The Rho1p Exchange Factor Rgf1p Signals Upstream from the Pmk1 Mitogen-activated Protein Kinase Pathway in Fission Yeast

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The *Schizosaccharomyces pombe* exchange factor Rgf1p specifically regulates Rho1p during polarized growth. Rgf1p activates the β -glucan synthase (GS) complex containing the catalytic subunit Bgs4p and is involved in the activation of growth at the second end, a transition that requires actin reorganization. In this work, we investigated Rgf1p signaling and observed that Rgf1p acted upstream from the Pck2p-Pmk1p MAPK signaling pathway. We noted that Rgf1p and calcineurin play antagonistic roles in Cl^- homeostasis; *rgf1* Δ cells showed the *vic* phenotype (viable in the presence of immunosuppressant and chlorine ion) and were unable to grow in the presence of high salt concentrations, both phenotypes being characteristic of knockouts of the MAPK components. In addition, mutations that perturb signaling through the MAPK pathway resulted in defective cell integrity (hypersensitivity to caspofungin and β -glucanase). Rgf1p acts by positively regulating a subset of stimuli toward the Pmk1p-cell integrity pathway. After osmotic shock and cell wall damage HA-tagged Pmk1p was phosphorylated in wild-type cells but not in *rgf1* Δ cells. Finally, we provide evidence to show that Rgf1p regulates Pmk1p activation in a process that involves the activation of Rho1p and Pck2p, and we demonstrate that Rgf1p is unique in this signaling process, because Pmk1p activation was largely independent of the other two Rho1p-specific GEFs, Rgf2p and Rgf3p.

INTRODUCTION

Fission yeast cells display a simple rod shape; after cytokinesis, growth is initiated monopolarly and occurs exclusively at the old end. After a point in G2, cells initiate growth from the new end in a process known as new end take-off (NETO), such that they grow in bipolar mode up to mitosis (Mitchison and Nurse, 1985). The spatial control of cell growth in the fission yeast *Schizosaccharomyces pombe* involves organization of the microtubule and actin cytoskeletons as well as that of the cell wall synthesis (Chang, 2001; Hayles and Nurse, 2001). A polarized actin cytoskeleton targets secretion to the growth sites, where probably specialized multienzyme complexes, consisting of both synthases and hydrolases, assemble the cell wall during the cell cycle (Motegi *et al.*, 2001; Win *et al.*, 2001; Mulvihill *et al.*, 2006). Both the cytoskeleton and the cell wall are dynamic structures that are constantly remodelled and reorganized in response to growth signals and environmental stresses in order to ensure the integrity of the yeast cell (Levin, 2005; Madrid *et al.*, 2006).

In mammals, the polarized assembly of the actin and microtubule cytoskeletons is regulated by site-specific activation of Rho-type GTPases (Jaffe and Hall, 2005; Ridley, 2006). In fission yeast cells, Rho1p is involved in cell wall synthesis (Arellano *et al.*, 1996), actin organization (Arellano *et al.*, 1997; Nakano *et al.*, 1997), stress responses, and exo-

cytosis. In both systems, one of the challenges is to figure out how the activity of Rho1p is regulated to control these different processes. Guanine nucleotide exchange factors (GEFs) activate signaling by promoting the exchange of GDP by GTP, and GTPase-activating proteins (GAPs) arrest signaling by stimulating GTP hydrolysis to GDP. This network (Rho GTPases, GEFs, and GAPs) relays a surprisingly large number of diverse extracellular signals to many morphological and functional responses. The regulation of Rho1p in fission yeast involves at least three GEFs (Rgf1p, Rgf2p, and Rgf3p) and several putative GAPs (Rga1p, Rga5p, and Rga8p). *rgf3*⁺ is an essential gene and the protein specifically activates Rho1p during cytokinesis (Tajadura *et al.*, 2004; Morrell-Falvey *et al.*, 2005; Mutoh *et al.*, 2005). Rgf1p and Rgf2p are the closest relatives, and they provide a redundant function for the activation of Rho1p (Mutoh *et al.*, 2005). Loss of Rgf1p function produces cell lysis, whereas loss of both Rgf1p and Rgf2p is lethal. In addition, Rgf2p may perform an essential function during the sporulation process (García *et al.*, 2006b). Among the negative regulators, none of them is essential for cell viability, although deletion of *rga1*⁺ causes a slow-growth defect and severe morphological abnormalities. Rga5p is involved in the regulation of GS activity and cell integrity, and Rga8p is a Shk1p (Cdc42/p21-activated kinase) substrate that negatively regulates Shk1p-dependent growth control pathways (Calonge *et al.*, 2003; Yang *et al.*, 2003).

In this study we focused on Rgf1p. Like most Rho-GEFs, Rgf1p contains a domain with strong similarity to the Dbl-family of exchange factors (residues aa 625–807, Dbl-homology domain [DH]) and a nearby pleckstrin-homology (PH) domain (residues 844–973). The DH-PH tandem is responsible for the activation of Rho-family GTPases in response to

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diverse extracellular stimuli (reviewed in Rossman *et al.*, 2005; Rossman and Sondek, 2005). Rgf1p also contains a *Dishevelled*, Egl-10, and pleckstrin (DEP) domain at its amino-terminus (residues 424–497), which in some G-protein receptors has been implicated in mediating the nature and sustainability of the response (Chen and Hamm, 2006). Finally, Rgf1p has a Citron and NIK1-like kinase homology-domain (CNH) at its carboxy-terminus (residues 997–1293). Its function is not clear, but in most cases it acts as a regulatory domain involved in macromolecular interactions (<http://www.genedb.org/genedb/pombe/index.jsp>).

We previously demonstrated that Rgf1p plays an important role in regulating the growth pattern of fission yeast cells. After cell division, cells initially grow in a monopolar manner, after which they initiate polarized growth at the second end in the G2 phase of the cell cycle. This transition to bipolar growth, termed NETO, relies on the localization of Rgf1p to the new end (García *et al.*, 2006a). In this process, the position of Rgf1p depends on Tea1p, which is necessary for NETO. Interestingly, although other mutants defective in bipolar growth, *tea1Δ*, *tea4Δ*, and *bud6Δ*, grow at wild-type rates, a novel aspect of the *rgf1Δ* is that its growth rate and viability are compromised. In *rgf1Δ* cells, failure to initiate bipolar growth coincides with cell lysis, thus coupling a growth polarity transition with cell wall biosynthesis. Our current model is that activation of Rho1p during bipolar growth is not achieved properly in *rgf1Δ* mutants, producing cell wall weakness. Rho1p localizes to sites of polarized growth and participates directly in the production of new cell wall by functioning as the regulatory subunit of the β -1,3-glucan synthase (GS) that synthesizes glucan, the main component of the cell wall (Arellano *et al.*, 1996, 1997; Nakano *et al.*, 1997). However, it has not been tested whether Rho1p also regulates the expression of cell integrity-related genes or actin genes via the Pmk1p mitogen-activated protein kinase (MAPK) cell integrity signaling pathway.

The Pmk1p MAPK from fission yeast is very similar to the budding yeast Mpk1p/Slt2p, which plays a central role in cell integrity signaling (Levin, 2005), and to the extracellular signal-regulated kinases, ERK1/2 (p42/p44), from animal cells that are activated by phorbol esters, cytokines, or osmotic stress (Roux and Blenis, 2004). In fission yeast, the Pmk1p MAPK pathway also regulates morphogenesis and ion homeostasis and becomes activated under multiple stresses, including hyper- or hypotonic conditions, the presence of cell wall-damaging compounds, glucose deprivation, and oxidative stress (Madrid *et al.*, 2006). The MAPK module is composed of MAPKKK Mkh1p (Sengar *et al.*, 1997), MAPKK Pek1p/Shk1p (Sugiura *et al.*, 1999; Loewith *et al.*, 2000), and MAPK Pmk1p/Spm1p (Toda *et al.*, 1996b; Zaitsevska-Carter and Cooper, 1997). Although this module was identified several years ago, little is known about its upstream components or the phosphorylation substrates activated by the cell integrity signaling pathway (Ma *et al.*, 2006; Takada *et al.*, 2007). The MAPKKs at the head of the module are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli (Roux and Blenis, 2004). In fission yeast, Rho1p binds directly to the Pck1p and Pck2p protein kinases of the PKC family, and it functions as a positive regulator for these kinases (Arellano *et al.*, 1999b; Sayers *et al.*, 2000). Recently it has been shown that Pck2p interacts with Mkh1p (MAPKKK) and activates the Pmk1p signaling pathway (Ma *et al.*, 2006). Here, we show that Rgf1p, a Rho1p-specific GEF, acts upstream from the Pck2-Pmk1p MAPK cell integrity signaling pathway. More importantly, our results suggest that

Rgf1p is necessary for the signal transduction of a subset of stimuli, in particular those related to changes in osmolarity and cell wall damage.

MATERIALS AND METHODS

Media, Reagents, and Genetics

The genotypes of the *S. pombe* strains used in this study are listed in Table 1. The complete yeast growth medium (YES), selective medium (MM) supplemented with the appropriate requirements, and sporulation medium (MEA) have been described elsewhere (Moreno *et al.*, 1991). Caspofungin (Csp; Vicente *et al.*, 2003) was stored at [minus]20°C in a stock solution (2.5 mg/ml) in H₂O and was added to the media at the corresponding final concentration after autoclaving. Crosses were performed by mixing appropriate strains directly on MEA plates. Recombinant strains were obtained by tetrad analysis or the “random spore” method. For overexpression experiments using the *nmt1* promoter, cells were grown in EMM containing 15 μ M thiamine up to logarithmic phase. Then, the cells were harvested, washed three times with water, and inoculated in fresh medium (without thiamine) at an OD₆₀₀ = 0.01 for 14, 16, or 18 h, depending on the experiment.

Plasmid and DNA Manipulations

pREP3X contains a thiamine-repressible *nmt1* promoter (full-strength, induction ratio, 300 $\times$), *Saccharomyces cerevisiae* LEU2, and *ars1*⁺ (Forsburg, 1993); to overexpress *rgf1*⁺, an XhoI-SmaI fragment containing the *rgf1*⁺ gene was ligated into the XhoI-SmaI sites of plasmid pREP3X (García *et al.*, 2006a). pREP3X-*pck2*⁺ (*nmt1-pck2*⁺) was made by inserting the entire ORF of *pck2*⁺ cloned by PCR into pREP3X. pREP3X-*pck1* (*nmt1-pck1*⁺) was made by inserting the entire ORF of *pck1*⁺ cloned by PCR into pREP3X. pREP41X-*pck2* (*nmt41x-pck2*) was made by inserting the entire ORF of *pck2*⁺ cloned by PCR into pREP41X (induction ratio, 25 $\times$; Arellano *et al.*, 1999b). For pREP3X-*rho1*, the ORF of Rho1p was amplified by PCR from a cDNA library and cloned into pREP3X; for pREP3X-*rho1*-G15V, the *rho1* ORF was mutagenized by site-directed mutagenesis and subcloned as a SalI-BamHI fragment into the same sites of pREP3X (Arellano *et al.*, 1997). *pck2*⁺ and *rho1*⁺ overexpression plasmids were kindly provided by P. Pérez (IMB, Salamanca, Spain). To make pART-*spm1*⁺, the Spm1p ORF was cloned by PCR behind the *adh* promoter in pART1. pART-*spm1*⁺ was kindly provided by J. Cooper (Washington University, St. Louis; Zaitsevska-Carter and Cooper, 1997).

Glucanase Sensitivity

The glucanase sensitivities of several mutant strains were determined as indicated in Carnero *et al.* (2000). Cells were grown in MM to an optical density at 600 nm (OD₆₀₀) of 1.0, washed in 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, and 1 mM β -mercaptoethanol, and incubated in the same buffer containing 20 μ g/ml β -glucanase (Zymolyase 100T; Seikagaku, Tokyo, Japan) per ml at 28°C with vigorous shaking. The OD₆₀₀ was monitored at the indicated times and was normalized relative to the absorbance of a control sample of each strain without enzyme at each time point.

Stress Treatments

Experiments designed to investigate Pmk1p activation under stress were performed using log-phase cell cultures (OD₆₀₀ of 0.5) grown at 28°C in YES medium and the appropriate stress treatment. In overexpression experiments, cells were first grown in MM medium plus thiamine, washed three times, and reinoculated into fresh medium (with or without thiamine) for 14, 16, or 18 h at 28°C, depending on the protein to be overexpressed. In glucose-deprivation experiments, cells were grown in YES medium with 7% glucose to an OD₆₀₀ of 0.5, recovered by filtration, and resuspended in the same medium without glucose but equilibrated osmotically with 3% glycerol. Hypotonic treatment was achieved by growing cells in YES medium plus 0.8 M sorbitol and then transferring them to the same medium without polyol. In all cases, 30 ml of culture was harvested by filtration and immediately frozen in liquid nitrogen for analysis.

Purification and Detection of Activated Pmk1p-HA6H and Sty1p-HA6H after Different Stresses

Cell homogenates were prepared under native conditions employing chilled acid-washed glass beads and lysis buffer (10% glycerol, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Nonidet P-40, plus a specific protease inhibitor cocktail: 100 μ M *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). The lysates were cleared by centrifugation at 13,000 $\times$ g for 10 min and Pmk1-HA6H was purified with Ni²⁺-NTA-agarose beads (Novagen, Madison, WI). The purified proteins were loaded on 10% SDS-PAGE gels, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and blotted to detect Pmk1-HA with 1:5000 diluted 12CA5 mAb as primary antibody (Roche, Indianapolis, IN), with polyclonal rabbit anti-phospho-p42/44 antibodies (1:2500; Cell Signaling, Beverly, MA), or with polyclonal rabbit anti-phospho-p38 antibodies (1:8000; Cell Signaling). The immunoreactive bands

Table 1. *S. pombe* strains used in this work

Strains	Genotypes
YSM180	h ⁻ 972
YS64	h ⁻ leu1-32 ade6M210 ura4D-18 his3D1
HVP54 ^a	h ⁻ leu1-32 ade6M210 ura4D-18
VT14	h ⁻ leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3 ⁺
VT18	h ⁺ leu1-32 ade6M210 ura4D-18 his3D1rgf1::his3 ⁺
PG65	h ⁻ leu1-32 ade6M210 ura4D-18 his3D1rgf1::kanMX6
MI200 ^b	h ⁺ pmk1-HA6his: ura4 ⁺ ade6M216 leu1-32 ura4d18
PG285	h ⁻ rgf1::kan pmk1-HA6his:ura ⁺ ura4D18 leu1-32 ade6M210
PG318	h ⁻ mkh1::ura4 ⁺ pmk1-HA6his:ura ⁺ ura4D18 leu1-32 ade6M210
PG332	h ⁻ pck2::kan pmk1-HA6his:ura ⁺ ura4D18 leu1-32
MS192 ^c	h ⁻ spm1::LEU2 leu1-32 ura4D18
MS189 ^d	h ⁺ skh1::ura4 ⁺ ura4D18 leu1-32 ade6M210
MS196 ^d	h ⁺ mkh1::ura4 ⁺ ura4D18 ade6M210
YKOB01 ^e	h ⁻ spm1::ura4 ⁺ ura4D18 leu1-32
PG335	h ⁻ spm1::ura4 ⁺ rgf1::kan ura4d18
PG337	h ⁻ mkh1::ura4 ⁺ rgf1::kan ura4d18
PG339	h ⁻ skh1::ura4 ⁺ rgf1::kan ura4d18
PG347	h ⁻ pmk1::ura4 ⁺ ura4d18
PG348	h ⁻ mkh1::ura4 ⁺ ura4d18
PG349	h ⁻ skh1::ura4 ⁺ ura4d18
PG77	h ⁺ rgf1::kan ade6M210
PG272 ^f	h ⁻ pck2::kan leu1-32 ura4D18
VT20 ^f	h ⁻ rho2::ura4 ⁺ ura4D18 leu1-32 ade6M210
PG1	h ⁻ rgf2::ura4 ⁺ ura4D18 leu1-32 ade6M210 his3D1
MS190 ^h	h ⁻ wis1::his3 ⁺ his3D1
MS194 ^h	h ⁻ spk1::ura4 ⁺ ura4D18 leu1-32
YS1295 ^f	h ⁺ pmp1::kan leu1-32 ade6M210 ura4D-18
PG351	h ⁻ pmp1::kan rgf1::kan leu1-32 ade6M210 ura4D-18
KS1489 ^g	h ⁺ spc1-HA6his:ura4 ⁺ leu1-32 ura4D-18
PG362	h ⁻ spc1-HA6his:ura4 ⁺ rgf1::kan leu1-32 ura4D-18
PG40	h ⁻ rgf1::his3 his3DI leu1-32 ade6M210 ura4D-18 leu1: rgf1 ⁺ -GFP
PG52	h ⁻ rgf1::his3 his3DI leu1-32 ade6M210 ura4D-18 leu1: rgf1 ⁺ (PTTRΔ)
PG199	h ⁻ rgf1::his3 his3DI leu1-32 ade6M210 ura4D-18 leu1: rgf1 ⁺ (PTTRΔ)-GFP
PPG160 ^f	h ⁻ rho1:HA leu1-32
PG378	h ⁻ rho1:HA leu1-32rgf1::kanMX6
PG380	h ⁻ rho1:HA leu1-32rgf1::kanMX6 leu1: rgf1 ⁺ (PTTRΔ)
PG287	h ⁻ pmk1-HA6his: ura4 ⁺ ade6M216 leu1-32 ura4d18 rgf2::ura4 ⁺
PG359	h ⁻ ehs2-1 pmk1-HA6his: ura4 ⁺ leu1-32 ura4d18
PG331	h ⁻ pmk1-HA6his: ura4 ⁺ leu1-32 ura4d18 his3D1rgf1::his3 ⁺ leu1: rgf1 ⁺ (PTTRΔ)

All strains were generated in this study except for the following strains: ^a from H. Valdivieso (IMB, University of Salamanca), ^b from J. Cansado (University of Murcia), ^c from J. Cooper (Washington University, St. Louis), ^d from D. Young (University of Calgary, Canada), ^e from C. R. Vazquez de Aldana (IMB, University of Salamanca), ^f from P. Perez (IMB, University of Salamanca), ^g from M. A. Rodriguez-Gabriel (UCM, University Complutense Madrid), and ^h from S. Moreno (CIC, University of Salamanca).

were revealed with anti-mouse or anti-rabbit HRP secondary antibodies (Bio-Rad, Hercules, CA) and the enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ).

Pulldown Assays for GTP-bound Rho Proteins

The expression vector pGEX-C21RBD (rho-kinase-binding domain; Reid *et al.*, 1996) was used to transform *Escherichia coli* cells. The fusion protein was produced according to the manufacturer's instructions and immobilized on glutathione-Sepharose 4B beads (Amersham). After incubation, the beads were washed several times, and the bound proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. The amount of GTP-bound Rho proteins was analyzed using the Rho-GTP pulldown assay modified from Ren *et al.* (1999). Briefly, extracts from 50-ml cultures of wild-type, *rgf1Δ*, and *rgf1-PTTRΔ* cells containing HA-*rho1*⁺ expressed from its own promoter were obtained using 200 μl of lysis buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM dithiothreitol, 1 mM NaCl, 2 mM MgCl₂, containing 100 μM *p*-aminophenyl methanesulfonyl fluoride, leupeptin, and aprotinin). GST-RBD fusion protein, 100 μg, coupled to glutathione-agarose beads was used to immunoprecipitate 1.5 mg of the cell lysates. The extracts were incubated with GST-RBD beads for 2 h. The beads were washed with lysis buffer four times, and bound proteins were blotted against 1:5000-diluted 12CA5 mAb as primary antibody to detect HA-Rho1p. The total amount of HA-Rho1p was monitored in whole-cell extracts (25 μg of total protein), which were used directly for Western blotting and were developed

with the 12CA5 mAb. Immunodetection was accomplished using the ECL detection kit (Amersham Biosciences).

Microscopy Techniques

The localization of Rgf1p-green fluorescent protein (GFP) was visualized in living cells under a DMRXA microscope (Leica, Wetzlar, Germany).

RESULTS

Pck2p Acts Downstream from Rgf1p

Previous studies have shown that Rgf1p is a Rho1p GEF (García *et al.*, 2006a). The Rgf1p deletion causes cell lysis, hypersensitivity to the antifungal drug Csp, and defects in the establishment of bipolar growth (García *et al.*, 2006a). Regarding the downstream targets of Rgf1p, we wondered whether the overexpression of either Pck1p or Pck2p might suppress hypersensitivity to Csp in the *rgf1Δ* mutant. In fission yeast, the protein kinase C homologues Pck1p and Pck2p are targets for Rho1p; both genes—*pck1*⁺ and *pck2*⁺—share overlapping roles in cell viability and partially com-

Figure 1. Pck2p acts downstream from Rgf1p. The Csp-hypersensitive growth phenotype of *rgf1Δ* mutants is suppressed by overexpression of *pck2*⁺. HVP54 (*rgf1*⁺) was transformed with pREP3X (empty vector) and VT14 (*rgf1Δ*) was transformed with pREP3X (empty vector), pREP3X-*pck1* (3X*pck1*⁺), pREP3X-*pck2* (3X*pck2*⁺), and pREP41x-*pck2* (41X*pck2*⁺). Transformants were spotted onto MM and MM plus 2 μg/ml caspofungin (Csp) plates as serial dilutions (8 × 10⁴ cells in the left row and then 4 × 10⁴, 2 × 10⁴, 2 × 10³, and 2 × 10² in each subsequent spot) and incubated at 28°C for 3 d.

plement each other (Toda et al., 1993). Pck2p plays a role in the regulation of the two main polymers of the cell wall, β- and α-glucans, whereas the function of Pck1p in cell integrity is not so well known (Arellano et al., 1999b; Calonge et al., 2000). To this end, the *rgf1Δ* strain (VT14) was transformed with the overexpression plasmids pREP3X-*pck1*⁺ and pREP3X-*pck2*⁺ (Arellano et al., 1999b), and the transformants were monitored for growth on Csp. Figure 1 shows that *pck2*⁺, but not *pck1*⁺, expressed from the plasmid containing the high-strength *nmf1* promoter (pREP3X) partially rescued the lysis and Csp hypersensitivity of the *rgf1Δ* cells. We realized that the strong overexpression of *pck2*⁺ in *rgf1Δ* mutant cells could be deleterious (Mazzei et al., 1993), so to avoid this problem we used *pck2*⁺ driven by the *P41nmf* promoter (medium level); we found a much better complementation of the hypersensitivity to Csp (Figure 1).

Cells Lacking *rgf1* Show the *vic* Phenotype

It has recently been shown that Rho2p and Pck2p act upstream from the Pmk1p MAPK signaling pathway, thereby resulting in the *vic* (viable in the presence of immunosuppressant FK506 and chloride ion) phenotype upon mutation (Ma et al., 2006). Because Rgf1p was linked to Pck2p, which in turn has been linked to the MAPK pathway, we decided to examine the functional relationship between Rgf1p and Pmk1p signaling by analyzing whether the *rgf1Δ* mutants showed the *vic* phenotype. The results clearly showed that *rgf1Δ*, as well as *pmk1Δ*, and *pek1Δ*, cells grew in the presence of FK506 and 0.2 M MgCl₂, whereas the wild-type cells were unable to grow in the same conditions (Figure 2A). It is known that calcineurin mutants (*ppb1Δ*) cannot grow in the presence of MgCl₂, whereas an additional mutation in a member of the MAPK pathway suppresses that phenotype. The addition of FK506 (a calcineurin-specific inhibitor) to wild-type cells mimics the calcineurin deletion and prevents growth in the presence of MgCl₂. The fact that the *rgf1Δ* mutants were able to grow in these conditions indicates that Rgf1p could play an antagonistic role for calcineurin mutants and is thus a strong candidate component of the Pmk1p-MAPK signaling pathway.

We further investigated the relationship between Rgf1p and the MAPK integrity pathway and we found that *rgf1Δ* cells shared other phenotypes with *pmk1Δ* mutants. *rgf1Δ* cells, like *pmk1Δ* cells, were hypersensitive to β-glucanase treatment (Figure 2B; Toda et al., 1996b; Sengar et al., 1997), and their growth was inhibited by high salt concentrations, a phenotype characteristic of the knockouts of the *pmk1*⁺, *mkh1*⁺, and *pek1*⁺ genes (Figure 2C). Moreover, we found that *rgf1Δ* cells required a significantly longer period of time to reenter the cell cycle upon reinoculation into fresh medium after prolonged stationary phase arrest compared with

Figure 2. Knockout of *rgf1*⁺ and the components of the Pmk1 MAPK pathway exhibits the *vic* phenotype, sensitivity to high osmolarity, and caspofungin (Csp) hypersensitivity. (A) *rgf1Δ* cells show the *vic* phenotype. Wild-type (YS64), *rgf1Δ* (VT14), *pmk1Δ* (MS192), and *pek1Δ* (MS189) cells were streaked onto YES or YES plus 0.5 μg/ml FK506 and 0.2 M MgCl₂ and incubated at 32°C for 3 d. (B) Sensitivity of *rgf1Δ* and *pmk1Δ* to β-glucanase. Cell lysis was measured at different times during treatment with β-glucanase by determining the OD₆₀₀ (see Materials and Methods). The strains examined were wt (YS64), *rgf1Δ* (VT14), and *pmk1Δ* (MS192). (C) Growth inhibition of *rgf1Δ*, *pmk1Δ*, and *mkh1Δ* mutants by KCl. Wild-type (YS64), *rgf1Δ* (VT14), *pmk1Δ* (MS192), and *mkh1Δ* (MS196) strains were grown in MM or in MM plus 1.4 M KCl for 5 d at 32°C. (D) Knockout of the components of the Pmk1 MAPK pathway (*pmk1*⁺, *mkh1*⁺, *pek1*⁺, *pck2*⁺, and *rho2*⁺) elicited Csp hypersensitivity. Wild-type (YS64), *wis1Δ* (MS190), *spk1Δ* (MS194), *pmk1Δ* (MS192), *pek1Δ* (MS189), *mkh1Δ* (MS196), *pck2Δ* (PG272), *rho2Δ* (VT20), *rgf1Δ* (VT14), and *rgf2Δ* (PG1) were streaked onto YES or YES plus 1 μg/ml Csp (CANCIDAS) and incubated at 32°C for 3 d.

Figure 3. The Csp-hypersensitive growth phenotype of *rgf1Δ* mutants is suppressed by overexpression of *pmk1*⁺. (A) VT14 (*rgf1Δ*) was transformed with pART1 (empty vector), *p41Xpck2* (*pck2*⁺), and *pARTpmk1* (*pmk1*⁺; Zaitsevskaya-Carter and Cooper, 1997). Transformants were spotted onto MM and MM plus 1 μg/ml Csp plates as serial dilutions (8×10^4 cells in the left row and then 4×10^4 , 2×10^4 , 2×10^3 , and 2×10^2 in each subsequent spot) and incubated at 28°C for 3 d. (B) *rgf1Δmkh1Δ*, *rgf1Δpek1Δ*, or *rgf1Δpmk1Δ* did not show synergism in sensitivity to Csp. Equal numbers of wild-type (YSM180) and *rgf1Δ* (PG77), *mkh1Δ* (PG348), *rgf1Δmkh1Δ* (PG337), *pek1Δ* (PG349), *rgf1Δpek1Δ* (PG339), *pmk1Δ* (PG347), and *rgf1Δpmk1Δ* (PG335) mutant strains were diluted (2×10^4 cells in the left row and then 2×10^3 , 2×10^2 and 2×10^1 in each subsequent spot) and were spotted onto YES plates with 0, 0.04, and 1 μg/ml Csp. Colony formation was analyzed after 4 d at 28°C.

the wild-type cells (not shown). This phenotype, also seen in *pmk1Δ* and *mkh1Δ* mutants, might represent a change in the response to stress conditions rather than a defect in cell integrity per se. Stationary-phase *rgf1Δ* cells spotted onto plates were protected osmotically (without lysis); however, they became visible 1 d after the wild-type colonies, and once this had occurred, they displayed a smaller colony size than the wild-type cells in the same conditions (not shown). We then examined whether knockout of the components of the protein kinase C-Pmk1p MAPK signaling pathway displayed hypersensitivity to Csp, an inhibitor of β -(1,3)-glucan synthase (Vicente *et al.*, 2003). As shown in Figure 2D (+1 μg/ml Csp), all knockouts of the components of the MAPK pathway were hypersensitive to Csp, whereas the growth of the wild type, *rgf2Δ* (deleted for another Rho1p GEF), *wis1Δ* (deleted for the stress-activated MAPKK), and *spk1Δ* (deleted for the MAPK involved in meiosis) was not inhibited. We also noticed that *rho2Δ* cells were not as sensitive to the antifungal agent as the other knockouts.

Overproduction of MAPK Pmk1p Suppresses the Hypersensitivity of *rgf1Δ* Cells to Caspofungin

We found that *Pck2p* overexpression suppressed the hypersensitive phenotype of Csp in *rgf1Δ* mutants. Accordingly, we examined whether up-regulation of the *Pck2p* effector, Pmk1p, might suppress the growth defect of the *rgf1Δ* cells in the presence of the antimycotic agent. As shown in Figure 3A, overexpression of Pmk1p under the control of the strong and constitutively active ADH promoter (Zaitsevskaya-Carter and Cooper, 1997) partially suppressed the growth defect of *rgf1Δ* cells in the presence of Csp.

We also examined the effect of an *rgf1Δ* mutation in combination with mutations affecting components of the MAPK integrity pathway, such as *Mkh1p*, *Pek1p*, and *Pmk1p*. The double mutants grew as well as the single mutants at 28°C (Figure 3B). We also compared the sensitivity to Csp of the

single and double mutants. The knockout of *mkh1*⁺, *pek1*⁺, and *pmk1*⁺ elicited a weaker sensitivity to Csp than *rgf1Δ*, because the *mkh1Δ*, *pek1Δ*, and *pmk1Δ* cells grew on YES plates supplemented with 0.3 μg/ml Csp, whereas the *rgf1Δ* cells failed to grow in the presence of 0.1, 0.08, and even 0.04 μg/ml Csp (Figure 3B). However, the *rgf1Δmkh1Δ*, *rgf1Δpek1Δ*, and *rgf1Δpmk1Δ* double knockout mutants failed to grow in the presence of 0.04 μg/ml Csp and did not show synergism in their sensitivity to Csp compared with the parental *rgf1Δ* single knockout (Figure 3B).

Rgf1p Is Involved in Pmk1p Activation Due to Hypertonic and Hypotonic Stress and Cell Wall Damage

To confirm that Rgf1p activates and transmits signaling through Pmk1p, we examined the level of Pmk1p phosphorylation upon different stresses in *rgf1Δ* mutants. It has been reported that MAP kinase Pmk1p activation is induced by multiple stressing situations, including hyper- and hypotonic conditions, the presence of cell wall damaging compounds, heat shock, glucose deprivation, and oxidative stress (Madrid *et al.*, 2006). The catalytic activity of this family of kinases depends on the phosphorylation of both the Thr-186 and the Tyr-188 residues, and can be detected by Western blotting with polyclonal anti-phospho-p42/44 antibody (see *Materials and Methods*; Zaitsevskaya-Carter and Cooper, 1997; Loewith *et al.*, 2000). First, we looked at Pmk1p activation after osmotic stress caused by KCl or sorbitol. To this end, cells from wild-type (MI200) and mutant *rgf1Δ* (PG285) strains with the genomic copy of *pmk1*⁺ tagged with HA6H (Madrid *et al.*, 2006) were grown at 28°C to the early log-phase. Extracts were obtained from samples taken before and after the addition of 0.6 M KCl or 1 M sorbitol for the times indicated. As shown in Figure 4A (KCl), whereas extracts from the wild-type showed a strong increase in Pmk1p signal intensity after treatment, this band was almost absent in the *rgf1Δ* mutant. Similarly, the Pmk1p phosphorylation seen after sorbitol treatment in *rgf1*⁺ cells was severely impaired in the *rgf1Δ* mutant (Figure 4A). In both situations, the induction of Pmk1 activity was not the result of an increase or decrease in Pmk1p protein levels, as observed after probing the same extracts with anti-hemagglutinin (HA) monoclonal antibodies (Figure 4A). We performed quantitative analysis by calculating the value termed induction-fold as the ratio between quantitative levels of Pmk1p phosphorylation at 15 min (treated cells) and Pmk1p phosphorylation at time 0 (untreated cells). All the single quantified values were also normalized with respect to their respective loading controls, and the results are shown below each panel (Figure 4). We also analyzed Pmk1p activation in control and *rgf1Δ* cells subjected to hypotonic stress, which induces a very rapid and transient phosphorylation of the MAPK in control cells (Madrid *et al.*, 2006; Figure 4B). As under osmotic stress, the Rgf1p deletion strongly decreased Pmk1p activation under hypotonic conditions (Figure 4B). These results therefore indicate a role for Rgf1p in osmotic stress sensing.

Hyper- and hypotonic stress initiates a variety of compensatory and adaptive responses, which serve to restore near-normal cell volumes and to reinforce the cell membrane and cell wall structure to withstand the physical challenge. The above results prompted us to test whether Rgf1p was also involved in the MAPK response to cell wall damage. As expected, we found that the phosphorylation of Pmk1p was markedly induced upon Csp treatment in wild-type cells, whereas in the absence of Rgf1p this activation had decreased considerably (Figure 4C).

We next wondered whether the role of Rgf1p was specific for stress conditions related to changes in the cellular volume that could involve the reorganization of the actin cy-

Figure 4. Rgf1p regulates Pmk1p activation induced by hyper- and hypotonic stress and by cell wall stress. The wild-type strain (*rgf1⁺*, MI200) or a mutant (*rgf1Δ*, PG285), both carrying a HA6H-tagged chromosomal version of *pmk1⁺*, were grown in YES medium to mid-log phase and subjected to different treatments for the indicated times. Pmk1-HA6H was purified by affinity chromatography under native conditions. Activated and total Pmk1p were detected by immunoblotting with anti-phospho-p42/44 or anti-HA antibodies, respectively. (A) For hypertonic stress, cells were subjected to 0.6 M KCl or 1 M sorbitol for 15 min. (B) For hypotonic stress, cells growing in YES medium supplemented with 0.8 M sorbitol were transferred to YES medium without sorbitol and collected at 0, 5, and 15 min. (C) For cell wall stress, cultures were supplemented with 1 μ g/ml Csp and grown for 60 min. (D) For thermal shock, cultures growing at 28°C were split and incubated at either 28 or 40°C for 40 min. (E) For oxidative stress, cultures were treated for 15 min with 6 mM H₂O₂. (F) For glucose starvation, cells growing in YES medium with 7% of glucose were shifted to the same medium without glucose but containing an equivalent osmotic concentration of glycerol. Each treatment was repeated at least three times. Relative units comparing the induction-fold of wild-type and mutant *rgf1Δ* cells in each individual experiment are shown below.

toskeleton or whether it was also involved in repairing other types of cell damage, such as oxidative stress or heat shock. As shown in Figure 4D, the intensity of Pmk1p phosphorylation after transferring the cultures from 28 to 40°C was not affected very much by deletion of the *rgf1⁺* gene. Similarly, the MAPK activation achieved with hydrogen peroxide in control cells was still evident in *rgf1Δ* cells (Figure 4E), and the induction-fold value upon comparing the stressed *rgf1Δ* cells with their controls (2/0.3) was higher than the induction seen in wild-type cells (4/1; Figure 4E). Thus, the heat-shock- and the oxidative stress-induced activations of Pmk1p in the fission yeast were largely independent of the presence or absence of Rgf1p. Next, we wondered whether Rgf1p was also involved in sensing nutrient limitation, a process that in the long-term also enhances the turnover of excess mass. Yeast cells starved for glucose dramatically down-regulate general protein synthesis and activate stress responses (Ashe *et al.*, 2000). In fission yeast cells, previous work has shown that stress caused by glucose depletion elicits a clearly delayed Pmk1p activation (Madrid *et al.*, 2006). We found that cells responded to glucose starvation by activating Pmk1p in a way totally independent of the presence or absence of Rgf1p (Figure 4F). Taken together, these data indicate that Rgf1p plays a substantial role in the osmotic and cell wall stress response by positively regulating the Pmk1p-cell integrity pathway.

Rgf1p Is Not Involved in Spc1p Activation Due to Hypertonic or Oxidative Stress

The above results led us to the hypothesis that Rgf1p could also be regulating the osmotic stress response through activation of the Spc1p/Sty1p MAPK signaling pathway

(Shiozaki and Russell, 1995b). The Spc1p kinase is activated by increases in osmolarity and by a wide range of environmental stresses. This activation involves the relocalization of Spc1p to the nucleus and phosphorylation of the transcription factor Atf1p, which results in changes in the expression of genes associated with the stress response (Takeda *et al.*, 1995). We investigated the effect of loss of the *rgf1⁺* gene on Spc1p activation after osmotic stress (KCl). We used cells from wild-type (KS1489) and mutant *rgf1Δ* (PG362) strains with the genomic copy of *spc1⁺* tagged with HA6H, allowing Spc1p purification with Ni²⁺-NTA and detection with anti-HA antibody (Shiozaki and Russell, 1995b). As previously reported (Degols *et al.*, 1996), exposure of the cells to high-osmolarity conditions, in this case YES medium containing 0.6 M KCl, led to a rapid increase in the tyrosine-phosphorylation of Spc1p (Figure 5). In *rgf1Δ* cells, the same treatment produced a similar increase in the Spc1p signal, indicating that Spc1p induction upon KCl application was completely independent of Rgf1p (Figure 5). We also examined whether the activation of the MAPK after oxidative stress was dependent on the presence of Rgf1p. As shown in Figure 5, bottom panel, the intensity of Spc1p phosphorylation after 0.3 mM H₂O₂ treatment was not affected very much by deletion of the *rgf1⁺* gene.

Rho1p Participates in the Activation of Pmk1p

GEFs are multidomain proteins, and previous studies have suggested that many of these domains are protein- or lipid-interaction domains, indicating that they serve as localization signals and/or as scaffolds for the formation of protein complexes (Yeh *et al.*, 2007). To determine whether Rgf1p function in Pmk1p signaling requires GEF activity, we used

Figure 5. Rgf1p-independent tyrosine phosphorylation of Spc1p by osmotic- and oxidative-stress signals. Cells expressing epitope-tagged Spc1p, wild-type (KS1489) and *rgf1Δ* (PG362) were grown to log phase in YES medium and then exposed to osmotic stress (15 min of exposure to YES plus 0.6 M KCl, top panel), or oxidative stress (15 min of growth in YES plus 0.3 mM H₂O₂, bottom panel). Spc1p was isolated by Ni²⁺-NTA-agarose affinity precipitation and then analyzed by immunoblotting with anti-phospho-p38 and anti-HA antibodies. Spc1p tyrosine phosphorylation increased in both strains after the two different types of stress.

a deletion mutant in the RhoGEF domain of Rgf1p (*rgf1-PTTRΔ*; García *et al.*, 2006a). The four amino acids deleted in the *rgf1-PTTRΔ* mutant (proline-threonine-threonine-arginine) have been predicted to be located on helix H8 (CR3), which is the most highly conserved region of the DH domain and is where many mutations that decrease nucleotide exchange activity map (Liu *et al.*, 1998; Soisson *et al.*, 1998). As expected, the *rgf1-PTTRΔ* mutant integrated in a single copy in *rgf1Δ* strain displayed a significantly reduced GEF activity toward Rho1p. In a pulldown binding assay, only a minor amount of GTP-Rho1p (active-Rho1p) was detected in the mutant strain *rgf1-PTTRΔ* (PG52) as compared with the levels of GTP-Rho1p in the wild-type strain (Fig. 6A). We then tagged the full-length wild-type and the mutant *rgf1-PTTRΔ* gene with GFP at the carboxy-end. These proteins were expressed at comparable levels and the mutated *rgf1-PTTRΔ*-GFP localized to the cell ends in the wild-type strain (Figure 6B). However, the *rgf1-PTTRΔ* mutant was completely nonfunctional in terms of Pmk1p activation after osmotic stress (Figure 6C). Thus, the role of Rgf1p in Pmk1p MAPK activation must depend on its GEF activity.

This result raised the possibility that either low or high GTP-Rho1p levels could be regulating MAPK activation in response to stress. To examine this option, we first tested whether overexpression of Rho1p or a dominant-active Rho1p mutant (Rho1G15V) might result in an increased phospho-Pmk1p signal in the absence of environmental stress. To this end, we transformed the Pmk1-HA6H-tagged strain MI200 with plasmids pREP3X, pREP3X-*rho1*⁺, and pREP3X-*rho1G15V*. These plasmids expressed the wild-type *rho1*⁺ and the hyperactive allele of *rho1*⁺ under the control of the high-strength thiamine-repressible promoter *nmt1* (Arellano *et al.*, 1996; Forsburg and Sherman, 1997). As shown in Figure 6D (left), the overexpression of both Rho1p and Rho1G15Vp (constitutively active) increased the phosphorylation levels of Pmk1p, similar to the increase obtained with the overexpression of Pck2p. As expected, cells

overexpressing the constitutively active allele of Rho1p activated the cascade earlier on in derepression than cells bearing the wild-type allele of Rho1p. We also found that a high level of Rgf1p, expressed from pREP3X-*rgf1*⁺, elicited the activation of Pmk1p (Figure 6D).

We next examined whether ectopic expression of Rho1p and Rgf1p caused direct activation of Pmk1p or whether it was being funnelled through the Mkh1p-Pek1p module. We constructed strains that expressed the Pmk1-HA6H fusion in an *mkh1Δ* background and transformed them with plasmids pREP3X-*rho1G15V* and pREP3X-*rgf1*⁺. Deletion of *mkh1*⁺ completely abolished the Pmk1p activation observed after overexpression of Rho1G15Vp or Rgf1p (Figure 6D). Moreover, we tested whether the overexpression of Rho1G15Vp and Rgf1p caused Pmk1p activation in a *pck2Δ* background and found that the lack of *pck2*⁺ completely abolished Pmk1p activation (Figure 6D, right). Taken together, these results strongly suggest that the Rgf1p-Rho1p-Pck2p cascade does regulate the activation of Pmk1p in *S. pombe*.

Involvement of Rgf2p and Rgf3p in Pmk1p Activation

Finally, we investigated what the contribution of the other Rho1p GEFs to Pmk1p activation might be. We have previously shown that overexpression of *rgf1*⁺, *rgf2*⁺ or *rgf3*⁺, driven by the *nmt1* promoter, produces a strong induction of the amount of GTP-bound Rho1p (active Rho1p; Tajadura *et al.*, 2004; García *et al.*, 2006a), and our unpublished results). However, in vivo the contribution of each GEF to the activation or Rho1p must be very different. The level of Rho1p-GTP is very diminished in *rgf1Δ* cells (Figure 6A (García *et al.*, 2006a)), whereas in the *rgf3* mutant the amount of Rho1p-GTP (activated) is almost the same as that of the wild type (Tajadura *et al.*, 2004).

We found that overexpression of either Rgf2p or Rgf3p, expressed from pREP3X-*rgf2*⁺ or pREP3X-*rgf3*⁺, respectively, elicited the activation of Pmk1p (Figure 7A). This result led us to wonder whether this was due to a real contribution of each GEF or whether it was merely a consequence of a high level of active Rho1p. To this end, we examined the effect of the loss of Rgf2p or the Rgf3p alone on the Pmk1p activation induced by different types of stress. Rgf3p is essential, so we used a strain with a TS mutation in *rgf3* (the *ehs2-1* mutant stands for echinocandin-hypersensitive). The *ehs2-1* cells showed a lytic thermosensitive phenotype at 37°C, which was suppressed when an osmotic stabilizer (1.2 M sorbitol) was added to the medium (Tajadura *et al.*, 2004). We first tested whether the *ehs2-1* mutant was impaired for the Pmk1p activation induced by osmotic stress or cell wall damage. As shown in Figure 7B, Pmk1p phosphorylation levels were similar in *ehs2-1* cells and in wild-type cells grown for 2 h at 37°C and then exposed to KCl (15 min.) or Csp (1 h) at the same temperature. Moreover, the *ehs2-1* mutant was not involved in repairing other types of cell damage, such as oxidative or heat shock. As shown in Figure 7B, the intensity of Pmk1p phosphorylation after treatment with H₂O₂ (6 mM) or heat shock at 40°C was not significantly affected in the *ehs2-1* mutant. We also tested Pmk1p activation in the presence of sorbitol (1 M) and under glucose starvation. The *ehs2-1* cells did not behave differently from the wild-type cells in either situation (not shown).

Our investigations with the *rgf2Δ* null mutant revealed that the intensity of Pmk1p phosphorylation after exposure to osmotic shock (1M sorbitol), cell wall damage (1 μg/ml Csp), oxidative stress (6 mM H₂O₂), or heat shock (40°C), and glucose starvation was not affected very much by deletion of the *rgf2*⁺ gene (Figure 7C).

Figure 6. Rho1p is involved in Pmk1p activation. (A) The *rgf1-PTTRΔ* mutant displayed significantly reduced GEF activity toward Rho1p. Wild-type (PPG160), *rgf1Δ* (PG378), and *rgf1-PTTRΔ* (PG380) cells expressing HA-*rho1*⁺ from its own promoter were precipitated with GST-RBD and blotted with anti-HA antibodies (12CA5). Total HA-Rho1p in cell lysates was visualized by Western blotting. Data are quantified and presented as percentages relative to the wild-type extracts run in the same experiment. (B) Early log-phase cells containing the *rgf1-PTTRΔ* allele fused to GFP (PG52) were visualized for GFP fluorescence and Nomarsky optics. The Rgf1-PTTR protein (mutated in the RhoGEF domain) localizes to the growing regions: one or both poles and the medial zone. (C) Rgf1p function in Pmk1p signaling requires GEF activity. The *rgf1*⁺ wild-type strain (MI200) or the *rgf1-PTTRΔ* mutant (PG331), both carrying an HA6H-tagged chromosomal version of *pmk1*⁺, were grown in YES medium to mid-log phase and treated with 0.6 M KCl, for 15 min. Pmk1-HA6H was purified by affinity chromatography under native conditions. Activated and total Pmk1p was detected by immunoblotting with anti-phospho-p42/44 or anti-HA antibodies, respectively. (D) High levels of Rho1p resulted in an increased phospho-Pmk1p signal. Left panels, the wild-type strain (*rgf1*⁺, MI200) carrying a HA6H-tagged chromosomal version of *pmk1*⁺ was transformed with plasmids for *rho1*⁺ (pREP3X-*rho1*⁺, pREP3X-*rho1G15V*), *pck2*⁺ (pREP-*pck2*⁺), *rgf1*⁺ (pREP3X-*rgf1*⁺), and with the empty plasmid (pREP3X). Transformants were grown in MM without thiamine for the indicated times. Purification and detection of active or total Pmk1 was performed as described above. Right panels, basal activation of Pmk1p produced by Rho1p and Rgf1p is channelled exclusively through Mkh1p and Pck2p. The wild-type, *mkh1Δ* (PG318) and *pck2Δ* (PG332) strains carrying a HA6H-tagged chromosomal version of *pmk1*⁺ were transformed with plasmids pREP3X, pREP3X-*rho1G15V* and pREP3X-*rgf1*⁺. For promoter derepression, transformants were grown in MM without thiamine for 16 h. Lines are labeled (–) for the empty plasmid pREP3X and (+) for the OP-plasmid, either pREP3X-*rho1G15V* or pREP3X-*rgf1*⁺. Purification and detection of active and total Pmk1p were performed as described above.

Thus, together these results indicate that among the Rho1p GEFs, Rgf1p contributes the greatest part by modulating the activity of the pathway after physical destabilization of the cell surface.

DISCUSSION

The main conclusion that can be drawn from the present work is that Rgf1p, a Rho1p-specific GEF, is a new member of the Pmk1p MAPK pathway in fission yeast. We provide genetic and biochemical evidence to support this view. First, mutants lacking *rgf1*⁺ exhibited the *vic* phenotype, which is strong indication of the involvement of the components of the Pmk1p signaling pathway (Ma *et al.*, 2006). Moreover, *rgf1Δ* cells were hypersensitive to β -glucanase treatment, and their growth was inhibited by high salt concentrations, both phenotypes being characteristic of knockouts in the *pmk1*⁺, *mkh1*⁺, and *pek1*⁺ genes. Second, knockout mutants of the components of the Pmk1p MAPK signaling pathway displayed hypersensitivity to Csp, a cell wall-damaging agent that specifically inhibits β -glucan biosynthesis. As expected, deletion of the components of the Pmk1p pathway did not exacerbate the hypersensitivity to Csp of strains lacking *rgf1*⁺. This observation supports the view that Rgf1p plays a role in the Pmk1p pathway. Third, Pmk1p MAPK

phosphorylation/activation in response to osmotic stress or cell wall damage depends on the Rgf1 protein. Our data provide new evidence to clarify the complex regulatory network modulating the level of activation of the Pmk1p. Although it seems likely that hypertonic and hypotonic stress and cell wall damage would be transduced by Rgf1p via Rho1p and Pck2p, other stimuli such as treatment with H₂O₂ and heat shock are largely independent of Rgf1p function.

MAP kinase cascades serve to amplify a small signal initiated at the cell surface and to convert a graded input into a highly sensitive, switch-like response (Ferrel, 1996). In budding yeast, the Rho1p effector pathway most studied is the Pkc1p-activated MAPK cascade. This is principally because mutants in this pathway display conditional cell lysis defects that render them genetically tractable. In this pathway, a linear series of protein kinases, known as a MAPK cascade, is responsible for amplification of the cell wall integrity (CWI) signal from Rho1p (Levin, 2005). In fission yeast, the cell integrity pathway contains a module of three kinases (Mkh1p, Skh1p/Pek1p, and Pmk1p/Spm1p) that regulate cell integrity and that, with calcineurin phosphatase, antagonize chlorine homeostasis. However, the involvement of this pathway in the gene expression related to cell wall remodelling or to the organization of the actin

Figure 7. Rgf2p and Rgf3p involvement in Pmk1p activation. (A) High levels of Rgf2p and Rgf3p resulted in an increased phospho-Pmk1p signal. The wild-type strain (*rgf1*⁺, MI200) carrying a HA6H-tagged chromosomal version of *pmk1*⁺ was transformed with plasmids for *rgf1*⁺, *rgf2*⁺, or *rgf3*⁺ overexpression (pREP3X-*rgf1*⁺, pREP3X-*rgf2*⁺, and pREP3X-*rgf3*⁺, respectively) and with the empty plasmid (pREP3X). For promoter derepression, transformants were grown in MM without thiamine for 16 h. (B) Rgf3p is not required for induction of Pmk1p phosphorylation in response to hypertonic stress or cell wall stress. Strains (*rgf3*⁺, MI200) and (*ehs2-1*, PG359) were grown to mid-log phase in YES medium and then incubated for 2 h at 37°C. For osmotic stress treatment, the cells were incubated for an additional 15 min in YES plus 0.6 M KCl. For cell wall stress, the cells were incubated for an additional hour in YES plus 1 μg/ml Csp, in both cases at 37°C. For oxidative stress, cultures were treated for 15 min with 6 mM H₂O₂. For thermal shock, after 2 h at 37°C the cells were incubated at 40°C for 30 min. (C) Rgf2p is not required for induction of Pmk1p phosphorylation in response to hypertonic stress or cell wall stress. The wild-type strain (*rgf2*⁺, MI200) or a mutant (*rgf2Δ*, PG287), both carrying a HA6H-tagged chromosomal version of *pmk1*⁺, were grown in YES medium to mid-log phase and then exposed to osmotic stress (15 min in YES plus 1 M sorbitol), cell wall damage (1 h of growth in YES plus 1 μg/ml Csp), oxidative stress (15 min growth plus 6 mM H₂O₂), heat shock (40°C for 40 min), or glucose starvation. Pmk1-HA6H was purified by affinity chromatography under native conditions. Activated and total Pmk1p were detected by immunoblotting with anti-phospho-p42/44 or anti-HA antibodies, respectively.

cytoskeleton is not very well understood. Deletion of *mkh1*, *pek1*, or *pmk1* did not significantly affect cell growth under standard conditions. However, the mutants are sensitive to β -glucanase treatment and to antifungal agents that interfere with cell wall biosynthesis (Toda *et al.*, 1996b; Sengar *et al.*, 1997). In the presence of hyperosmotic medium, high temperatures or nutrient starvation, a number of cells (30%) exhibit filamentous, multiseptated growth, and the cells are swollen (Sengar *et al.*, 1997; Zaitsevskaia-Carter and Cooper, 1997; Sugiura *et al.*, 1999). This phenotype is accompanied by thickened cell walls and prominent septa, and this might indicate that these cells are defective in cell wall biosynthesis or degradation under stressful conditions.

The finding that Rho2p and Pck2p are upstream components of the Pmk1p MAPK pathway supports the notion of the participation of this module of kinases in the cell remodelling necessary to reinforce the cell wall structure or to reorganize the cytoskeleton in order to withstand osmotic stress or other physical challenges (Ma *et al.*, 2006). Rho2p is involved in the control of cell morphogenesis (Hirata *et al.*, 1998) and regulates cell wall α -glucan biosynthesis through the protein kinase Pck2p (Calonge *et al.*, 2000). Pck2p is also related to cell wall β -glucan; although *pck2Δ* mutants have less cell wall, the mutants that overexpress Pck2p show an increase in the β -glucan content and a higher β -glucan synthase activity (Arellano *et al.*, 1999b).

Rho2p acts upstream from Pck2p, regulating the Pmk1p MAPK pathway (Ma *et al.*, 2006). Moreover, it has been recently shown that Rho2p is critical for Pmk1p activation in the presence of KCl, sorbitol and hypotonic stress, whereas it is dispensable for heat shock, H₂O₂, or glucose starvation (Barba *et al.*, 2008). It is possible that one or more elements could contribute to Pmk1p activation through Pck2p, alternatively to Rho2p (Barba *et al.*, 2008). A good candidate for Pmk1p activation via Pck2p is the essential GTPase Rho1p.

Rho1p interacts with Pck1p and with Pck2p, and this interaction stabilizes these kinases, raising their concentrations precisely in the areas of growth (Arellano *et al.*, 1999b; Sayers *et al.*, 2000). Our results indicate that Rgf1p contributes to Pmk1p signaling only when Rho1p is working properly. In this sense, we found that both low and high levels of GTP-bound-Rho1p had an impact on the proper functioning of the cascade.

We observed that a deletion mutation in a highly conserved region of the Rgf1p-DH-domain produced a lack of function phenotype in terms of the induction of Pmk1p in response to osmotic stress. The amount of GTP-Rho1p in the *rgf1-PTTRΔ* mutant was minute compared with the wild-type *rgf1*⁺, suggesting that wild-type levels of activated Rho1p are important for Pmk1p activation. Although it seems evident that Rgf1p could act as a GEF for Rho1p, we cannot rule out the possibility of its acting as a GEF for Rho2p.

However, our results do not favor this hypothesis because no changes in the levels of Rho2p bound to GTP were observed in strains with different levels of Rgf1p, either with or without osmotic stress (not shown). In addition, Rho2p overexpression did not suppress the hypersensitivity to Csp or sensitivity to KCl in the *rgf1Δ* mutant cells (García *et al.*, 2006a and data not shown, respectively). We also found that high levels of Rho1p increased the phosphorylation level of Pmk1p, similarly to that obtained by overexpression of Pck2p. As expected, overexpression of the constitutively active allele of Rho1p activated the cascade earlier on in derepression than cells that carried the wild-type allele of Rho1p. In both situations, Pck2p and Mkh1p activity was necessary for MAPK activation, suggesting that the stimulus was being funnelled through Pck2p and the three kinases.

We have previously shown that overexpression of both Rgf1p and Rgf3p raises the amount of GTP-bound Rho1p

and β -glucan synthase activity (Tajadura *et al.*, 2004; García *et al.*, 2006a). We reasoned that if Rho1p was activating Pmk1p, then it would be expected that ectopic activation of Rho1p by any of its GEFs would also activate Pmk1p. This is in fact the case, because the overproduction of Rgf2p or Rgf3p activated Pmk1p to a similar extent to Rgf1p. Rgf2p is required for spore wall maturation, and the protein shows a low expression profile in vegetatively growing cells (our unpublished observation). The *rgf3⁺* gene is essential, and the protein has been proposed to specifically activate Rho1p function during cytokinesis (Tajadura *et al.*, 2004). Nevertheless, there exists the possibility that these GEFs could be activating the pathway by themselves. It seems very unlikely that Rgf2p would be involved in the signal transduction of the Pmk1p cascade. The knockout of Rgf2p did not elicit the characteristic *vic* phenotype and was not hypersensitive to Csp treatment or sensitive to high-salt concentrations either (not shown); even more conclusively, Pmk1p activation after osmotic stress, oxidative stress, heat shock, or Csp treatment was not impaired in the *rgf2 Δ* mutant. Regarding Rgf3p, we observed that the activation of Pmk1p in response to a number of different stresses was not affected in the *ehs2-1* (mutated in *rgf3⁺*) at the restrictive temperature. In sum, the Pmk1p activation seen after overexpression of Rgf2p and Rgf3p is probably due to a large increase in activated Rho1p levels. Our data also show that Rgf1p, but not Rgf2p or Rgf3p, activity is involved in Pmk1p activation during osmotic or cell wall stress, strongly suggesting that the expression, localization, and/or activity of different GEFs are regulated in vivo to mediate the function of Rho1p in different cellular processes.

In *S. pombe*, Rho1p signaling is required to maintain cell integrity. Here we show that the Rgf1p-Rho1p duo is involved in osmotic and cell wall stress signaling through the Pmk1p cell integrity cascade. The finding that the up-regulation of Pck2p and Pmk1p suppressed, at least partially, the Csp defect of *rgf1 Δ* mutants suggests that Pck2p signals to the cell wall integrity pathway via the MAPK cascade. It has been shown that Atf1p is phosphorylated by Pmk1p under cell wall stress, but the consequences of such activation are unknown (Takada *et al.*, 2007). Besides its role in the cell integrity pathway, Rho1p acts by regulating the biosynthesis of β (1,3)-glucan and the cell wall in general, and it is also required for actin polymerization. Its role as a GS activator seems to be at least partially independent of its role in the MAPK cascade. The cells that overexpressed either Rgf1p or Rho1G15V (dominant-active allele of Rho1p) showed aberrant depositions of Cfw-stainable material and a huge increase in GS activity, and finally the cells' death. We found that deletion of Pmk1p did not release the lethal phenotype seen after the overproduction of Rgf1p or Rho1G15V; moreover, the GS level was not diminished in the *pmk1⁺* deletion mutants. It is likely that Rho1p would regulate GS at two levels: direct regulation of the enzyme itself and, through the MAPK cascade, regulation of the expression of the enzyme.

Our work demonstrates that a specific GEF regulates a subset of Rho1p functions, specifically linking the stimulus-induced signaling to cell wall and/or cytoskeletal remodeling. This supports the hypothesis that GEFs play specialized roles and is consistent with the hypothesis that the excess of regulators for GTPases would have evolved to exert spatiotemporal regulation or adaptive responses that enable GTPases to accomplish diverse cellular roles.

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