Activation of Srk1 by the Mitogen-activated Protein Kinase Sty1/Spc1 Precedes Its Dissociation from the Kinase and Signals Its Degradation

Sandra López-Avilés,*† Eva Lambea,*† Alberto Moldón,‡ Maribel Grande,*§ Alba Fajardo,* Miguel A. Rodríguez-Gabriel,‖ Elena Hidalgo,‡ and Rosa Aligue*

*Departament de Biologia Cellular, Universitat de Barcelona. Institut d’Investigacions Biomèdiques August Pi i Sunyer, 08036 Barcelona, Catalunya, Spain; †Departament de Ciències Experimentalss i de la Salut, Universitat Pompeu Fabra. 08003 Barcelona, Catalunya, Spain; and ‡Departamento de Microbiologia II. Universidad Complutense, 28040 Madrid, Spain

Submitted July 5, 2007; Revised January 9, 2008; Accepted February 1, 2008
Monitoring Editor: Daniel Lew

Control of cell cycle progression by stress-activated protein kinases (SAPKs) is essential for cell adaptation to extracellular stimuli. The Schizosaccharomyces pombe SAPK Sty1/Spc1 orchestrates general changes in gene expression in response to diverse forms of cytotoxic stress. Here we show that Sty1/Spc1 is bound to its target, the Srk1 kinase, when the signaling pathway is inactive. In response to stress, Sty1/Spc1 phosphorylates Srk1 at threonine 463 of the regulatory domain, inducing both activation of Srk1 kinase, which negatively regulates cell cycle progression by inhibiting Cdc25, and dissociation of Srk1 from the SAPK, which leads to Srk1 degradation by the proteasome.

INTRODUCTION

In response to extracellular stimuli, cells induce an elaborated program that includes changes in transcription and translation as well as cell cycle progression to allow cells to adapt. Activation of the stress-activated protein kinases (SAPKs) is essential to this response. In the fission yeast, Schizosaccharomyces pombe, the main elements of the SAPK pathway are the mitogen-activated protein kinase (MAPK) Sty1/Spc1, the MAPKK Wis1, and the MAPKKks Wak1 and Win1 (reviewed in Hohmann, 2002). Several of the SAPK pathway components were isolated in screenings designed to identify new genes regulating cell cycle division (Millar et al., 1995; Shiozaki and Russell, 1995; Shiozaki et al., 1997), signifying the link between environmental stress response and cell cycle control. In S. pombe spc1/sty1 mutants were identified in two different genetic analyses, spc1 mutant as a recessive suppressor of lethality caused by loss of phosphatase 2C (Shiozaki and Russell, 1995) and sty1 mutant as a recessive suppressor of lethality caused by the simultaneous inactivation of pyp1 and pyp2 phosphatases (Millar et al., 1995). sty1/spc1 mutants have a G2 delay that is greatly exacerbated by growth in high osmolality. In addition, a lethal interaction of spc1/sty1 and cdc25 mutations shows that Spc1/Sty1 promotes the onset of mitosis (Shiozaki and Russell, 1995).

A number of effectors of Sty1/Spc1 MAP kinase have been identified, including the Atf1 transcription factor, which is homologue to mammalian ATF-2 and c-Jun (Shiozaki and Russell, 1996; Wilkinson et al., 1996). In addition, Sty1/Spc1 binds and phosphorylates two downstream kinases, Cmk2 and Srk1 (Sty1-regulated kinase), which are related to the mammalian calmodulin-dependent kinases, MAPKAP kinases and to the budding yeast RCK1 and RCK2 kinases, which were identified as suppressors of a checkpoint mutant (Dahlkist et al., 1995; Alemany et al., 2002; Sanchez-Piris et al., 2002; Smith et al., 2002).

Recently, a molecular link between the SAPK pathway and the G2-M transition was described through the Srk1 kinase (Lopez-Aviles et al., 2005). The Srk1 kinase was initially identified because it is transcriptionally up-regulated after activation of the Sty1 MAPK pathway (Smith et al., 2002). It is present in a complex with the Sty1 MAPK and is directly phosphorylated by Sty1 (Smith et al., 2002). In addition, it is also shown that Srk1 controls mitotic entry by directly phosphorylating and inhibiting Cdc25 during normal cell cycle (Lopez-Aviles et al., 2005). Here we show that, in response to stress, Sty1 phosphorylates Srk1 and induces various effects; Srk1 is rapidly activated and dissociated from Styl, which leads to the inactivation of mitosis entry by translocating Cdc25 from the nucleus to the cytoplasm; in addition, Srk1 released from its interaction to Styl is targeted to degradation by the proteasome, as a negative feedback.

MATERIALS AND METHODS

Fission Yeast Strains, Media, and Techniques

The strains used are listed in Table 1. Media and genetic methods for studying S. pombe were performed as described by Moreno et al. (1991). Where indicated hydroxyurea (HU; 10 mM final concentration, Sigma, St. Louis, MO),

This article was published online ahead of print in MBC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–07–0639) on February 13, 2008.
† These authors contributed equally to this work.
§ Present address: Institut de Biologia Molecular de Barcelona (IBMB-CSIC), Parc Cientific de Barcelona, C/Josep Samitier 1-5, 08028 Barcelona, Catalunya, Spain.

Address correspondence to: Rosa Aligue (aliguerosa@ub.edu).
Abbreviations used: GST, glutathione S-transferase.
MG132 (50 μM final concentration, StressGen, San Diego, CA), and cycloheximide (100 μg/ml final concentration, Sigma) were added to liquid cultures.

**Plasmid and Strain Construction**

Strain construction, Srk1 tagging, and mutagenesis of residue lysine 153 to alanine to obtain Srk1-KA were as described elsewhere (Lopez-Aviles et al., 2005). Plasmid plNV-DEGRON-HA-Sty1 was constructed as described by Iaconov et al. (1999). The fragments GST-Srk11-403-KA and GST-Srk11-403-420-KA were made by restriction enzyme digestion. GST-Srk1 was used to obtain GST-Srk1-KA-T463A, the GST-Srk1 was used to obtain GST-Srk1-T463A and GST-Srk1-T463D by restriction enzyme digestion. GST-Srk1 was used to obtain GST-Srk1-KA-T463A, the GST-Srk1 was used to obtain GST-Srk1-T463A

Strain construction, Srk1 tagging, and mutagenesis of residue lysine 153 to alanine to obtain Srk1-KA were as described elsewhere (Lopez-Aviles et al., 2005). Plasmid pINV-DEGRON-HA-Sty1 was constructed as described by Iaconov et al. (1999). The fragments GST-Srk11-403-KA and GST-Srk11-403-420-KA were made by restriction enzyme digestion. GST-Srk1 was used to obtain GST-Srk1-KA-T463A, the GST-Srk1 was used to obtain GST-Srk1-T463A and GST-Srk1-T463D by restriction enzyme digestion. GST-Srk1 was used to obtain GST-Srk1-KA-T463A, the GST-Srk1 was used to obtain GST-Srk1-T463A and GST-Srk1-T463D.

Strain construction, Srk1 tagging, and mutagenesis of residue lysine 153 to alanine to obtain Srk1-KA were as described elsewhere (Lopez-Aviles et al., 2005). Plasmid pINV-DEGRON-HA-Sty1 was constructed as described by Iaconov et al. (1999). The fragments GST-Srk11-403-KA and GST-Srk11-403-420-KA were made by restriction enzyme digestion. GST-Srk1 was used to obtain GST-Srk1-KA-T463A, the GST-Srk1 was used to obtain GST-Srk1-T463A and GST-Srk1-T463D by restriction enzyme digestion. GST-Srk1 was used to obtain GST-Srk1-KA-T463A, the GST-Srk1 was used to obtain GST-Srk1-T463A and GST-Srk1-T463D.

**Immunoprecipitation and Western Blotting**

The Srk1-HA protein was immunoprecipitated from cell extracts with 2 μg of monoclonal anti-HA antibody and using 30 μl of protein A Sepharose beads (Pierce, Rockford, IL). Western blot analysis was performed with the following primary antibodies: monoclonal anti-HA (12CA5, Roche, Indianapolis, IN; 1/1000); anti-actin (1/2000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-PSTAIR (1/1000, Upstate Biotechnology, Lake Placid, NY); anti-Hog1 (1/1000, Santa Cruz Biotechnology), and anti-phospho pS8 (1/1000, Cell Signaling Technology, Beverly, MA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Bio-Rad, Richmond, CA) were used as secondary antibodies. Membranes were developed by enhanced chemiluminescence (ECL kit, Amersham-Pharmacia, Piscataway, NJ).

**In Vitro Kinase Assays**

Glutathione S-transferase (GST) fusion proteins were expressed and purified as described (Lopez-Aviles et al., 2005). Purified GST-Sty1 and GST-Sty1-KA were preincubated with GST-PBS2-EE in a kinase buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM β-mercaptoethanol, 50 μM ATP) for 20 min at 30°C. Next, GST-Srk1 or GST-Srk1-KA was added to the reaction together with GST-Cdc2530-420 when indicated, and 0.1 μCi/ml [γ-32P]ATP for 20 min at 30°C. Preincubation of purified GST-Sty1-KA and GST-Srk1 was performed when indicated, in a kinase buffer without ATP for 30 min at 30°C. Labeled proteins were resolved by SDS-PAGE and detected by autoradiography.

**Table 1. Schizosaccharomyces pombe strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA0104</td>
<td>h&quot; leu1-32 ura4-D18</td>
<td>Lab stock</td>
</tr>
<tr>
<td>JM160</td>
<td>h sty1::ura4 leu1-32 ura4-D18</td>
<td>I. Millar</td>
</tr>
<tr>
<td>RA0770</td>
<td>h&quot; wis1::ura4 leu1-32 ura4-D18</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA1102</td>
<td>h' srk1::kanMX6 leu1-32</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA1449</td>
<td>h' srk1::kanMX6 att::ura4 leu1-32 ura4-D18</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA0772</td>
<td>h gln3-4 ura4 leu1-32 ura4-D18</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA1380</td>
<td>h' srk1-463A:HA:kanMX6</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA1648</td>
<td>h' srk1-463A:HA:kanMX6 sty1::ura4 leu1-32 ura4-D18</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA1737</td>
<td>h srk1-T463A::HA:kanMX6</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RA1715</td>
<td>h srk1-T463D-30-145::HA:kanMX6 leu1-32 ura4-D18</td>
<td>Lab stock</td>
</tr>
<tr>
<td>KGY4337</td>
<td>h&quot; cdc25-GFP::kan5 ura4-D18 ad6-M210 leu1-32</td>
<td>K. Gould</td>
</tr>
<tr>
<td>RA1726</td>
<td>h&quot; cdc25-GFP::kan5 srk1::ura4 ura4-D18 ad6-M210 leu1-32</td>
<td>Lab stock</td>
</tr>
<tr>
<td>S1299</td>
<td>h&quot; cdc25-9A leu1-32</td>
<td>P. San-Segundo (H. Pienwica-Worms)</td>
</tr>
</tbody>
</table>
Furthermore, we created a point mutant version to substitute threonine 463 for alanine (Srk1-T463A) and tested it for phosphorylation by Sty1. Again, the catalytically inactive version of Srk1, Srk1-KA-T463A, was used to avoid auto-

**Figure 1.** Positive regulation of Srk1 by Sty1 kinase. (A) In vitro Sty1 kinase activity. Equal concentrations of purified GST-Sty1 (lanes 2, 4, and 6) and Sty1-KA (lane 5), previously incubated with budding yeast PBS2-EE (lanes 1 and 3–6), were assayed with Atf1 (lanes 2–5) as substrate and GST as a control (lane 6). (B) Activity of Srk1 after phosphorylation by Sty1. The purified GST-Srk1 (lanes 1–6) was incubated with Sty1 (lanes 2 and 3) or Sty1-KA (lanes 5 and 6) and then the fragment of Cdc25 (Cdc2556–145), lanes 3–5. As a control, the fragment of Cdc25 (Cdc2556–145) (lane 7) or GST (lane 8) were incubated with Sty1 (lanes 7 and 8). (C) Diagram of Srk1 protein kinase (top) and the truncated forms of Srk1 (bottom). In dark, Srk1 catalytic domain where K indicates lysine from the ATP binding loop. SP and TP indicate the putative phosphorylating sites for Sty1. (D) The full-length GST-Srk1-KA (lanes 1–4) and the two truncated forms, GST-Srk1^30–420 (lane 5) and GST-Srk1^1–403–KA (lane 6), were used as substrates of purified Sty1 (lanes 2 and 4–6) or Sty1-KA (lane 3), as a control in the kinase assay. (E) The GST-Srk1-KA-T463A inactive and containing a point mutation corresponding to threonine 463 to alanine (lanes 1 and 3), the inactive GST-Srk1-KA (lanes 2 and 4) and the full-length GST-Srk1 (lane 5), were assayed in a kinase reaction with purified GST-Sty1 (lanes 1 and 2) as in D.
phosphorylation. As shown in Figure 1E, phosphorylation of Srk1 was abolished in the mutant version.

Together these results show that Sty1 phosphorylates and activates Srk1 at threonine 463.

**Sty1 Protein, But Not Its Activity, Is Essential for Srk1 Stability**

Once established that Srk1 is activated by phosphorylation at threonine 463, we wondered whether this activation was totally dependent on Sty1. The sty1-deleted cells were exposed to osmotic stress and Srk1 activity was examined. Srk1 was immunoprecipitated at different times after stress exposure, and its activity was analyzed using Cdc256-145 fragment as substrate. As expected, there was no Srk1 activity detected when compared with control (Figure 2A, top). However, it was observed that no Srk1 was purified from these samples (Figure 2A, bottom). The same assay was performed again to compare in parallel the expression of Srk1 from wild-type and Δsty1 cells exposed to osmotic stress, and indeed, the Srk1 protein is not present in Δsty1 cells (Figure 2B). This result suggests that Sty1 regulates Srk1 stability in addition to its activity. To confirm this observation, Srk1 protein stability was analyzed using a different approach. We analyzed Srk1 protein expression in a Δsty1 strain where sty1 gene was expressed under the control of the invertase (inv1) inducible promoter (Iacovoni et al., 1999) in a plasmid bearing the “heat-inducible degron cassette” (Sanchez-Diaz et al., 2004) fused at the N-terminal of the sty1 gene (pINV1-DEGRON:Sty1-HA). A shift from glucose- to sucrose-based culture medium leads to a very rapid induction of the inv1 promoter, and therefore of Sty1 (Iacovoni et al., 1999). In addition, the “heat-inducible degron cassette” caused Sty1 degradation by a specific ubiquitin-mediated pathway when cells were shifted from 25 to 37°C (Sanchez-Diaz et al., 2004). The Δsty1 cells with the endogenous srk1 gene fused to the epitope hemagglutinin (HA) containing the pINV1-DEGRON:Sty1-HA were shifted to 37°C, and both the Sty1 and Srk1 proteins were detected by Western blot at different times after the shift. We observed that Srk1 protein vanished in parallel with Sty1 degradation induced by the DEGRON cassette at 37°C (Figure 2C).

To determine whether Srk1 stability was dependent on Sty1 activity, Srk1 protein levels were analyzed from Δwis1 cells, whose Sty1 protein is not activated under stress. As shown in Figure 2D, Srk1 protein was detected in Δwis1 cells under both normal and stress conditions, indicating that Srk1 stability is dependent on Sty1 protein and independent on its activity.

To investigate further whether a lack of Sty1 protein promotes the loss of Srk1 by degradation, Δsty1 cells were treated with a proteasome inhibitor, MG132, and Srk1 protein was analyzed at different times after treatment. As shown in Figure 2E, the Srk1 protein level was recovered when degradation was prevented, suggesting that the binding of Sty1 to Srk1 avoids Srk1 degradation.

**Phosphorylation of Srk1 at Threonine 463 Promotes Its Degradation**

To study the in vivo effect of threonine 463 phosphorylation, we examined the level of Srk1 protein in cells in which the endogenous srk1 gene was HA tagged (srk1::HA) or replaced by srk1-T463A (srk1-T463A-HA) or by srk1-T463D (srk1-T463D-HA). As shown in Figure 3A, the levels of Srk1-T463D protein were lower than those of Srk1 (Figure 3A, lanes 1 and 2). In contrast, the levels of Srk1-T463A protein did not change compared with Srk1 (Figure 3A, lanes 4 and

**Figure 2.** Sty1 stabilizes Srk1 protein. (A) Δsty1 cells containing endogenous tagged srk1::HA were exposed to 1 M KCl and Srk1 was immunoprecipitated with anti-HA antibody from samples taken at indicated time points. In vitro kinase assay was performed with the immunoprecipitates, using Cdc25 (Cdc256-145) as a substrate (top). The last lane corresponds to Srk1 activity from wild-type cells. Western blots of the immunoprecipitated samples were probed with anti-HA to monitor the presence of Srk1 (bottom). (B) Wild-type and Δsty1 cells, both with endogenous srk1::HA, were exposed to 1 M KCl and Western blots of protein extracts were prepared at the indicated times and probed with anti-HA to detect Srk1 protein (top). The same membrane was probed with anti-PSTAIR antibodies to detect Cdc2 protein as a loading control (bottom). (C) srk1-HA Δsty1 cells were transformed with pINV1-DEGRON-Sty1 plasmid expressing DEGRON-Sty1 under the control of the INV1 gene promoter at 25°C. Cells were transferred to 37°C, samples were taken at the indicated time points and Western blots were probed with anti-HA to detect DEGRON-Sty1 (middle) and anti-PSTAIR antibodies to detect Cdc2 protein as a loading control (bottom). (D) Wild-type, Δsty1, and Δwis1 cells containing endogenous srk1 gene tagged with HA (srk1::HA) were exposed to 1 M KCl for 30 min. Western blots of protein extracts were prepared and probed with anti-HA antibodies to detect Srk1 protein (top). The same membrane was probed with anti-actin antibody as a loading control (bottom). (E) Δsty1 cells carrying endogenous srk1 gene tagged with HA (srk1::HA) were exposed to MG132 drug (50 μM final concentration) and samples were collected at the indicated time points. Srk1 protein levels were monitored by Western blot with anti-HA antibody (top). Control levels of Srk1 protein were shown from wild-type cells carrying endogenous srk1 gene tagged with HA (srk1::HA) (lane 1). The same membrane was probed with actin antibody as a control (bottom).

Vol. 19, April 2008
5). To ascertain whether the loss of Srk1-T463D protein levels was due to degradation, cells carrying the srk1-T463D-HA allele were treated with MG132. In this case the levels of Srk1-T463D were recovered (Figure 3A, lane 3), indicating that phosphorylation at threonine 463 promotes instability and degradation of Srk1.

To confirm that Srk1 phosphorylation by Sty1 promotes Srk1 instability, we analyzed Srk1 protein levels in response to stress once it is phosphorylated by Sty1. Cells carrying the endogenous srk1 gene tagged with the HA epitope (srk1-HA) were treated with cycloheximide (100 µg/ml) and exposed to osmotic stress (0.8 M KCl). Samples were processed to monitor the level of Srk1 protein with anti-HA antibody (top) and quantified and normalized with the control level of Cdc2 protein (bottom) detected with anti-PSTAIR antibody.

**The Srk1 Phosphorylated at Threonine 463 Dissociates from Sty1**

The observations that Srk1 stability requires Sty1 binding and that the Srk1-T463D protein becomes unstable, suggest that the phosphorylation of Srk1 by Sty1 induces, in addition to activation, dissociation of both proteins. To determine whether phosphorylated Srk1 is separated from Sty1, we examined the binding between Sty1 and Srk1 phosphomutants. The Srk1, Srk1-T463D, and Srk1-T463A proteins were overexpressed and immunoprecipitated to test their binding to Sty1 by Western blot. As shown in Figure 4A, Srk1-T463D had less affinity than Srk1 and Srk1-T463A for Sty1 protein, indicating that Srk1 phosphorylation induces...
Sty1 dissociation. To confirm this observation, we performed the same experiment immunoprecipitating the Srk1, Srk1-T463D, and Srk1-T463A proteins from cells under stress conditions. Wild-type, ∆srk1 cells and cells containing srk1-T463D allele or srk1-T463A allele were plated on 0.8 M KCl media to induce osmotic stress. As shown in Figure 4D, cells carrying srk1-T463D were viable, whereas cells carrying srk1-T463A were sensitive to stress in the same way as the ∆srk1 cells were (Figure 4D). Furthermore, the kinase activity of purified Srk1 and the phospho-mutants, Srk1-T463A and Srk1-T463D was assayed in vitro. As shown in Figure 4E, the activity of Srk1-T463D was highest, whereas Srk1-T463A was as active as the wild-type Srk1. All these results indicate that it is not sufficient for the Srk1 kinase to be catalytically active; it has to be activated by Sty1 to respond to stress.

**Srk1 Activity Is Necessary for G2/M Inhibition in Stress Response**

In fission yeast, Srk1 negatively regulates the onset of mitosis (Lopez-Aviles et al., 2005). To determine whether Srk1 activation by Sty1 was to inhibit the G2/M transition after osmotic stress, we analyzed cell cycle progression of srk1-deleted cells under stress conditions. Wild-type and ∆srk1 were synchronized in S phase with HU and released in presence of KCl (0.6 M KCl). Both wild-type and ∆srk1 cells showed the same cell cycle progression pattern. They presented a temporal arrest before mitosis when exposed to osmotic stress (data not shown). We believe that the osmotic stress-signaling pathway, like the DNA damage-signaling pathway, controls two responses, one delaying the cell cycle and the other allowing response to stress. Thus, to determine the effect of ∆srk1 on the kinetics of cell cycle delay per se, experiments were done again in a genetic background that prevents adaptation to osmotic stress. Exposure of yeast to increases in external osmolarity induces synthesis and activation of the Atf1 transcription factor responsible for the synthesis of several genes necessary for the response to stress and adaptation (Shiozaki and Russell, 1996; Wilkinson et al., 1996). Therefore, the atf1 gene was deleted to avoid immediate adaptation. The atf1 deleted cells (Δatf1) and the double mutant Δatf1Δsrk1 were synchronized in S phase with HU and released in presence of 0.6 M KCl, as described above. We observed that Δatf1 showed a delay in G2/M transition, as described for wild-type cells (Figure 5A), whereas the double mutant progressed throughout the cell cycle, albeit to a lesser extent than in unstressed conditions (Figure 5A). This result indicates that Srk1 is necessary for inhibiting G2/M transition in the presence of osmotic stress.

Srk1 kinase activity was assayed from cells bearing an endogenously tagged Srk1 exposed to different doses of osmotic stress (0.6 and 0.8 M KCl) and using a phosphorylatable fragment of Cdc25 (Cdc2556-145) as a substrate (Figure 5B). As expected, Srk1 was dose-dependent activated by osmotic stress and able to phosphorylate Cdc25. The same assay was performed in cells bearing the catalytic-inactive Srk1 (Srk1-KA) to confirm that Cdc25 phosphorylation is due to Srk1 (Figure 5B).

Next, we studied the sensitivity to osmotic stress of cells containing the cdc25-9A allele (nine consensus phosphorylatable sites for Srk1 are mutated to alanine), which express a Cdc25-9A protein unable to be phosphorylated by Srk1 (Lopez-Aviles et al., 2005). The cdc25-9A cells showed some sensitivity to grow on medium containing 0.8 M KCl than wild-type cells, but not as sensitive as cells lacking Srk1 or Sty1 MAP kinase (Figure 4D).

**Cdc25 Nuclear Export Is Dependent on Srk1 under Stress Conditions**

Cdc25 phosphatase is a direct substrate of Srk1 (Lopez-Aviles et al., 2005). Srk1 phosphorylates and promotes the

---

**Figure 4 (cont).** Preincubated with Sty1-KA were assayed with the fragment of Cdc25 (Cdc2556-145) as a substrate (top). SDS-PAGE gels from the kinase assay were stained with Coomassie blue (bottom). (D) Serial dilution of log phase cultures of wild-type, ∆atf1, Δsrk1, cdc25-9A, srk1-T463D, and srk1-T463A. Cells were plated in rich medium (YES) and rich medium containing KCl 0.8 M (YES + 0.8 M KCl). Colony formation was analyzed after 2 days of growth at 30°C. (E) In vitro kinase assay from Strk1 purified phospho-mutants. GST-Srk1, -srk1-T463A, and -srk1-T463D were assayed and auto-phosphorylation was measured (top). SDS-PAGE gels from the kinase assay were stained with Coomassie blue (bottom).

**Figure 5.** The G2/M delay caused by osmotic stress needs Srk1 protein. (A) The Δatf1 and the double mutant Δatf1Δsrk1 cells were synchronized with HU and released in presence or absence of 0.6 M KCl. The seption index, an indication of the percentage of cells that have completed mitosis, was measured at different times corresponding to minutes after release from HU and addition of KCl. (B) Srk1 activated in response to stress phosphorylates Cdc25. Cells were exposed to different doses of KCl (0.6 or 0.8 M) for 15 min and Srk1 (left panels) or Srk1-KA (right panels) were immunoprecipitated with anti-HA antibody (top), and its kinase activity was assayed using the Cdc25 fragment (GST-Cdc2556-145) as a substrate (bottom).
cytosolic localization of Cdc25 and consequent mitotic arrest. Therefore, we analyzed whether the subcellular localization of Cdc25 during stress response depended on Srk1. Wild-type and Δsrk1 cells carrying the endogenous cdc25 gene tagged with the GFP, Cdc25-GFP, were synchronized in S phase with HU, released and exposed to KCl (0.6 M KCl; Supplementary Figure 2A). Cdc25-GFP localization was imaged in living cells by time-lapse confocal microscopy. In wild-type cells, we found that Cdc25 localization was already cytosolic 10 min after osmotic stress, and this was maintained in the cytosol up to 40 min after the osmotic stress exposure (Figure 6A; Movie 6A and Supplementary Figure 2B). In contrast, in srk1-deleted cells, Cdc25 was generally found in the nucleus during the entire period of osmotic stress (Figure 6B; Movie 6B and Supplementary Figure 2B). Therefore, we can conclude that Cdc25 protein is exported from the nucleus under stress conditions in a Srk1-dependent manner.

**DISCUSSION**

Activation of the Sty1 MAPK is a key step for the generation of adaptive responses that allow cell survival to stress. Modulation of cell cycle progression is essential for adaptation to stress. Here we show the multiple effect of Srk1 phosphorylation by the MAPK Sty1 during stress response, and we present the Srk1 kinase as a component that coordinates stress response and cell cycle control.

**Multiple Effect of Srk1 Phosphorylation by the MAPK Sty1**

Srk1 kinase was identified as a protein kinase whose expression is up-regulated upon exposure to stress in a Sty1-dependent manner. Srk1 is present in a complex with Sty1 and is directly phosphorylated by MAPK (Smith et al., 2002). We observed that Srk1 stability is dependent on the presence of the Sty1 protein and independent of Sty1 activity. Interestingly, similar observations have been made in mammalian cells for MK2 (the human homolog of Srk1) and p38 MAPK. Both proteins bind and form a stable complex. Elimination of either of the two proteins reduces the remaining partner’s level (Kotlyarov et al., 2002). The effect of Sty1 on Srk1 protein stability can also be attributed to the regulation of srk1 transcription. However, Srk1 disappears in parallel with Sty1 protein degradation, as shown in Figure 2C, and the Srk1 degradation can be reverted by the addition of a proteasome inhibitor (Figure 2E). Moreover, phosphorylation at threonine 463 by activated Sty1 induces both Srk1 activation and protein instability, as observed from the low level of the endogenous Srk1-T463D protein and its recovery by the addition of a proteasome inhibitor (Figure 3A). These results indicate that Srk1 stability requires Sty1 binding and moreover, the Srk1 phosphorylated protein (Srk1-T463D) becomes unstable. Thus, the phosphorylation of Srk1 by Sty1 induces, in addition to activation, the dissociation of the Srk1-Sty1 protein complex and the destabilization of the Srk1. In support of this hypothesis, Srk1-T463D showed less affinity to Sty1 protein than was shown by unphosphorylated Srk1 and Srk1-T463A (Figure 4A).

Sty1 phosphorylates and activates Srk1 at threonine 463 (Figures 1, B and E, and 4E). However, it seems that phosphorylation at threonine 463 activates Srk1, because it allows Srk1 to separate from Sty1. Thus, dissociation from Sty1 is sufficient to activate Srk1. This conclusion is supported by the observation of Srk1 phospho-mutants activities. Srk1-T463A is catalytically active in a kinase assay in vitro at a level similar to that of Srk1 (Figure 4E). All the same, the in vitro Srk1 activity can be inhibited by allowing Srk1 binding to Sty1 (Figure 4C). Nevertheless, analysis of the in vivo effect of the Srk1 phospho-mutants showed that mutation of the threonine 463 to alanine (Srk1-T463A) makes cells sensitive to osmotic stress similarly to srk1 deletion. This indicates that the catalytic activity of Srk1-T463A is not sufficient for Srk1 function in stress response and supports the fact that threonine 463 has to be phosphorylated to activate Srk1 function in stress response. Collectively, these observations point to an additional mechanism of Srk1 regulation by Sty1.

**Srk1 Kinase as a Component That Coordinates Stress Response and Cell Cycle Control**

Significantly, Srk1 is rapidly and highly activated in response to stress. In a previous study, we demonstrated that the Srk1 kinase is activated during G2/M transition of the cell cycle (Lopez-Aviles et al., 2005). However, the cell cycle activity of Srk1 is lower than its activity detected at stress response. This is also reflected in the phenotype of srk1-deleted cells, which showed a semi- wee phenotype (Lopez-Aviles et al., 2005). The simplest interpretation of these observations is that Srk1 activity has a short requirement during a normal cell cycle, where its function is to maintain the inhibited status of Cdc25 phosphatase emerging during G2 phase. Therefore, low activity of Srk1 is sufficient to inhibit the Cdc25 protein expressed throughout the G2 phase. On the other hand, in stress response, as the function of Srk1 is essential to maintain cell integrity, its activation has to be high to guarantee the correct response.

Furthermore, upon stress, Srk1 translocates from the cytoplasm to the nucleus in a process that is dependent on the Sty1 MAPK (Smith et al., 2002). We showed that, 10 min after osmotic stress, the Cdc25 protein translocates to the cytoplasm, concurrent with Sty1 and Srk1 activation. Significantly, this nuclear export depends on the Srk1 kinase activity. This observation also agrees with our previous demonstration that the association between Cdc25 and Rad24 is highly induced under stress conditions (Lopez-Aviles et al., 2005). Thus, in response to stress, Srk1 controls G2/M transition by phosphorylating Cdc25 and promoting its binding to Rad24 and its subsequent translocation to the cytoplasm.

In fission yeast, stress induces a temporal arrest at the G2/M transition, which, it has been reported, was not dependent on the DNA-damage checkpoint or mitotic spindle checkpoint (Kawasaki et al., 2006). The results shown here indicate that the temporal G2/M arrest of the stress response is dependent on Srk1 kinase. Moreover, Δsrk1 cells are sensitive to osmotic stress similar to cells bearing cdc25-9A allele, which is unable to be phosphorylated by Srk1, strengthening the role of Srk1 regulating the cell cycle in stress conditions through Cdc25 protein. Of note, Δsrk1 cells are more sensitive than cdc25-9A, suggesting an additional role for Srk1 in stress response. To examine the effect ofSrk1 protein on the kinetics of the cell cycle after osmotic stress, we used a mutant that was defective in its stress adaptation response, Δatf1. The assumption behind this approach was that osmotic stress-signaling pathways control two responses, one delaying the cell cycle and the other allowing response and adaptation to stress. This idea is consistent with similar studies done in S. cerevisiae (Alexander et al., 2001), where the glycerol-based osmoregulation mutants were used to analyze the role of Sse1 and Hoq1 in cell cycle regulation after hyperosmotic stress. Taken together, the results indicate that Srk1 is not part of the rapid stress-adaptation response, but that it is necessary to coordinate cell cycle progression under stress.
It has been previously reported that Srk1 is regulated by Sty1 with two different mechanisms, transcriptional and posttranscriptional (Smith et al., 2002). As shown here, in response to stress, Sty1 regulates the activity of Srk1 on cell cycle control by phosphorylation on threonine 463. Moreover, the expression of srk1 gene is up-regulated in response to stress dependent of Sty1 (Smith et al., 2002). We do not yet understand the physiological meaning of Sty1 inducing Srk1

![Figure 6](image-url)
instability and degradation in addition to activation, while concomitantly promoting srk1 transcription-activation. A simple explanation as to why Sty1 induces Srk1 degradation might be to ensure Srk1 activity in cell-cycle control, within a narrow window. Thus, the Sty1-dependent activation of srk1 transcription might serve to reestablish Srk1 levels, which are constant throughout the cell cycle (Lopez-Aviles et al., 2005).

Overall, Srk1 is present in a complex with Sty1 in un-stressed conditions, whereas stress-activated Sty1 kinase phosphorylates Srk1 at threonine 463 inducing the dissociation of Srk1 and Sty1 proteins, which appears to be necessary for the full activation of Srk1 (Figure 7). As a result, phosphorylation at threonine 463 leads to Srk1 activation and the inhibition of cell cycle progression through the Cdc25 cytoplasmic translocation. The single phosphorylation of Srk1 turns the Srk1 protein unstable and promotes its degradation (Figure 7), whereas active Sty1 kinase activates transcription of CESR (core environmental stress response) genes.

REFERENCES


