. D. and Fink, G. R. (1998). The control of filamentous differentiation and virulence in fungi. *Trends Cell Biol.* **8**, 348-353.

Nakafuku, M., Obara, T., Kaibuchi, K., Miyajima, I., Miyajima, A., Itoh, H., Nakamura, S., Arai, K., Matsumoto, K. and Kaziro, Y. (1988). Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: Studies on its structure and possible functions. *Proc. Natl. Acad. Sci. USA* **85**, 1374-1378.

Ohkuni, K. and Yamashita, I. (2000). A transcriptional autoregulatory loop for *KIN28-CCL1* and *SRB10-SRB11*, each encoding RNA polymerase II CTD kinase-cyclin pair, stimulates the meiotic development of *S. cerevisiae*. *Yeast* **16**, 829-846.

Ozier-Kalogeropoulos, O., Malpertuy, A., Boyer, J., Tekaia, F. and Dujon, B. (1998). Random exploration of the *Kluyveromyces lactis* genome and comparison with that of *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **26**, 5511-5524.

Pan, X. and Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19**, 4874-4887.

Reynolds, T. B. and Fink, G. R. (2001). Bakers' yeast, a model for fungal biofilm formation. *Science* **291**, 878-881.

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.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E. and Sigler, P. B. (1996). Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1 Å resolution. *Nature* **379**, 369-374.

Tamaki, H., Miwa, T., Shinozaki, M., Saito, M., Yun, C.-W., Yamamoto, K. and Kumagai, H. (2000). *GPR1* regulates filamentous growth through *FLO11* in yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **267**, 164-168.

Thevelein, J. M. and de Winde, J. H. (1999). Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **33**, 904-918.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G. and Wigler, M. (1987a). Cloning and characteriztion of *BCY1*, a locus encoding a regulatory subunit of the cAMP dependent protein kinase in yeast. *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.

Toda, T., Cameron, S., Sass, P. and Wigler, M. (1988). *SCH9*, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes Dev.* **2**, 517-527.

Wall, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G. and Sprang, S. R. (1995). The structure of the G protein $G_i\alpha_1\beta_1\gamma_2$. *Cell* **83**, 1047-1058.

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

Yun, C.-W., Tamaki, H., Nakayama, R., Yamamoto, K. and Kumagai, H. (1997). G-protein coupled receptor from yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **240**, 287-292.

Yun, C.-W., Tamaki, H., Nakayama, R., Yamamoto, K. and Kumagai, H. (1998). Gpr1p, a putative G-protein coupled receptor, regulates glucose-dependent cellular cAMP level in yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **252**, 29-33.