Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast

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Rck2, a yeast Ser/Thr protein kinase homologous to mammalian calmodulin kinases, requires phosphorylation for activation. We provide evidence that in budding yeast, this step can be executed by the osmostress-activated mitogen-activated protein kinase Hog1. Rck2 phosphorylation was transiently increased during osmostress or in mutants with a hyperactive high osmolarity glycerol (HOG) pathway. This modification depended on catalytically active Hog1 kinase and two putative mitogen-activated protein kinase phosphorylation sites in Rck2. Immunokinase assays showed that Hog1 can directly phosphorylate Rck2 to stimulate its enzymatic activity toward translation elongation factor 2. We demonstrate that Hog1 and Rck2 are necessary for attenuation of protein synthesis in response to osmotic challenge and show that modification of elongation factor 2 induced by osmostress depends on Rck2 and Hog1 *in vivo*. Therefore, we propose that the transient down-regulation of protein synthesis after osmotic shock is a response not to damage but to an extracellular signal mediated by Hog1 and Rck2.

Yeast cells maintain osmotic homeostasis through the function of a mitogen-activated protein (MAP) kinase pathway called the high osmolarity glycerol (HOG) pathway (1), which closely resembles the p38 (also known as SAPK2) pathway in higher eukaryotes (2, 3). Osmotic stress evokes a large number of physiological responses, including well characterized changes in the transcriptional program (4–6). As made clear from genetic data, cellular events other than the transcriptional response must also be under the influence of the HOG signaling system (6). The activity of the pathway needs to be tightly controlled, because its inappropriately high continuous activation is lethal. This observation has greatly facilitated genetic analysis and thereby the elucidation of the architecture of the pathway (7). In mammalian cells, MAP kinase p38 is activated by various stress stimuli. On activation, p38 accumulates in the nucleus to induce the stress adaptation program of the cell (3). In addition to transcription factors, cellular targets of p38 are the MAP kinase-activated protein kinases (MAPKAP) 2 and 3, which normally reside in the nucleus but translocate into the cytoplasm on phosphorylation by p38 (8, 9). In budding yeast, the MAP kinase Hog1 behaves comparably to p38. After osmostress-induced phosphorylation, Hog1 accumulates rapidly in the nucleus (10, 11). Until recently, however, the substrates of Hog1 have remained elusive.

Rck1 and Rck2 were first identified by reverse genetics to study Ca^{2+} signaling in yeast (12). Despite their high similarity to Ca^{2+}/calmodulin-dependent kinases, Rck2 does not respond to Ca^{2+} signals, and its regulatory inputs have remained unknown (12). The kinases were also discovered serendipitously by complementation of *Schizosaccharomyces pombe* mutants deficient in the DNA damage pathway (13). In a search for Hog1-interacting proteins, we used two-hybrid screens with Hog1 as bait and discovered several potential binding partners, including Rck2. Further analysis clearly supported the notion that Rck2 is directly targeted and enzymatically activated by the Hog1 MAP kinase. A comparison to the mammalian p38–MAPKAP kinase system (3, 8) revealed surprising parallels in important regulatory features. We also found a connection between MAP kinase signaling and regulation of protein synthesis, which closely resembles the situation in mammalian cells. In these cells, stress-induced regulation of protein synthesis is mediated by phosphorylation either of eukaryotic initiation factor eIF4E by Mnk1 (14) or of the translation elongation factor 2 (EF-2) by calmodulin kinase II (15). Our results demonstrate that Rck2 provides a similar link in yeast by connecting osmostress-induced signal systems to the protein synthesis apparatus.

Materials and Methods

Yeast Strains and Plasmids. All yeast strains used in this study have *W303–1A* genetic background (*MAT*a *ade2–1 can1–100 his3–11 leu2–3 trp 1–1 ura 3–1*), except the two-hybrid strain L40, which is described in ref. 16. The *W303–1A* (wild type) and *hog1*Δ strains are described in ref. 11, the *gpd1*Δ and *hot1*Δ strains in ref. 6, and the *rck1*Δ, *rck2*Δ, and *rck1,2*Δ strains in ref. 13. The complete coding region of the *HOG1* gene was deleted by the microhomology PCR method (17) in the *rck1*Δ, *rck2*Δ strain background, and the deletion was verified by PCR analysis of chromosomal DNA.

For the yeast two-hybrid screen, the *HOG1* coding region was cloned as a *Bam*HI fragment into the vector pBTM116 (16). C-terminally hemagglutinin (HA)-tagged Hog1 was constructed by cloning of a *Not*I fragment containing six copies of the HA epitope into the plasmid pVR50 described in ref. 11. The *RCK1* and *RCK2* genes were obtained by PCR from genomic yeast DNA. Promoter fragments (800 bp) were cloned as a *Hin*dIII-*Bam*HI fragment into the yeast vector YCp33 (18). The coding regions were amplified separately and cloned into the pBluescript vector (Stratagene), and the constructs were verified by DNA sequencing. Proteins were C-terminally tagged with six copies of the HA epitope or an enhanced green fluorescent protein (19), cloned as a *Not*I cassette. The kinase inactive RCK2 mutant (K201R) was generated by PCR, introducing a functionally silent analytical *Bgl*II site. Phosphorylation site mutants T379A and S520A were also generated by PCR, containing a diagnostic *Kpn*I site (T379A) and a *KspI* site (S520A). Oligonucleotides used for PCR amplification are published as supplemental data on the PNAS web site, www.pnas.org. The double mutant (T379A S520A) was constructed by replacing an *Eco*RI-*Nco*I fragment of *RCK2* containing the T379A mutation. For expression in yeast, all constructs were fused with the endogenous 800-bp promoter and introduced into YCp33. Constructs for two-hybrid analysis were obtained from the screen or generated by subcloning of a *Bam*HI *Eco*RI fragment of *RCK2* in the

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Abbreviations: EF-2, translation elongation factor 2; HA, hemagglutinin; HOG, high osmolarity glycerol; MAP, mitogen-activated protein; MAPKAP, MAP kinase-activated protein kinase; BD, binding domain.

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yeast two-hybrid vector pGAD10 (CLONTECH). Plasmids containing *FUS3* and *KSS1* were described in ref. 20.

Yeast Two-Hybrid Screen and Other Growth Assays. A two-hybrid screen was performed with Hog1p as DNA-binding bait, cloned in the vector pBTM116 (16). The screen was carried out in strain L40 by using a yeast cDNA library (21). Transformants (560,000) were screened for histidine prototrophy on minimal medium containing 2 mM 3-aminotriazol. Histidine-positive clones were further tested for activation of the *LacZ* reporter gene in filter-lift assays. Library plasmids were obtained by electroporation of yeast DNA into *Escherichia coli* strain HB101 and selection on M9 medium without leucine. Quantitative two-hybrid assays were done with cell extracts and 2 mM *o*-nitrophenyl *β-D* galactopyranoside as substrate. Interaction was calculated as specific β-galactosidase activity in units/milligram of protein.

Yeast strains were tested for their ability to suppress the lethal phenotype of a constitutive hyperactive HOG pathway, as described in ref. 6. Briefly, N-terminally truncated *SSK2* was ectopically expressed under control of an inducible *GAL* promoter, and survival of strains was checked by spotting serial dilutions of logarithmically growing cultures on glucose and galactose plates.

Phosphorylation Assays. Phosphorylation of Rck2p was monitored by Western blot analysis of HA-tagged Rck2p. The gene was expressed from plasmids either in an *rck2*Δ strain or in an *rck1*Δ *rck2*Δ *hog1*Δ strain background. Cells were lysed by vortexing with glass beads in lysis buffer (50 mM Tris·HCl, pH 7.5/100 mM NaCl/10 mM imidazole/1 mM EDTA/1 mM EGTA/0.2 mM Na_3VO_4/10 mM NaF), supplemented with protease inhibitors (Complete, Boehringer). Dephosphorylation of the samples (10 μg protein/μl) was performed by treatment with λ phosphatase (0.5 units/μl, New England Biolabs) for 15 min at 37°C. Specificity of phosphatase treatment was checked by addition of vanadate (5 mM final concentration). Cell extracts containing 40 μg of total protein were separated on SDS/7.5% polyacrylamide gel and blotted onto nitrocellulose membranes, and Rck2p was detected with a monoclonal HA antibody by using the enhanced chemiluminescence Western blot detection system (Pierce).

For *in vitro* kinase assays, C-terminally HA epitope-tagged Hog1p (or Rck2p) was immunoprecipitated from yeast cell extracts with HA antibody bound to Pan mouse IgG Dynabeads (Dynal, Great Neck, NY) for 2 h at 4°, before and after activation of Hog1p by salt shock (5 min 0.4 M NaCl). Each precipitate was washed twice with lysis buffer and then once with 2 × kinase buffer (100 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/2 mM DTT). The washed beads were aliquoted and mixed with substrates for kinase reaction (10 μl beads/5 μl 5 × kinase buffer/10 μg purified protein/3 μl H_2O/ 2 μl [^{32}P]ATP). Reaction was carried out for 15 min at room temperature and stopped by addition of 15 μl 5 × SDS loading buffer. To test specificity of Hog1 kinase activity, the p38 inhibitor SB 203580 (Calbiochem) was added (10 μM and 30 μM final concentration). Sample phosphorylation was detected by autoradiography after SDS/PAGE.

EF-2 modification was assayed by two-dimensional gel electrophoresis by using polyclonal antisera directed against EF-2 for immunodetection. Total protein extracts were prepared before and 15 min after osmotic shock, as described. Total protein (50 μg) was separated on pH 3–10 nonlinear gradients (Immobiline Dry Strip, Amersham Pharmacia) with a stepwise gradient (100 V, 30 min; 500 V, 15 min; 1,500 V, 15 min; and 3,500 V, 1 h, at a maximum current of 1 mA). Subsequently, strips were equilibrated in 50 mM Tris·HCl, pH 8.8/3% SDS/5 mM DTT and applied to SDS/PAGE and Western blotting, as described.

Table 1. Results of the two-hybrid screen with Hog1 as a bait

	Number of clones		2-H interaction	
Gene	Total	Different	HIS3	Lac-Z
RCK2	12	4	+++	+++
RNR4	2	2	++	++
GLO3	1	1	++	++
ADH3	2	2	++	++
others	8	7	++	++

A yeast cDNA library was screened with Hog1p as bait. Transformants (560,000) were screened, and clones, which were positive for lac-Z and HIS3 reporter genes, were sequenced.

Leucine Incorporation Assays. Incorporation of ^{3}H-labeled leucine into trichloroacetic acid-precipitable material was determined as described in ref. 22. To enable equal growth of the strains, wild-type (W303–1A) and *hog1*Δ strains (W303–1A *HOG1*::*TRP1*) were transformed with the plasmid YCp111, which contains the *LEU2* gene. Cells (50 ml) were grown in synthetic medium (leucine) to an OD of 0.5, concentrated by centrifugation, and resuspended in 10 ml of medium. ^{3}H-leucine (75 μ Ci, 140 Ci/mmol) was added to each culture 5 min before osmotic challenge (0.4 M NaCl). Concurrent with stress application, the culture was chased with unlabeled leucine (final concentration of 0.12 mg/ml). At time points 0, 5, 15, 30, 45, and 60 min, 1-ml cells were collected, resuspended in 200 μl lysis buffer (see above), supplemented with 10 μg/ml cycloheximide, and frozen in liquid nitrogen. Cells were broken with glass beads, and total protein was precipitated with ice-cold acetone. Protein pellets were washed two times with 25% trichloric acid and once with 70% acetone and resolved in 1-ml urea buffer (5 M urea/0.2% SDS/50 mM Tris·HCl, pH 10) for scintillation counting.

For measuring amino acid uptake, cells (wild type, *hog1*Δ, and *rck2*Δ) were grown as described. The culture was divided in two parts, and one-half was challenged by osmotic shock (0.4 M NaCl), followed immediately by the addition of ^{3}H-leucine. Cells were harvested 5 and 12 min after leucine addition, washed once with cold medium, and recentrifuged. Cell pellets were dissolved in scintillation mixture, and radioactivity was measured by liquid scintillation. Leucine uptake after salt shock was calculated as percentage of uptake without stress. The value obtained after 12 min without stress was used as reference (100%) for each strain.

Results

Hog1 Interacts with the Rck2 Kinase in a Two-Hybrid Screen. In a general search for Hog1-interacting proteins, we used a Hog1-LexA-binding domain (BD) fusion as a bait for a library containing cDNA fusions to the Gal4 activation domain. In the set of positive clones (Table 1), we found independent fusions containing sequences of the following three genes: *RCK2*, *GLO3*, and *RNR4*. Rck2 has been previously identified as a Ser/Thr protein kinase (12, 13). Glo3 is thought to act as GTPase-activating protein for ADP ribosylation factor Arf1, which is involved in vesicular transport (23). Rnr4 is the small subunit of ribonucleotide reductase and is thought to be involved in regulation of its enzyme activity (24).

Here we provide evidence that the Rck2 kinase is a genuine and physiologically relevant Hog1 substrate. Two-hybrid analysis revealed a strong interaction between the Hog1-BD and Rck2-activation domains (ADs) (300 units/mg β-galactosidase activity in quantitative two-hybrid assays). Other yeast MAP kinase fusions, such as Fus3-BD and Kss1-BD, did not result in strong interactions with Rck2-AD (12 and 15 units/mg, respectively). The interaction between Hog1-BD and Rck1-AD, which has 52% identity to Rck2 but is expressed at much lower levels, was significantly weaker (68 units/mg). Rck2 belongs to a family of

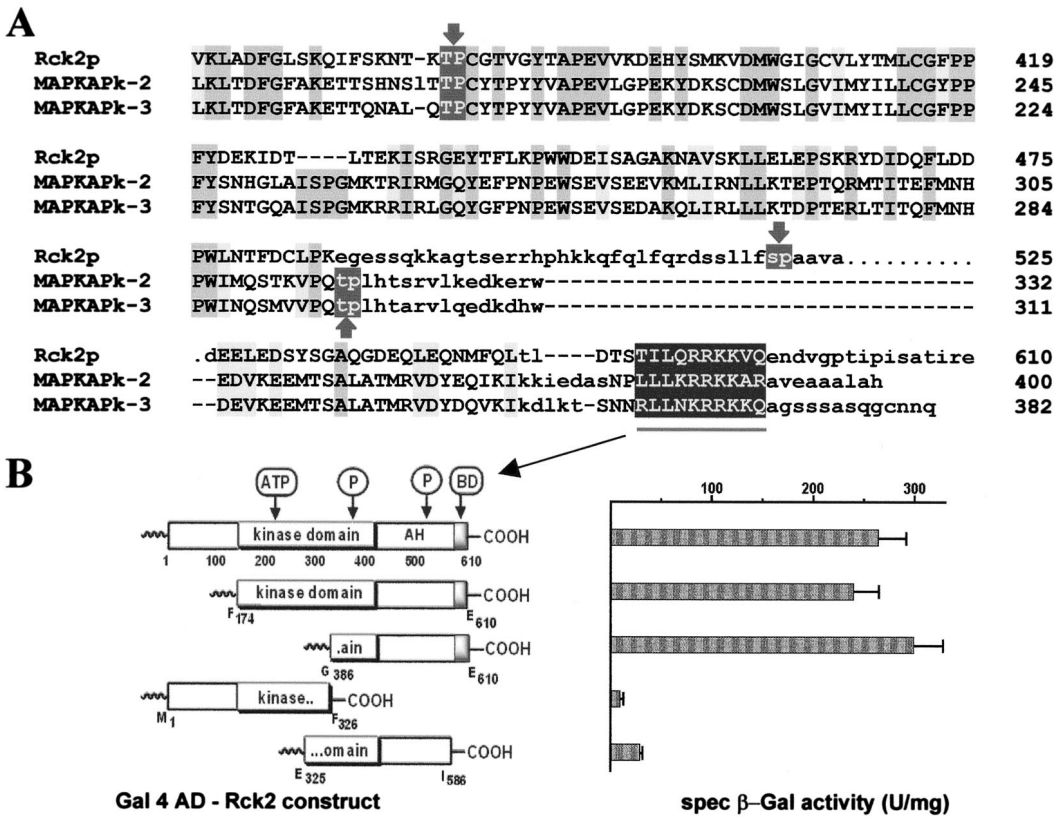

Fig. 1. Sequence alignment and two-hybrid assays. (*A*) Sequence alignment between yeast Rck2 and mammalian MAPKAP kinases. Phosphorylation sites for proline-directed kinases are indicated by arrows. MAP kinase-binding domain at the C terminus. (*B*) Mapping of the MAP kinase-binding domain in Rck2. ATP, ATP-binding domain; P, potential phosphorylation sites; BD, MAP kinase-binding domain. Different truncated forms of Rck2 shown (*Left*) were tested, and the resulting two-hybrid interaction is displayed (*Right*).

protein kinases that contain a large regulatory domain at the C terminus. In mammalian MAPKAP kinase 2, this domain is regulated and modified by p38 (Fig. 1*A*). The two-hybrid data show that the interaction between Hog1 and Rck2 is mainly mediated through the C-terminal regulatory domain of Rck2 (Fig. 1*B*). A very basic amino acid sequence motif found at the C terminus further suggests that the interaction between Hog1 and Rck2 might be directed via the proposed universal MAP kinase homing motif (25). Deletion of this motif abolished the two-hybrid signal normally obtained with the second half of the protein (Fig. 1*B*). This result further supports the notion that the interaction between the two kinases is direct and specific.

Rck2 Phosphorylation Increases During Osmostress. Assuming that Rck2 could be regulated by phosphorylation events, we assayed a functional epitope-tagged version of the protein by PAGE. Western analysis showed that the protein migrates in at least two forms, whose overall ratio differs between osmotically stressed and unstressed cells (Fig. 2*A*). The slower migrating species must represent a phosphorylated product, because only one protein band was detectable after treatment with λ-protein phosphatase, whereas two bands remained when a phosphatase inhibitor was added (Fig. 2*A*). A time-course experiment showed that the transient increase in the slower migrating form of Rck2 correlates well with the transient activation of Hog1 kinase during stress (Fig. 2*B*). This correlation does not signify just parallel and independent activation, because the ectopic induction of a hyperactive MAPKKK (ΔN-SSk2) also caused a corresponding increase of Rck2 phosphorylation (Fig. 2*C*). In contrast, if cells were stressed in the absence of Hog1, the modified form of Rck2 did not appear (Fig. 2*A*). Both results support the view that osmostress-induced phosphorylation of Rck2 must be mediated by the Hog1 kinase.

Rck2 Is a Substrate of Hog1 MAP Kinase. To prove that Rck2 is a direct substrate of Hog1, we generated and purified the protein from *E. coli* extracts as glutathione *S*-transferase (GST) fusion and tested it as substrate in Hog1-specific immuno kinase assays. Because Rck2 exhibits significant autophosphorylation activity (M.T., unpublished work, and ref. 12), we used a kinase inactive product (Rck2-K201R) for these assays. Variants of HA epitope-tagged Hog1 were absorbed from yeast cell extracts prepared from normal and osmostressed cells. ^{32}P became preferentially incorporated into GST-Rck2-K201R when Hog1 originated from stress-induced cells (Fig. 2*D*). The increase in signal was at least comparable to increases found with myelin basic protein as substrate (not shown). When a catalytically inactive allele of Hog1 was used, only background signals were found even on salt induction. Similarly, the p38 kinase-specific inhibitor SB203580 (26) lowered the incorporation of radioactive phosphate into Rck2 to the uninduced level.

MAPKAP kinase 2 function has been shown to rely on two phosphorylation events mediated by activated p38. One phosphorylation site is located within kinase domain VIII and the second in a hinge region in the C-terminal autoinhibitory domain. Phosphorylation in the C-terminal extension controls availability of a nuclear export signal as well as activation of a repressive autoregulatory domain (9). Rck2 also contains putative MAP kinase phosphorylation sites. One of them is in the catalytic domain at a position (T379) that corresponds to the modification site of MAPKAP kinase 2 (Fig. 1*A*). The second one is in the middle of the C-terminal domain with supposed

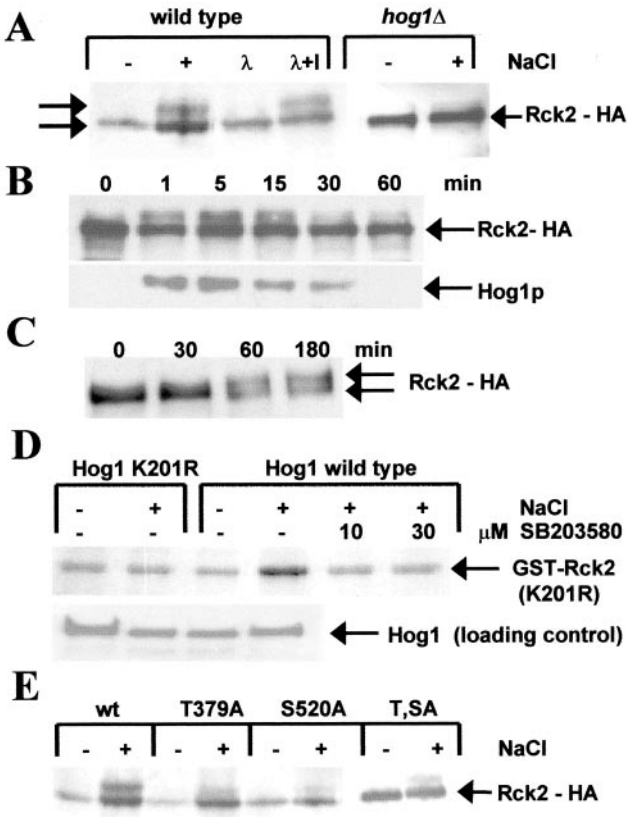

Fig. 2. Phosphorylation of Rck2 by Hog1 *in vivo* and *in vitro*. (*A*) Western Blot of HA epitope-tagged Rck2 before (−) and 10 min after (+) a mild osmotic shock with 0.4 M NaCl. The two forms of Rck2 are indicated by arrows. Samples from lane 2 (+) were treated with λ phosphatase without (λ) and with inhibitor (λ + I). (*B*) Time course of Rck2 phosphorylation after osmotic shock. HA epitope-tagged Rck2 was assayed by Western blot analysis at the indicated time points. (*Lower*) Double-phosphorylated Hog1 is detected with the p38 antibody (New England Biolabs). (*C*) Rck2 phosphorylation after induction of the HOG pathway by galactose-induced expression of a N-terminally truncated SSK2 allele. Samples taken at the indicated time points after induction were assayed by Western blot analysis by using the HA antibody. (*D*) *In vitro* kinase assays with bacterially expressed glutathione *S*-transferase-Rck2 (kinase inactive K201R mutant) and immunoprecipitated Hog1 kinase. C-terminally HA epitope-tagged Hog1 was precipitated from yeast cells before (−) and after (+) activation by 0.4 M NaCl for 5 min, and ^{32}P incorporation was detected by autoradiography. The kinase inactive (K52R) Hog1 mutant was compared with wild type. Additionally, wild-type Hog1 kinase activity was blocked by the specific p38 inhibitor SB 203580 (Calbiochem) at the indicated concentrations. (*E*) Phosphorylation of Rck2 mutant proteins. The phosphorylation site mutants T379A, S520A, and T379A S520A were tested for phosphorylation, as described for *A*, by Western blot analysis after a mild osmotic shock (0.4 M NaCl) for 10 min.

autoinhibitory function (S520). Although two more Ser/Pro sites can be found in the N-terminal portion of the protein, we concentrated on the analysis of T379 and S520.

Point mutations T379A, S520A, and T379A S520A of HA epitope-tagged Rck2 were generated and tested for modification after osmotic shock *in vivo* (Fig. 2*E*). Both single substitution mutations T379A and S520A abolished the Hog1-dependent modification of Rck2, as measured by its migration on PAGE. However, a minor stress-induced form of Rck2 still appeared, comparable to the pattern found in *hog1* mutants (Fig. 2*A*). Surprisingly, the migration pattern of the double mutant showed no further differences to the single mutants. Therefore, either the modification of the sites must be interdependent, or the slower-migrating form represents only the double-phosphorylated protein. Because the integrity of both putative modification sites is clearly necessary for the appearance of the slower-migrating form of Rck2, we propose that modification events similar to those at MAPKAP kinase 2 are driving the activation of the yeast kinase.

Lethality of the Hyperactive HOG Pathway Is Suppressed by Deletion of *RCK2*. To investigate the physiological relevance of the putative phosphorylation cascade, we obtained deletion mutants in *RCK2* and *RCK1*. In contrast to a *hog1* strain, neither single nor double mutants exhibited obvious growth retardation under chronic osmostress conditions (i.e., growth in media containing 0.4 M NaCl; not shown). This result suggested that Rck2 may participate only in a limited nonessential response. To test this hypothesis, we measured the activity of two enzymes known to be induced by the HOG pathway. Indeed, we found that glycerol 3-phosphate dehydrogenase (27) and catalase activity (28) were induced in *rck1 rck2* mutants 30 min after osmotic shock to the same level as in wild-type strains (not shown). However, physiological influences of the HOG pathway can be observed not only through its loss of function but also under conditions where activity is deregulated. Permanent hyperactivation of the MAP kinase is detrimental, as shown by the fact that cells with a constitutive MAPKKK are unable to grow. The effects of Hog1 hyperactivation are ameliorated to the same extent in *rck2 rck1* double mutants and *rck2* single mutants but not in *rck1* single mutants (Fig. 3*A*). Ectopic expression of wild-type Rck2 reverses this effect (Fig. 3*B*) but not expression of the inactive kinase (Rck2-K201R) or of the phosphorylation site mutants Rck2-T379A, Rck2-S520A, and Rck2-T379A, S520A, respectively. This observation strongly suggests that Rck2 has a physiological role in stress adaptation as a specialized effector for Hog1 and that both phosphorylation sites and kinase activity are necessary for this process.

Rck2 Mediates Regulation of Protein Synthesis During Adaptation to Osmostress. In mammals, highly specific Ca^{2+}/calmodulin-dependent protein kinases regulate the activity of translation EF-2 by phosphorylation. Phosphorylation of EF-2 leads to its decreased binding affinity to ribosomes and consequently to a lower level of protein synthesis (15). We therefore asked whether a similar function could be assigned to Rck2 during an osmotic imbalance in yeast. We measured the incorporation of ^{3}H-labeled leucine into high-molecular-weight products under normal growth conditions and under conditions of mild osmostress. As shown in Fig. 3*C*, wild-type cells were clearly affected by stress conditions. Osmotically challenged cells exhibited much lower rates of leucine incorporation into high-molecular-weight product than untreated cells. The same assay showed a remarkably different outcome for *hog1* as well as *rck2* mutants. First, the overall level of synthesis appears to be enhanced in mutant backgrounds, compared with wild type. More importantly, the rate of leucine incorporation remained largely unaffected by high osmolarity in both types of mutants. These effects also depended on Rck2 kinase activity and phosphorylation (Fig. 3*D*).

To rule out that our results were biased by changes in amino acid uptake, we measured ^{3}H-leucine uptake directly after osmotic shock in wild-type cells in the *hog1*Δ and *rck2*Δ strains. ^{3}H-leucine was added after stress exposure, and its uptake into cells was measured after 5 and 12 min, respectively. We obtained similar reductions in leucine uptake after stress for all three strains. As compared with nonstressed cells after 12 min, leucine uptake was 37% (wild type), 36% (*hog1*), and 26% (*rck2*) after 5 min, and 49% (wild type), 52% (*hog1*), and 41% (*rck2*) after 12 min, respectively.

Rck2 Modulates EF-2 Phosphorylation *in Vitro* and *in Vivo*. To test directly whether yeast EF-2 serves as a substrate of Rck2, we

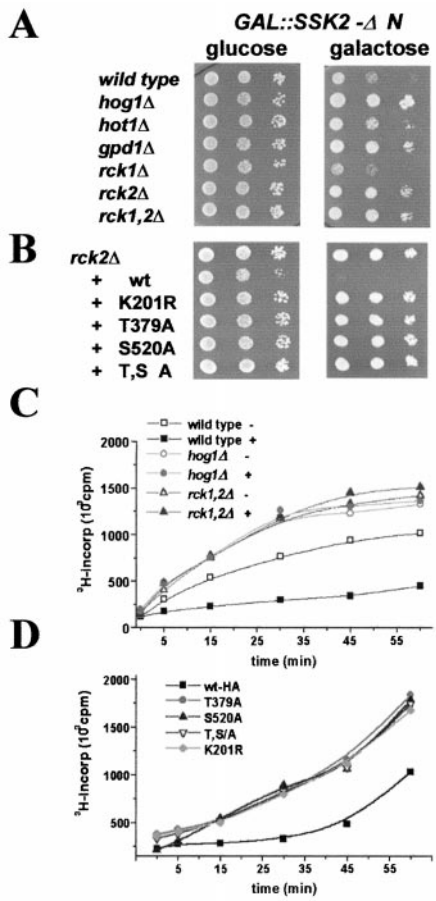

Fig. 3. Physiological responses of *hog* and *rck* mutants. (*A*) Suppression of hyperactive HOG pathway by deletion of downstream targets. The HOG pathway was activated by ectopic expression of a N-terminally truncated SSK2 allele, and the ability of different deletion strains to suppress the resulting lethal phenotype was tested. (*B*) Different Rck2 point mutations were tested in the same assay after retransformation of the *rck2* deletion strain and compared with the Rck2 wild-type form. (*C*) ^{3}H-leucine incorporation into trichloroacetic acid (TCA)-precipitable compounds with (+) and without (−) mild osmotic shock in different yeast strains. Cells were grown for 2 h in minimal medium without leucine before the label (75 μCi) was added. Uptake was allowed for 5 min before the shock was applied and cold leucine was added. Protein was TCA precipitated and resolved for scintillation counting as described in *Materials and Methods*. (*D*) ^{3}H-leucine incorporation after mild osmotic shock in different Rck2 mutants. The *rck2* deletion strain was retransformed with different Rck2 plasmids to test the influence on protein synthesis after osmostress.

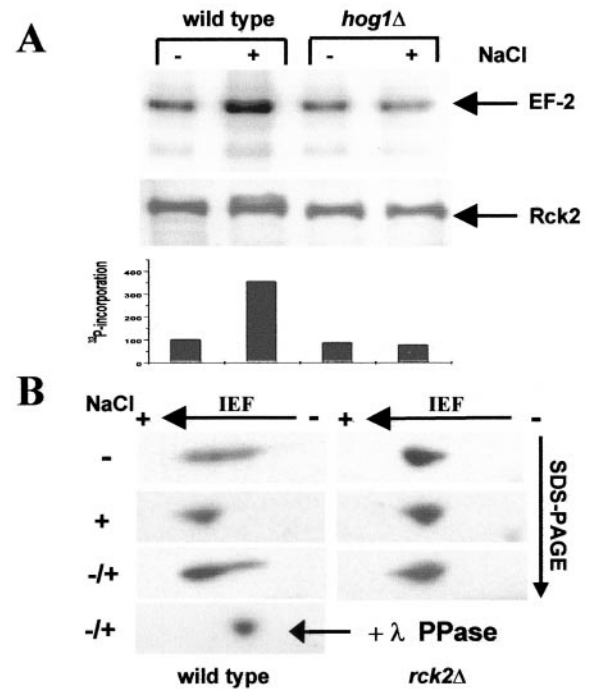

Fig. 4. EF-2 phosphorylation *in vitro* and *in vivo*. (*A*) *In vitro* kinase assay with Rck2 and EF-2 as substrate. C-terminally HA epitope-tagged Rck2 from wild type and a *hog1* deletion strain was immunoprecipitated before (−) and after (+) induction by osmotic shock as described in *Materials and Methods*. The amounts of precipitated Rck2 kinase were tested by Western blotting, and incorporation of ^{32}P was quantified with a PhosphorImager (Molecular Dynamics). (*B*) Two-dimension gel analysis of EF-2 modification *in vivo*. Protein extracts were prepared before (−) and 15 min after osmotic shock (+) and separated on two-dimensional gels as described in *Materials and Methods*; (−/+) mixture of both extracts; + λ PPase, mixture treated with λ phosphatase. EF-2 was detected with polyclonal antibodies.

performed an *in vitro* kinase assay with biochemically purified yeast EF-2 (kindly donated by Angus Nairn, The Rockefeller University, New York) and immunoabsorbed Rck2 from yeast cells before and after a mild osmotic shock. When Rck2 was isolated from stressed Hog1-proficient strains, we could observe increased phosphorylation of EF-2, whereas no such effect was found with *hog1* cells (Fig. 4*A*).

To test whether relevant changes in EF-2 modification could be detected *in vivo*, we tried to analyze extracts from stressed and unstressed cells by two-dimensional gel electrophoresis (Fig. 4*B*). EF-2 was detected with a polyclonal antibody (kindly provided by Angus C. Nairn). In unstimulated wild-type cells, EF-2 separated into at least two spots with roughly equal intensity. In osmotically stressed cells, we observed a dramatic shift toward the more negatively charged spot. A mixture of both samples confirmed the presence of different isoforms, which collapsed into one spot after treatment with λ phosphatase. Therefore, we concluded that the stress-induced changes in EF-2 modification are caused by phosphorylation. In the same assay, extracts from *rck2* strains behaved quite differently. EF-2 always migrated in one spot whose position did not change after osmotic challenge (Fig. 4*B*). These results clearly support the notion that the state of EF-2 is influenced by activated Rck2 *in vivo*.

Discussion

From our work, the outline of a linear signaling cascade emerges whose activation by osmotic imbalances at the plasma membrane triggers the attenuation of protein synthesis. One central observation in this context is clearly the physical interaction between the osmostress-activated MAP kinase Hog1 and the calmodulin kinase relative Rck2. Starting from the positive signal discovered through a two-hybrid assay, much additional data indicate that this physical interaction is direct and functionally relevant. For example, the presence of a consensus MAP kinase-binding motif in Rck2 was found to be necessary for a strong two-hybrid signal. Furthermore, two putative phosphorylation motifs for proline-dependent kinases such as MAP kinases are of crucial importance for Rck2 function, and this dependence could be seen at the biochemical as well as the physiological level. In addition, it is clear that Rck2 becomes transiently modified *in vivo* by phosphorylation during osmostress. The extent of this modification depends on Hog1, and the kinetics of Rck2 phosphorylation correlates well with that of Hog1 activation. Finally, according to *in vitro* kinase assays, the Hog1-modified phosphorylation status of Rck2 has direct implications for its protein kinase activity. So, even if we cannot exclude activating inputs

into Rck2 that are independent of osmostress, Rck2 must be one of the main effectors for Hog1 during cellular adaptation to external hyperosmolarity.

A two-hybrid connection has recently been noted between Hog1 and the Rck2 homologue Rck1 (29). However, in our hands, the Rck1 connection seems to be of little significance, in quantitative as well as physiological terms. For example, *rck1* single mutants, in contrast to *rck2* mutants, do not allow survival at continuous Hog1 activation. Therefore, even if Rck2 and Rck1 share substrates, we reason that only Rck2 is able to efficiently transmit the osmotically induced signal.

During the preparation of this manuscript, interactions between Rck2 and Hog1 have also been reported by Bilsland-Marchesan *et al.* (30), who identified S520 as a functionally important phosphorylation site that is directly modified by Hog1. Our data confirm and extend these results, suggesting that a second site is also involved in activation. In this respect, our observations hint at a surprisingly tight conservation between the p38–MAPKAP kinase 2 relay and the yeast HOG system.

What could the target of Rck2 be? Melcher and Thorner (12) speculated that Rck2 is most likely involved in translational regulation, because they found that Rck2 could phosphorylate elongation factor 2. Their observation that overexpression of a C-terminally truncated and thereby deregulated form of Rck2 inhibits growth lent some weight to this idea. Our present work not only further substantiates this idea but also provides a clear physiological basis for it. First, phosphorylation of EF-2 by Rck2 is significantly induced if the kinase is isolated from osmostressed cells. Second, EF-2 modification after osmotic shock *in vivo* depends on Rck2 and Hog1. Finally, incorporation of ^{3}H-leucine into trichloroacetic acid-precipitable material is severely reduced after osmoshock, a response that can be clearly detected in wild-type cells but not in *hog1* or *rck2* mutants. Rapid down-regulation of protein synthesis as well as depression of amino acid uptake after osmotic shock have been described (31, 32), but no mutants were analyzed in those studies. We believe that our experimental setup allows us to differentiate between these effects. First, by precharging cells with ^{3}H-leucine, effects caused by amino acid transport should be negligible. Second, because *hog1* and *rck2* mutants exhibit the same depressed rates of amino acid uptake after stress as do wild-type cells, the effect of these mutations on leucine incorporation into protein precipitates can only reflect a loss of translational attenuation. Such an interpretation of our data would fit nicely with findings in mammalian cells in which phosphorylation of EF-2 has been shown to cause an inhibition of protein synthesis (15). Our results further demonstrate that changes in translational efficiency are the consequence not of stress damage but of a signaling event.

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