KlRHO1 and KlPKC1 are essential for cell integrity signalling in Kluyveromyces lactis

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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 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...
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, KILMPK1 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 oomycetemial lysis phenotype, deletion of the ROM2 gene is rescued by the presence of ROM1. In contrast, Klbck1 and Klmpk1 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 (Dong et al., 1996; Qadota et al., 1996). 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 (YE PD) 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 OD\(_{600}\) 0.25, and grown for another 4-7 h. Tenfold dilutions were then prepared using the growth medium, and 3-5 µl 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 MgCl\(_2\)) in 12 ml plastic tubes. To each cell pellet, 0-5 g glass beads (2-0-5 mm diameter; Roth) were added and vigorously shaken at 4 °C for 7 min (Vibrax VXK 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 µl 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.

**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 DH5α 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 (YE PD). 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 (YE PD) 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.

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*Uncharacterized allele of K. lactis gene.

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**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 μl RHI (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 μl *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 μl volume, 10 μl 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 μl 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 μl was added to the PCR reaction mixture (Robzyk & Kassir, 1992).

**Genomic library and cloning of genes.** The *KIPKCI* gene was cloned from a *K. lactis* genomic library constructed by Wesołowski-Louvel et al. (1988) in the vector KEP6 (Bianchi et al., 1991) which carries *URA3* as a selection marker. The *S. cerevisiae* strain RH1802 (pck1Δ::HIS3; Table 1) was used as a recipient for heterologous complementation of the *his1* phenotype under osmotically non-stabilizing conditions. Plasmid KEPKIPKCI (= pJJH817) was shown to carry the *KIPKCI* 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 of 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 *KIPKCI* gene and its flanking regions was excised from KEPKIPKCI as a *SalI* fragment and cloned into YEp352 (Hill et al., 1986) to yield plasmid YEp352-KIPKCI, and the *KIRHO1* coding region was contained within a *HindIII* fragment in YEp352-KIRHO1. For expression analyses in *K. lactis*, the described DNA fragments (*KIPKCI*-SalI and *KIRHO1*-HindIII) were cloned into the shuttle vectors pCXJ22 (*URA3*) and pCXJ24 (*LEU2*), respectively, to give pCXJ22-KIPKCI 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 *KIPKCI*, first a *HIS3*-PGAL1 cassette was amplified by PCR using plasmid pPA6a-His3MX6PGAL1 as template (Longtime et al., 1998) and employing the oligonucleotide

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pair GALpKIPKC1-F4/GALpKIPKC1-R2 (Table 2). The resulting fragment was co-transformed with plasmid pCXJ22-KlPKC1 into S. cerevisiae strain HD56-5A, selecting for histidine prototrophy. Plasmid pCXJ22-pGAL1KlPKC1 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 GALpKlRHO1-F4/GALpKlRHO1-R2 (Table 2). The PCR product was then co-transformed with plasmid pCXJ24-KlRHO1 into S. cerevisiae strain HD56-5A, selecting for G418 resistance on YEPD medium. Plasmid pCXJ24-pGAL1KlRHO1 isolated from yeast transformants showed the correct recombination by restriction analyses and sequencing. Activated alleles of Klrho1 were obtained by in vitro mutagenesis, as described below for the activated KlRHO1 alleles introduced into the two-hybrid vectors, although the geranyl-geranylization site (C205) was left intact. Activated alleles of KlRHO1 were obtained by in vitro mutagenesis. Starting with pJJH833 (pUK1921-KlRHO1) as template and the oligonucleotides KlRHO1Q70Hneu and KlRHO1Q70Hrevneu, plasmid pUK1921-KlRHO1Q70H was obtained using the Quick Change Site-Directed-Mutagenesis kit (Stratagene). From this, plasmids pCXJ24-KlRHO1Q70H and pCXJ24-pGAL1KlRHO1Q70H were constructed, as described above for the wild-type KlRHO1 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 BglII–HindIII fragment containing the GAL1 promoter was obtained by PCR using oligonucleotides GALpKIRHO1-F4 and HindR2 (Table 2) on the template pFA6a-KanMex-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 KIPKCI 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 KIPKCI coding region with the oligonucleotides KIPKCI1atg and KIPKCI1mid from genomic S. cerevisiae DNA, and co-transformed the product with pGAD424 linearized with BstNI into S. cerevisiae HD56-5A. The PCR product carried the 5′ half of the ORF with a BstNI site inserted right in front of the ATG translation start codon, and lacked the 3′ sequence that encodes the kinase domain (KIPKCI1-328). 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 XhoI and SaII, and the 3.5 kb fragment, containing most of the KIPKCI 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 KIPKCI coding sequence (KIPKCI1-298), comprising the HR1 sequences, fused to that of the Gal4 transcription activation domain, called pJJH910, was obtained by digestion with XhoI/SaII 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 EcoRI/PstI and cloned into pUK1921 to yield pJJH905. From the latter, a sequence leading to a C205S substitution, which destroys the translation start codon, and lacked the 3′ sequence that encodes the kinase domain (KIPKCI1-328). After in vitro mutagenesis, as described above for the overexpression plasmids. The activated KIRHO1Q70H-C205S allele was excised by digestion with EcoRI/PstI 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 Jürg Jacoby, Anderson Cancer Center, Houston, TX, together with pHS49 with the S. cerevisiae PKC1 gene was amplified by PCR from YDpU (Berben et al., 1999) and subcloned into YIplac128 (Gietz & Sugino, 1988). The plasmid, termed pYEp-Klpkc1d, was digested with XhoI/KIRHO1Q70H-C205S and religated. The resulting plasmid was called pJJH916E. 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 EcoRI/PstI and fused to the Gal4 DNA-binding domain in the vector pGBT9 vector (Bartel et al., 1993). In addition, pTDT1 (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-KanMex6-pGAL1-GFP as template (Longtine et al., 1998) and the oligonucleotide pair KIRHO1HA4/ KIRHO1gpR5 (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 YEPlac128. The resulting fragment co-transformed with 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 KIRHO1GFPrev and KIRho15sor, and the resulting fragment co-transformed with pCXJ24-pGAL1-GFPKIRHO1 linearized with MluI 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, KIPKcl was labelled at its C-terminal end with GFP, using pFA6a-GFP-KanMX6 as a template and the primers KIPKClmCf2 and KIPKClmCr1. The PCR product was co-transformed with plasmid pCXJ22-KIPKCI for in vivo recombination in S. cerevisiae strain HD56-5A. Plasmid pRRO80 (pCXJ22- KIPKCI1GFP) 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 fusion was shown by complementation of lethality of a rho1 deletion when expressed in multicopy. For this purpose, the 3.6 kb HindIII fragment from pRRO82 carrying GFPKIRHO1 with its flanking sequences was subcloned into the respective site of Yeplac33 (Hill et al., 1986) to yield plasmid pJL934. This was introduced into strain DH5ΔArho1 (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 YEPlac, 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 DH5Δ (Kirchrath 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 KIPKCI gene (Fig. 1A) the internal 1.45 kb BglII–NsiI fragment of the gene was subcloned into YIpplac128 (Gietz & Sugino, 1988). The plasmid, termed YIplp-Klpkc1d, was digested with XhoI prior to transformation into the diploid strain KLD1 obtained by crossing of MW270-TB and MW309-5B (Table 1). Transformants were selected on SCD lacking leucine. Correct integration was checked by PCR, using the oligonucleotide pairs KIPKCI1dR5/KIPKCI1term2, KIPKCI1dR5/u40ccwcmu and universal -47/KIPKCI1term2. 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 KIRho1delta5′ and KIRho1delta3′-neo (Table 2). The HindIII fragment with KIRHO1 was subcloned from pJJH833 into Yeplac33 (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 3′-flanking sequence, the HindIII fragment with the KIRho1::URA3 construct was cloned into pUK1921 to yield pJJH845, digested with XbaI and religated. The resulting plasmid was called pJJH853 and digested with HindIII/XbaI prior to
transformation of the diploid \textit{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 \textit{KIPKC1} and \textit{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 \textit{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 \textit{in vivo} fluorescence microscopy consisted of a Zeiss Axiosplan2e microscope equipped with a ×100 alpha-Plan Fluar 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**

**\textit{KIPKC1} and \textit{KIRHO1} complement the respective \textit{S. cerevisiae} defects**

In \textit{S. cerevisiae}, deletion of the unique \textit{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 (\textit{pkc1::HIS3}), was used as recipient for cloning of \textit{KIPKC1} by heterologous complementation with a genomic library of \textit{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 insert of approximately 10-5 kb of genomic \textit{K. lactis} sequence. Subcloning of various fragments into YEp352 and transformation into the \textit{pck1::HIS3} recipient narrowed down the complementing sequence to a \textit{SalI} fragment of 7-4 kb (Fig. 1A, C).

From that, a 4-3 kb fragment, which spanned the entire \textit{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. \textit{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 \textit{KIRHO1} gene was isolated by heterologous hybridization with a Sc\textit{RHO1} probe on a 2-3 kb genomic \textit{HindIII} fragment, as described in Methods (Fig. 2A). Sequence analysis demonstrated that the putative \textit{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 \textit{S. cerevisiae} resulted in an overall identity of 82% (Fig. 2B). A rho\textit{1}′ strain from \textit{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 \textit{S. cerevisiae} mutant at the restrictive temperature, although transformants grew quite slowly compared to a wild-type strain. Interestingly, \textit{KIRHO1} was not able to complement a \textit{Srho1} deletion if carried on a single-copy vector (pCJ24-KIRHO1) which was introduced into a diploid strain heterozygous for the deletion (\textit{RHO1}::\textit{rho1}::\textit{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 \textit{rho1}::\textit{kanMX}/YEp352-KIRHO1. A total of 10 segregants that were G418 resistant were invariably prototrophic for uracil, indicating that episomal \textit{KIRHO1} is able to complement if expressed at sufficiently high levels. In contrast to \textit{RHO1} wild-type cells carrying the vector, such segregants did not grow on medium containing 5-FOA, i.e. plasmid loss was lethal.

**\textit{KIPKC1} and \textit{KIRHO1} are essential genes**

To investigate the role of the cloned genes in \textit{K. lactis}, we constructed null mutants starting with the diploid strain KLD1. For \textit{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 \textit{KIPKC1} gene (Fig. 1A). One transformant that showed the correct integration by PCR was subjected to sporulation and tetrad analysis. Germination was allowed on YE PD 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
disrupted \textit{Klpkc1} copy. This result strongly suggested that the wild-type gene is essential in \textit{K. lactis}.

A \textit{Klrho1} null mutant was obtained in the diploid strain KLD1 by substitution of the complete ORF with the \textit{URA3} gene from \textit{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 \textit{KlRHO1}, too, is essential in \textit{K. lactis}.

\textbf{Overexpression of \textit{KIRHO1}, but not of \textit{KIPKC1}, affects growth of \textit{K. lactis}}

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

We have previously used the \textit{ScGAL1} promoter in the investigation of overexpression of the \textit{KIROM2} gene encoding a GDP/GTP exchange factor in \textit{K. lactis} (Lorberg et al., 2003). Here, we used the \textit{lacZ} reporter construct to measure \(\beta\)-galactosidase activity in strain MW190-9B (\textit{lac4}) transformed with pXW3-pGAL1lacZ (ScGAL1p-lacZ), as described in Methods. Specific \(\beta\)-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 \(\beta\)-galactosidase activities to below detectable levels after 72 h of incubation (data available upon request). Thus, the heterologous \textit{ScGAL1} promoter is properly regulated in \textit{K. lactis} and can be employed for conditional expression.

To test the effects of \textit{KIRHO1} overexpression in \textit{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 \textit{ScGAL1p-KIRHO1} allele for all media and conditions tested, as compared to the strain containing just
the vector control or \textit{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 \textit{ScGAL1} promoter in \textit{K. lactis} described above. It should be noted that we were unable to retrieve the plasmids carrying the \textit{GAL1} promoter constructs (neither pCXJ24-pGAL1KlRHO1 nor pCXJ22-pGAL1KlPKC1, described below) from transformants, even when grown on glucose media lacking leucine or uracil, respectively. Moreover, different \textit{K. lactis} strains showed different degrees of growth inhibition on galactose medium with pCXJ24-pGAL1KIRHO1, i.e. were phenotypically more heterogeneous than the \textit{S. cerevisiae} strains overexpressing \textit{KIRHO1} (see section on \textit{S. cerevisiae} \textit{KIRHO1} overexpression below).

The results with \textit{KIRHO1} prompted us to test also the effects of \textit{KIPKCI} overexpression in \textit{K. lactis}. There, the serial dilution patch tests showed that growth was not affected on galactose media in the strain carrying the plasmid pCXJ22-pGAL1KIPKCI containing the \textit{KIPKCI} gene under the \textit{ScGAL1} promoter, compared to the strain carrying just the vector (Fig. 3B).
Overexpression of *KIRHO1* also affects growth of *S. cerevisiae*

As discussed above, transformants of the *S. cerevisiae rho1* 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-pGAL1*KIRHO1* and pCXJ24-KRHO1 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-pGAL1*KIRHO1*, 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-pGAL1*KIPKC1*, 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 **K**IRho1 and ScRho1**

In order to identify possible interactions between the *K. lactis* proteins KIPkc1 and KRho1 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,
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...
KlPkc1 and KlRho1Q70HC205S were significantly higher than those detected for the controls and the KlPkc1 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-Q70HC205S 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/
ScRh01) 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 DHD5Δrho1, 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 KLrho1 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 KlPkc1. 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 KlPkc1 also supports this assumption. Moreover, since C1B and HR1A are thought to mediate interaction with Rh1 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 KlPkc1 and KlRh1, 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 KlRh1 with the full-length KlPkc1 fusion protein provides further evidence for the functional homology between the Rh1 proteins from the two yeast species, since we substituted both the residue 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 ScRh1 and the KlPkc1 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 Rh1 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 KIRH1 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 (Hellwell 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 Rh1 be lethal? In S. cerevisiae, Rh1 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., 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., 2001), consistent with its function as a regulatory subunit of the glucan synthase complex (Drgonova et al., 1996). 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 \textit{S. cerevisiae} mutants, we lack direct evidence for the involvement of KIRho1 and KIPkc1 in cell integrity signalling in \textit{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 \textit{K. lactis} proteins to those of their \textit{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 \textit{et al.}, 1999). Here, we found KIRho1–GFP to be more evenly distributed at the plasma membrane in \textit{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 \textit{K. lactis}, both of which are also likely to serve a variety of other essential functions. The observed differences from the homologues of \textit{S. cerevisiae} both in primary sequence and in protein function may serve to elucidate novel regulatory networks in \textit{K. lactis}.

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