KIRHO1 and KIPKC1 are essential for cell integrity signalling in *Kluyveromyces lactis*

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Cell integrity in yeasts is ensured by a rigid cell wall whose synthesis is triggered by a MAP kinase-mediated signal-transduction cascade. Upstream regulatory components of this pathway in *Saccharomyces cerevisiae* involve a single protein kinase C, which is regulated by interaction with the small GTPase Rho1. Here, two genes were isolated which encode these proteins from *Kluyveromyces lactis* (*KIPKC1* and *KIRHO1*). Sequencing showed ORFs which encode proteins of 1161 and 208 amino acids, respectively. The deduced proteins shared 59 and 85 % overall amino acid identities, respectively, with their homologues from *S. cerevisiae*. Null mutants in both genes were non-viable, as shown by tetrad analyses of the heterozygous diploid strains.

Overexpression of the *KIRHO1* gene under the control of the *ScGAL1* promoter severely impaired growth in both *S. cerevisiae* and *K. lactis*. On the other hand, a similar construct with *KIPKC1* did not show a pronounced phenotype. Two-hybrid analyses showed interaction between Rho1 and Pkc1 for the *K. lactis* proteins and their *S. cerevisiae* homologues. A green fluorescent protein (GFP) fusion to the C-terminal end of KIPkc1 located the protein to patches in the growing bud, and at certain stages of the division process also to the bud neck. N-terminal GFP fusions to KIRho1 localized mainly to the cell surface (presumably the cytoplasmic side of the plasma membrane) and to the vacuole, with some indications of traffic from the former to the latter. Thus, KIPkc1 and KIRho1 have been shown to serve vital functions in *K. lactis*, to interact in cell integrity signalling and to traffic between the plasma membrane and the vacuole.

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INTRODUCTION

The advances of whole-genome-sequencing projects have dramatically increased the pace of molecular genetics. This is especially true for bacteria and unicellular eukaryotes, such as different yeast species. A number of yeast genome sequencing projects have been completed in recent years (Dujon *et al.*, 2004), including one for the milk yeast, *Kluyveromyces lactis* (Sherman *et al.*, 2004). These approaches contribute considerably to our understanding of genome evolution and help to identify protein coding sequences. Although homologues within the genome of baker's yeast, *Saccharomyces cerevisiae* (the first eukaryotic genome to be completely sequenced; Goffeau, 2000), are readily detected by homology searches, it remains to be

proven that their products are also functionally equivalent *in vivo*.

This is also true for proteins involved in signal-transduction networks. Thus, our studies of the cell integrity pathway in *K. lactis* have so far revealed a great deal of similarity to the signal transduction pathway in *S. cerevisiae*, but also some important differences. In baker's yeast, cell integrity is ensured by a central MAP kinase (MAPK) module (reviewed by Heinisch *et al.*, 1999; Heinisch, 2005; Levin, 2005), consisting of the MAPKKK encoded by *BCK1*, a dual pair of MAPKKs encoded by *MKK1* and *MKK2*, and the MAPK encoded by *MPK1* (= *SLT2*). The cascade is activated by protein kinase C, encoded by the sole *PKC1* gene of *S. cerevisiae*. The activation of this kinase upon cell surface stress is mediated by the interaction with Rho1, a small GTPase active in its GTP-bound form. The nucleotide exchange factors (GEF) generating this form of Rho1 are encoded either by *ROM2* or, to a minor extent, by *ROM1*. Stress at the cell surface is detected by transmembrane sensors of the Wsc-protein family or by Mid2. Wsc1 (= *Slg1*) and Mid2 have been shown to interact intracellularly with

Abbreviations: 5-FOA, 5-fluoroorotic acid; GFP, green fluorescent protein.

The GenBank/EMBL/DDBJ accession numbers for the *KIPKC1* and *KIRHO1* sequences reported in this paper are AY129673 and AY129674, respectively.

Rom2p to generate the signal and activate the MAPK cascade, as described above. Finally, genes encoding cell wall biosynthetic enzymes are transcriptionally activated by the phosphorylated form of the transcription factor Rlm1.

Of these components, we have previously characterized the *K. lactis* homologues *KIBCK1*, *KIMP1* and *KIROM2* (Jacoby *et al.*, 1999; Kirchrath *et al.*, 2000; Lorberg *et al.*, 2003). Whereas null mutants of the first two in *S. cerevisiae* show an osmoremedial lysis phenotype, deletion of the *ROM2* gene is rescued by the presence of *ROM1*. In contrast, *Klbc1* and *Klmp1* null mutants lack a distinct lysis phenotype under normal growth conditions. However, they still show sensitivity to a variety of substances known to affect cell integrity pathway mutants in *S. cerevisiae*, such as SDS, Calcofluor white and caffeine. *Klrom2* null mutants have proved to be non-viable, presumably because of the lack of a *ROM1* homologue in *K. lactis* (Lorberg *et al.*, 2003).

This discrepancy between the strict dependence of *K. lactis* cells on upstream components of the pathway and the comparatively weak phenotype in mutants lacking the downstream components prompted us to further investigate the signal cascade in this yeast. A central player in the signal-transduction pathway in *S. cerevisiae* is the protein kinase C (Pkc1), which can be considered a prototypic form of several mammalian isoenzymes (reviewed by Schmitz & Heinisch, 2003). As stated above, Pkc1 has been reported to be activated by the small GTPase Rho1 in its GTP-bound form (Kamada *et al.*, 1996). Pkc1–Rho1 interactions can occur at two different sites of the kinase, apparently with different outputs (the central C2 domain and the N-terminal HR1A sequence (Schmitz *et al.*, 2002; Schmitz & Heinisch, 2003). In addition, Rho1 has been reported to localize to sites of polarized cell growth and is also directly involved in cell wall biogenesis, acting as a subunit of the glucan synthase complex (Drgonova *et al.*, 1996; Qadota *et al.*, 1996). Further reported functions for the protein in *S. cerevisiae* are the organization of the cytoskeleton by interaction with Bni1 (Fujiwara *et al.*, 1998; Kohno *et al.*, 1996; Dong *et al.*, 2003) and with the polarisome (Kono *et al.*, 2005), and the involvement in the oxidative stress response by interaction with Skn7 (Alberts *et al.*, 1998). In addition, Rho1 is recruited to the sites of mating (Bar *et al.*, 2003), and has been shown to be localized at the peroxisomal surface (Marelli *et al.*, 2004). Rho1 has also been shown to interact with the exocyst component Sec3 and to maintain the polarized localization of the latter (Guo *et al.*, 2001). Moreover, liberation of chitin synthase from internal stores has been shown to be dependent on Rho1/Pkc1 (Valdivia & Schekman, 2003). Altogether, these data tightly link Rho1 function with protein delivery to sites of cell wall synthesis and secretion.

We report here on the isolation of *KIPKC1* through functional complementation of a *pkc1* deletion mutant in *S. cerevisiae*. *KIRHO1* originally isolated through its homology to *ScRHO1* also showed complementation of the respective mutants in baker's yeast. Deletion analysis

showed both genes to be essential in *K. lactis*. Two-hybrid analyses showed interaction between KIPkc1 and KIRho1, and also with the corresponding *S. cerevisiae* proteins. Finally, green fluorescent protein (GFP) fusions were employed for intracellular localization studies of the proteins in *K. lactis*.

METHODS

Strains used. *K. lactis* and *S. cerevisiae* strains used in this work are listed in Table 1. For *in vitro* mutagenesis, *Escherichia coli* strain XL1 (Stratagene) was used. For all other genetic manipulations in bacteria, *E. coli* DH5 α was employed (Invitrogen).

Media, growth conditions and enzymic analyses. Rich media were based on 1% yeast extract and 2% peptone, and supplemented with 2% glucose (YEPD). Synthetic medium consisted of 0.67% yeast nitrogen base without amino acids and was supplemented with 2% glucose (SCD). Amino acids, adenine and uracil were added according to Scherman *et al.* (1986). Other carbon sources employed, where indicated, were 2% galactose, 2% lactose, 2% (v/v) glycerol and 3% ethanol. For growth on plates, 1.5% agar was added to the media. One molar sorbitol was used for osmotic stabilization. For selection of transformants, uracil, adenine, tryptophan, leucine or histidine was omitted as required. When *kanMX* was used as selection marker (Wach *et al.*, 1994), G418 was added to rich medium (YEPD) at a final concentration of 200 mg l⁻¹ (*S. cerevisiae*) or 100 mg l⁻¹ (*K. lactis*). For mating of *K. lactis* strains, solid media with 3% malt extract and 5% agar were used. Sporulation of *S. cerevisiae* and *K. lactis* was done on 1.5% agar plates containing 1% potassium acetate, adjusted to pH 6.0.

For serial dilution patch tests, cells were grown overnight in selective medium, diluted to OD₆₀₀ 0.25, and grown for another 4–7 h. Tenfold dilutions were then prepared using the growth medium, and 3–5 μ l was spotted onto plates. Growth was documented after the indicated incubation times.

For preparation of crude extracts and determination of specific β -galactosidase activities, cells were grown in 20 ml of selective media. Crude extracts were prepared by washing twice with 3 ml water and once with 3 ml lacZ buffer (0.1 M potassium phosphate buffer, pH 7.0, 10 mM KCl, 1 mM MgCl₂) in 12 ml plastic tubes. To each cell pellet, 0.5 g glass beads (0.2–0.5 mm diameter; Roth) were added and vigorously shaken at 4 °C for 7 min (Vibrax VXR basic shaker; IKA). After the addition of 1 ml ice-cold lacZ buffer, samples were transferred to microfuge tubes and cell debris was pelleted at maximum speed for 10 min. The supernatant was transferred to new microfuge tubes and used as crude extract (with intermediate storage at –20 °C, if necessary). One millilitre of ONPG (2 mg ml⁻¹ in lacZ buffer) was used as substrate and allowed to react with appropriate amounts of crude extract at 30 °C for 5–120 min, until a yellow colour developed (Guarente, 1983). Reactions were stopped by the addition of 0.5 ml sodium carbonate solution (0.5 M). Specific activities were calculated as micromoles of substrate converted per minute and per milligram of protein. The method of Zamenhoff (1957) was used to determine protein concentrations in crude extracts, using BSA as standard.

X-Gal plate assays were performed by growing cells on plates with selective medium adjusted to pH 7.0 with 10 mM potassium phosphate buffer (pH 7.0) and with the addition of 200 μ l of a 10 mg X-Gal ml⁻¹ stock solution. Blue colour development was monitored after incubating the plates overnight at 30 °C and for another 24 h at 37 °C.

Table 1. Yeast strains employed in this work

Strain	Genotype	Source
<i>K. lactis</i> strains		
MW190-9B	<i>MATα lac4-8 Klura3 Rag⁺</i>	M. Wésolowski-Louvel
MW270-7B	<i>MATα uraA1-1 leu2 met1-1</i>	M. Wésolowski-Louvel
MW309-5B	<i>MATα lac4-8 uraA1-1 met2-2 his2-2 ade2-1</i>	M. Wésolowski-Louvel
KLD1	<i>MATα/MATα uraA1-1/uraA1-1 leu2/leu2 met1-1/MET1 met2-2/MET2 his2-2/HIS2 ade2-1/ADE2 lac4-8/LAC4</i>	Diploid obtained from a cross between MW270-7B and MW309-5B; Jacoby <i>et al.</i> (1999)
KB6-2C	<i>MATα ura3-12 his3-35 ade*</i>	K. Breunig
KMP1	<i>MATα ura3-12 leu2-137 trp1-11 ura*</i>	M. von Pein
KHO1-5C	<i>MATα ura3-12</i>	From KB6-2C X KMP1
<i>S. cerevisiae</i> strains		
HD56-5A	<i>MATα ura3-52 leu2-3,112 his3-11,15 MAL3 SUC2 GAL</i>	Arvanitidis & Heinisch (1994)
DHD5 Δ rho1	<i>MATα/MATα isogenic diploid to HD56-5A with <i>RHO1/rho1::kanMX</i></i>	Kirchrath <i>et al.</i> (2000); this work
PJ69-4A	<i>MATα trp1-901 ura3-52 leu2-3,112 his3-200 gal4Δ gal80Δ LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
RH1802	<i>MATα ura3-52 leu2-3,112 his3Δ-200 trp1-901 ade2-101 pkc1Δ::HIS3</i>	Kindly provided by Gerhard Braus, Göttingen
HNY21	<i>MATα ura3-52 leu2-3,112 his3Δ-200 trp1-901 ade2-101 rho1-104 (=rho1^{ts})</i>	Yamochi <i>et al.</i> (1994)

*Uncharacterized allele of *K. lactis* gene.

Nucleic acid manipulations. Competent *E. coli* cells were prepared according to Hanahan *et al.* (1995). Plasmid DNA was isolated by the method of Birnboim & Doly (1979) or using commercial plasmid isolation kits (Qiagen or Roche).

Yeast cells were transformed by the method of Klebe *et al.* (1983). In the case of *K. lactis*, 5 μ l RFI (10 mM MOPS, pH 6.8, 10 mM rubidium chloride, 75 mM calcium chloride, 15 % v/v, glycerol; Hanahan *et al.*, 1995) was added together with the DNA to frozen cells. Chromosomal DNA was prepared from yeast using the Qiagen isolation kit. For isolation of plasmid DNA, cells from 3 ml cultures were collected by centrifugation, washed once with 1 ml water and transferred to microfuge tubes. After another centrifugation, 250 μ l *E. coli* lysis buffer (plasmid isolation kit; Roche) and 0.2 g glass beads were added to the pellet, and cells were broken by vigorous shaking for 5 min at 4 °C. The supernatant was transferred to a fresh tube and further treated as described for the bacterial plasmid isolation kit by the manufacturer (Roche). After elution from the column in a 50 μ l volume, 10 μ l was used for transformation of *E. coli* with selection for ampicillin resistance. Southern analysis was performed following the standard methods described in Sambrook *et al.* (1989).

Genomic DNA templates for PCR reactions from yeasts were obtained by suspension of a small aliquot of cells scratched from plates into 50 μ l 0.02 M NaOH and heating in a microwave oven for 1 min. The suspension was allowed to stand at room temperature for 5 min and 2 μ l was added to the PCR reaction mixture (Robzyk & Kassir, 1992).

Genomic library and cloning of genes. The *KIPKC1* gene was cloned from a *K. lactis* genomic library constructed by Wesolowski-Louvel *et al.* (1988) in the vector KEp6 (Bianchi *et al.*, 1991) which carries *URA3* as a selection marker. The *S. cerevisiae* strain RH1802 (*pkc1 Δ ::HIS3*; Table 1) was used as a recipient for heterologous complementation of the lysis phenotype under osmotically non-stabilizing conditions. Plasmid KEpKIPKC1 (=pJJH817) was shown to carry the *KIPKC1* coding sequence and its flanking regions.

The *KIRHO1* gene was isolated by heterologous hybridization. For this purpose, genomic DNA was prepared from the *K. lactis* strain KB6-2C. A Southern analysis was performed with a hybridization temperature of 55 °C, using a PCR fragment carrying the *ScrHO1* gene obtained with the oligonucleotides RHO1-ATG and RHO1-TAG (Table 2) as a DIG-labelled probe (Roche). A 2.3 kb *HindIII* fragment was identified as specifically hybridizing to the probe. Therefore, genomic DNA of KB6-2C was digested with *HindIII*, and the fragments of approximately 2.3 kb were isolated from an agarose gel and ligated into YEp352 (Hill *et al.*, 1986). *E. coli* transformants generating white colonies on X-Gal plates were inoculated in mixtures of five colonies per sample and used for plasmid preparations. The isolated DNA was then digested with *HindIII* and, after separation in an agarose gel and blotting, hybridized to the *ScrHO1* probe described above. Plasmids were then prepared from each the five *E. coli* clones that gave a positive signal, and again used for heterologous hybridization. The *HindIII* fragment of the positive clone was subsequently subcloned into pUK1921 (Heinisch, 1993) to yield pJJH833. Sequencing of the complete insertion revealed the presence of an ORF termed *KIRHO1*.

PCR reactions and plasmid constructions. All oligonucleotides employed are listed in Table 2. For heterologous complementation studies in *S. cerevisiae*, the complete *KIPKC1* gene and its flanking regions was excised from KEpKIPKC1 as a *Sall* fragment and cloned into YEp352 (Hill *et al.*, 1986) to yield plasmid YEp352-KIPKC1, and the *KIRHO1* coding region was contained within a *HindIII* fragment in YEp352-KIRHO1. For expression analyses in *K. lactis*, the described DNA fragments (*KIPKC1-Sall* and *KIRHO1-HindIII*) were cloned into the shuttle vectors pCXJ22 (*URA3*) and pCXJ24 (*LEU2*), respectively, to give pCXJ22-KIPKC1 and pCXJ24-KIRHO1. They behave as multicopy plasmids in *K. lactis* and contain a *CEN/ARS* sequence for *S. cerevisiae* (Chen, 1996).

Plasmids for the expression of the genes under the *GAL1* promoter were constructed by *in vivo* recombination. For *KIPKC1*, first a *HIS3-PGAL1* cassette was amplified by PCR using plasmid pFA6a-His3MX6PGAL1 as template (Longtine *et al.*, 1998) and employing the oligonucleotide

Table 2. Oligonucleotides used in this work

Sequences introduced not corresponding to the yeast genes are depicted in underlined upper-case type. Restriction sites introduced by the oligonucleotide are in lower-case type. Single nucleotides underlined and in bold type correspond to substitutions leading to amino acid exchanges or the introduction of new restriction recognition sites. Bold upper-case type designates translational stop codons introduced by the oligonucleotide.

Name	Sequence (5'–3')
833v	GAAATGTCAGGGAGGGATGG
833z	GGACTGTACTTCACAGCACAGGG
GALpKIPKC1-F4	GGATACCAAAAACTAAAGGAAAGAACGACTATAAAACAACGGTTTATTAAGAATTTCGAGCTCGTTTAAAC
GALpKIPKC1-R2	GTAGATGTCCTGTTCAACAGGGAATGCAGTGTGTTTACGATCTTCATCATTTTGTAGATCCGGGTTTT
GALpKIRHO1-F4	CAGGTTATTGTTGTGAGTGATTGAGTATACGGACGGTACTAAATCATAGGAATTTCGAGCTCGTTTAAAC
GALpKIRHO1-R2	CAATAACTAGCTTTCTTCTAATGCTGGCAACATTACCAACAGCTTGAGACATTTTGTAGATCCGGGTTTT
HindR2	<u>GGT</u> GaagcttGCAGTCATTTTGTAGATCCGGGTTTT
KIPKC1atg	GATGATGAAGATACCCCAACCAACCAAAAAAGAGATCGAATTCCCGgatccATGGATGAAGATCGTA-AACACACTG
KIPKC1mid	GCGGGGTTTTTCAGTATCTACGATTTCATAGATCTCTGCAGgtcgac TTACTA AGGCGTAGTTGGCTTGATAACGG
KIPkc1rd5	GCAACGCAGTACCATACTTGG
KIPKC1term2	GGTAGGAAACGCAATGGGAAGG
KIPKCmycF2	GAGGAATTCGGTGGCTTTTCTTCATGCCGAAGACTTGCAGTTACGGATCCCCGGGTAAATTAA
KIPKCmycR1	GCGGTAGGAAACGCAATGGGAAGGAACGTTTCGTGGGTAATCGTACGGAATTCGAGCTCGTTTAAAC
KIRHO1C205S	<u>GGCG</u> ctgcagTTACAAGACGACAG ACTT CCTTTTTTTGCTAGACTTAG
Klrho1delta3' neu	GGGTGAAGAGTGGGTTTGGGGCGTGCGTGTCTTTATTACAAGACGACACACGACCATGATTACGGCTAGC-TTGG
Klrho1delta5'	ATGTCTCAAGCTGTTGGTAATGTTGCCAGCATTAGAAGAAAGCTAGTTATTGATTAAGTTGGGTAACGCCAGGG
KIRHO1eco	<u>GGCG</u> gaattcATGTCTCAAGCTGTTGGTAATGTTG
KIRho1gfpR5	CTAGCTTTCTTCTAATGCTGGCAACATTACCAACAGCTTGAGACATTTTGTATAGTTCATCCATGC
KIRHO1GFPprev	GGGACAACTCCAGTGAAGTTCTTCTCCTTTACTGTTAATTAAAGACATTATGGAAAGGGTTAAAAACAAATC-AAATC
KIRHO1HAF4	GAGGTTTGATTTTTATTTTCGGGTTGTTTTATTTTCTATTTTAACGAATTCGAGCTCGTTTAAAC
KIRho15vor	GAGGTTTGATTTTTATTTTCGGGTTGTTTTATTTTCTATTTTAACGCATGGCTTAGACGTGGCTGCTG
KIRho1905raus	CTTGCTTTGCCACAACCCAAGCGC
KIRHO1Q70Hneu	AATTGGCGTTGTGGGATACTGCTGGCCAC GAGG ATTATGATAGATTGAGA
KIRHO1Q70Hrevneu	TCTCAATCTATCATAAT CTCT CTGGCCAGCAGTATCCCAACAGCCAATT
Rho1-ATG	GCGGGGATCCGAATCGCTGTTAGAGG
Rho1-TAG	GGGCAGTATCTGCCA
Rho1-5'	ggaatgggagagtcgacg
Rho1-3'	CTGAGGTTGGAGGCTCAG
u40ccwneu	GCAGCCTGAATGGCGAATGG
universal -40	GTTTCCAGTCACGAC
universal -47	CGCCAGGGTTTTCCAGTCACGAC
URA3-5r	CAGTCAAGATATCCACATGTG
URA3-3r	GGGAAGGGATGCTAAGGTAGAGGG

pair GALpKIPKC1-F4/GALpKIPKC1-R2 (Table 2). The resulting fragment was co-transformed with plasmid pCXJ22-KIPKC1 into *S. cerevisiae* strain HD56-5A, selecting for histidine prototrophy. Plasmid pCXJ22-pGAL1KIPKC1 was isolated from yeast transformants and checked for correct recombination by restriction analyses and sequencing. Similarly, a *KanMX-PGAL1* cassette was amplified by PCR using plasmid pFA6a-KanMx-pGAL1 as a template (Longtine *et al.*, 1998), and employing the oligonucleotide pair GALpKIRHO1-F4/GALpKIRHO1-R2 (Table 2). The PCR product was then co-transformed with plasmid pCXJ24-KIRHO1 into *S. cerevisiae* strain HD56-5A, selecting for G418 resistance on YEPD medium. Plasmid pCXJ24-pGAL1KIRHO1 isolated from yeast transformants showed the correct recombination by restriction analyses and sequencing.

Activated alleles of *KIRHO1* were obtained by *in vitro* mutagenesis, as described below for the activated *KIRHO1* alleles introduced into the two-hybrid vectors, although the geranyl-geranylation site (C205) was left intact. Activated alleles of *KIRHO1* were obtained by *in vitro* mutagenesis. Starting with pJJH833 (pUK1921-KIRHO1) as template and the oligonucleotides KIRHO1Q70Hneu and KIRHO1Q70Hrevneu, plasmid pUK1921-KIRHO1Q70H was obtained using the Quick Change Site-Directed-Mutagenesis kit (Stratagene). From this, plasmids pCXJ24-KIRHO1Q70H and pCXJ24-pGALKIRHO1Q70H were constructed, as described above for the wild-type *KIRHO1* alleles.

To investigate the adequacy of the *ScGAL1* promoter for conditional expression studies in *K. lactis*, plasmid pXW3-pGAL1lacZ was

constructed. For this purpose, a *Bgl*II–*Hind*III fragment containing the *GAL1* promoter was obtained by PCR using oligonucleotides GALpKIRHO1-F4 and HindR2 (Table 2) on the template pFA6a-KanMX-pGAL1 (Longtine *et al.*, 1998) and cloned in-frame to *lacZ* into pXW3 (Chen *et al.*, 1992).

For two-hybrid analyses, several plasmids were constructed. Three different *KIPKC1* fragments were fused to the sequence encoding the Gal4 activation domain in the vector pGAD424 (Bartel *et al.*, 1993). First, we PCR-amplified a part of the *KIPKC1* coding region with the oligonucleotides KIPKC1atg and KIPKC1mid from genomic *K. lactis* DNA, and co-transformed the product with pGAD424 linearized with *Bam*HI into *S. cerevisiae* HD56-5A. The PCR product carried the 5' half of the ORF with a *Bam*HI site inserted right in front of the ATG translation start codon, and lacked the 3' sequence that encodes the kinase domain (*KIPKC1*_{1–600}). After *in vivo* recombination, the resulting plasmid pJJH909 was isolated and amplified in *E. coli*. To obtain a vector carrying the complete ORF, pJJH817 (originally isolated from a genomic library as described above) was digested with *Xho*I/*Sal*I, and the 3.5 kb fragment, containing most of the *KIPKC1* coding region and the 3'-flanking sequences, was used to substitute the respective fragment from pJJH909. The resulting plasmid was called pJJH915. A two-hybrid construct only carrying the 5' end of the *KIPKC1* coding sequence (*KIPKC1*_{1–228}), comprising the HR1 sequences, fused to that of the Gal4 transcription activation domain, called pJJH910, was obtained by digestion with *Xho*I/*Sal*I and religation of the large fragment. Sequencing confirmed that the regions introduced by PCR contained neither a translation stop codon nor errors leading to non-conservative amino acid exchanges.

For cloning of *KIRHO1* sequences into a two-hybrid vector, the coding region was first PCR-amplified from pJJH833 with the oligonucleotides KIRHO1eco and universal -40. The resulting fragment was digested with *Eco*RI/*Pst*I and cloned into pUK1921 to yield pJJH905. From the latter, a sequence leading to a C205S substitution, which destroys the putative geranyl-geranyl modification site of KIRho1, was introduced by inverse PCR using the oligonucleotides KIRHO1C205S and KIRho1905raus, followed by digestion with *Pst*I and religation. The resulting plasmid was called pJJH916. From that, pJJH917HB (=pUK1921-KIRHO1Q70H-C205S) was obtained by *in vitro* mutagenesis, as described above for the overexpression plasmids. The activated *KIRHO1Q70H-C205S* allele was excised by digestion with *Eco*RI/*Pst*I and fused to the Gal4 DNA-binding domain in the vector pGBD-C1 (James *et al.*, 1996) digested with the same enzymes to give pJJH918HB.

pGADScPKC containing the *S. cerevisiae* *PKC1* gene fused to the coding sequence for the Gal4 activation domain was kindly provided by Jörg Jacoby, Anderson Cancer Center, Houston, TX, together with pHPS49 with the *ScrHO1Q68HC206S* allele fused to the sequence encoding the Gal4 DNA-binding domain in the pGBT9 vector (Bartel *et al.*, 1993). In addition, pTD1 (Li & Fields, 1993) and pVA3 (Iwabuchi *et al.*, 1993) were used as controls for a positive interaction.

For intracellular localization studies, KIRho1 was tagged at its N-terminal end with GFP. For this, a marker cassette was amplified by PCR using plasmid pFA6a-KanMX6-pGAL1-GFP as template (Longtine *et al.*, 1998) and the oligonucleotide pair KIRHO1HAF4/KIRho1gfpR5 (Table 2). The PCR product was then co-transformed with plasmid pCXJ24-KIRHO1 into *S. cerevisiae* strain HD56-5A selecting for resistance to G418 on YEPD medium. Plasmid pCXJ24-pGAL1-GFPKIRHO1 isolated from colonies growing on this medium showed the correct recombination by restriction analyses. To obtain a GFP fusion under the control of the original *KIRHO1* promoter, the respective region was amplified by PCR from pJJH833 using the oligonucleotide pair KIRHO1GFPprev and KIRho15vor, and the resulting fragment co-transformed with pCXJ24-pGAL1-GFPKIRHO1

linearized with *Mlu*I into *S. cerevisiae* HD56-5A. Plasmid pRRO82 (pCXJ24-GFPKIRHO1) showing the expected *in vivo* recombination was isolated from transformants selected for leucine prototrophy, amplified in *E. coli* and introduced into *K. lactis* KMP1, again selecting for the *LEU2* marker. Similarly, KIPkc1 was labelled at its C-terminal end with GFP, using pFA6a-GFP-KanMX6 as a template and the primers KIPKCmycF2 and KIPKCmycR1. The PCR product was co-transformed with plasmid pCXJ22-KIPKC1 for *in vivo* recombination in *S. cerevisiae* strain HD56-5A. Plasmid pRRO80 (pCXJ22-KIPKC1GFP) was demonstrated to carry the desired fusion. To test for the *in vivo* function of the GFP fusions, pRRO80 was shown to complement the lysis phenotype of the *S. cerevisiae* strain RH1802 (Table 1) on medium lacking uracil and osmotic support. The *GFPKIRHO1* function was shown by complementation of lethality of a *rho1* deletion when expressed in multicopy. For this purpose, the 3.6 kb *Hind*III fragment from pRRO82 carrying *GFPKIRHO1* with its flanking sequences was subcloned into the respective site of YE352 (Hill *et al.*, 1986) to yield plasmid pJJ934. This was introduced into strain DHD5Δrho1 (described below and in Table 1), selecting for uracil prototrophy. One of the resulting clones was sporulated and subjected to tetrad analysis. Most of the 18 tetrads analysed yielded three viable spores on YEPD, and at least one spore of each tetrad was G418 resistant (indicative of the *rho1* deletion). The latter were invariably prototrophic for uracil and sensitive to 5-fluoroorotic acid (5-FOA), demonstrating that they were inviable without the complementing function provided by the plasmid.

Restriction maps and sequences of all the constructs described above are available upon request.

Construction of null mutants. A heterozygous *rho1* deletion in *S. cerevisiae* was obtained by amplifying the *kanMX* cassette from genomic DNA of a *rho1::kanMX* deletion strain kindly provided by Michael Hall, University of Basel, using the flanking oligonucleotides Rho1-5' and Rho1-3' (Table 2). The fragment obtained was introduced into the diploid strain DHD5 (Kirchraht *et al.*, 2000) with selection for G418 resistance. Tetrad analysis showed a 2:2 segregation for viability, as expected from the lethal phenotype of a *rho1* deletion.

To construct a *K. lactis* strain carrying a disruption of the *KIPKC1* gene (Fig. 1A) the internal 1.45 kb *Bgl*II–*Nsi*I fragment of the gene was subcloned into YIplac128 (Gietz & Sugino, 1988). The plasmid, termed YIp-Klpkc1d, was digested with *Xho*I prior to transformation into the diploid strain KLD1 obtained by crossing of MW270-7B and MW309-5B (Table 1). Transformants were selected on SCD lacking leucine. Correct integration was checked by PCR, using the oligonucleotide pairs KIPkc1rd5/KIPKC1term2, KIPkc1rd5/u40ccwneu and universal -47/KIPKC1term2. A strain yielding the expected fragments was sporulated and subjected to tetrad analysis.

The *KIRHO1* deletion was obtained by first replacing the coding region on a yeast multicopy plasmid with a *URA3* marker by *in vivo* recombination and substitution of the genomic wild-type copy using a linear fragment derived from this. For this purpose, the *URA3* marker gene was amplified by PCR from YDpU (Berben *et al.*, 1991) with the oligonucleotides Klrho1delta5' and Klrho1delta3'neu (Table 2). The *Hind*III fragment with *KIRHO1* was subcloned from pJJH833 into YE351 (Hill *et al.*, 1986) to yield pJJH840. The latter was co-transformed with the PCR product into HD56-5A. Transformants were selected for uracil prototrophy, and the recombinant plasmid pJJH841 was isolated, amplified in *E. coli* and checked by restriction analyses. For later PCR verification we shortened the construct as follows. To reduce the size of the 5'-flanking sequence, the *Hind*III fragment with the *Klrho1::URA3* construct was cloned into pUK1921 to yield pJJH845, digested with *Xba*I and religated. The resulting plasmid was called pJJH853 and digested with *Hind*III/*Xba*I prior to

transformation of the diploid *K. lactis* strain KLD1. Transformants were selected for uracil prototrophy and checked by PCR with the oligonucleotides 833v, 833z, URA3-3r and URA3-5r for correct substitution of one of the chromosomal copies. A transformant displaying the right fragments was sporulated and subjected to tetrad analysis.

Sequence analyses and accession numbers. DNA sequences were obtained from SeqLab employing custom-made oligonucleotides from MWG Biotech. The sequences for *KIPKC1* and *KIRHO1* were determined for both DNA strands and deposited in GenBank under the accession numbers AY129673 and AY129674, respectively. Note that no single difference could be detected to the respective sequences obtained when the complete *K. lactis* genome was published (Sherman *et al.*, 2004; Dujon *et al.*, 2004). Sequence analyses, including alignments, were performed using the functions of Clone Manager Suite 7.0 (Scientific and Educational Software) with standard settings. Specific domains were identified with the SMART program (Schultz *et al.*, 1998) using the default thresholds.

Fluorescence microscopy. For fluorescence microscopy, cells were grown overnight in selective synthetic medium and then inoculated for another 6 h in fresh medium with shaking at 30 °C. From these cultures, 2 µl was observed on a standard microscope slide covered with a cover slip and sealed with Fixogum (Marabuwerke). The setup used for *in vivo* fluorescence microscopy consisted of a Zeiss Axioplan2e microscope equipped with a ×100 alpha-Plan Fluor objective and differential-interference-contrast. Images were acquired using a Photometrics CoolSNAP HQ camera (Roper Scientific). Fluorescence was excited with a xenon lamp, and filter set 41-001 (AHF) was used to adjust excitation and emission wavelengths. The setup was controlled by Metamorph v6.2 software (Universal Imaging Corporation). Brightfield images were acquired as single planes using differential-interference-contrast. To visualize fluorescence of Pkc1, only three planes were taken. For Rho1, up to 12 Z planes from top to bottom of the cell were acquired. Deconvolution was performed on the acquired stacks using Huygens Essential Software (Scientific Volume Imaging). Default parameters were used, except for numerical aperture (set to 1.45). Results were imported into Metamorph and used for maximum projection and scaling of images.

RESULTS

KIPKC1 and *KIRHO1* complement the respective *S. cerevisiae* defects

In *S. cerevisiae*, deletion of the unique *PKC1* gene is lethal in some genetic backgrounds and can be partially rescued by osmotic stabilization in others. One strain of the latter type, RH1802 (*pkc1::HIS3*), was used as recipient for cloning of *KIPKC1* by heterologous complementation with a genomic library of *K. lactis* (see Methods for details). Transformants were first selected for growth on synthetic medium lacking uracil and containing 1 M sorbitol. Replica-plating onto SCD without uracil and lacking osmotic stabilization, and incubation at 30 °C, yielded one positive clone from which plasmid KEpKIPKC1 was recovered. It contained an insertion of approximately 10.5 kb of genomic *K. lactis* sequence. Subcloning of various fragments into YEp352 and transformation into the *pkc1::HIS3* recipient narrowed down the complementing sequence to a *SalI* fragment of 7.4 kb (Fig. 1A, C).

From that, a 4.3 kb fragment, which spanned the entire *KIPKC1* coding sequence and its flanking regions, was sequenced for both strands. An ORF of 3483 bp was found, which encoded a deduced protein of 1161 amino acids. After completion of the genome sequence, this fragment could be detected on chromosome V. *S. cerevisiae* Pkc1 has a variety of functional domains, as deduced from homology to the mammalian isozymes and functional analyses in yeast (Schmitz & Heinisch, 2003). These are also conserved in Klpkc1 with an overall identity to ScPkc1 of 59 % (see Fig. 1B for a schematic representation).

The *KIRHO1* gene was isolated by heterologous hybridization with a *ScRHO1* probe on a 2.3 kb genomic *HindIII* fragment, as described in Methods (Fig. 2A). Sequence analysis demonstrated that the putative *KIRHO1* ORF consisted of 624 bp, which encoded a deduced protein of 208 amino acids and was located on chromosome II. An alignment with the protein of *S. cerevisiae* resulted in an overall identity of 82 % (Fig. 2B). A *rho1^{ts}* strain from *S. cerevisiae* was used as a recipient for transformation with a multicopy vector carrying the respective fragment to confirm functional complementation. As shown in Fig. 2(C), plasmid YEp352-KIRHO1 conferred growth on the *S. cerevisiae* mutant at the restrictive temperature, although transformants grew quite slowly compared to a wild-type strain. Interestingly, *KIRHO1* was not able to complement a *Scrho1* deletion if carried on a single-copy vector (pCXJ24-KIRHO1) which was introduced into a diploid strain heterozygous for the deletion (*RHO1/rho1::kanMX*) and subjected to tetrad analysis. There, a maximum of two segregants each from 18 tetrads segregated were viable, none of which was G418 resistant. Nevertheless, transformation with a multicopy vector yielded viable segregants of the type *rho1::kanMX/YEp352-KIRHO1*. A total of 10 segregants that were G418 resistant were invariably prototrophic for uracil, indicating that episomal *KIRHO1* is able to complement if expressed at sufficiently high levels. In contrast to *RHO1* wild-type cells carrying the vector, such segregants did not grow on medium containing 5-FOA, i.e. plasmid loss was lethal.

KIPKC1 and *KIRHO1* are essential genes

To investigate the role of the cloned genes in *K. lactis*, we constructed null mutants starting with the diploid strain KLD1. For *KIPKC1*, various deletion constructs with different markers were prepared. Despite numerous attempts, none of the transformants obtained on selective media showed a correct recombination, as judged by specific PCR reactions. Therefore, we constructed a strain carrying a disruption of the *KIPKC1* gene (Fig. 1A). One transformant that showed the correct integration by PCR was subjected to sporulation and tetrad analysis. Germination was allowed on YEPD with 1 M sorbitol for osmotic stabilization. Out of 13 tetrads separated, all but two generated a maximum of two viable spores. All viable segregants (including the two tetrads yielding three spores with viable progeny) failed to grow on medium lacking leucine, the marker for the

Fig. 1. Characterization of *KIPKC1*. (A) *KIPKC1* restriction map and disruption construct. See Methods for details on cloning and gene disruption. (B) Comparison of domain structures of KIPkc1 and ScPkc1. A schematic representation of sequence similarities is shown. (C) Heterologous complementation in *S. cerevisiae*. Strain RH1802 was used as a recipient for the plasmids indicated, and growth on rich medium was assessed after 2 days of incubation. Three independent transformants carrying the *KIPKC1* gene on a *SalI* fragment were tested.

disrupted *Klpkc1* copy. This result strongly suggested that the wild-type gene is essential in *K. lactis*.

A *Klrho1* null mutant was obtained in the diploid strain KLD1 by substitution of the complete ORF with the *URA3* gene from *S. cerevisiae* (Fig. 2A). One transformant that had the correct integration as validated by PCR was sporulated. Again, segregants were allowed to germinate on YEPD/1 M sorbitol medium. All of 12 tetrads segregated with a maximum of two viable spores. The fact that all segregants required uracil indicated that *KIRHO1*, too, is essential in *K. lactis*.

Overexpression of *KIRHO1*, but not of *KIPKC1*, affects growth of *K. lactis*

In order to allow for a phenotypic analysis, we first wanted to assess the effect of overexpression of *KIRHO1* in *K. lactis*. For this purpose, plasmid pCXJ24-pGAL1KIRHO1, which propagates with multiple copies in *K. lactis* and as a centromeric plasmid in *S. cerevisiae* (Chen, 1996), and carries *KIRHO1* under the control of the *S. cerevisiae* *GAL1* promoter, was constructed by *in vivo* recombination. As a control, the *KIRHO1* gene with its original promoter was cloned into the same vector.

We have previously used the *ScGAL1* promoter in the investigation of overexpression of the *KIROM2* gene encoding a GDP/GTP exchange factor in *K. lactis* (Lorberg *et al.*, 2003). Here, we used the *lacZ* reporter construct to measure β -galactosidase activity in strain MW190-9B (*lac4*) transformed with pXW3-pGAL1lacZ (*ScGAL1p-lacZ*), as described in Methods. Specific β -galactosidase activities reached approximately 5 U (mg protein)⁻¹ after growth on galactose, with half-maximum activity obtained after 5 h of induction. A shift to repressing conditions (i.e. growth on 5 % glucose) eventually decreased β -galactosidase activities to below detectable levels after 72 h of incubation (data available upon request). Thus, the heterologous *ScGAL1* promoter is properly regulated in *K. lactis* and can be employed for conditional expression.

To test the effects of *KIRHO1* overexpression in *K. lactis*, plasmids pCXJ24-pGAL1KIRHO1 and pCXJ24-KIRHO1 were transformed into MW270-7B, selecting for leucine prototrophy on media with glucose as the sole carbon source. Subsequent serial dilution patch tests showed that growth was clearly impaired on galactose in the strain carrying the *ScGAL1p-KIRHO1* allele for all media and conditions tested, as compared to the strain containing just

Fig. 2. Characterization of *KIRHO1*. (A) *KIRHO1* restriction map and deletion construct. See Methods for details on cloning and construction of the deletion. (B) Alignment of the deduced amino acid sequences of KIRho1 and ScRho1. Amino acid exchanges to introduce an activated allele (Q70H) or to avoid membrane localization in the two-hybrid experiments (C205S) are indicated by arrows. (C) Complementation of the *Scrho1*^{ts} mutant phenotype by *KIRHO1*. Strain HNY21 was used as a recipient for the plasmids indicated, and growth on selective medium was assessed after 2 days of incubation at the indicated temperatures. Four independent transformants were tested, each carrying either the *KIRHO1* gene (YEpl352-KIRHO1) or the vector without insertion (YEpl352).

the vector control or *KIRHO1* expressed from its own promoter (Fig. 3A). No differences were found on glucose medium among the different transformants in this experiment, in agreement with the data obtained for the tight regulation of the *ScGAL1* promoter in *K. lactis* described above. It should be noted that we were unable to retrieve the plasmids carrying the *GAL1* promoter constructs (neither pCXJ24-pGAL1KIRHO1 nor pCXJ22-pGAL1KIPKC1, described below) from transformants, even when grown on glucose media lacking leucine or uracil, respectively. Moreover, different *K. lactis* strains showed different degrees of growth inhibition on galactose medium with

pCXJ24-pGAL1KIRHO1, i.e. were phenotypically more heterogeneous than the *S. cerevisiae* strains overexpressing *KIRHO1* (see section on *S. cerevisiae* *KIRHO1* overexpression below).

The results with *KIRHO1* prompted us to test also the effects of *KIPKC1* overexpression in *K. lactis*. There, the serial dilution patch tests showed that growth was not affected on galactose media in the strain carrying the plasmid pCXJ22-pGAL1KIPKC1 containing the *KIPKC1* gene under the *ScGAL1* promoter, compared to the strain carrying just the vector (Fig. 3B).

(A) *K. lactis* MW270-7B **(B) *K. lactis* MW309-5B** **Overexpression of *KIRHO1* also affects growth of *S. cerevisiae***

As discussed above, transformants of the *S. cerevisiae* *rho1*^{ts} mutant with the *KIRHO1* gene grew slowly. Since the high degree of identity between *KIRHO1* and *ScRHO1* argues against a partial defect in complementation, one explanation would be that the overexpression of the *K. lactis* gene from a multicopy vector is also detrimental to *S. cerevisiae*. To investigate this hypothesis, plasmids pCXJ24-pGAL1KIRHO1 and pCXJ24-KIRHO1 were transformed into the *S. cerevisiae* strain HD56-5A (*leu2 RHO1*) with selection for leucine prototrophy. Serial dilution patch tests were performed on various media (Fig. 3C). Growth was slightly impaired on galactose at 30 °C (i.e. under inducing conditions) in the strain carrying plasmid pCXJ24-pGAL1KIRHO1, compared to strains carrying pCXJ24-KIRHO1 or the empty vector. This phenotype was stronger at 37 °C and was not osmoremedial. Growth was further inhibited by addition of caffeine, Calcofluor white, Congo red or sodium chloride on galactose media (Fig. 3C and data not shown).

In *S. cerevisiae*, it has been demonstrated that a Q68H substitution results in a Rho1 conformation mimicking the GTP-bound (i.e. active) state. Here, we here constructed the corresponding *K. lactis* allele (Q70H) and tested the effect of its overexpression in *S. cerevisiae*. As shown in Fig. 3(C), the presence of such an allele did not affect growth when expressed from its own promoter, although growth was

clearly inhibited on galactose in all media and conditions tested. Thus, high-level expression of the activated *KIRHO1* allele from the *GAL1* promoter resulted in a much stronger phenotype than that observed for the wild-type allele.

We also tested the effects of *KIPKC1* overexpression in *S. cerevisiae*. As observed in *K. lactis*, the serial dilution patch tests showed that growth was not affected on galactose media in the strain carrying the plasmid pCXJ22-pGAL1KIPKC1, compared to those carrying just the vector or those in which *KIPKC1* was expressed from its native promoter (Fig. 3D). Only the addition of Calcofluor white resulted in a marginal growth inhibition under induction conditions.

KIPkc1 interacts with both KIRho1 and ScRho1

In order to identify possible interactions between the *K. lactis* proteins KIPkc1 and KIRho1 we performed two-hybrid analyses. For this purpose, we constructed fusions carrying sequences encoding either the entire KIPkc1 or two C-terminally truncated versions of the kinase with the activation domain of the Gal4 protein. Since ScPkc1 interacts with ScRho1 only if the latter is in its GTP-bound state, we employed *KIRHO1*Q70HC205S (presumably encoding a GTPase constitutively locked in that state and lacking the geranyl-geranyl modification site at the C-terminal end) fused to the Gal4p DNA-binding domain. Combinations of these constructs as well as the vectors without insertions were then tested in strain PJ69-4A,

(C) *S. cerevisiae* HD56-5A **(D) *S. cerevisiae* HD56-5A**

Fig. 3. Effects of homologous and heterologous overexpression of *KIRHO1* and *KIPKC1* on growth. Cells carrying plasmids with the genes as indicated were grown in selective media overnight, diluted to OD₆₀₀ 0.1, and 10-fold serial dilutions were spotted onto the media indicated, selecting for plasmid maintenance. Growth was monitored after 2–3 days of incubation. Caffeine was added at a concentration of 5 mM and Calcofluor white at 10 mg l⁻¹. (A) Effects of overexpression of *KIRHO1* in *K. lactis* MW270-7B. Serial dilutions were spotted onto synthetic medium lacking leucine, with either 2% glucose or 2% galactose as a sole carbon source. (B) Effects of overexpression of *KIPKC1* in *K. lactis* MW309-5B. Serial dilutions were spotted onto synthetic medium lacking uracil, with either 2% glucose or 2% galactose as a sole carbon source. (C) Effects of overexpression of *KIRHO1* in *S. cerevisiae* HD56-5A. Serial dilutions were spotted onto synthetic medium lacking leucine, with either 2% glucose or 2% galactose as a sole carbon source. (D) Effects of overexpression of *KIPKC1* in *S. cerevisiae* HD56-5A. Serial dilutions were spotted onto synthetic medium lacking uracil, with either 2% glucose or 2% galactose as a sole carbon source. pCXJ22, pCXJ24, vectors without insertion. *Activated allele of *KIRHO1* leading to a substitution of Q70H in the encoded protein.

carrying the reporter genes *ADE2*, *HIS3* and the bacterial *lacZ* under the control of promoters with Gal4p-binding sites (James *et al.*, 1996). Transformants were first tested for their ability to confer growth on media lacking either adenine or histidine, or containing X-Gal. Those expressing fusions of both KIPkc1 and KIRho1Q70HC205S grew well on all media and turned blue on X-Gal plates (Fig. 4A). The activated *KIRHO1* allele showed some transcriptional activation on its own, resulting in a slow growth on such media and a light-blue colony colour on X-Gal plates.

Strains carrying either KIPkc1 (1–600) or KIPkc1 (1–228) in conjunction with KIRho1Q70HC205S did not show stronger interactions in these tests than the activated *KIRHO1* allele alone.

In order to obtain more quantitative results, we grew the transformants in selective liquid media and determined the specific β -galactosidase activities in crude extracts (Fig. 4B). In agreement with the data obtained above, the β -galactosidase activities found in transformants with

(A)

Control	-ade	-his	X-Gal	Binding domain	Activation domain
				pVA3	pTD1
				<i>KIRHO1*</i>	<i>KIPKC1</i>
				Vector	Vector
				Vector	<i>KIPKC1</i>
				<i>KIRHO1*</i>	Vector
				<i>KIRHO1*</i>	<i>KIPKC1</i> (1–600)
				<i>KIRHO1*</i>	<i>KIPKC1</i> (1–228)
				<i>KIRHO1*</i>	Vector
				<i>ScRHO1*</i>	<i>KIPKC1</i>
				<i>ScRHO1*</i>	<i>KIPKC1</i> (1–600)
				<i>ScRHO1*</i>	<i>KIPKC1</i> (1–228)
				<i>ScRHO1*</i>	Vector
				Vector	Vector
				Vector	<i>ScPKC1</i>
				Vector	<i>KIPKC1</i>

(B)

Binding domain	Activation domain	Specific β -gal activity
pVA3	pTD1	65.0 $\pm$ 25.0
<i>KIRHO1*</i>	<i>KIPKC1</i>	21.6 $\pm$ 2.0
Vector	Vector	3.6 $\pm$ 0.5
Vector	<i>KIPKC1</i>	4.5 $\pm$ 0.1
<i>KIRHO1*</i>	Vector	7.2 $\pm$ 0.2
<i>KIRHO1*</i>	<i>KIPKC1</i> (1–600)	9.7 $\pm$ 0.5
<i>KIRHO1*</i>	<i>KIPKC1</i> (1–228)	7.7 $\pm$ 0.5
<i>KIRHO1*</i>	<i>ScPKC1</i>	14.2 $\pm$ 0.5
<i>ScRHO1*</i>	<i>KIPKC1</i>	21.8 $\pm$ 1.0
<i>ScRHO1*</i>	<i>KIPKC1</i> (1–600)	9.4 $\pm$ 0.1
<i>ScRHO1*</i>	<i>KIPKC1</i> (1–228)	7.1 $\pm$ 0.3
<i>ScRHO1*</i>	<i>ScPKC1</i>	9.5 $\pm$ 0.8
<i>ScRHO1*</i>	Vector	4.7 $\pm$ 0.2

Fig. 4. Two-hybrid interactions. (A) Interaction between KIRho1 and KIPkc1 in the *S. cerevisiae* two-hybrid system: growth on different media. Transformants were grown overnight in selective medium (i.e. synthetic medium with 2% glucose lacking tryptophan and leucine). Five microlitres each of the original culture and of a 1:100 dilution were spotted onto the media indicated. For the colorimetric determination of β -galactosidase activity on X-Gal plates, only the undiluted spot is represented. –ade, minus adenine; –his, minus histidine. (B) Quantitative assessment of specific β -galactosidase (β -gal) activities (including heterologous interactions). Transformants were grown overnight under selective pressure for plasmid maintenance, and specific β -galactosidase activities were determined in crude extracts. Values given are means $\pm$ SD of at least three independent experiments each.

KIPkc1 and KIRho1Q70HC205S were significantly higher than those detected for the controls and the KIPkc1 truncations. Nevertheless, the latter consistently resulted in slightly higher activities than the negative controls.

We also tested heterologous interactions between the *K. lactis* and *S. cerevisiae* proteins. As shown in Fig. 4, no

significant difference was found when *KIRHO1*-Q70H-C205S was substituted by the corresponding *ScRHO1* activated allele in conjunction with *KIPKC1*. However, interactions between ScPkc1 and KIRho1-Q70H-C205S were significantly lower than those detected for the full-length KIPkc1 construct. Interestingly, homologous interactions between the two proteins from *S. cerevisiae* (ScPkc1/

Fig. 5. Intracellular distribution of GFP–KIRho1 and KIPkc1–GFP. (A) Localization of KIPkc1 in *K. lactis* KMP1; (B) localization of KIRho1 in *K. lactis* KHO1-5C. See Methods for details of the construction of GFP fusion vectors, and image acquisition and processing. Bars, 5 μ m.

ScRho1) also appeared to be weaker than those from *K. lactis* (KIPkc1/KIRho1).

Intracellular protein localization

To determine the intracellular localization of KIPkc1 and KIRho1 we employed the GFP fusion constructs described in Methods. We used the pCXJ22 and pCXJ24 derivatives, respectively, to express the two fusions under the control of their native promoters. That both GFP fusions were functional was demonstrated by complementation of the osmoremedial lysis phenotype of the *Scpkc1* deletion in strain RHO1802 (Table 1) and of the lethal phenotype of a *Scrho1* deletion after transformation of YEp352-GFPKIRHO1 followed by tetrad analysis in strain DHD5 Δ rho1, respectively (data not shown). pCXJ22-KIPKC1GFP was then introduced into *K. lactis* strain KHO1-5C (*ura3-12*) and pCXJ24-GFPKIRHO1 into KMP1 (*leu2-137*). Strains were grown under selective pressure for plasmid maintenance to exponential phase, and *in vivo* GFP fluorescence was observed. As is evident from Fig. 5(A),

KIPkc1 can localize to the bud neck in the later stages of cell division, in patches to the tip of the growing bud, and may also be present in the nucleus. On the other hand, GFP–KIRho1 appears predominantly at the cell surface and in the vacuole, as shown by nearest neighbour analysis (Fig. 5B). Frequently observed fluorescent connections between the two structures may indicate an active endocytotic turnover (note that exposure times of 1 s do not allow for resolution of such traffic).

DISCUSSION

Although the components of the cell integrity pathway generally seem to be conserved between *S. cerevisiae* and *K. lactis*, we have previously found that the phenotypes of null mutants may differ significantly between the two yeast species (Jacoby *et al.*, 1999; Kirchrath *et al.*, 2000; Lorberg *et al.*, 2003). We therefore followed up this work by the characterization of two central components of this signalling pathway, i.e. the molecular switch constituted by the small GTPase KIRho1 and the protein kinase C (KIPkc1).

First evidence for the functional identity of the two proteins to their homologues from baker's yeast was provided by the observation that both genes complemented the mutant phenotypes when expressed in the respective *S. cerevisiae* strains. This assumption is further substantiated for KIRho1 by the extremely high identity of the deduced amino acid sequence to that of ScRho1 (82 %). Despite the lower overall degree of identity between the protein kinase C homologues of the two yeasts, all domains to which a function has been attributed in ScPkc1 are also strongly conserved in KIPkc1. The lower overall conservation can basically be attributed to a strong divergence in the central interdomain region, where identities of less than 35 % are observed. The two C1 repeats and the kinase domain display more than 85 % identity. The latter conservation indicates that similar target proteins may be phosphorylated in *K. lactis* and *S. cerevisiae*. The complementation of the osmo-sensitive phenotype of the *Scpkc1* null mutant by KIPkc1 also supports this assumption. Moreover, since C1B and HR1A are thought to mediate interaction with Rho1 in *S. cerevisiae* (Nonaka *et al.*, 1995; Kamada *et al.*, 1996; Schmitz *et al.*, 2002), similar regulatory mechanisms may act on the two kinases. An interaction of KIPkc1 and KIRho1, similar to the one just cited for the enzymes from baker's yeast, is indicated by the two-hybrid data reported in this work. The interaction of an activated variant of KIRho1 with the full-length KIPkc1 fusion protein provides further evidence for the functional homology between the Rho1 proteins from the two yeast species, since we substituted both the residue that leads to the protein being locked in the active conformation and the one that is presumably modified for membrane interaction according to the data reported for the *S. cerevisiae* homologue (Inoue *et al.*, 1999). Here, in the heterologous interaction tests, we found a stronger interaction between the activated form of ScRho1 and the KIPkc1 fusion than vice versa. This may indicate that the stronger interaction is contributed by the protein kinase C, rather than the GTPase.

Given the various functions of Rho1 observed in *S. cerevisiae* mentioned in the Introduction, and the high degree of identity to its counterpart from *K. lactis* discussed above, it is not surprising that *KIRHO1* proved to be essential in the latter also. Nevertheless, we conclude from this phenotype that none of the other small GTPases likely to be encoded in the *K. lactis* genome (Sherman *et al.*, 2004) is sufficient to take over its specific functions. We infer from the two-hybrid analyses discussed above that one of these functions is the activation of *KIPKC1*. Although the latter may well serve a major function in cell integrity signalling in *K. lactis* (see below), the fact that null mutants are not viable even in the presence of osmotic stabilization indicates that the enzyme also affects other vital cellular processes. In *S. cerevisiae*, evidence for a bifurcated pathway in cell integrity signalling has been presented, with both branches being dependent on protein kinase C activity (reviewed by Levin, 2005). Moreover, ScPkc1 has been implicated in the regulation of the actin cytoskeleton (Helliwell *et al.*, 1998) and of oligosaccharyl transferase activity (Park & Lennarz, 2000), as well as cell cycle control

(reviewed by Levin, 2005), to name just a few functions. In some strains of *S. cerevisiae*, a *pkc1* deletion is viable in the presence of osmotic stabilization, indicating that some of the kinase functions either are not essential or can be served at least in part by other protein kinases. Our results indicate that at least one of these functions cannot be substituted in *K. lactis* by its endogenous enzymes.

Overexpression of *K. lactis* genes and the *lacZ* reporter construct from the *ScGAL1* promoter confirms previous observations of a striking similarity in the regulation of galactose/lactose metabolism in the two yeast species (Zenke *et al.*, 1999). As observed here, full induction takes approximately 24 h, whilst full repression may take up to 3 days.

In our serial dilution drop tests employed to assess the effects of overexpression, these time scales can be neglected, since colonies arise from single cells growing under inducing (galactose) or repressing (glucose) conditions. In these tests, we did not observe a pronounced effect of overexpression of *KIPKC1* in *K. lactis* or *S. cerevisiae*. This is consistent with the domain structure of the enzyme, which indicates a complex control of the kinase activity by various effectors (reviewed by Schmitz & Heinisch, 2003). Thus, a mere increase in intracellular protein concentration will likely not result in a simultaneous increase in kinase activity. However, overexpression of *KIRHO1* did result in a dramatic growth inhibition on galactose media. In line with these results, ScRho1 in baker's yeast has also recently been found to result in growth inhibition (Queralt & Igual, 2005). The growth-inhibitory effect was not observed when the gene was simply placed on a multicopy vector and introduced into *K. lactis*. Rather, expression from the strong *ScGAL1* promoter was needed to cause the phenotype. It should be noted that analyses in *K. lactis* were obstructed by a pronounced phenotypic heterogeneity between different strains, and by the fact that both the *ScGAL1* promoter constructs tended to recombine heavily, as deduced from the inability to recover plasmids from such strains. Several attempts to reisolate the plasmid from several different *K. lactis* recipients failed (data not shown). Thus, for those recipients that lacked phenotypic effects, we cannot be sure that they carried the desired promoter fusion at all. On the other hand, those showing growth defects can be assumed to carry at least some part of the fusion, possibly integrated somewhere into the genome.

Why would overproduction of Rho1 be lethal? In *S. cerevisiae*, Rho1 has been implicated in redirecting cell wall synthesis to sites of lesions upon external stress (Saka *et al.*, 2001), consistent with its function as a regulatory subunit of the glucan synthase complex (Drgonova *et al.*, 1996). It has also been shown to localize to peroxisomes and to take part in the reaction to oxidative stress (Marelli *et al.*, 2004; Alberts *et al.*, 1998). Furthermore, ScRho1 seems to regulate the traffic of endocytotic vesicles, presumably by cooperating with the formin Bni1 (Kaksonen *et al.*, 2005). Although the small GTPases function as molecular switches and are believed to be active only in their GTP-bound state,

one can assume that overproduction will lead to a titration of the regulatory factors (i.e. GDP/GTP exchange factors and GTPase activating proteins), influencing their local concentrations. This will interfere with the subcompartmental functions of the switch and thus result in the observed growth defects.

Apart from the striking similarities in the protein characteristics and the functional complementation of the respective *S. cerevisiae* mutants, we lack direct evidence for the involvement of KIRho1 and KIPkc1 in cell integrity signalling in *K. lactis*. As discussed above, both null mutants proved to be non-viable. Nevertheless, the localization of the two GFP fusion proteins provides further evidence for the similarity of the functions of the *K. lactis* proteins to those of their *S. cerevisiae* counterparts. Thus, we found the KIPkc1–GFP fusion at sites of bud growth, in the bud neck during cell division, and possibly in the nucleus. A recent study has attributed these subcellular localizations to the function of different domains in ScPkc1, such as the HR1, C1 and C2 sequences, as well as the kinase domain itself (Denis & Cyert, 2005). This is consistent with all of these domains being highly conserved in the KIPkc1 sequence.

ScRho1 is predominantly localized in patches at the cell surface, consistent with its role in polarisome organization (Ayscough *et al.*, 1999). Here, we found KIRho1–GFP to be more evenly distributed at the plasma membrane in *K. lactis*. However, a substantial amount of fluorescence could be found within the vacuole. We suspect that this is due to the overproduction of the fusion protein from a multicopy vector, causing a certain amount of degradation. Supporting this notion, we frequently observed bands of fluorescence between the cell surface and the vacuole in the long-exposure images.

In summary, here we characterized two further components of the cell integrity pathway of *K. lactis*, both of which are also likely to serve a variety of other essential functions. The observed differences from the homologues of *S. cerevisiae* both in primary sequence and in protein function may serve to elucidate novel regulatory networks in *K. lactis*.

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