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Rck2 Kinase Is a Substrate for the Osmotic Stress-Activated Mitogen-Activated Protein Kinase Hog1

ELIZABETH BILSLAND-MARCHESAN,[1] JOAQUÍN ARIÑO,[2] HARUO SAITO,[3]
PER SUNNERHAGEN,[1] AND FRANCESC POSAS[2,4]*

*Department of Cell and Molecular Biology, Lundberg Laboratory, Göteborg University, S40530 Göteborg, Sweden[1];
Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115[3]; and Departament de
Bioquímica i Biologia Molecular, Facultat de Veterinària, Universitat Autònoma de Barcelona,
08193 Barcelona,[2] and Cell Signaling Unit, Departament de Ciències Experimentals
i de la Salut, Universitat Pompeu Fabra (UPF), Barcelona E-08003,[4] Spain*

Exposure of yeast cells to increases in extracellular osmolarity activates the Hog1 mitogen-activated protein kinase (MAPK). Activation of Hog1 MAPK results in induction of a set of osmoadaptive responses, which allow cells to survive in high-osmolarity environments. Little is known about how the MAPK activation results in induction of these responses, mainly because no direct substrates for Hog1 have been reported. We conducted a two-hybrid screening using Hog1 as a bait to identify substrates for the MAPK, and the Rck2 protein kinase was identified as an interactor for Hog1. Both two-hybrid analyses and coprecipitation assays demonstrated that Hog1 binds strongly to the C-terminal region of Rck2. Upon osmotic stress, Rck2 was phosphorylated in vivo in a Hog1-dependent manner. Furthermore, purified Hog1 was able to phosphorylate Rck2 when activated both in vivo and in vitro. Rck2 phosphorylation occurred specifically at Ser519, a residue located within the C-terminal putative autoinhibitory domain. Interestingly, phosphorylation at Ser519 by Hog1 resulted in an increase of Rck2 kinase activity. Overexpression of Rck2 partially suppressed the osmosensitive phenotype of $hog1\Delta$ and $pbs2\Delta$ cells, suggesting that Rck2 is acting downstream of Hog1. Consistently, growth arrest caused by hyperactivation of the Hog1 MAPK was abolished by deletion of the *RCK2* gene. Furthermore, overexpression of a catalytically impaired (presumably dominant inhibitory) Rck2 kinase resulted in a decrease of osmotolerance in wild-type cells but not in $hog1\Delta$ cells. Taken together, our data suggest that Rck2 acts downstream of Hog1, controlling a subset of the responses induced by the MAPK upon osmotic stress.

Mitogen-activated protein kinase (MAPK) cascades are common signaling modules found in both higher and lower eukaryotic cells. A typical MAPK cascade is composed of three tiers of protein kinases, a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (39). Yeast cells have several distinct MAPK cascades that transduce distinct extracellular stimuli (e.g., mating pheromone, high osmolarity, low osmolarity, and nitrogen starvation) (16, 14). Budding yeast (*Saccharomyces cerevisiae*) responds to increases in osmolarity in the extracellular environment by activating the Hog1 MAPK cascade. This cascade is essential for the survival of yeast in high-osmolarity environments (5, 6). Because a major outcome of the activation of this MAPK pathway is the elevated synthesis of glycerol, this pathway is referred to as the HOG (high-osmolarity glycerol response) pathway (1, 6).

The yeast HOG pathway is activated by two independent mechanisms. The first mechanism involves a two-component osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins (29), which activate the Ssk2 and Ssk22 MAPKKKs (26). Once activated, those MAPKKKs phosphorylate the Pbs2 MAPKK. Pbs2 phosphorylation can also be achieved by a second mechanism, which involves the transmembrane protein Sho1, the MAPKKK Ste11, and the Ste11 binding protein Ste50 (20, 25, 28). Thus, both independent activation pathways converge at the level of Pbs2, and either the Ssk2, Ssk22, or Ste11 MAPKKK

can activate Pbs2 by phosphorylation. Once activated, the Pbs2 MAPKK phosphorylates the Hog1 MAPK, which induces diverse stress responses.

There are a great number of genes known to be induced by the Hog1 MAPK in response to osmotic stress. Among them are genes responsible for osmotic adaptation, such as those for enzymes involved in glycerol synthesis (i.e., *GPD1*, *GPP2*, and *GLO1*), and genes involved in general stress responses like those for cytosolic catalase (*CTT1*), heat shock proteins (*HSP12*, *HSP70*, and *HSP104*), and a DNA damage-induced protein (*DDR2*) (14). The mechanism of gene regulation through activated Hog1 is still unknown, mainly because no definitive substrates for Hog1 have been reported. Several candidates, however, have been described. Those are the transcription factors Msn2 and Msn4, which bind to the promoter element defined as STRE, found in several stress-response induced genes (31), and the bZIP-type protein Sko1, which binds to CREB-like sequences (23, 30). Although these transcription factors seem to be controlled by Hog1, no direct interaction with Hog1 has been reported to date.

It is known that there exist other substrates for MAPK apart from transcription factors. In yeast, the cyclin-dependent kinase inhibitor Far1 is phosphorylated by the Fus3 MAPK in the mating pathway, which results in cell cycle arrest (24), and the tyrosine phosphatases Ptp2 and Ptp3, which are responsible for dephosphorylation and inactivation of Hog1 and Fus3, may be targets for their respective MAPKs (40, 41). In mammalian cells, several kinases involved in a number of cellular processes have been identified as substrates for MAPKs. This is the case, for example, for p90/rsk and MAPKAP kinase 2,

* Corresponding author. Mailing address: Unitat de Senyalització Cellular, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra (UPF), C/Doctor Aiguader 80, Barcelona E-08003, Spain. Phone: 34-93-542 2848. Fax: 34-93-542 2802. E-mail: francesc.posas @cexs.upf.es.

substrates for the ERK kinases and p38 stress-activated MAPK (32, 34).

To understand the complex mechanism required for osmotic stress adaptation, it is necessary to identify substrates for the Hog1 MAPK. We conducted a two-hybrid screening to identify targets for Hog1. We found that Rck2, a protein kinase previously isolated by virtue of its sequence similarity to the yeast and mammalian calmodulin kinases (21) and as a suppressor of *Schizosaccharomyces pombe* checkpoint mutants (9), interacts with Hog1 and that Rck2 kinase activity is regulated by phosphorylation by the Hog1 MAPK.

MATERIALS AND METHODS

Yeast strains. The following yeast strains were used: W303-1A (*MATa leu2-3,112 ura3-1 his3-11 trp1-1a can100*), EBΔR2Wa (*MATa rck2::kanMX4 leu2-3,112 ura3-1 his3-11 trp1-1a can100*), *hog1Δ* (*MATa hog1::LEU2 leu2- 3,112 ura3-1 his3-11 trp1-1a can100*), EBΔHR2Wa (*MATa hog1::LEU2 rck2::kanMX4 leu2-3,112 ura3-1 his3-11 trp1-1a can100*), DMΔpWa (*MATa pbs2::kanMX4 leu2-3,112 ura3-1 his3-11 trp1-1a can100*) (all strains up to this point are derivatives of W303-1A), L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*), PJ69-4a (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1:HIS3 GAL2:ADE2 met2::GAL7:lacZ*), PJ69-4α (*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1:HIS3 GAL2:ADE2 met2::GAL7:lacZ*), and FY1679 (*MATa/α ura3-52/ura3-52 his3-Δ200/+ leu2-Δ1/+ trp1-Δ63*). Genomic disruptions were made by long-flanking-homology PCR-based gene disruption (38).

Buffers and media. Buffer A is 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 5 μg of leupeptin per ml. Buffer B is 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 5 μg of leupeptin per ml. Buffer C is 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.2% Triton X-100, 0.1 mM PMSF, and 0.5 mM DTT. Kinase buffer is 50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, and 2 mM DTT. Phosphatase inhibitor mixture contains 10 mM NaF, 1 mM sodium vanadate, and 1 mM sodium pyrophosphate. Sodium dodecyl sulfate (SDS) loading buffer is 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. YPD medium contains, per 1 liter, 10 g of yeast extract, 20 g of peptone, and 20 g of dextrose. Selective medium contains 1.7 g of yeast nitrogen base (Difco) per liter, 5 g of (NH$_4$)$_2$SO$_4$ per liter, 20 g of dextrose per liter, and supplements (100 mg each of amino acids, uracil, or adenine per liter, as appropriate, except where indicated). All yeast growth was at 30°C. Osmotolerance in liquid cultures was evaluated by diluting fresh cultures with medium (to achieve an optical density of 0.005). Two hundred microliters of the diluted cultures in the presence of different NaCl concentrations was placed in 96-well microtiter plates. Cells were grown for 16 h with shaking at 30°C, and growth was determined by measuring the optical density of the cultures at 630 nm. Relative growth was calculated as the ratio between growth in the presence and that in the absence of added NaCl and expressed as a percentage.

Plasmids. Plasmid pRSR2 carries a 5.4-kbp *Bam*HI/*Xho*I fragment containing *RCK2* in the multicopy plasmid pRS426 (Stratagene). pCMR2 was constructed by inserting the entire wild-type coding *RCK2* sequence into pCM262, a derivative of pCM190 (3, 13) kindly provided by E. Herrero, by homologous recombination in yeast (22). The *RCK2* fragment was PCR amplified from YEpRCK2 using *Pwo* DNA polymerase and hybrid primers EbtetRCK2F and EbtetRCK2R (Table 1). The resulting PCR product was cotransformed with pCM262 digested with *Not*I and *Pst*I into yeast strain FY1679, followed by selection for uracil prototrophy. The desired construct was recovered by transformation of *Escherichia coli* DH5α to ampicillin resistance. This plasmid encodes RCK2 carboxy terminally fused to three hemagglutinin (HA) tags and one (His)$_6$ tag, under transcriptional control of the Tet promoter. Its authenticity was verified by restriction mapping; we also verified, by Western blotting with anti-HA antibodies, that the full-length tagged protein was produced in yeast. pCMkdR2 was constructed in an analogous way, except that a Lys201→Met mutation was introduced by PCR using the mutagenic primers EbkdR2F and EbkdR2R (Table 1). The plasmid construct was finalized by cotransformation in yeast and recovery in *E. coli* as described above.

The bacterial expression plasmids pET-16b and pRSETB (Stratagene) allow the expression of His-tagged proteins in *E. coli*. Full-length wild-type and several mutant *RCK2* alleles were cloned into the pET-16b and pRSETB plasmids. Mutations in Ser519→Ala and Lys201→Met were made by PCR and verified by either DNA sequencing or digestion with specific restriction enzymes. The yeast expression vector YCpIF16 (P$_{GAL1}$-HA *TRP1 CEN*) allowed the expression of HA fusion proteins using the *GAL1* promoter (12). *HOG1* was cloned into the *Bam*HI site of YCpIF16 for expression of HA-tagged HOG1. *HOG1* was cloned into the *Bam*HI site of the pBTM116 plasmid (37) to fuse it to the LexA binding domain. The LexA-HOG1 fusion protein fully complemented the osmosensitivity observed for a *hog1Δ* strain. pACTII-RCK2 was obtained by fusion of the full-length *RCK2* with the *GAL4* activation domain (AD) in pACTII (18). A

fusion of full-length *HOG1* with the *GAL4* AD was made by cotransformation of yeast strain PJ69-4a with pACTII cut with *Bam*HI and *Sac*I and full-length *HOG1* PCR product from the hybrid primers EBHOG1F and EBHOG1R (Table 1). Similarly, various fragments of *RCK2* were fused with the *GAL4* DNA binding domain (DB) by cotransformation of PJ69-4α with pGBT9 (2) cut with *Eco*RI and *Bam*HI and PCR products from hybrid primers DMRCK2B-F1 through DMRCK2B-R3 (Table 1). Yeast expression plasmid pGal-PBS2^{DD} has been described previously (40).

Two-hybrid analysis. The two-hybrid analysis was carried out essentially according to the method of Durfee et al. (11), using pACTII (18) and pBTM116 (37), as the AD plasmid and the LexA DB plasmid, respectively. A yeast cDNA library in the pACTII plasmid (ATCC 87293) was screened for proteins that interact with LexA-HOG1 using the L40 reporter strain. One million clones were analyzed by growth in histidine-deficient plates containing 40 mM 3-aminotriazole (3-AT) (Sigma). 3-AT was included in the screening plates to abolish any transactivator activity of the LexA-HOG1 bait. Positive clones were selected and further tested for β-galactosidase activity as follows. Cells (~5 × 10^6) were spotted onto YPD plates and incubated for 5 h at 30°C, and the cells were replicated onto nitrocellulose membranes. β-Galactosidase activity was visualized in situ using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described previously (37). For analysis of interactions between various fragments of *RCK2*, cloned in a DB vector, and *HOG1*, cloned in an AD vector, constructs were introduced into the same diploid cell by mating (4). Taking advantage of the *GAL1:HIS3* and *GAL2:ADE2* reporters of the host PJ69-4a/α strains (17), the strength of interactions was then assayed on selective medium lacking histidine and containing 3 mM 3-AT and 2 μg of adenine per ml.

In vivo coprecipitation assay. Cells in mid-log phase (10 ml) were collected by brief centrifugation at 4°C. The pellet was washed in 1 ml of buffer C containing 1 tablet of Complete protease inhibitor mix (Roche) per 25 ml of buffer and resuspended in 80 μl of the same buffer. Five hundred microliters of glass beads was added, and cells were disrupted in a FastPrep 120 apparatus (Bio 101) at speed 4 for 15 s. One milliliter of buffer C containing 0.25% Nonidet P-40 was added, followed by a 10-min centrifugation at 10,000 ×*g* in a chilled microcentrifuge. The extract was precleared by addition of 20 μl of Pansorbin (formalin-fixed *Staphylococcus aureus* cells) followed by head-over-head incubation for 2 h at 4°C. After 2 min of centrifugation at 10,000 × *g*, 4 μl of the precipitating antibody was added followed by a 4-h head-over-head incubation. After addition of 10 μl of Pansorbin, incubation was continued overnight. The extract was then centrifuged for 2 min at 10,000 × *g*, and the pellet was washed twice in 500 μl of buffer C containing 0.25% NP-40 and finally resuspended in 500 μl of SDS loading buffer prior to electrophoresis.

Expression and purification of epitope-tagged proteins. His-tagged wild-type and mutant *RCK2* alleles were constructed using pET-16b, expressed in *E. coli* BL21(DE3) cells (33), purified using TALON metal affinity resin (Clontech), and eluted using imidazole buffer, according to the manufacturer's instructions. RCK2 (434–610) was constructed using pRSETB (Invitrogen) and expressed as described above. Glutathione *S*-transferase (GST) fusion proteins encoding PBS2(EE) and HOG1 were constructed using pGEX-4T (Pharmacia), expressed in *E. coli* DH5, and purified using glutathione-Sepharose beads (Pharmacia) in buffer B as described previously (29). HA-tagged HOG1 was expressed in yeast, and purification was carried out by immunoprecipitation with anti-HA monoclonal antibody 12CA5 and protein A-Sepharose beads (Roche). Beads were washed extensively with buffer A plus 150 mM NaCl and resuspended in kinase buffer.

In vivo RCK2 phosphorylation assays. Wild-type, *pbs2Δ*, and *hog1Δ* cells were grown in synthetic complete (sc) medium and subjected or not to a brief osmotic shock (0.4 M NaCl, 5 to 10 min). Cell extracts were prepared as described above but in the presence of buffer A without EGTA and EDTA. Extracts were treated with or without λ phosphatase (300 U for 60 min) in the presence or absence of phosphatase inhibitors. Rck2 was detected by immunoblotting using partially purified polyclonal antibodies against Rck2.

In vitro kinase assays. HA-HOG1 was purified as described above from untreated yeast cells or cells treated with a brief osmotic shock (0.4 M NaCl for 10 min). One microgram of recombinant GST-HOG1 from *E. coli* was activated by phosphorylation using 0.5 μg of GST-PBS2(EE) in the presence of kinase buffer and ATP. After 15 min at 30°C, 5 μg of His-tagged Rck2 proteins, purified from *E. coli*, was added to the reaction mixture together with [γ-^{32}P]ATP (0.2 μCi/μl). The mixture was then incubated for 5 min at 30°C, and the reactions were terminated by the addition of 2× SDS loading buffer.

The induction of Rck2 activity by Hog1 phosphorylation was monitored by the following in vitro kinase assay. One microgram of recombinant GST-HOG1 or GST-HOG1(KN) from *E. coli* was phosphorylated by using 0.5 μg of GST-PBS2(EE) in the presence of kinase buffer and ATP. After 15 min at 30°C, 5 μg of wild-type or mutant versions of Rck2 were added to the mixture and incubation was maintained at 30°C for 15 min. GST-HOG1 and GST-HOG1(KN) were removed from their mixture by affinity chromatography using glutathione-Sepharose beads, and Rck2 proteins were further incubated for 15 min in the presence of kinase buffer and radioactive ATP.

Labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography using dried gels or after transfer of the proteins to Immobilin P membranes (Millipore). His-tagged proteins were

TABLE 1. Oligonucleotides used

Primer name	Homology[a]	Sequence (5' to 3')[b]	Use
AD8	RCK2 −25 to −1	GAG AGG ATC AAA CAA AAT CTC TTC G	RCK2 disruption
EBL2RCK2	kanMX4 −409 to −433, RCK2 331 to 312	GGG GAT CCG TCG ACC TGC AGC GTA CCA CTA CTT TCA TGG ACA TCG	RCK2 disruption
EBL3RCK2	kanMX4 1052 to 1076, RCK2 1443 to 1462	AAC GAG CTC GAA TTC ATC GAT GAT ACG ATT GTT TAC CAA AGG AGG	RCK2 disruption
EBL4RCK2	RCK2 1749 to 1731	CGT ATC CAG CGT TAA TTG G	RCK2 disruption
EBPBS2L1	PBS2 −225 to −208	GGA CAC AGA GAT AAC TGC	PBS2 disruption
EBPBS2L2	kanMX4 −409 to −433, PBS2 22 to 3	GGG GAT CCG TCG ACC TGC AGC GTA CGG TTA GCA AAC TTG TCT TCC	PBS2 disruption
EBPBS2L3	kanMX4 1052 to 1076, PBS2 1947 to 1964	AAC GAG CTC GAA TTC ATC GAT GAT AGG AAC GTG GTG AGA ATG G	PBS2 disruption
EBPBS2L4	PBS2 2342 to 2326	TTC GAG GAG TCG ATG GC	PBS2 disruption
EBHOG1F	pACTII 947 to 964, HOG1 1 to 20	GAG GCC CCG GGG ATC CGA ATG ACC ACT AAC GAG GAA TT	HOG1-GAL4 AD fusion
EBHOG1R	pACTII 985 to 968, HOG1 1308 to 1288	ATA GAT CTC TCG AGC TCG TTA CTG TTG GAA CTC ATT AGC	HOG1-GAL4 AD fusion
DMRCK2B-F1	pGBT9 845 to 874, RCK2 1 to 17	AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG ATG CTT AAA ATA AAG GC	RCK2-GAL4 DB fusions
DMRCK2B-R1	pGBT9 932 to 903, RCK2 507 to 488	TAA GAA ATT CGC CCG GAA TTA GCT TGG CTG CTA GAT TTT ATT GAT CAG CTT G	RCK2-GAL4 DB fusions
DMRCK2B-F2	pGBT9 845 to 874, RCK2 504 to 519	AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG ATC GGT GAA GGT GCT TT	RCK2-GAL4 DB fusions
DMRCK2B-R2	pGBT9 932 to 903, RCK2 1401 to 1383	TAA GAA ATT CGC CCG GAA TTA GCT TGG CTG CTA GTA TCT TTT AGA CGG CTC	RCK2-GAL4 DB fusions
DMRCK2B-F3	pGBT9 845 to 874, RCK2 1395 to 1413	AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG AGA TAC GAC ATT GAC CAG	RCK2-GAL4 DB fusions
DMRCK2B-R3	pGBT9 932 to 903, RCK2 1833 to 1816	TAA GAA ATT CGC CCG GAA TTA GCT TGG CTG CTA TTC CCT GAT AGT GG	RCK2-GAL4 DB fusions
EbkdR2R	RCK2 619 to 589 (mutation at 602 and 603)	CTG CCT TTT TGA TAA **CCA** TAA TGG CAA CAG C	RCK2 KD mutation
EbkdR2F	RCK2 589 to 619 (mutation at 602 and 603)	GCT GTT GCC ATT **ATG** GTT ATC AAA AAG GCA G	RCK2 KD mutation
5RCK2SA	RCK2 1544 to 1578 (mutation at 1558 and 1563)	CCT CGC TAC TGT **TTG** CAC **CGG** CTG CTG TTG CTA TG	RCK2 SA mutation
3RCK2SA	RCK2 1544 to 1578 (mutation at 1558 and 1563)	CAT AGC AAC AGC CGG **TGC** AAA CAG TAG CGA GG	RCK2 SA mutation
EbtetRCK2F	pCM262 2492 to 2522, RCK2 1 to 27	CCG GAT CAA TTC GGG GGA TCA GTT TAA ACG CAT GCT TAA AAT AAA GGC CCT TTT CTC G	pCMR2, pCMkdR2 overexpression
EbtetRCK2R	pCM262 2551 to 2520, RCK2 1830 to 1811	CCT GGT GAT CCG TCG ACC TGC AGG CGG CCG CGT TCC CTG ATA GTG GCG CTT A	pCMR2, pCMkdR2 overexpression

[a] For genes, nucleotide 1 is defined as the A of the start codon. For vectors, nucleotide 1 is defined according to the published sequence. For the kanMX module (38), nucleotide 1 is defined as the A of the start codon of the E. coli Kan^r gene.

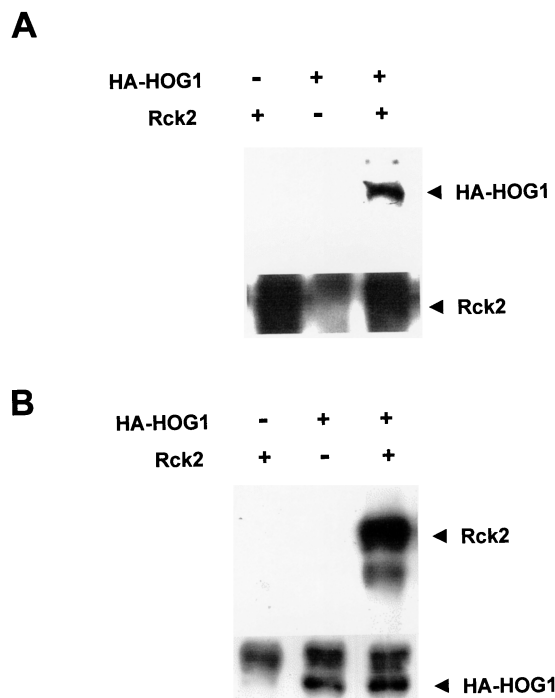[b] For mutagenic primers, mutated positions are in boldface.

probed by immunoblotting with the anti-His monoclonal antibody BMG-His-1 (Roche).

RESULTS

The Hog1 MAPK binds to the C-terminal regulatory domain of the Rck2 kinase. Osmotic stress induces activation of the Hog1 MAPK, which mediates induction of a set of osmoadaptive responses. Full understanding of Hog1-mediated responses requires the identification of direct substrates for Hog1, a goal, however, not achieved so far. To identify substrates for this MAPK, a two-hybrid screening was conducted. A yeast library constructed with a *GAL4* activator domain vector was screened using as bait a fusion construct between the LexA DB and the full-length *HOG1*. The LexA-HOG1 fusion protein used in this screening fully complemented the osmosensitive phenotype of a *hog1*-deficient strain and showed the same time-dependent phosphorylation pattern after osmotic stress as that of the wild-type protein (data not shown). Fourteen yeast cDNA clones that interacted with Hog1 were isolated from the screening. Of these clones, six were overlapping clones encoding Rck2, a 610-residue protein which resembles the yeast and mammalian calmodulin kinases as well as *S. cerevisiae* protein kinase Dun1 (10, 21). In addition to the catalytic domain, Rck2 contains a 132-residue C-terminal extension speculated to play an autoinhibitory role (21). Interestingly, the clones obtained from the two-hybrid screening allowed us to approximately map the domain of interaction of Rck2 with Hog1 (Fig. 1). A partial cDNA clone encoding amino acid residues 391 to 610 was able to interact with Hog1 as efficiently as did the full-length clone (Fig. 1A). To extend these analyses and further confirm the specific domain in Rck2 that is essential for binding to Hog1, various segments of Rck2 were fused to the GAL4 activator domain, and their interaction with the full-length Hog1 protein was tested. Figure 1A shows typical results, and Fig. 1B summarizes the interaction between several Rck2 segments and Hog1. The results obtained with both orientations of AD and DB fusions were completely consistent, indicating that Rck2 residues 466 to 610 are sufficient for binding to Hog1. We thus designated this segment the HOG1 binding domain.

The interaction between Hog1 and Rck2, concluded on the basis of the two-hybrid data, was confirmed by in vivo immunoprecipitation experiments. Yeast *rck2Δ* mutant cells were cotransformed with plasmids that express HA-tagged HOG1 and a multicopy plasmid carrying wild-type Rck2. Rck2 was immunoprecipitated using specific polyclonal antibodies against this protein, and the presence of HA-HOG1 in the precipitates was determined with an anti-HA monoclonal antibody. As shown in Fig. 2A, Rck2 was able to coprecipitate Hog1. Conversely, when HA-HOG1 was immunoprecipitated using monoclonal antibodies against HA, and the presence of Rck2 in the precipitates was determined with specific antibodies against Rck2, Hog1 was able to coprecipitate Rck2 (Fig. 2B). Thus, these in vivo binding assays confirmed the conclusion of the two-hybrid analyses indicating that Hog1 interacts with Rck2.

Rck2 is phosphorylated after osmotic stress in a Hog1-dependent manner. When yeast cells are exposed to osmotic stress, Hog1 MAPK is rapidly phosphorylated and activated (20). We decided to test whether Rck2 was also phosphorylated after osmotic stress. Wild-type, *hog1Δ*, and *pbs2Δ* strains were subjected to a brief osmotic shock, and wild-type Rck2 protein was monitored by Western blotting using anti-Rck2 antibodies. As shown in Fig. 3A, Rck2 was detected as multiple bands under nonstress conditions (lane 1). When subjected to

FIG. 1. Identification by two-hybrid analysis of the HOG1 binding domain in Rck2. (A) Interactions of various Rck2 fragments fused to the GAL4 activator domain with the full-length Hog1 fused to the LexA DB were examined. Representative filter β-galactosidase assays demonstrating interactions between Hog1 and Rck2 are shown. Positions of the Rck2 fragments included in the constructs are indicated in parentheses. Proteins encoded by the control plasmids pLexA-RAS^{V12} and pACT-RAF, which are known to interact with each other (37), are shown for comparison. (B) Summary of the two-hybrid interaction analysis between Rck2 and Hog1. The Rck2 segments included in the AD (denoted as A) or DB (denoted as D) constructs are schematically shown on the left, with their precise amino acid positions indicated on the right. The presence or absence of interaction is depicted by a plus or minus sign, respectively.

osmotic stress, the mobility pattern of Rck2 was altered (Fig. 3A, lane 2). Mainly, the slower band shifted to a faster-migrating form. The exact nature of the upper band is currently unknown. However, we believe that the band represents a phosphorylated form of Rck2 and that its disappearance is caused by additional phosphorylation events as suggested by the following results. The mobility change of RCK2 was induced by phosphorylation because, when extracts from osmotically stressed cells were treated with λ phosphatase, the mobility pattern could be reversed (Fig. 3A, lane 3). In control experiments using phosphatase inhibitors, Rck2 mobility was not affected by λ phosphatase treatment (lane 4). Thus, after osmotic stress Rck2 is phosphorylated. Interestingly, when osmotic stress Rck2 phosphorylation was studied with mutant cells deficient in the HOG pathway (*pbs2Δ* and *hog1Δ* strains) no differences in Rck2 mobility were observed after osmotic stress compared with the wild-type strain (Fig. 3A, right panel). Therefore, Rck2 is phosphorylated after osmotic stress in a HOG-dependent manner.

A

B

FIG. 2. In vivo association of Hog1 and Rck2 proteins. *rck2Δ* yeast strain EBΔR2Wa was transformed with a plasmid expressing HA-HOG1 and a multicopy plasmid carrying *RCK2*. (A) Hog1 coprecipitates with Rck2. Rck2 was immunoprecipitated using specific antibodies against this kinase (lower panel), and the presence of HA-HOG1 in the precipitates was determined with an anti-HA antibody (upper panel) (B) Rck2 coprecipitates with Hog1. HA-HOG1 was immunoprecipitated using anti-HA antibody (lower panel), and the presence of Rck2 was determined with specific antibodies against Rck2 (upper panel).

Both in vivo- and in vitro-activated Hog1 phosphorylates Rck2. We then tested whether activated Hog1 was able to phosphorylate Rck2. For this purpose, yeast cells expressing HA-tagged Hog1 were treated with a brief osmotic shock. HA-HOG1 was then immunoprecipitated by using monoclonal anti-HA antibodies and protein A-Sepharose beads. The activation of HA-HOG1 was assessed by Western blotting using the monoclonal antibody 4G10 against phosphorylated tyrosine residues. Immunoprecipitated Hog1 was incubated together with a catalytically inactive, His-tagged Rck2 in the presence of [γ-^{32}P]ATP. The use of a kinase-deficient Rck2 protein, termed RCK2(KD), which contains a Lys201→Met mutation, was necessary because Rck2 has a background kinase activity that results in autophosphorylation. As shown in Fig. 3B, RCK2(KD) phosphorylation significantly increased when the protein was incubated with activated Hog1 from osmotically stressed cells.

We then tested whether Rck2 phosphorylation was carried out directly by the Hog1 MAPK, using purified proteins in an in vitro kinase assay. For this purpose, Hog1 and a constitutively activated version of Pbs2 [PBS2(EE)] (EE denotes Ser514→Glu and Thr518→Glu mutations in the activation loop of the Pbs2 MAPKK) were purified as GST fusion proteins from *E. coli*. In the first step of the reaction, Hog1 was activated by phosphorylation in the presence of PBS2(EE) and ATP. Then, RCK2(KD), purified from *E. coli* as a His-tagged protein, and [γ-^{32}P]ATP were added to the reaction. As shown in Fig. 3C, only when RCK2(KD) was incubated with preactivated Hog1 did phosphorylation of Rck2 result. Taken together, these results indicate that Rck2 is a direct substrate for the MAPK Hog1.

A

B

C
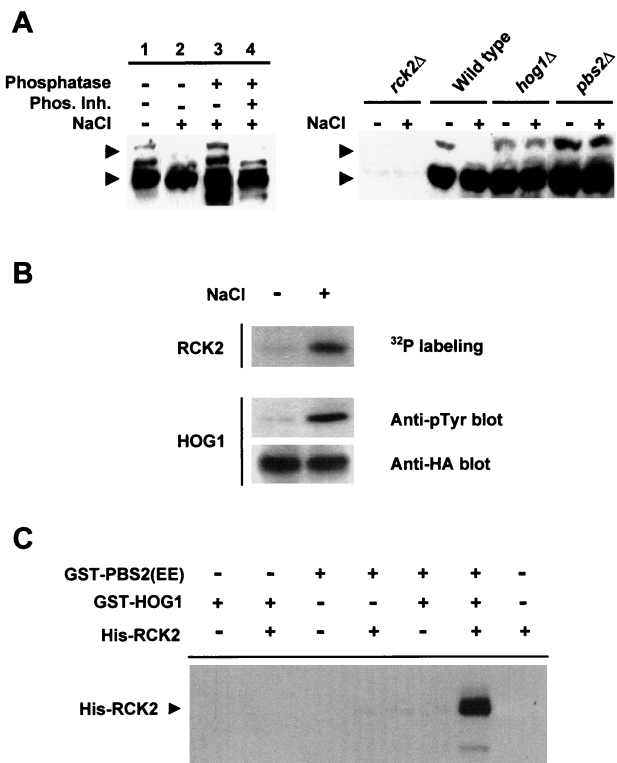
FIG. 3. In vivo and in vitro phosphorylation of Rck2 by Hog1. (A) Dependence of the HOG pathway for the in vivo osmotic stress-induced phosphorylation of Rck2. Wild-type cells were grown in sc medium and subjected (+) or not (−) to a brief osmotic shock (0.4 M NaCl, 10 min). Cell extracts were prepared and treated with (+) or without (−) λ phosphatase in the presence (+) or absence (−) of a mixture of phosphatase inhibitors as described in Materials and Methods (left panel). Rck2 was detected by immunoblotting using anti-Rck2 antibodies (arrowheads), and its phosphorylation state was monitored by noting changes in the electrophoretic mobility of the protein. Results from several strains subjected (+) or not (−) to brief osmotic stress (0.4 M NaCl, 5 min) are shown in the right panel. Relevant genotypes are depicted. Extracts from *rck2Δ* cells are included in order to monitor the specificity of the anti-Rck2 antibodies. (B) In vivo-activated Hog1 phosphorylates Rck2. HA-HOG1 was immunoprecipitated by using anti-HA monoclonal antibody from yeast cells before (−) or after (+) the addition of NaCl to a final concentration of 0.4 M. The presence of HA-HOG1 in the precipitates was detected with an anti-HA antibody, and Hog1 activation was monitored by immunoblot analysis using a monoclonal antibody specific to phosphotyrosine (4G10) (middle panel). After immunoprecipitation, HA-HOG1 was incubated with purified His-tagged RCK2(KD) in the presence of kinase buffer and radioactive ATP. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography. (C) In vitro phosphorylation of Rck2 by Hog1. Recombinant tagged proteins were purified from *E. coli* as described in Materials and Methods. Hog1 and the constitutively activated Pbs2 allele [PBS2(EE)] were incubated in the presence of kinase buffer and ATP. Rck2 was then added (when indicated) in the presence of radioactive ATP. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. The position of tagged Rck2 is indicated on the left.

Hog1 phosphorylates Ser519 at the autoinhibitory domain of Rck2. To map the phosphorylation site for Hog1 in Rck2, we created several truncated *RCK2* alleles and expressed them as His-tagged proteins in *E. coli*. It was found, however, that removal of the C-terminal region of Rck2 results in proteins with a very high in vitro kinase activity (data not shown), which is consistent with the putative autoinhibitory role of this region. For this reason, the Lys201→Met mutation (referred to as KD) was used to create catalytically deficient enzymes. Truncated forms of RCK2(KD) expressed and purified from *E. coli* were subjected to in vitro phosphorylation by activated Hog1 (as described above). A 63-residue C-terminal deletion had no effect on Rck2 phosphorylation compared to the full-
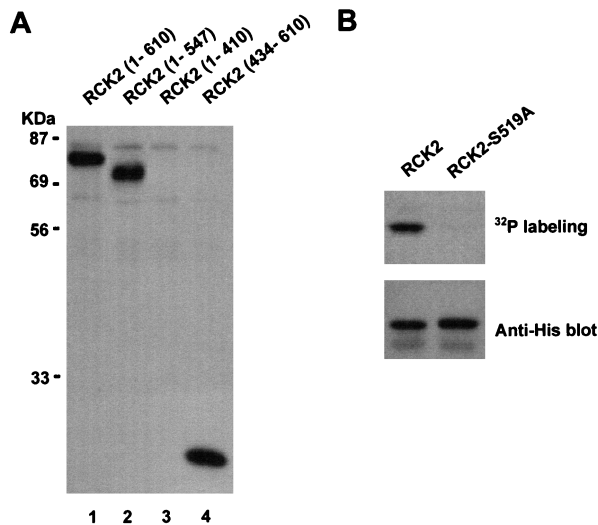
FIG. 4. Hog1 phosphorylates Ser519 at the regulatory domain of Rck2. (A) Phosphorylation of different fragments of Rck2 by Hog1. Various Rck2 fragments were tested for their ability to be phosphorylated by an in vitro-activated Hog1 (as described in Materials and Methods). After the in vitro kinase assay, phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. Positions of the Rck2 fragments included in the constructs are indicated in parentheses. Proteins were His tagged and contained a Lys201→Met mutation which results in catalytically inactive enzymes, to avoid autophosphorylation. (B) Mutation of Rck2 Ser519→Ala abolishes Hog1 phosphorylation. The full-length RCK2(KD) and its Ser519 mutant form were tested for Hog1 phosphorylation as described for panel A. After phosphorylation, proteins were resolved by SDS-PAGE and transferred to a nylon membrane. Phosphorylated proteins were detected by autoradiography (upper panel). His-tagged Rck2 proteins were detected by immunoblotting by using the anti-His monoclonal antibody BMG-His-1 (lower panel).

FIG. 5. Induction of Rck2 activity by Hog1 phosphorylation. (A) Purified wild-type Rck2 protein or the indicated mutant forms of Rck2 were incubated with (+) or without (−) wild-type GST-HOG1 or the catalytically inactive mutant version GST-HOG1(KN), in the presence of kinase buffer and cold ATP. After 15 min at 30°C, HOG1 was removed by affinity chromatography and Rck2 was further incubated in the presence of kinase buffer and radioactive ATP. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography. The position of Rck2 is indicated on the left. A representative experiment is shown. (B) The intensity of autophosphorylation was quantified with a phosphorimager (Fuji BAS1000). Samples from three independent experiments were measured, and the intensity of each band was normalized to that of lane 2. Error bars indicate standard errors of the means.

length protein (Fig. 4A, lanes 1 and 2). However, a 200-residue C-terminal deletion completely abolished Rck2 phosphorylation (Fig. 4A, lane 3). Furthermore, when a C-terminal polypeptide (amino acids 434 to 610) was tested, it was phosphorylated by Hog1, suggesting that the carboxy-terminal regulatory domain and not the kinase domain of Rck2 was the target for Hog1 phosphorylation. Thus, phosphorylation of Rck2 by Hog1 occurs in a region which is coincident with the HOG1 binding domain.

A sequence corresponding to the consensus phosphorylation site for MAPK (Ser-Pro) is present in the region between residues 434 and 546 (corresponding to the protein sequence LLFSP). We created a point mutant version to replace Ser519 with Ala (RCK2-Ser519A) and tested it for phosphorylation by Hog1. As shown in Fig. 4B, phosphorylation of Rck2 was completely abolished in the mutant version, indicating that Ser519 at the regulatory domain of Rck2 is the unique phosphorylation site for Hog1.

Phosphorylation of Rck2 by Hog1 results in induction of Rck2 activity. To test if Rck2 kinase activity can be regulated by phosphorylation, we developed the following in vitro kinase assay. Because no substrates are known for this kinase, we based the in vitro kinase assay on the fact that Rck2 is able to autophosphorylate (reference 21 and our results). Therefore, we assayed Rck2 activity by measuring its level of autophosphorylation. To test the role of Hog1 phosphorylation in Rck2 kinase activity, we incubated wild-type Rck2, the catalytically impaired RCK2(KD), or the unphosphorylatable RCK2(SA) mutant with activated GST-HOG1 in the presence of nonradioactive ATP. After incubation, Hog1 was removed from the mixture by affinity chromatography and Rck2 autophosphory-

lation was assayed in the presence of kinase buffer and radioactive ATP. As expected, the catalytically impaired Rck2 was unable to autophosphorylate (Fig. 5, lane 4) and increased its level of phosphorylation slightly when incubated with Hog1, as a result of Hog1 activity (Fig. 5, lane 5). Interestingly, when the wild-type Rck2 was incubated with Hog1, a very marked increase in phosphorylation was observed which could not be accounted for by Hog1 phosphorylation alone (Fig. 5, lane 2). When the catalytically impaired HOG1(KN) mutant was used, Rck2 phosphorylation was not induced (Fig. 5, lane 3). Similarly, when wild-type Hog1 was incubated with Rck2 but no ATP was present in the reaction mixture, Rck2 autophosphorylation was not increased (data not shown). This clearly indicates that Hog1 phosphorylation is required for the induction of Rck2 activity. The Ser519 mutant of Rck2, which cannot be phosphorylated by Hog1, still has some autophosphorylation activity, but it did not show any increase in phosphorylation when incubated with Hog1 (Fig. 5, lanes 6 and 7). Taken together, our results suggest that, after incubation with active Hog1, Rck2 is phosphorylated at Ser519 in the autoinhibitory domain, and this phosphorylation markedly increases Rck2 kinase activity.
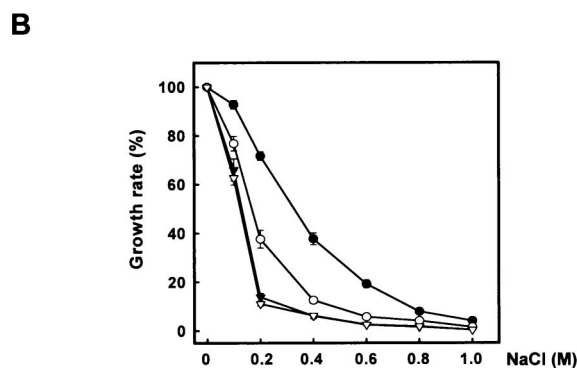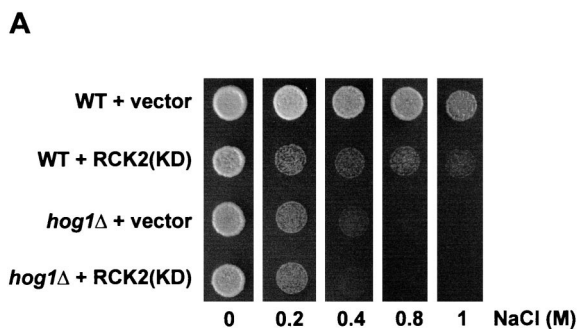
A

B

FIG. 6. Cellular osmosensitivity induced by overexpression of a catalytically inactive RCK2(KD) allele. (A) Wild-type (WT) and *hog1*Δ cells transformed with an empty vector (pCM262) or a plasmid expressing the catalytically inactive RCK2(KD) (pCMkdR2) were grown on YPD or YPD containing NaCl at different concentrations, in the absence of doxycycline (allowing full expression from the Tet promoter). Growth in plates was scored after 3 days at 30°C. (B) Wild-type cells transformed with pCM262 (●) or pCMkdR2 (○) and *hog1*Δ cells transformed with pCM262 (▼) or pCMkdR2 (▽) were grown in liquid medium in the presence of different concentrations of NaCl, and the effect of stress on cell growth was determined as described in Materials and Methods. Results are means ± standard errors of the means from three independent experiments.

FIG. 7. Suppression of *hog1*Δ and *pbs2*Δ cell osmosensitivity by *RCK2* over-expression. Yeast cells deficient in the *HOG1* or *PBS2* gene were transformed with either an empty vector (pRS426) or a multicopy plasmid carrying wild-type (WT) *RCK2* (pRSRCK2). Cells were grown in YPD, YPD containing NaCl at a concentration of 0.4 M, or sorbitol at 1 M. Growth was scored after 3 days at 30°C.

Overexpression of a dominant negative *RCK2* allele results in osmosensitivity. It was reported previously that *RCK2* disruption does not cause osmosensitivity (9, 21). Prompted by our current findings, we have reexamined osmotic stress-related phenotypes of *rck1*, *rck2*, and *rck1 rck2* mutants in different strain backgrounds but again found no difference compared to the wild type (data not shown). However, the absence of an evident osmosensitive phenotype can still be compatible with the notion of Rck2 being involved in the HOG pathway. Thus, we tested whether overexpression of a catalytically impaired *RCK2* mutant [RCK2(KD)] was able to alter activation of the osmotic stress responses driven by Hog1. Because RCK2(KD) does not have any detectable kinase activity (as shown in Fig. 5), overexpression of RCK2(KD) might inhibit the Hog1 signal transduction pathway by causing RCK2(KD) to abortively interact with Hog1 and/or downstream elements.

Yeast cells overexpressing RCK2(KD) were cultured on plates in the absence or presence of various NaCl concentrations. As shown in Fig. 6A, wild-type cells expressing the *RCK2(KD)* allele grew slower than cells carrying an empty vector in the presence of virtually any NaCl concentration tested. A similar effect was observed when sorbitol was used instead of NaCl (data not shown). This behavior was consistent for different plasmid isolates tested and reproducible in different genetic backgrounds. A nonspecific toxic effect of overexpression of the *RCK2(KD)* allele was ruled out, as, on normal-
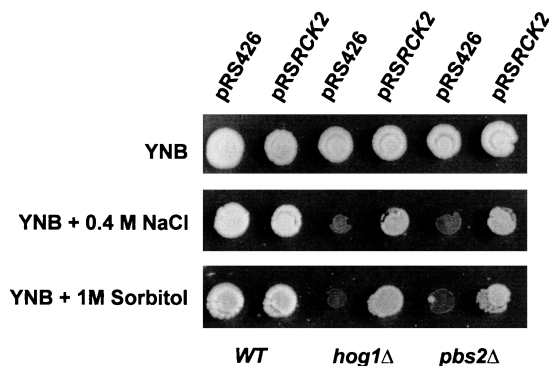
osmolarity medium, the growth rate was similar (Fig. 6A). Interestingly, when RCK2(KD) was overexpressed in *hog1*Δ cells, no effect on osmosensitivity was observed compared to the *hog1*Δ cells transformed with an empty vector.

To further characterize this phenomenon and to obtain a more quantitative estimate of the effect of the *RCK2(KD)* allele on cellular osmosensitivity, cell growth in the presence of various NaCl concentrations was measured in liquid medium. Wild-type cells expressing RCK2(KD) were much more sensitive to the presence of NaCl than were cells carrying an empty vector (Fig. 6B, open circles versus solid circles). For instance, at 0.4 M NaCl, the growth rate of cells carrying the *RCK2(KD)* allele was reduced to less than 50% compared to the same cells carrying an empty vector. Again, no alterations in osmosensitivity were observed in *hog1*Δ cells expressing the *RCK2(KD)* allele (Fig. 6B, open triangles versus solid triangles). Thus, the dominant inhibitory effect of RCK2(KD) appears specific to the stress-induced activation of the HOG pathway.

Overexpression of *RCK2* suppresses the osmotic sensitivity of *hog1* and *pbs2* mutants. A possible explanation for the osmosensitivity observed with overexpression of the dominant negative RCK2(KD) is the involvement of Rck2 in the Hog1 osmotic stress signal transduction. To confirm this possibility, we tested whether overexpression of wild-type *RCK2* was able to suppress the *hog1*Δ and *pbs2*Δ mutant osmosensitive phenotype. Overexpression of *RCK2* from a multicopy vector in *hog1*Δ mutants resulted in an over 100-fold increase in the survival rate on high-NaCl-containing medium. Moreover, a comparable effect was seen for sorbitol-containing plates (Fig. 7), indicating general suppression of the osmotic sensitivity of this mutant. For a strain disrupted in the *PBS2* gene, which encodes the Hog1-activating MAPKK, the outcome was similar (Fig. 7). By contrast, overexpression of the *RCK2*-related gene *RCK1* (10) did not affect osmosensitivity of these mutants (data not shown). Therefore, these data also support the notion of a link between Hog1 and Rck2 kinases to control osmotic stress responses in yeast.

Deletion of *RCK2* suppresses cell lethality caused by hyperactivation of the HOG pathway. To further confirm the genetic data on the involvement of Rck2 in HOG signaling, we decided to test whether deletion of *RCK2* alters HOG signaling. It has been reported previously that hyperactivation of the HOG pathway results in cell lethality (20, 29). Hyperactivation of Hog1 can be achieved by deletion of the *SLN1* osmosensor (a
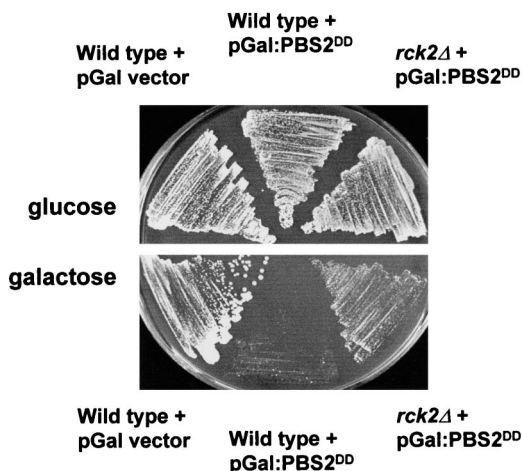
FIG. 8. Deletion of the *RCK2* gene suppresses the lethality of the PBS2[DD] mutation in yeast. The wild-type and *rck2Δ* yeast strains were transformed with either pGal control vector or pGal:PBS2[DD]. Yeast cells were grown at 30°C on sc plates containing glucose or galactose. Growth was scored after 4 days.

negative regulator of the pathway) or by overexpression of constitutive alleles of the genes encoding MAPKKK Ssk2 and Ssk22 or Pbs2 MAPKK (20). This lethality can be prevented by overexpression of protein phosphatases Ptp2 and Ptc1 or by deletion of downstream elements such as the Hog1 MAPK. Based upon this observation, we have tested whether cell lethality caused by the constitutive allele of the gene for Pbs2 (PBS2[DD]) could be suppressed by deletion of *RCK2*. As shown in Fig. 8, deletion of the *RCK2* gene suppressed cell lethality caused by hyperactivation of the Hog1 MAPK by PBS2[DD], providing additional evidence that Rck2 is a new element of the HOG pathway, acting downstream of the Hog1 MAPK.

DISCUSSION

Yeast cells respond to increases in osmolarity in the extracellular environment by activating the HOG pathway. While the sensing and signal transduction mechanism required for activation of the Hog1 MAPK is well established (27), the proteins that are immediate targets of the Hog1 MAPK, necessary for the generation of osmostress responses, are not known. To identify substrates for the Hog1 MAPK, we undertook a two-hybrid screening using Hog1 as bait. In this report, we report that the Rck2 kinase interacts with Hog1 and that its kinase activity is regulated by the Hog1 MAPK, suggesting that Rck2 is one such substrate, the first one for the Hog1 MAPK identified so far.

The results of this study demonstrate by two-hybrid and in vivo coprecipitation experiments that Hog1 binds Rck2. Furthermore, Hog1 phosphorylates a specific serine residue at the noncatalytic C terminus of Rck2. These results, together with earlier findings, allow us to speculate on the molecular mechanism of Rck2 activation by Hog1. It was proposed previously that the C-terminal extension of the Rck2 kinase could play an inhibitory role, based on the observation that overexpression of a C-terminally truncated form of Rck2 is more toxic to the cell than is the wild-type allele (21). Consistent with these results, we observed that deletion of the C-terminal 63 residues results in an extremely active Rck2 enzyme (data not shown). Because osmotic stress-activated Hog1 binds and phosphorylates the C-terminal regulatory domain of Rck2, it can be proposed that Hog1 phosphorylation results in Rck2 activation most probably

as a result of a release from the constraint imposed by the autoinhibitory domain. Once active, Rck2 autophosphorylates as well as phosphorylating its substrates, thus allowing the control of a subset of the osmotic stress responses.

There are a number of independent lines of evidence for an involvement of Rck2 in yeast osmotic stress responses. First, Rck2 is phosphorylated in vivo upon osmotic stress, and its phosphorylation is *HOG1* dependent. It is worth noting that phosphorylation of Rck2 results in its activation as shown by in vitro kinase assays. Second, multicopy expression of *RCK2* partially suppresses the osmosensitive phenotype of mutant cells defective in the HOG MAPK cascade, such as *hog1Δ* or *pbs2Δ* cells. Third, although the exact mechanism of the dominant negative effect of the RCK2(KD) allele is unknown at the moment, a high-level expression of the kinase-impaired RCK2(KD) causes osmosensitivity in a wild-type background but not in a *hog1Δ* background. However, the formal possibility that some of the effects of Rck2 in osmoregulation could be controlled by a distinct pathway that does not involve Hog1 cannot be excluded. Furthermore, cell lethality caused by hyperactivation of the HOG pathway can be suppressed by deletion of *RCK2*. In the light of these findings, it is noteworthy that the *rck2* disruption does not cause osmosensitivity even if deleted in a background lacking the related Rck1 kinase (9, 21). A model that accommodates these observations could involve a situation for Rck2 analogous to that found for the MAPK Kss1, whose role in filamentous growth was long overlooked, since the *kss1Δ* mutant was not affected in this function. More detailed analysis (8, 19) showed, however, that the Kss1 kinase has two opposite functions in the filamentous growth pathway: activation in its phosphorylated form and inhibition in its unphosphorylated form. In the *kss1Δ* null mutant, these two effects cancel each other out. Other models are also compatible with the data, such as a model that invokes the presence of multiple redundant downstream targets for the Hog1 MAPK. This would explain the dominant inhibitory effect observed with overexpression of the RCK2(KD) protein, whereas no effect in osmosensitivity is observed for the *rck2* deletion.

A scenario in which a protein kinase is under the control of a stress-activated MAPK is not unique. This is the case, for example, for the p38 pathway in mammals. The p38 pathway is the signal transduction pathway homologous to the HOG pathway. Both pathways are activated by osmotic stress, and not only do they have very similar components, but also the mammalian proteins can functionally complement the osmosensitive phenotype observed with the corresponding yeast mutants. This is the case for the p38 MAPK, which complements osmosensitivity of a *hog1Δ* strain (15), and the human MTK1 MAPKKK, which complements an *ssk2Δ ssk22Δ* deficient strain (35). Activation of the p38 pathway results in induction of a set of response genes by direct phosphorylation of several transcription factors by the MAPK (i.e., CHOP, MEF2C, ATF-2, and ELK-1) (7, 36). However, some of the responses induced by the p38 pathway are mediated by kinases lying downstream of and directly regulated by the p38 MAPK. These include kinases such as MNK, MAPKAP-K2/3, PRAK, and MSK (7, 36). Activation of those kinases results in a number of responses such as regulation of transcription factors (i.e., CREB) and essential proteins such as HSP27 and eIF4e (36). Those proteins mediate a subset of stress responses of the p38 MAPK. A similar scenario could be imagined for yeast, where Hog1 would be regulating transcription factors and kinases (i.e., Rck2) to complete the whole network of the osmotic stress-induced responses.

In summary, Rck2 protein kinase is a mediator of the os-

motic stress signal transduction pathway, which is under the direct control of the MAPK Hog1. Discovery of other proteins under the control of Hog1 and Rck2 will result in a better understanding of osmotic stress responses in both yeast and mammalian cells.

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