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Protein Kinase Ymr291w/Tda1 Is Essential for Glucose Signaling in *Saccharomyces cerevisiae* on the Level of Hexokinase Isoenzyme ScHxk2 Phosphorylation*[S]

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Background: Monomer-dimer equilibrium, substrate affinity, and subcellular localization of yeast hexokinase ScHxk2 depend on the state of phosphorylation of serine 15.
Results: Serine/threonine protein kinase Ymr291w/Tda1 is essentially required for ScHxk2-S15 phosphorylation.
Conclusion: Ymr291w/Tda1 is the ScHxk2-S15 kinase or an upstream regulatory enzyme.
Significance: The analysis of Ymr291w/Tda1 function(s) is indispensable for understanding glucose signaling in yeast.

The enzyme ScHxk2 of *Saccharomyces cerevisiae* is a dual-function hexokinase that besides its catalytic role in glycolysis is involved in the transcriptional regulation of glucose-repressible genes. Relief from glucose repression is accompanied by the phosphorylation of the nuclear fraction of ScHxk2 at serine 15 and the translocation of the phosphoenzyme into the cytosol. Different studies suggest different serine/threonine protein kinases, Ymr291w/Tda1 or Snf1, to accomplish ScHxk2-S15 phosphorylation. The current paper provides evidence that Ymr291w/Tda1 is essential for that modification, whereas protein kinases Ydr477w/Snf1, Ynl307c/Mck1, Yfr014c/Cmk1, and Ykl126w/Ypk1, which are co-purified during Ymr291w/Tda1 tandem affinity purification, as well as protein kinase PKA and PKB homolog Sch9 are dispensable. Taking into account the detection of a significantly higher amount of the Ymr291w/Tda1 protein in cells grown in low-glucose media as compared with a high-glucose environment, Ymr291w/Tda1 is likely to contribute to glucose signaling in *S. cerevisiae* on the level of ScHxk2-S15 phosphorylation in a situation of limited external glucose availability. The evolutionary conservation of amino acid residue serine 15 in yeast hexokinases and its phosphorylation is illustrated by the finding that *YMR291W*/*TDA1* of *S. cerevisiae* and the homologous *KLLA0A09713* gene of *Kluyveromyces lactis* allow for cross-complementation of the respective protein kinase single-gene deletion strains.

The adaptation of metabolism to changing nutrient availability is based on complex sensing and signaling mechanisms. In the case of the Crabtree-positive yeast *Saccharomyces cerevisiae*, the regulatory phenomenon of glucose repression allows the preferential uptake and utilization of glucose, which is trapped inside the cell and activated for metabolism via phosphorylation (1). The genome of *S. cerevisiae* encodes three enzymes that are capable of catalyzing glucose phosphorylation: the differentially regulated hexokinases ScHxk1[3] and ScHxk2 and the glucokinase ScGlk1 (2, 3). ScHxk2 is the predominating hexose kinase when glucose is abundantly available (4). This enzyme belongs to a class of dual-function hexokinases, which in addition to their contribution to the uptake and initiation of metabolism of glucose, fructose, and mannose, are involved in glucose-dependent signal transduction (5). Dual-function hexokinases are capable of translocating from the cytoplasm to the nucleus to mediate glucose repression of transcription in yeast (6) and plants (7) or to participate in mechanisms finally leading to glucose homeostasis in higher organisms (8). Studies on glucose signaling in *S. cerevisiae* revealed that repression by glucose of Mig1-regulated genes specifically requires the concurrent translocation of ScHxk2 into the nucleus and the recruitment of the Mig1-interacting corepressor complex Cyc8(Ssn6)-Tup1 (9).

The hexokinases of *S. cerevisiae* are phosphoenzymes *in vivo* (10). In the case of ScHxk2, the phosphorylation was found to be carbon-source dependent, being more extensive during growth on carbon sources such as galactose, raffinose, and ethanol, whereas high external glucose promoted the dephosphorylation of the phosphohexokinase (11). Two phosphorylation sites were identified: the *in vivo* phosphorylation site serine 15 (12), which is considered in the current paper, and the autophosphorylation-inactivation site serine 158 (13), which remains unregarded here. ScHxk2 forms a monomer-homodimer equilibrium (14), and serine 15 is likely to be part of the intersubunit interface, explaining monomer formation as a consequence of serine 15 phosphorylation (15). Keeping in mind that serine 15 is also part of the nuclear localization signal

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[3] The abbreviations used are: ScHxk1, *Saccharomyces cerevisiae* hexokinase isoenzyme 1; 1NM-PP1, 4-amino-1-tert-butyl-3-(1′-naphthylmethyl)pyrazolo[3,4-d]pyrimidine; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; hrCNE, high resolution clear native electrophoresis; KlHxk1, *Kluyveromyces lactis* hexokinase isoenzyme 1; TAP, tandem affinity purification.

Lys^{7}-Met^{16} of ScHxk2 (16), its phosphorylation is likely to play a key role in ScHxk2-dependent glucose signaling. Indeed, the interaction of ScHxk2 with the karyopherins Kap60/Kap95 and Xpo1 (Crm1) is influenced by the phosphorylation state of serine 15 (and thereby indirectly by the availability of glucose) (17, 18). The regulatory significance of ScHxk2-S15 phosphorylation is further supported by the finding that the interaction of ScHxk2 and ScMig1 is mediated through the N-terminal Lys^{7}-Met^{16} decapeptide of the hexokinase and serine residue 311 of the transcriptional repressor (19, 20). Independent evidence for a physiological role of ScHxk2-S15 phosphorylation came from enzymatic studies indicating that this modification affects substrate affinity and inhibition by ATP of the enzyme (21).

The discovery that phosphorylation at serine 15 is key to the regulation of ScHxk2 function initiated the search for the respective enzyme(s). Although Glc7/Reg1 was identified as the responsible phosphoprotein phosphatase (11), the identity of the ScHxk2-S15 kinase is still a matter of debate. Serine 15 is part of a protein kinase A consensus phosphorylation sequence (22) and, indeed, the catalytic subunit Tpk1 of PKA is capable *in vitro* of phosphorylating that residue (12). There is no experimental evidence, however, that PKA is required for serine 15 phosphorylation *in vivo* (10). Similarly, reports on the role of protein kinase Snf1 in ScHxk2-S15 phosphorylation are contradictory (11, 23). By contrast, a recent analysis of protein kinase single-gene deletion strains of *S. cerevisiae* indicated that the absence of the protein kinase Ymr291w/Tda1 (subsequently referred to as Ymr291w) is accompanied by a total loss of ScHxk2 *in vivo* phosphorylation (24). To further explore the molecular basis of ScHxk2 phosphorylation, Ymr291w was enriched by tandem affinity purification and subjected to *in vitro* phosphorylation analysis. In addition, four protein kinases that co-purified with Ymr291w as well as protein kinases PKA, Sch9, and Snf1, which are all involved in glucose-responsive pathways in yeast (25), are considered in the present study. Finally, the evolutionary conservation of metabolic regulation on the level of Ymr291w-dependent hexokinase phosphorylation is illustrated by the results of cross-complementation studies performed in *S. cerevisiae* and *K. lactis*.

EXPERIMENTAL PROCEDURES

Strains and Growth—The *Escherichia coli* strain DH5α (Invitrogen) was used for DNA amplification. The *S. cerevisiae* wild type strains W303 (accession number 20000A) and BY4741 (Y00000) and single-gene deletion strains BY4741 Δ*ymr291w* (Y00878), BY4742Δ*snf1* (Y14311), BY4741Δ*ynl 307c* (Y01137), BY4741Δ*yfr014c* (Y05693), and BY4741Δ*ykl 126w* (Y04976) were obtained from Euroscarf (Germany). PKA mutant strain Y3175 (*MATα*; *ura3–1*; *trp1–1*; *leu2–3,112*; *his3–11,15*; *ade2–1*; *can1–100*; *tpk1*(M164G) GAL; *tpk2*::*KAN*; *tpk3*::*TRP1*) is described in Ref. 26. The *Kluyveromyces lactis* wild type strain used was JA6 (*MATα*; *ade1–600*; *adeT-600*; *trp1–11*; *ura3–12* (27)). Strain DH5α was grown in LB medium (0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl). *S. cerevisiae* was cultivated in YP (1% (w/v) yeast extract, 2% (w/v) peptone) or minimal medium (0.69% (w/v) yeast nitrogen base, 0.2% (w/v) casamino acids), the latter medium supplemented with tryptophan (20 mg/liter). The initial glucose concentration was 0.05% (w/v) or 0.1% (w/v) in low-glucose media and 2% (w/v) in the high-glucose medium. The 0.05% low-glucose medium was supplemented with 3% (v/v) glycerol. *K. lactis* was propagated in YNB medium (0.69% (w/v) yeast nitrogen base without amino acids, pH 5.5, supplemented with adenine, uracil, and amino acids according to Ref. 28) at an initial galactose concentration of 2% (w/v). All cell cultures including those subjected to nephelometric growth analysis were agitated at 200 rpm and 37 °C (*E. coli*) or 30 °C (yeast).

Construction of Plasmid p416YMR291W—This single-copy plasmid was constructed and verified as described in Ref. 24.

Construction of Strain BY4741YMR-TAP—The strain expressing chromosomally encoded C-terminally TAP-tagged Ymr291w was generated using a gene fusion cassette containing the TAP tag DNA sequence (29) and the *K. lactis URA3* gene (*KlURA3*) as a selection marker, flanked by sequences complementary to the 3′-terminal 40 bp of the *YMR291W* open reading frame and to 40 bp downstream of the *YMR291W* stop codon. The TAP tag sequence (909 bp) and the *KlURA3*-encoding region (804 bp) were amplified using primers TAPG1f, TAPG2r, TAPG3f, and TAPG4r (Table 1), and the amplicons were joined by overlap extension PCR (30). For homologous recombination, the purified construct was transformed into *S. cerevisiae* strain BY4741 using the lithium acetate method (31). Correct integration was confirmed by DNA sequencing. Throughout this study, DNA manipulation work was performed as described in Ref. 32.

Construction of Plasmid p426YMR291W-TAP—The plasmid encoding C-terminally TAP-tagged Ymr291w was generated using multicopy vector p426GPD (33). The ScHxk2 promoter was amplified from position −599 relative to the first nucleotide of the ATG start codon using primers TAP1f and TAP2r (Table 1). The *YMR291W* gene (1,761 bp) and the DNA sequence encoding the TAP tag (909 bp) (29) were amplified using primers TAP3f, TAP4r, TAP5f, and TAP6r (Table 1) and the three amplicons were joined by overlap extension PCR (30). The purified construct was ligated into plasmid p426GPD at its SacI and ClaI restriction sites thus replacing the *GPD* promoter. The recombinant plasmid was amplified in *E. coli*, verified by DNA sequencing, and transformed into *S. cerevisiae* strain BY4741Δ*ymr291w* using the lithium acetate method (31).

Construction of Strain BY4741Δsch9—The Δ*sch9* mutant strain was generated by amplifying a cassette containing the *KlURA3* gene flanked by DNA sequences complementary to 45 bp upstream and 38 bp downstream of the *SCH9* open reading frame using primers SCH9f and SCH9r (Table 1). For homologous recombination, the purified cassette was transformed into *S. cerevisiae* strain BY4741 using the lithium acetate method (31). Correct integration was confirmed by DNA sequencing.

Construction of Plasmid pKA-TUC-KLLA0A09713—The plasmid encoding the Ymr291w homolog Klla0a09713p of *K. lactis* was generated from single-copy vector pKA-TUC (34). The *KLLA0A09713* gene including 498 bp upstream and 482 bp downstream was amplified using primers KLL7f and KLL8r (Table 1). The amplicon was purified and ligated into plasmid pKA-TUC (34) at its SacI and NotI restriction sites. The recombinant plasmid was amplified in *E. coli*, verified by DNA sequencing, and trans-

TABLE 1
Oligonucleotide primers used for PCR

Name	Sequence (5′→3′)
KLL1f	GCATCGATGAGCTCCAGGCGCTCCTTGAATAACC
KLL2r	CTATAGACATATCCTGGATGTGTATTTTTCTTTTTG
KLL3f	CCAGGATATGTCTATAGCAACTTCTGATTATTC
KLL4r	CATTCAACATCAGTCATCTTCCCCGAG
KLL5f	GATGACTGATGTTGAATGATAATTCACCTTTTTTTTC
KLL6r	ATCGATGCGGTACCGCTAAATGATCACTGGGCTG
KLL7f	GCATCGATGAGCTCCTGACAGACACTTCCC
KLL8r	GCATCGATGCGGCCGCGATTATCTACAAGCATAAGAG
KLL9r	GTAGTCATATTTCTCCGGATTTTTTTATGC
KLL10f	GGAGAAATATGACTACAGCTAGTTCTTC
KLL11r	CATAATGTTTCTATATCTGCATGGTTTCATC
KLL12f	CAGATATAGAAACATTATGGATAGACTTTAAAAC
NAT1f	GACCTGACAGATTGGAAAG
NAT2r	CCTTAATTAACCCGGGGATCCGGCAAAGGAAGAACCAGTCTC
NAT3f	GAGACTGGTTCTTCCTTTGCCGGATCCCCGGGTTAATTAAGG
NAT4r	GTTATACGGAACCACAAGTGGAATTCGAGCTCGTTTAAACTG
NAT5f	CAGTTTAAACGAGCTCGAATTCCACTTGTGGTTCCGTATAAC
NAT6r	GACCTGGTTTTAAGCTACC
SCH9f	GAATTATACTCGTATAAGCAAGAAATAAAGATACGAATATACAATGCTTGATATCGAATTCCTGCAGC
SCH9r	GGAAAAGAAGAGGAAGGGC AAGAGGAGCGATTGAGAAACGACTCACTATAGGGCGAATTG
TAP1f	GGGCTCCAGAGCTCCACATTGGTGACCC
TAP2r	GAACTAGCTGTAGTCATTTTATTTAATTAGCG
TAP3f	CGCTAATTAAATAAAATGACTACAGCTAGTTC
TAP4r	CCTGCAGCGTACGAAGCTATCTGCATGGTTTC
TAP5f	GAAACCATGCAGATAGCTTCGTACGCTGCAGG
TAP6r	CCGATACGATCGATTTATTCTTTGCTCACCGAAGG
TAPG1f	GAATGTTGATGATTCTGATGGTG
TAPG2r	GATATCAAGCTCATTATTCTTTGCTCACCGAAGG
TAPG3f	GAGCAAAGAATAATGAGCTTGATATCGAATTCCTGCAGC
TAPG4r	GAATAATTAATCGTAAATTTCATTAGTTAGAATGTCGTCCCGACTCACTATAGGGCGAATTG

formed into *K. lactis* strain JA6Δ*klla0a09713* using the lithium acetate method (31).

Construction of Plasmid pKA-TUC-YMR291W—This plasmid encodes a construct in which the *YMR291W* gene of *S. cerevisiae* is controlled by the promoter and terminator of the homologous *KLLA0A09713* gene of *K. lactis*. To amplify the *KLLA0A09713* promoter region from position −498 relative to the first nucleotide of the ATG start codon, the coding sequence of the *YMR291W* gene (1,761 bp) and the *KLLA0A09713* terminator region (482 bp), the primers KLL7f, KLL9r, KLL10f, KLL11r, KLL12f, and KLL8r (Table 1) were used. The amplicons were joined by overlap extension PCR (30), and the purified construct was ligated into plasmid pKA-TUC (34) at its SacI and NotI restriction sites. The recombinant plasmid was amplified in *E. coli*, verified by DNA sequencing, and transformed into *K. lactis* strain JA6Δ*klla0a09713* using the lithium acetate method (31).

Construction of Plasmid p416KLLA0A09713—This plasmid encodes a construct in which the *KLLA0A09713* gene of *K. lactis* is controlled by the promoter and terminator of the homologous *YMR291W* gene of *S. cerevisiae*. To amplify the *YMR291W* promoter region from position −497 relative to the first nucleotide of the ATG start codon, the coding sequence of the KLLA0A09713 gene (1,674 bp) and the *YMR291W* terminator region (385 bp), the primers KLL1f, KLL2r, KLL3f, KLL4r, KLL5f, and KLL6r (Table 1) were used. The amplicons were joined by overlap extension PCR (30), and the purified construct was ligated into single-copy plasmid p416ADH (33) at its SacI and KpnI restriction sites thus replacing the *ADH* promoter. The recombinant plasmid was amplified in *E. coli*, verified by DNA sequencing, and transformed into *S. cerevisiae* strain BY4741Δ*ymr291w* using the lithium acetate method (31).

Construction of Strain JA6Δklla0a09713—The Δ*klla0a09713* mutant strain was generated by use of a fusion cassette containing the *NAT* gene of *Streptomyces noursei* coding for nourseothricin *N*-acetyltransferase as a selection marker, flanked by DNA sequences complementary to 500 bp upstream and 500 bp downstream of the *KLLA0A09713* ORF. To amplify the upstream complementary region, the NAT coding sequence (35), and the downstream complementary region, primers NAT1f, NAT2r, NAT3f, NAT4r, NAT5f, and NAT6r (Table 1) were used. The amplicons were joined by overlap extension PCR (30), and the purified construct was transformed into *K. lactis* strain JA6 using the lithium acetate method (31). Correct integration was confirmed by DNA sequencing.

Nephelometric Growth Analysis—The growth of yeast strains W303 and Y3175 was monitored in a NEPHELOstar Galaxy laser-based microplate nephelometer (BMG LABTECH, Germany) equipped with 96-well Cellstar® suspension culture plates (Greiner BioOne, Germany) by the detection of particulate matter via forward light scattering. The cells were grown to stationary phase in 5 ml of minimal medium containing 2% (w/v) glucose, washed twice with and resuspended in minimal medium without carbon source to adjust an optical density (600 nm, 1 cm) of 2. This suspension was used to inoculate minimal medium containing 2% (w/v) glucose and different concentrations (0, 0.1, 0.5, 2, 5, and 10 μM) of the cell-permeable protein kinase inhibitor 1NM-PP1. Typically, 5 μl of the cell suspension and 200 μl of the medium were applied per well. Evaporation and condensation was avoided by sealing the plates with gas-permeable Breathe-Easy sealing membrane (Carl Roth, Germany).

Preparation of Protein Extracts for High Resolution Clear Native Electrophoresis—Yeast cells were grown stepwise in YP or minimal medium with 2% (w/v) glucose in pre-cultures of 5 ml for 16 h followed by cultivation in 25 ml for 4 h. The cells

were washed twice using medium without carbon source, resuspended in 25 ml of YP or minimal medium supplemented with the indicated carbon source at an initial optical density (600 nm, 1 cm) of 1, cultivated for another 2 h, and harvested at an optical density (600 nm, 1 cm) of ~2 by centrifugation (3,500 × *g*, 4 °C, 5 min). The cells were washed with ice-cold distilled water, resuspended in 150 μl of lysis buffer A (50 mM imidazole, 5 mM aminocaproic acid, 50 mM NaCl, pH 7.0, supplemented with 1 × EDTA-free protease inhibitor mixture, 1 mM AEBSF and phosphatase inhibitor cocktails II + III (1:100)) and subjected to glass bead disruption. For this purpose, 200 mg of glass beads (0.5 mm diameter; BioSpec Products) were added, and the cells vigorously shaken 5 times for 1 min at 25 Hz in a Mixer Mill MM 200 (Retsch, Germany). Between subsequent shaking steps, the suspension was kept on ice for 1 min, respectively. The supernatant obtained by centrifugation (5,000 × *g*, 4 °C, 5 min) was used for further analysis.

Tandem Affinity Purification of Ymr291w—Cells of strain BY4741Δ*ymr291w*/p426*YMR291W-TAP* were grown stepwise in minimal medium with 2% (w/v) glucose in pre-cultures of 5 ml for 16 h, 25 ml for 8 h, and 500 ml for 16 h. Final cultivation started at an initial optical density (600 nm, 1 cm) of 0.6 in minimal medium containing 2% (w/v) glucose and continued for 6 h. The cells were harvested by centrifugation (3,500 × *g*, 4 °C, 5 min), resuspended in 1 ml of lysis buffer B (10 mM Tris-HCl, 150 mM NaCl, 1% (w/v) dodecyl-β-D-maltoside, 1 × EDTA-free protease inhibitor mixture, 1 mM AEBSF, pH 7.4) per gram of wet cell pellet, and disrupted twice in a 40K cell of a French pressure cell press (SLM Instruments) at 20,000 p.s.i. (138 MPa). The supernatant obtained by centrifugation (5,000 × *g*, 4 °C, 5 min) was fractionated by applying the TAP procedure (36). In detail, 10 ml of supernatant was incubated for 2 h at 4 °C with 400 μl of IgG-Sepharose 6 Fast Flow (GE Healthcare, UK) equilibrated with lysis buffer B, and the loaded matrix was subsequently washed three times with 10 ml of washing buffer (10 mM Tris-HCl, 150 mM NaCl, 1% (w/v) dodecyl-β-D-maltoside, 1 mM AEBSF, pH 7.4). Tobacco etch virus protease cleavage was performed overnight at 4 °C in 1 ml of cleavage buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) dodecyl-β-D-maltoside, 1 mM DTT, 0.5 mM EDTA, 1 mM AEBSF, pH 8.0) containing 100 μg of tobacco etch virus protease (37). The flow-through was incubated for 2 h with 400 μl of calmodulin-Sepharose 4B (GE Healthcare, UK) equilibrated with calmodulin buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% (w/v) dodecyl-β-D-maltoside, 1 × EDTA-free protease inhibitor mixture, 1 mM AEBSF, pH 7.4), and the loaded matrix was subsequently washed three times with 10 ml of the same buffer. The matrix-bound proteins were eluted with 2 mM EGTA in calmodulin buffer, concentrated by methanol/chloroform precipitation (38), and analyzed by SDS-PAGE. The concentrated mixture of eluted protein is subsequently referred to as TAP eluate.

Protein Kinase Assay—The phosphorylation of ScHxk2 was analyzed by incubating 0.5 μg of purified unphosphorylated enzyme (13) or 0.5 μg of purified ScHxk2(S15A) mutant hexokinase (12) with 0.2 μg of TAP eluate for 2 h in 30 mM Tris-HCl buffer containing 5 mM ATP and 20 mM $MgCl_2$ at pH 7.4 and 30 °C in a final volume of 30 μl. ScHxk2(S15A) which is non-phosphorylatable at amino acid position 15 was included as a control. The assay mixture was dialyzed against lysis buffer A using the QuixSep microdialysis system (Membrane Filtration Products) equipped with a 12-kDa cut-off membrane and further analyzed by hrCNE.

Protein Phosphatase Assay—Purified unphosphorylated ScHxk2 was phosphorylated *in vitro* and dialyzed as described above. The phosphoenzyme was incubated with 400 units of λ-phosphatase in the supplied PMP buffer in the absence or presence of phosphatase inhibitors (5 mM EDTA plus 1× phosphatase inhibitor cocktails II + III) for 1 h in a final volume of 15 μl at 30 °C. The samples were analyzed by hrCNE.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—SDS-PAGE was performed essentially as described in Ref. 39 using 10% (w/v) polyacrylamide gels. The PageRuler prestained protein ladder (Thermo Scientific) served as molecular weight marker. The gels were either stained with colloidal Coomassie Brilliant Blue G-250 (40) or the separated proteins were blotted on a PVDF membrane (Millipore) using the Mini Protean II tank blot system (Bio-Rad). The TAP tag of the Ymr291w-TAP protein was detected with HRP-coupled polyclonal rabbit anti-goat IgG antibody (Dianova, Germany) using the Immobilon™ Western Chemiluminescent HRP detection system (Millipore). α-Tubulin served as a control and was detected with monoclonal rat anti-tubulin IgG (Lifespan Biosciences) and HRP-coupled sheep anti-mouse IgG (Dianova, Germany). For quantitative signal analysis, the ImageQuant TL 7.0 software (GE Healthcare, UK) was applied.

High Resolution Clear Native Electrophoresis—The hrCNE method was performed according to Ref. 41 using a cathode buffer containing 0.01% (w/v) dodecyl-β-D-maltoside and 0.05% (w/v) desoxycholate. The high molecular weight calibration kit for native electrophoresis (GE Healthcare) was used as the molecular weight marker. The gels were either stained with colloidal Coomassie Brilliant Blue G-250 (40) or the separated proteins were blotted on a PVDF membrane (Millipore) using the Trans-Blot SD semi-dry transfer cell (Bio-Rad). ScHxk2 was detected with a polyclonal rabbit anti-ScHxk2 antibody (BioGenes GmbH, Germany) and visualized with HRP-coupled donkey anti-rabbit IgG (Dianova) using the Immobilon™ Western Chemiluminescent HRP detection system (Millipore). For quantitative signal analysis, ImageQuant TL 7.0 software (GE Healthcare, UK) was applied.

Phosphate Affinity Polyacrylamide Gel Electrophoresis—The polyacrylamide-bound dinuclear Mn^{2+}-Phos-tag™ complex (42) was utilized to separate and identify phosphorylated and unphosphorylated KlHxk1 present in protein extracts. SDS-polyacrylamide gels (7.5% (w/v)) were prepared as described in Ref. 39. The procedure was modified by adding Phos-tag™ and $MnCl_2$ to adjust a final concentration of 15 μM, respectively. The gels were run at 2 mA for 16 h, equilibrated with 1 mM EDTA for 15 min, and the separated proteins were blotted on a PVDF membrane (Millipore) using the Mini Protean II tank blot system (Bio-Rad). KlHxk1 was detected with a polyclonal rabbit anti-KlHxk1 antibody (43) and visualized with HRP-coupled donkey anti-rabbit IgG (Dianova) using the Immobilon Western Chemiluminescent HRP detection system (Millipore).

Mass Spectrometric Peptide and Protein Analyses—The proteins present in the TAP eluate of strain BY4741Δ*ymr291w*/p426*YMR291W-TAP* were converted to peptides by applying a two-step digestion procedure using endopeptidases Lys-C (Wako, Japan) and trypsin (Promega) as described in Ref. 44. The peptides were purified following the stage-tip protocol (45) and separated on an in-house-packed 20-cm analytical reverse-phase column with a 75-μm inner diameter containing Reprosil-AQ Pur 3-μm C18 reverse-phase beads (Dr. Maisch GmbH, Germany) using a linear 10–50% acetonitrile gradient on a Proxeon Easy-nLC system (Proxeon Biosystems, Denmark). The separated peptides were directly sprayed into a Q-Exactive mass spectrometer (Thermo Scientific), and the recorded spectra were analyzed using the MaxQuant software package version 1.2.2.5 (46, 47) by matching the data to the UniProt *Saccharomyces cerevisiae* database (version of 06 May, 2012) with a false discovery rate of 1% for proteins and peptides allowing a maximum of two missed cleavages. Variable modifications were set to "oxidation of methionines" and "acetylation of N termini," whereas fixed modifications were set to "carbamidomethylation of cysteines." All other parameters were set to the default values of the software. To identify phosphopeptides, the protein preparations were treated and measured as described in the above setting, however, the parameters for the MaxQuant analysis were "oxidation of methionines," "acetylation of N termini," and "phosphorylation of serines, threonines, and tyrosines." Fixed modifications, selected database, and false discovery rate were set as before. The serine-to-alanine mutation in ScHxk2(S15A) was confirmed by adding the FASTA file of the Ygl253w amino acid sequence containing a serine-to-alanine exchange at position 15 leaving all other settings unchanged. The spectra were inspected manually and annotated using the Viewer application of the MaxQuant package.

Reagents—The following reagents were purchased from these providers: AEBSF, DTT, and HCl, AppliChem (Germany); peptone, tryptone, and yeast extract, BD Biosciences; 1NM-PP1 (4-amino-1-tert-butyl-3-(1′-naphthylmethyl)pyrazolo[3,4-d]pyrimidine), Calbiochem (Germany); amino acids, galactose, glucose, and yeast nitrogen base, ForMedium (United Kingdom); high molecular weight calibration kit for native electrophoresis, GE Healthcare; dodecyl-β-D-maltoside, Glycon (Germany); Platinum® *Pfx* DNA polymerase, Invitrogen; nourseothricin, Jena Bioscience (Germany); methanol, Merck (Germany); restriction enzymes and λ-phosphatase, New England Biolabs; T4 DNA ligase, Promega; ATP, EDTA-free protease inhibitor mix, Roche Applied Science; acrylamide-bisacrylamide solution, 30% (w/v), ratio 37.5:1, ammonium peroxodisulfate, ammonium sulfate, glycerol, glycine, phosphoric acid, Ponceau S, and Tris, Roth (Germany); Coomassie Brilliant Blue G-250 and Tween 20, Serva (Germany); PageRuler™ prestained protein ladder, Thermo Scientific; and Phos-tag™, Wako Chemicals (Japan). All other reagents used were Sigma products.

RESULTS

Tandem Affinity Purification of Ymr291w—The TAP procedure was applied to enrich Ymr291w and remove ScHxk2 as well as protein kinases and phosphoprotein phosphatases that potentially could interfere with the *in vitro* analysis of ScHxk2 phosphorylation by Ymr291w. The functionality of the C-terminally TAP-tagged Ymr291w protein kinase was examined by transformation of *S. cerevisiae* strain BY4741Δ*ymr291w* with multicopy plasmid p426*YMR291W-TAP* and subsequent hrCNE analysis (Fig. 1). The latter method (41) exploits the finding that phosphorylation of ScHxk2 at serine 15 results in the dissociation of the unphosphorylated homodimeric enzyme (*D* in *lanes 1–4*) (24) exhibiting a calculated molecular mass of ~107 kDa (Uniprot P04807). The appearance of a protein band that corresponds within the analytical limits of native gel electrophoresis to the ScHxk2 phosphomonomer (*M* in *lane 4*) indicates a functional complementation of the *ymr291w* deletion strain. By contrast, no monomer was formed in control strains BY4741Δ*ymr291w* (*lane 2*) and BY4741Δ*ymr291w*/p426GPD (*lane 3*). Strain BY4741 was included as a physiological reference that contains both hexokinase species (*lane 1*). To examine the suitability of the TAP eluate to study the catalytic action of Ymr291w *in vitro*, the eluted proteins were identified by in-solution digestion and LC-MS/MS (supplemental Table S1). The data clearly identify Ymr291w as the predominant protein and document the absence of ScHxk2 and phosphoprotein phosphatases in the TAP eluate. Despite modifications of the TAP procedure introduced to enhance stringency and improve Ymr291w recovery, four of the overall 128 protein kinases encoded by the *S. cerevisiae* genome were identified in the TAP eluate: the serine/threonine protein kinases Ydr477w/Snf1, Yfr014c/Cmk1, and Ykl126w/Ypk1 as well as the dual-specificity serine/threonine/tyrosine protein kinase Ynl307c/Mck1. It should be noted, however, that the cumulated intensities for all peptides of each of these co-purified enzymes was at least ~1.000-fold lower than that of Ymr291w suggesting that these proteins represent minor contaminants (supplemental Table S1).

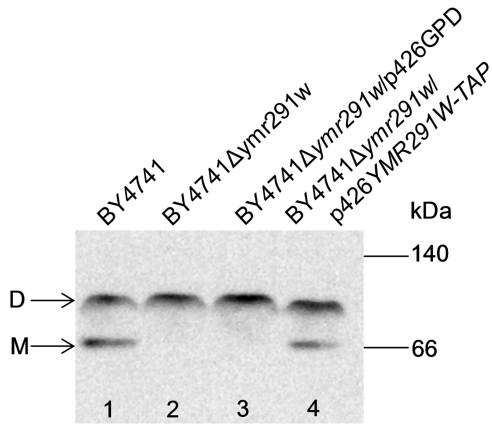

FIGURE 1. **Functional complementation of *S. cerevisiae* deletion strain BY4741Δ*ymr291w* with multicopy plasmid p426*YMR291W-TAP* encoding C-terminally TAP-tagged Ymr291w protein kinase.** Strains BY4741, BY4741Δ*ymr291w*, BY4741Δ*ymr291w*/p426GPD, and BY4741Δ*ymr291w*/p426*YMR291W-TAP* were grown in high-glucose minimal medium. Cells were disrupted as described under "Experimental Procedures". Samples containing 25 μg of extracted protein were subjected to hrCNE in 5 to 20% (w/v) polyacrylamide gradient gels. The separated proteins were blotted on a PVDF membrane and probed with polyclonal rabbit anti-ScHxk2 antibody followed by immunodetection with HRP-conjugated donkey anti-rabbit IgG using the Immobilon™ Western Chemiluminescent HRP detection system. *D,* ScHxk2 homodimer; *M,* ScHxk2 monomer.

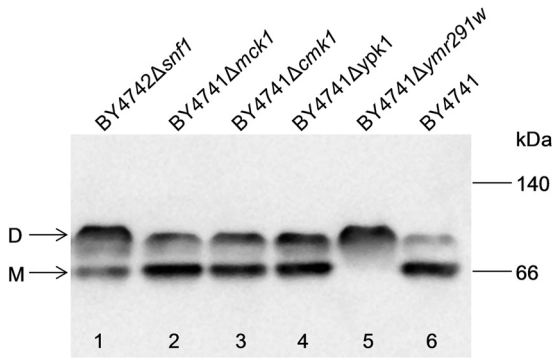

FIGURE 2. **ScHxk2 *in vivo* phosphorylation in protein kinase single-gene deletion strains of *S. cerevisiae*.** Strains BY4742Δ*snf1*, BY4741Δ*mck1*, BY4741Δ*cmk1*, BY4741Δ*ypk1*, and BY4741Δ*ymr291w* were grown in high-glucose YP medium. Strain BY4741 was included as a physiological control. For cell disruption, electrophoresis and immunodetection, see the legend to Fig. 1. *D*, ScHxk2 homodimer; *M*, ScHxk2 monomer.

Role of Protein Kinases Snf1, Mck1, Cmk1, and Ypk1 in ScHxk2 in Vivo Phosphorylation—The potential involvement in hexokinase phosphorylation of the four protein kinases that co-purified with Ymr291w during tandem affinity purification was studied by hrCNE analysis of protein extracts from the respective single-gene deletion strains (Fig. 2). The protein patterns in *lanes 1–4* demonstrate that the absence of the respective enzyme did not prevent phosphorylation as indicated by the presence of a band corresponding to the monomeric enzyme. In contrast, no monomeric hexokinase was detected in the absence of Ymr291w (*lane 5*). Interestingly, the protein pattern of strain BY4741, which was used as a physiological reference (*lane 6*), was similar to the patterns obtained with protein extracts of the *mck1*, *cmk1*, and *ypk1* protein kinase single-gene deletion strains (*lanes 2–4*), whereas the extent of monomer formation was always lower in strain BY4742Δ*snf1* (*lane 1*), as if Snf1 was positively modulating ScHxk2 phosphorylation. These data indicate an indispensable function of Ymr291w in ScHxk2 *in vivo* phosphorylation and support the view that the *YMR291W* gene product either represents the terminal ScHxk2 kinase or an upstream regulatory enzyme without excluding a role for the co-purifying protein kinases.

Ymr291w-dependent ScHxk2 in Vitro Phosphorylation—The unphosphorylated ScHxk2 protein was purified according to Ref. 13 and employed in a protein kinase assay as a substrate to analyze the function of Ymr291w *in vitro* (Fig. 3). In line with expectations, the Ymr291w-enriched TAP eluate caused the appearance of the protein band corresponding to the hexokinase monomer (Fig. 3*A*, *lane 3*), whereas only the dimer was detected in the negative controls (*lanes 1* and *4*). The missing detection of the ScHxk2 protein in the TAP eluate (Fig. 3*A*, *lane 2*) is in accordance with the mass spectrometry data (supplemental Table S1). Strain BY4741 was included as a physiological reference to localize both hexokinase species in the native gel (Fig. 3*A*, *lane 5*). To determine the site of Ymr291w-dependent phosphorylation, purified unphosphorylated ScHxk2 was phosphorylated *in vitro* with TAP eluate followed by hrCNE separation and protein staining (Fig. 3*B*). The protein present at the position of the monomer in *lane 3* (framed band *M*) was identified by mass spectrometry as ScHxk2 phosphorylated on residue serine 15 (Fig. 3*C*), whereas the homodimeric enzyme (*D*) in the same lane did not contain any detectable phosphopeptide. There is an additional diffuse protein band located between phosphomonomer and homodimer containing ScHxk2 (Fig. 3*B*, *lane 3*) whose molecular nature and formation remain to be characterized. It should be noted that due to the unclear role of Snf1 in ScHxk2 phosphorylation (11, 23), the TAP eluate was substituted by a TAPΔ*snf1* eluate prepared from single-gene deletion strain BY4742Δ*snf1* (Y14311). The essentially identical outcome of this experiment (data not shown) as compared with the protein pattern in Fig. 3*A* together with the existence of monomeric ScHxk2 in strain BY4741Δsnf1 (Fig. 2) indicates the dispensability of Snf1 for ScHxk2 phosphorylation.

Reversibility of Ymr291w-dependent ScHxk2 Phosphorylation—The reversibility of this modification as a basic regulatory principle was demonstrated by λ-phosphatase treatment of the *in vitro* phosphorylated enzyme (Fig. 4*A*). Due to dephosphorylation of the phosphomonomer, the lower hexokinase band disappeared, whereas the intensity of the upper band correspondingly increased (*lane 3*). No effect was observed in the presence of phosphatase inhibitors (*lane 4*). Protein extract from wild type strain BY4741 served as a control (*lane 1*). To verify serine 15 as the site of Ymr291w-dependent phosphorylation, the purified ScHxk2(S15A) mutant hexokinase, which is non-phosphorylatable at amino acid position 15 was treated with TAP eluate (Fig. 4*B*). The reproducible detection of a single protein band at a position slightly below that of the homodimer (but clearly above that of the phosphomonomer) irrespective of the presence or absence of TAP eluate (Fig. 4*B*, *lanes 2–4*) is compatible with the identification of serine 15 as the Ymr291w-dependent phosphorylation site (Fig. 3) without excluding a possible requirement of residue 15 for phosphorylation at another site. The increased electrophoretic mobility of ScHxk2(S15A) compared with that of the unphosphorylated wild type enzyme is in agreement with a hydrodynamic study indicating that substitution of serine 15 by alanine promotes the dissociation of the ScHxk2 homodimer (14).

Influence of Carbon Source on ScHxk2 Phosphorylation—Due to the controversial findings regarding the roles of Ymr291w and Snf1 in ScHxk2 phosphorylation reported in Refs. 11, 23, 24, and the present study, the influence of the carbon source during yeast cultivation was experimentally addressed. For this purpose, single-gene deletion strains BY4741Δ*ymr291w* and BY4742Δ*snf1* were analyzed by hrCNE following growth in media differing in glucose concentration and presence of glycerol (Fig. 5*A*). In accordance with Fig. 2, strain BY4741 (*lanes 1–3*) included as a physiological reference and BY4742Δ*snf1* (*lanes 7–9*) display bands of both phosphorylated and unphosphorylated hexokinase in all growth conditions. By contrast, the phosphomonomer band is completely missing in the protein patterns of strain BY4741Δ*ymr291w* (*lanes 4–6*). The significantly lower relative amount of phosphomonomer detected in the Δ*snf1* mutant as compared with wild type (Fig. 5*B*) suggests a positive influence of Snf1 on phosphorylation and monomer formation. The above findings confirm the absolute requirement of Ymr291w for ScHxk2-S15 phosphorylation and rule out different growth conditions as the cause for the different

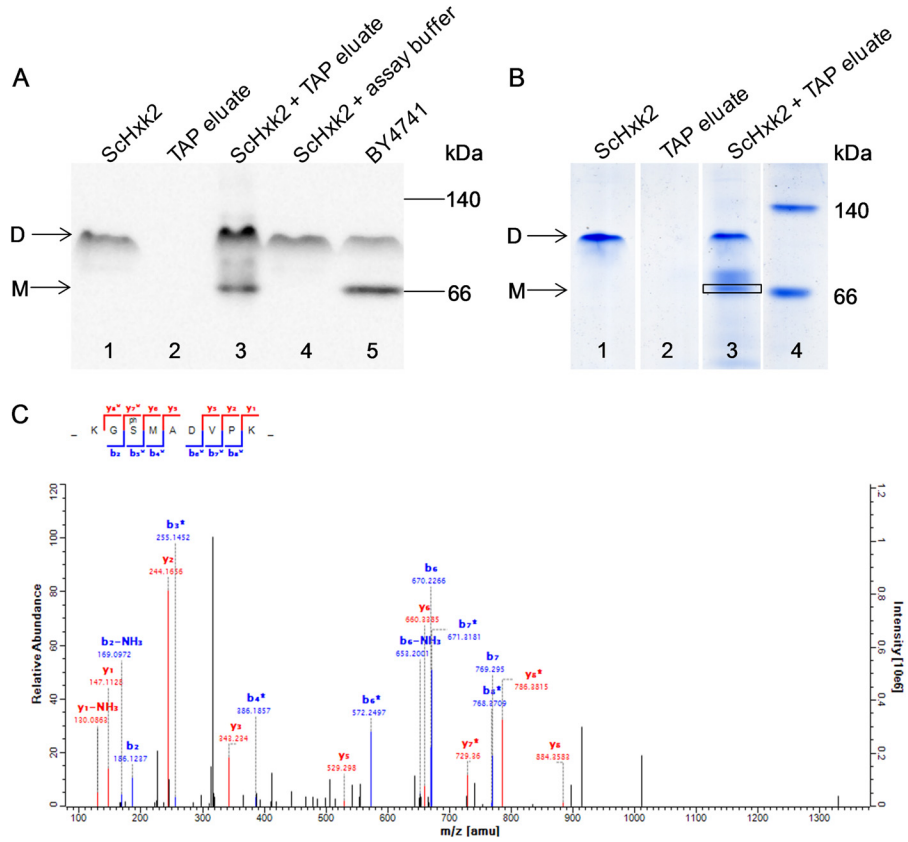

FIGURE 3. **ScHxk2 *in vitro* phosphorylation with TAP-enriched Ymr291w protein kinase.** *A,* immunodetection. Purified unphosphorylated ScHxk2 was incubated for 2 h with TAP eluate in 30 mM Tris-HCl buffer containing 5 mM ATP and 20 mM $MgCl_2$ at pH 7.4 and 30 °C (*lane 3*). The unphosphorylated hexokinase (*lanes 1* and *4*) and TAP eluate (*lane 2*) were included as negative controls. BY4741 protein extract (*lane 5*) was analyzed as a positive control to indicate the positions of the ScHxk2 monomer and dimer in the gel. Samples containing 25 ng of purified ScHxk2 (*lanes 1*, *3*, and *4*), 10 ng of TAP eluate (*lanes 2* and *3*), and 10 μg of BY4741 protein extract (*lane 5*) were applied. For cell disruption, electrophoresis, and immunodetection, see the legend to Fig. 1. *B,* sample preparation for mass spectrometric analysis of Ymr291w-dependent ScHxk2 *in vitro* phosphorylation. The experiment described in *A* was modified by increasing the amounts of ScHxk2 (5 μg; *lanes 1* and *3*) and TAP eluate (0.2 μg; *lanes 2* and *3*) followed by protein staining with colloidal Coomassie Brilliant Blue G-250. *C,* identification of a peptide harboring phosphoserine 15 of the *in vitro* phosphorylated ScHxk2. The protein present in the framed band *M* (*panel B*) was subjected to in-gel digestion using sequencing grade trypsin and analyzed by LC-MS/MS. The spectrum shows the ScHxk2 peptide KGS(phos)MADVPK. The sequence of this peptide was verified by the presence of complementary B ions (NH_2 terminus-derived fragment ions) and Y ions (COOH terminus-derived fragment ions). *D*, ScHxk2 homodimer; *M*, ScHxk2 monomer.

functional assignments made in Refs. 11, 23, and 24 with respect to the role of Snf1 and Ymr291w in that modification.

Involvement of Protein Kinase Sch9 in ScHxk2 Phosphorylation—Protein kinase B homolog Sch9 contributes to the glucose regulation of cell growth and has overlapping substrate specificities with PKA (48) whose contribution to ScHxk2 phosphorylation is still unclear (10, 12). To reveal a potential role of Sch9 in ScHxk2 phosphorylation, single-gene deletion strain BY4741Δ*sch9* was grown in high-glucose and low-glucose media, followed by hrCNE analysis (Fig. 5*A*). Similar to the situation in the *snf1* mutant strain (*lanes 7–9*), the absence of Sch9 did not prevent phosphomonomer formation (*lanes 10–12*). This finding precludes an essential role of Sch9 in ScHxk2-S15 phosphorylation, but leaves open the question regarding a modulatory function because the extent of phosphomonomer formation was reproducibly lower in strain BY4741Δ*sch9* compared with wild type following growth in the high-glucose medium (Fig. 5*B*, *lane 10 versus 1*).

Involvement of Protein Kinase A in ScHxk2 Phosphorylation—The N terminus of ScHxk2 contains a consensus sequence for protein kinase A-dependent phosphorylation (22) and has been shown to be phosphorylated at serine 15 by this enzyme *in vitro* (12). In contrast, genetic studies in *S. cerevisiae* suggested a negative role of PKA in ScHxk2 phosphorylation (10). As cells with null mutations of the three genes encoding the catalytic subunits *TPK1-TPK3* of PKA are not viable (49), PKA mutant strain Y3175 was used to evaluate the role of PKA in ScHxk2 phosphorylation (Fig. 6). The suitability of this strain is based on deletions of the *TPK2* and *TPK3* genes and on the presence of a functionally silent active-site mutation in *TPK1* that sensitizes to the cell-permeable ATP-analogous target-specific protein kinase inhibitor 1NM-PP1 (25, 50). The effective concentration of 1NM-PP1 was determined in nephelometric growth studies indicating that 10 μM is sufficient to almost completely inhibit growth of the mutant both in low-glucose and high-glucose media leaving wild type cells unaffected (Fig. 6*A*). The analysis of protein extracts from mutant strain Y3175 indicated that ScHxk2 phosphorylation occurs regardless of the presence of 1NM-PP1 in high-glucose and low-glucose media (Fig. 6*B*, *lanes 4–9*). Notably, the phosphomonomer represented the predominant enzyme species in the protein extracts from wild type and mutant cells when grown in low-glucose media. The above data support the view that PKA is dispensable for ScHxk2 *in vivo* phosphorylation.

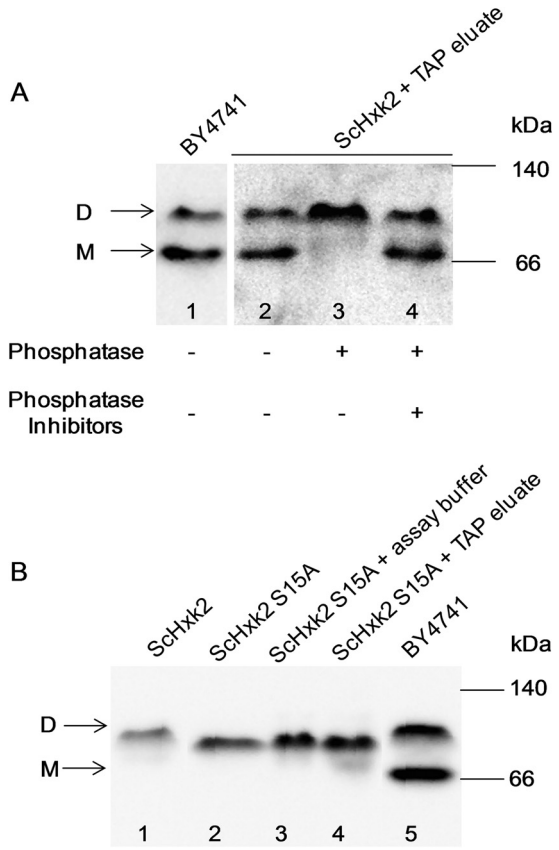

FIGURE 4. **Reversibility and site specificity of ScHxk2 phosphorylation.** *A,* dephosphorylation of *in vitro* phosphorylated ScHxk2 by λ-phosphatase. Purified ScHxk2, phosphorylated by the TAP-enriched Ymr291w protein kinase (*lanes 2–4*), was incubated for 1 h at 30 °C with λ-phosphatase in the absence (*lane 3*) or presence (*lane 4*) of phosphatase inhibitors. The BY4741 protein extract was used as a physiological reference (*lane 1*). Samples containing 25 ng of purified ScHxk2 plus 10 ng of TAP eluate (*lanes 2-4*) and 10 μg of extracted protein (*lane 1*) were applied. *B,* analysis of ScHxk2(S15A) *in vitro* phosphorylation. The purified mutant enzyme (*lanes 2* and *3*) was incubated for 2 h with TAP eluate in 30 mM Tris-HCl buffer containing 5 mM ATP and 20 mM $MgCl_2$ at pH 7.4 and 30 °C (*lane 4*). Purified unphosphorylated ScHxk2 (*lane 1*) and protein extract from strain BY4741 (*lane 5*) were included as controls. Samples containing 25 ng of purified ScHxk2 (*lane 1*), 25 ng of purified ScHxk2(S15A) (*lanes 2–4*), 10 ng of TAP eluate (*lane 4*), and 10 μg of protein extract (*lane 5*) were applied. For electrophoresis and immunodetection, see the legend to Fig. 1. *D,* ScHxk2 homodimer; *M,* ScHxk2 monomer.

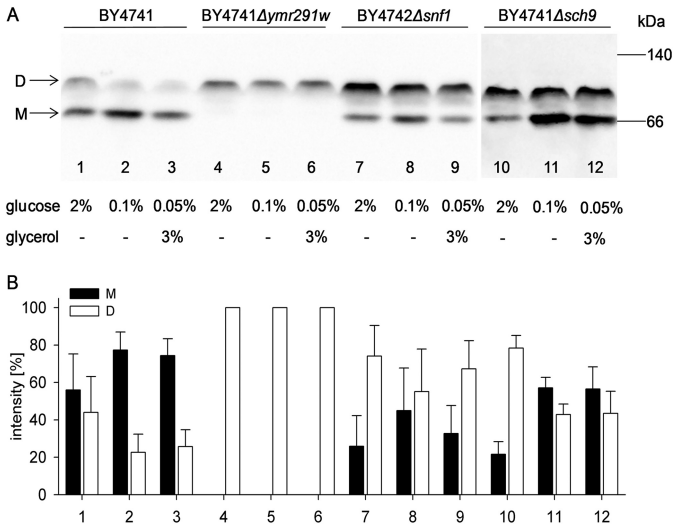

FIGURE 5. **ScHxk2 *in vivo* phosphorylation in selected protein kinase single-gene deletion strains of *S. cerevisiae* and influence of carbon source.** *A,* hrCNE analysis of ScHxk2 *in vivo* phosphorylation. Strains BY4741Δ*ymr291w*, BY4742Δ*snf1,* and BY4741Δ*sch9* were grown in YP medium containing 2% (w/v) glucose, 0.1% (w/v) glucose, or 0.05% (w/v) glucose + 3% (v/v) glycerol. Reference strain BY4741 was included as a control. For cell disruption, electrophoresis, and immunodetection, see the legend to Fig. 1. *B,* quantitation of monomeric and dimeric ScHxk2. Relative intensities of the immunodetected monomeric and dimeric ScHxk2 were calculated using the ImageQuant TL 7.0 software. The total intensity per lane was set 100%. Results were calculated from 18 (strains BY4741 and BY4741Δ*ymr291w*) or 9 (strains BY4742Δ*snf1* and BY4741Δ*sch9*) independent experiments. *p* values resulting from paired *t* tests were: *lane 1 versus 2*, $p = 0.000034$; *1 versus 3*, $p = 0.00070$; *1 versus 7*, $p = 0.00048$; *2 versus 8*, $p = 0.0024$; *3 versus 9*, $p = 0.000015$; *1 versus 10*, $p = 0.00016$; *2 versus 11*, $p = 1.1 \times 10^6$; *3 versus 12*, $p = 0.0085$. *D*: ScHxk2 homodimer; *M,* ScHxk2 monomer.

Effect of External Glucose Concentration on Ymr291w Steady-state Concentration—In view of the finding that phosphorylation at serine 15 of the overexpressed ScHxk2 protein was stimulated (and/or the dephosphorylation of the phosphoenzyme inhibited) under glucose-limited conditions (12), the influence of the initial glucose concentration of the growth medium on the level of Ymr291w in the protein extract was investigated. Strain BY4741/*YMR-TAP* expressing chromosomally encoded C-terminally TAP-tagged Ymr291w was used, and the protein kinase was detected via its protein A domain (Fig. 7). The position of the protein band recognized by the anti-protein-A antibody corresponds to the expected molecular mass of 100 kDa of the TAP-tagged Ymr291w protein kinase. Comparison of the staining intensity of Ymr291w-TAP after cultivation in high-glucose and low-glucose medium indicated in five independent experiments a suppression of *YMR291W-TAP* expression and/or an enhanced degradation of Ymr291w-TAP in the presence of high glucose conditions. In contrast, immunodetection of α-tubulin (50 kDa) used as a control did not reveal any glucose-dependent differences. The above findings are consistent with the conclusion supported by the data in Fig. 5*B* that the extent of ScHxk2-S15 phosphorylation in wild type yeast is inversely correlated with the availability of glucose in the growth medium.

Cross-complementation of Yeast Hexokinase Kinase Mutants—The genome of the Crabtree-negative yeast *K. lactis* encodes the gene *KLLA0A09713* whose predicted translation product Klla0a09713p exhibits 44% identity with the Ymr291w protein of *S. cerevisiae* reference strain ATCC 204508/S288c. In addition, the dual-function hexokinase KlHxk1 of *K. lactis* is sharing 73% identity with ScHxk2 and is also phosphorylated at the conserved amino acid residue serine 15 *in vivo* (43). Based on these findings, cross-complementation studies of single-gene deletion strains lacking Ymr291w and its homolog Klla0a09713p, respectively, were performed (Fig. 8). KlHxk1 phosphorylation in *K. lactis* was analyzed by phosphate affinity SDS-PAGE (Fig. 8, *panel A*). The latter method was applied because KlHxk1, although forming in the purified state a monomer-homodimer equilibrium like the homologous ScHxk2 enzyme (51), is detected in the monomeric form only when protein extracts are analyzed by hrCNE (data not shown). This observation most likely is due to a different homodimer-phosphomonomer ratio of the two hexokinases in the different protein extracts as purified KlHxk1 is clearly separated by hrCNE into monomeric and dimeric enzyme species (15). Comparison of the protein patterns in Fig. 8, *panel A*, *lanes 1*

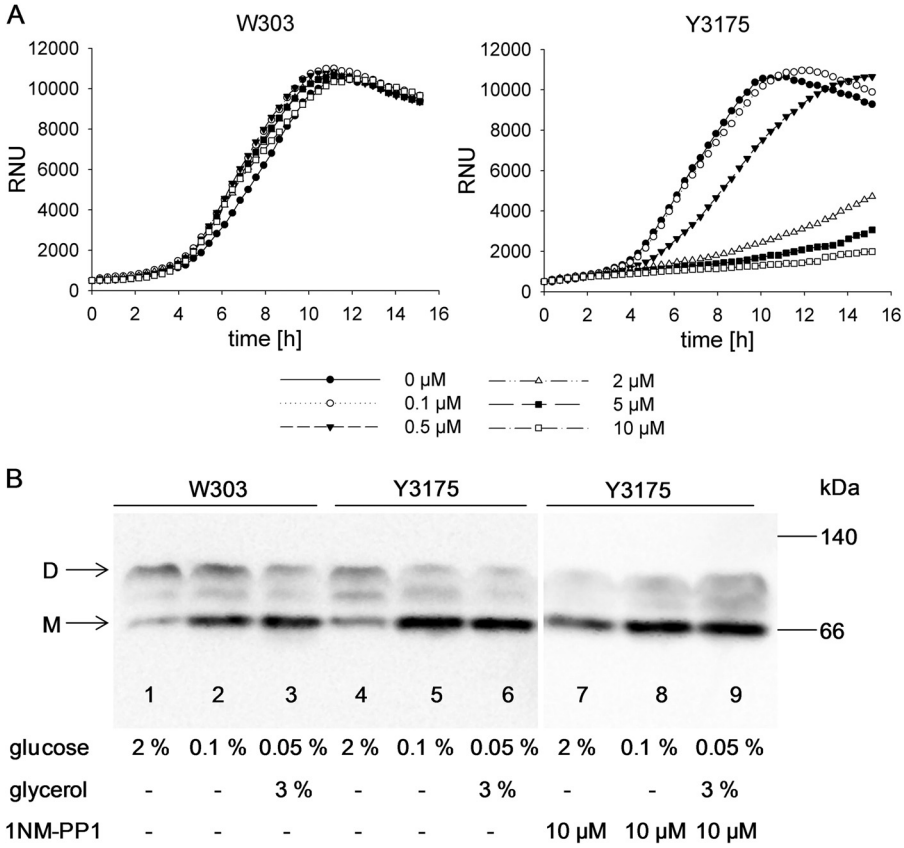

FIGURE 6. **ScHxk2 *in vivo* phosphorylation in the absence of functional protein kinase A and influence of carbon source.** *A,* effect of the ATP-analogous PKA inhibitor 1NM-PP1 on the growth of the conditional mutant Y3175 (Δ*tpk2* Δ*tpk3*) carrying a functionally silent active-site mutation in *TPK1*. Growth was monitored in high-glucose minimal medium at different micromolar concentrations of 1NM-PP1 in a laser-based microplate nephelometer (NEPHELOstar Galaxy) as described under "Experimental Procedures." Cell density is given in relative nephelometric units (*RNU*). Data are based on three independent experiments each of which consisting of assays performed in triplicate. *TPK* wild type strain W303 was used as a physiological reference. *B,* hrCNE analysis of ScHxk2 *in vivo* phosphorylation. Strains W303 and Y3175 were grown in minimal medium supplemented with 2% (w/v) glucose, 0.1% (w/v) glucose, or 0.05% (w/v) glucose + 3% (v/v) glycerol. The conditional mutant (Y3175) was analyzed following cultivation in the absence/presence of 10 μM 1NM-PP1 in the main cell culture. For cell disruption, electrophoresis, and immunodetection, see the legend to Fig. 1. *D,* ScHxk2 homodimer; *M,* ScHxk2 monomer.

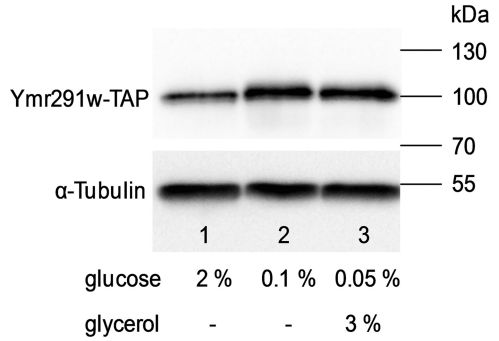

FIGURE 7. **Dependence of Ymr291w steady-state concentration on external glucose availability.** Strain BY4741/*YMR-TAP* expressing chromosomally encoded C-terminally TAP-tagged Ymr291w was grown in YP medium at initial concentrations of 2% (w/v) glucose, 0.1% (w/v) glucose, and 0.05% (w/v) glucose + 3% (v/v) glycerol, respectively. The cells were disrupted as described under "Experimental Procedures." Samples containing 100 μg of extracted protein were subjected to SDS-PAGE in 10% (w/v) polyacrylamide gels and the separated proteins were blotted on a PVDF membrane. Ymr291w was visualized with a polyclonal HRP-conjugated rabbit anti-goat IgG antibody using the Immobilon™ Western Chemiluminescent HRP detection system. α-Tubulin was used as a control and detected by use of monoclonal rat anti-tubulin IgG and HRP-coupled sheep anti-mouse IgG.

(*K. lactis* wild type), *4* (complementation of strain JA6 Δ*klla0a09713* by the authentic Klla0a09713p protein), and *5* (complementation of strain JA6Δ*klla0a09713* by the Klla0a09713p-homologous Ymr291w protein) reveals that both proteins allow for KlHxk1 phosphorylation in *K. lactis* as indicated by the upper band corresponding to the retained phosphoenzyme. Similarly, the hrCNE protein patterns in Fig. 8, *panel B*, *lanes 1* (*S. cerevisiae* wild type), *4* (complementation by the authentic Ymr291w protein), and *5* (complementation by the Ymr291w-homologous Klla0a09713p protein) demonstrate the functional equivalence of the homologous protein kinases with respect to ScHxk2 phosphorylation in *S. cerevisiae*.

DISCUSSION

Phosphorylation and dephosphorylation of proteins represent a universal mechanism of covalent modification that enables cells to modulate structural and functional properties, subcellular localization, and complex formation with physiological ligands of their constituent proteins and enzymes. In *S. cerevisiae*, about 50% of the metabolic proteins were reported to be targets of the protein kinase/phosphoprotein phosphatase signaling network (52–54), among them the hexokinases ScHxk1 and ScHxk2 (10). The phosphorylation of ScHxk2 at serine 15 has three consequences: (i) displacement of the monomer-homodimer equilibrium in favor of the monomeric species (14), (ii) increase of substrate affinity and inhibition by ATP (21), and (iii) stimulation of nucleocytoplasmic translocation of

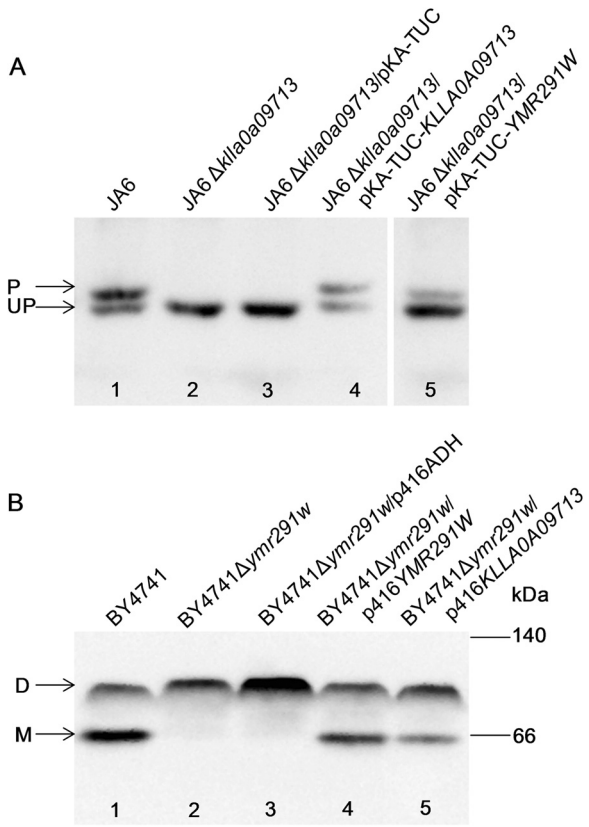

FIGURE 8. **Cross-complementation of single-gene deletion strains of *S. cerevisiae* and *K. lactis* lacking *YMR291W* and its homolog *KLLA0A09713*, respectively.** *A,* complementation of *K. lactis* strain JA6Δ*klla0a09713* (*lane 2*) with the Ymr291w protein kinase of *S. cerevisiae* encoded by single-copy plasmid pKA-TUC-*YMR291W* (*lane 5*). Strains JA6 (*lane 1*), JA6Δ*klla0a09713*/pKA-TUC (*lane 3*), and JA6Δ*klla0a09713*/pKA-TUC-*KLLA0A09713* (*lane 4*) were included as controls. Cells were grown in YNB medium supplemented with 2% (w/v) galactose. Protein extracts were prepared as described under "Experimental Procedures." The extracted proteins (10 μg) were separated by phosphate affinity SDS-PAGE in a 7.5% (w/v) polyacrylamide gel containing 15 μM Phos-tag™, blotted on a PVDF membrane, and probed with polyclonal rabbit anti-KlHxk1 antibody followed by immunodetection with HRP-conjugated donkey anti-rabbit IgG using the Immobilon™ Western Chemiluminescent HRP detection system. *UP*, unphosphorylated KlHxk1; *P,* phosphorylated KlHxk1. *B,* complementation of *S. cerevisiae* strain BY4741Δ*ymr291w* (*lane 2*) with the Ymr291w-homologous protein Klla0a09713p of *K. lactis* encoded by single-copy plasmid p416ADH-*KLLA0A09713* (*lane 5*). Strains BY4741 (*lane 1*), BY4741Δ*ymr291w*/p416ADH (*lane 3*), and BY4741Δ*ymr291w*/p416*YMR291W* (*lane 4*) were included as controls. Cells were grown in high-glucose minimal medium. For cell disruption, electrophoresis, and immunodetection, see the legend to Fig. 1. *D,* ScHxk2 homodimer; *M,* ScHxk2 monomer.

the phosphoenzyme (23). Despite comprehensive efforts to explore the mechanism and regulatory significance of the *in vivo* phosphorylation of ScHxk2, the identity of the responsible protein kinase(s) is still a matter of debate (11, 23, 24). This situation reflects the complexity of experimental work on protein phosphorylation in *S. cerevisiae,* which encodes more than 128 protein kinases that in many cases phosphorylate multiple targets (55).

In a previous study, single-gene deletion strains of selected serine/threonine protein kinases with known functions in glucose metabolism or with unknown functions but known localization in the cytoplasm and/or the nucleus of *S. cerevisiae* were screened for ScHxk2 *in vivo* phosphorylation (24). The outcome, although clearly identifying Ymr291w as the only kinase that essentially was required for ScHxk2 phosphorylation, was limited by the fact that pleiotropic kinases like Snf1, PKA, and Sch9 were not considered in the mutant screen. In addition, the putative involvement of Mck1 (a dual-specificity serine/threonine/tyrosine protein kinase involved in control of chromosome segregation and regulation of entry into meiosis (56)), Cmk1 (a calmodulin-dependent serine/threonine protein kinase involved in stress response (57)), and Ypk1 (a serine/threonine protein kinase involved in sphingolipid-mediated signaling (58)) that co-purified with Ymr291w during tandem affinity purification (this work) called for their experimental consideration. The respective results (Figs. 2 and 5) demonstrate that each of these protein kinases is singly dispensable for ScHxk2 *in vivo* phosphorylation when Ymr291w is present. The same conclusion applies to protein kinase A (Fig. 6) despite the existence of a PKA consensus phosphorylation sequence in ScHxk2 (22) and the capability of purified Tpk1 catalytic subunit to phosphorylate ScHxk2 at serine 15 *in vitro* (12). This apparent contradiction may be related to the different experimental conditions applied and/or to a general limitation of gene deletion studies.

In the case of Snf1 playing a central role in glucose signaling by contributing to the adaptation to glucose limitation and growth on carbon sources that are less preferred than glucose (59) the situation is different in part: Snf1 is not essential for ScHxk2 *in vivo* phosphorylation as the modification takes place in cells lacking this protein kinase; however, its absence was reproducibly correlated with a lower phosphomonomer level suggesting a positive role (Fig. 5). This observation is not simply appropriate to confirm the statement given in Ref. 23 that ScHxk2 is a substrate for Snf1, but rather excludes the possibility of Snf1 being the kinase of ScHxk2 as stated in Ref. 11. It should be noted that the consequences of *SCH9* deletion are similar to those reported above for Snf1 deficiency when cells were grown in high-glucose medium: ScHxk2 phosphorylation takes place, but to a lesser degree compared with wild type (Fig. 5).

The evidence obtained by mass spectrometry that ScHxk2 is phosphorylated specifically at serine 15 by Ymr291w or by a Ymr291w-dependent protein kinase present in the TAP eluate (Fig. 3) together with the absence of the phosphohexokinase band in the protein pattern of the *ymr291w* single-gene deletion strain (Fig. 1) strongly suggest that serine 15 represents the site of that modification also in wild type yeast. This conclusion is in line with data from the PhosphoPep 2.0 database (55), indicating that the R.KGS*MADVPK peptide of ScHxk2 carrying phosphorylated serine 15 is 5.9-fold down-regulated in a *ymr291w* deletion strain. Additional support for a physiological role of Ymr291w in ScHxk2-S15 phosphorylation *in vivo* comes from the finding that the steady-state level of this protein kinase is higher in cells grown in low-glucose media (Fig. 7), where according to Ref. 23 the degree of serine 15 phosphorylation is expected to be higher than in a high-glucose environment. The latter observation is supported by the detection of a significantly higher relative amount of phosphomonomer in wild type cells during glucose limitation (Fig. 5*B*) when phosphorylation at serine 15 is required for the relief from glucose repression and the Xpo1-dependent translocation of the hexokinase from

the nucleus into the cytosol. When glucose is abundantly available, ScHxk2 is dephosphorylated by Glc7-Reg1, thus allowing its nuclear import via interaction with Kap60/Kap95 (23) to mediate glucose repression. Referring to the data in Fig. 5*B*, it should be noted that variations in the technically sensitive steps of this work are balanced by the number of 18 replicates. In addition, variations of the protein load during hrCNE would essentially affect the ScHxk2 monomer-homodimer equilibrium according to the law of mass action only if such variations exceeded at least one order of magnitude (21).

The present paper is focused on the functional role with respect to ScHxk2-S15 phosphorylation of a limited number of protein kinases, which by far do not comprise the entirety of *S. cerevisiae* protein kinases. This situation provokes the speculation that the observed absence of the modification in the *ymr291w* deletion strain (Fig. 1) might be due to a lack of activation of a so far unidentified terminal hexokinase kinase by Ymr291w. Indeed, the identification within current mass spectrometric detection limits of Snf1, Mck1, Cmk1, and Ypk1 in the TAP eluate (supplemental Table S1) is appropriate to support a scenario in which two of the latter kinases independently of each other catalyze ScHxk2-S15 phosphorylation following their activation, thereby preventing an unequivocal functional assignment. This consideration applies to other protein kinases including PKA and Sch9 as well and illustrates the limitations of gene deletion studies. The absence of the latter two kinases and the finding that the individual levels of Snf1, Mck1, Cmk1, and Ypk1 in the TAP eluate did not exceed 0.1% of the Ymr291w protein, however, strongly supports the view of a direct role of Ymr291w in ScHxk2-S15 phosphorylation. The additional finding that no phosphoprotein phosphatase was present in the TAP eluate (supplemental Table S1) contradicts the alternative hypothesis of an inhibition of phosphohexokinase dephosphorylation by Ymr291w or by an Ymr291w-dependent phosphatase. Despite the remaining uncertainty regarding a direct role of Ymr291w in ScHxk2-S15 phosphorylation, the regulation of Ymr291w expression and activity as well as its interaction with physiological ligands represent promising experimental challenges to further explore the mechanism of glucose repression. Keeping in mind the finding that only four of 128 protein kinases (supplemental Table S1) encoded by the *S. cerevisiae* genome co-purified with Ymr291w during tandem affinity purification, future experiments should also consider the putative existence of a protein kinase complex mediating glucose-dependent ScHxk2-S15 phosphorylation.

Evaluation of the physiological significance of the present work has to take into account that in yeast as much as 65% of the detected phosphorylation sites might be non-functional and caused by off-target activity of protein kinases, particularly in disordered regions of their substrate proteins (60). In comparison, the molecular and functional consequences of ScHxk2-S15 phosphorylation are complex as outlined above and not unique because the equivalent modification of the homologous hexokinase KlHxk1 in glucose-repressible *K. lactis* is accompanied in a similar way by a stimulation of homodimer dissociation and an increase of substrate affinity and enzyme activity (15, 43, 51). The evolutionary conservation of serine 15 phosphorylation is also reflected by the finding that the *YMR291W* gene of *S. cerevisiae* and the homologous *KLLA0A09713* gene of *K. lactis* (the latter exhibiting on the protein level 44% identity with the Ymr291w protein of *S. cerevisiae* reference strain ATCC 204508/S288c) allow for cross-complementation of corresponding protein kinase single-gene deletion strains (Fig. 8). This situation together with the availability of crystal structures of monomeric and dimeric unphosphorylated and serine 15-phosphorylated KlHxk1 (15, 61) exhibiting 73% identity with ScHxk2 represent a promising basis to understand and further investigate the structural and functional aspects of hexokinase *in vivo* phosphorylation at the conserved residue serine 15 in yeast. Prospective studies on that issue should also address the poorly considered role of phosphorylation of hexokinase isoenzyme ScHxk1 and the significance of genetic differences between the various yeast strains employed in glucose repression analysis.

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