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Importance of Polarisome Proteins in Reorganization of Actin Cytoskeleton at Low pH in *Saccharomyces cerevisiae*

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The actin cytoskeleton of the yeast *Saccharomyces cerevisiae* can be altered rapidly in response to external cues. We reported previously that *S. cerevisiae* responds to low-pH stress by transiently depolarizing its actin cytoskeleton, and that this step requires a mitogen-activated protein kinase, high osmolarity glycerol 1 (Hog1p). This study further investigated the components involved in this actin reorganization at pH 3.0. Gene deletions on the Sln1p branch of the HOG pathway completely blocked actin depolarization, suggesting that Hog1p activation depends mainly on the osmosensor Sln1p. The protein-synthesis inhibitor cycloheximide did not influence the time course of actin depolarization, suggesting that the depolarization is a direct effect of the HOG pathway. Deletion of the scaffolding protein, Spa2p, or the Spa2p-interacting protein Pea2p, markedly inhibited the depolarization, and further deletion of the formin protein, Bni1p, notably delayed actin repolarization. Our results suggest the involvement of polarisome proteins, such as Spa2p, Pea2p and Bni1p, but not Bud6p, in Hog1p-dependent reorganization of the yeast actin cytoskeleton at low pH.

Key words: actin cytoskeleton, Hog1, low pH, polarisome, *Saccharomyces cerevisiae*.

The budding yeast *Saccharomyces cerevisiae* is a widely used model for studying cell adaptation and survival under different forms of stress. Low pH is a commonly encountered environmental stress for all micro-organisms. Low pH induces various adaptations in cell-wall composition and structure. Under low-pH conditions, *S. cerevisiae* cells become highly resistant to Zymolyase (β-1,3-glucanase) and use more extensively the alkali-sensitive linkage between integral cell wall proteins and β-1,3-glucan (*1*). These cell-wall structural changes depend on activation of high osmolarity glycerol 1 (Hog1) mitogen-activated protein kinase (MAPK) signalling pathway (*1*). The low pH-induced resistance to glucanase may be vital under natural conditions such as growth on acidic environments such as rotting fruit that comprise many cell-wall-active hydrolytic enzymes (*1*).

We reported previously that *S. cerevisiae* transiently depolarizes its actin cytoskeleton at low pH, and that this step requires Hog1p (*2*). This effect is probably the first step by which the organism reorganizes its cell wall in response to damage (*2*). However, the mechanisms regulating such dynamic changes in the actin cytoskeleton remain uncharacterized.

Saccharomyces cerevisiae undergoes polarized cell growth during vegetative growth. The cells in early G1 grow isotropically and insert new cell-wall material around the entire cell surface until they reach a critical size, at which time activation of the G1-cyclin-dependent kinase (Cdc28p-Clnp) initiates cytoskeletal polarization and bud emergence (*3*). During bud growth, actin filaments assemble into cortical patches that cluster in the expanding bud and into cables that extend along the axis of growth (*4*). The cortical patches are implicated in endocytosis (*5*), and their assembly depends on the actin-nucleating Arp2/3 complex (*6*). Actin cables guide the polarized movement of secretory vesicles containing growth components (e.g. new cell wall and membrane) to the growing bud (*7*), and their assembly dependents on the formins, Bni1p and Bnr1p (*8*, *9*).

GTPase Cdc42p is activated through the action of Cdc28p-Clnp (*10*) and then it catalyzes the translocation of bud-site components like Spa2p to the incipient bud site (*11*, *12*). Spa2p is involved in a wide variety of responses that require dynamic organization of the actin cytoskeleton (*13*, *14*). Although the molecular function of Spa2p in cell polarity is unknown at present, it is known to interact with a number of proteins involved in cell polarity and signalling. Spa2p interacts with the cell polarity proteins Pea2p, Bud6p and Bni1p in two-hybrid and co-immunoprecipitation assays, and Spa2p, Pea2p and Bud6p co-fractionate on sucrose gradients as a 12S complex termed the 'polarisome' (*14*, *15*). Cells with deletion of any of these proteins are viable but show defective bud emergence and response to pheromones, pseudo-hyphal growth and correct bud site selection in diploid cells (*16*). Furthermore, Spa2p co-immunoprecipitates with Myo1p, Myo2p, Pan1p and the protein product encoded by YFR016c, which are proteins involved in cell polarity and/or actin function (*17*). In addition, Spa2p interacts with components of the pheromone-response MAPK module (Ste11p and Ste7p) and MEKs of the PKC cell-integrity pathway (Mkk1p and Mkk2p) in the two-hybrid assay (*14*). Spa2p also co-immunoprecipitates with Ssk2p (MAPKKK of the HOG pathway) in osmotically stressed cells (*18*). The Mpk1p–MAPK module

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localizes to sites of polarized growth in a Spa2p-dependent manner (*19*). Spa2p may function as a scaffold protein for the cell wall integrity pathway during polarized growth (*19*).

The HOG pathway (Fig. 1A) is initiated from two membrane proteins, Sho1p and Sln1p, which respond to high external osmolarity (*20*, *21*). Signals from each are transduced by unique components and converge to activate the MAPKK Pbs2p. The Sho1 branch requires the yeast PAK homologue Ste20p in complex with Cdc42p, to activate the MAPKKK Ste11p (*22*, *23*). High osmolarity suppresses Sln1p histidine kinase activity, leading to accumulation of non-phosphorylated Ssk1p (*24*). The non-phosphorylated form of Ssk1p in turn activates the MAP kinase cascade, which comprises the redundant Ssk2p and Ssk22p MAPKKKs, the Pbs2p and Hog1p (Fig. 1A). The activated Hog1p translocates to the nucleus, where, with phosphorylation of at least three separate transcription factors (Sko1p, Hot1p and Smp1p), it can alter gene expression, most notably upregulating genes involved in glycerol synthesis (*25–30*).

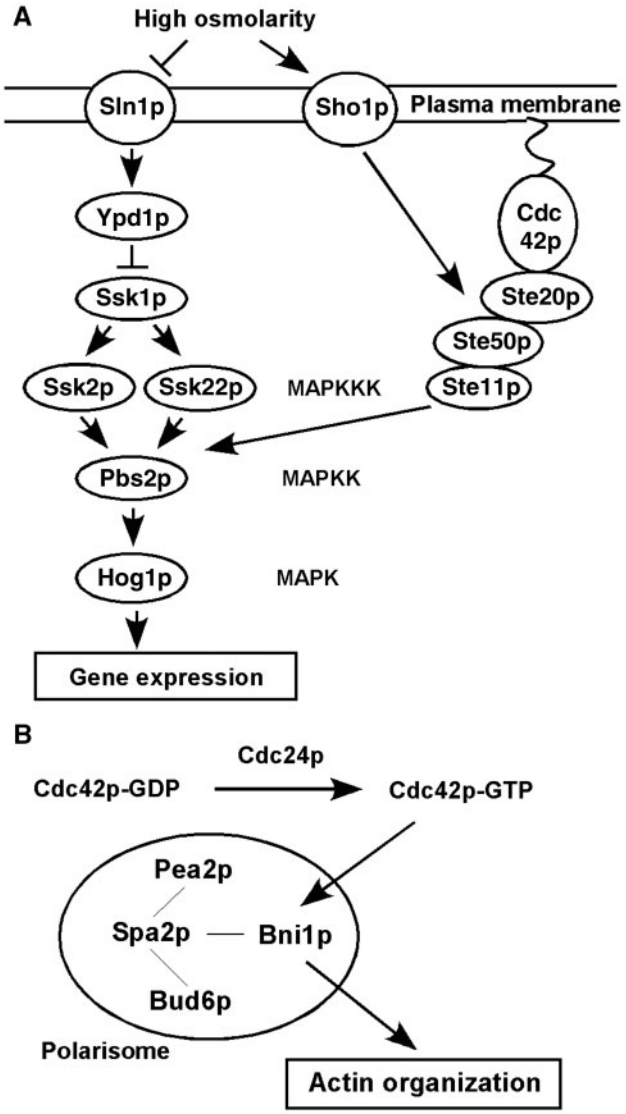

Fig. 1. **The *S. cerevisiae* HOG signal transduction pathway (A).** Model depicting the role of polarisome in polarized growth in *S. cerevisiae* (B). See INTRODUCTION section for details.

The present study looked specifically for components involved in yeast actin reorganization at low pH. The results showed that Hog1p is activated mainly through the Sln1 branch of the pathway and the actin cytoskeleton is depolarized without *de novo* protein synthesis. The results further showed that polarisome proteins Spa2p, Pea2p and Bni1p are required for reorganization of the actin cytoskeleton.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The *Saccharomyces* strains used in this study were isogenic derivatives of BY4741 or BY22286 (Table 1) obtained from Euroscarf (Institute of Molecular Biosciences, University of Frankfurt, Frankfurt, Germany) or the Yeast Genetic Resource Center (Osaka University, Osaka, Japan), respectively. Yeast cells were cultured at 30°C in YPD medium (2% peptone, 1% yeast extract and 2% glucose). Cells in 1 ml of stationary-phase culture (about 1×10^8 cells) were harvested by centrifugation at 4°C and then grown in 5 ml of YPD medium at 30°C for 4 h to exponential phase (5×10^7 cells/ml).

Fluorescence Microscopy—To examine the F-actin architecture in the yeast, cells were incubated in rhodamine-conjugated phalloidin, as described by Adams and Pringle (*4*), with minor modifications. Briefly, cells in 1 ml of the exponential-phase culture were harvested by centrifugation at 4°C and then incubated at 30°C in 1 ml of YPD medium, pH 3.0 (adjusted from pH 6.5 with HCl and then filtered through a sterile 0.2-μm filter). After incubation for various times, the cells were fixed by adding 9 ml of 4.2% formaldehyde/0.1 M cacodylate buffer (pH 6.5) to the medium. After 1 h, the fixative was removed by washing twice with phosphate-buffered saline (PBS, in millimoles: 53 Na_2HPO_4, 13 NaH_2PO_4 and 75 NaCl) then the cells were permeabilized for 20 min using 0.2% Triton X-100 in PBS. After two washes in PBS, the cells were incubated for 2 h with rhodamine–phalloidin at room temperature. After several washes in YPD medium, the cell pellet was re-suspended in mounting medium (p-phenylenediamine/90% glycerol) and viewed under an Olympus BX50 fluorescence microscope. Actin was examined in small- and medium-budded cells that normally exhibit a polarized actin cytoskeleton (*4*). Individual cells in the section figures were classified either as polarized actin cytoskeleton (cells with more actin patches in the mother cell than in the bud) or a depolarized actin cytoskeleton (cells with more actin patches in the bud).

For studies examining Hog1p localization, yeast green fluorescent protein (GFP) clone was purchased from Invitrogen (Carlsbad, CA, USA). In this yeast strain (YLR113W), the genomic copy of Hog1p is replaced with an allele that expresses Hog1p fused at its C-terminus to GFP under an endogenous promoter. After incubation for various times in YPD medium, pH 3.0 at 30°C, the cells were fixed in 3.7%

Table 1. ***Saccharomyces cerevisiae*** **strains used in the present study.**

Strain	Genotype	Source
BY22286	*MATa leu2 his3 trp1 ura3*	Yeast Genetic Resource Center
sho1Δ	BY22286; *sho1::TRP1*	Yeast Genetic Resource Center
sln1Δ ssk1Δ	BY22286; *sln1::URA3 ssk1::LEU2*	Yeast Genetic Resource Center
ssk1Δ	BY22286; *ssk1::HIS3*	Yeast Genetic Resource Center
BY4741	*MATa leu2 his3 met15 ura3*	EUROSCARF
bni1Δ	BY4741; *bni1::KanMX4*	EUROSCARF
bnr1Δ	BY4741; *bnr1::KanMX4*	EUROSCARF
pea2Δ	BY4741; *pea2::KanMX4*	EUROSCARF
spa2Δ	BY4741; *spa2::KanMX4*	EUROSCARF
ssk2Δ	BY4741; *ssk2::KanMX4*	EUROSCARF

formaldehyde/0.1 M cacodylate buffer (pH 6.5). After 30 min, the cells were permeabilized for 20 min using 0.2% Triton X-100 in PBS. After two washes in PBS, the cells were incubated for 5 min with 4′, 6′-diamidino-2-phenylindole (DAPI) to stain nuclei. After centrifugation, the cell pellet was re-suspended in mounting medium and viewed by fluorescence microscopy.

Cycloheximide Treatment—Yeast cells in 2 ml of the exponential-phase culture were harvested and then incubated at 30°C in 2 ml of YPD medium, pH 3.0, containing cycloheximide at 10 μg/ml. Samples were removed at different time points, and actin distribution was determined as described. To determine the effect of inhibition of protein synthesis on yeast colony-forming activity (CFA), a known number of cells was plated and cultured on YPD agar plates, at 30°C, and colonies were counted after 2 days.

RESULTS

Involvement of the Sln1 Branch in Activating Hog1p—We showed previously that *S. cerevisiae* responds to low pH by transiently depolarizing its actin cytoskeleton through the action of Hog1p (*2*). The HOG pathway (Fig. 1A) comprises signal transduction *via* both Sln1 and Sho1 branches in response to external stress such as changes in osmolarity. To determine the branch used in the low-pH response, several deletion mutants were incubated at pH 3.0. At different time points, aliquots were removed, and the cells were fixed and stained with rhodamine–phalloidin to visualize polymerized (F) actin. Actin was examined in small- and medium-budded cells that normally exhibit a polarized actin cytoskeleton (*4*). Cells with more actin patches in the mother cell than in the bud were classified as depolarized. Wild-type cells showed actin depolarization with no visible cables and randomly distributed patches between mother and daughter cells, peaking at 30 min after pH shift from 6.5 to 3.0, and decreasing after 45 min (Fig. 2A and B). The *ssk1Δ* and *ssk1Δ sln1Δ* deletion mutants showed no actin depolarization throughout the incubation (Fig. 2A, E and F), whereas the *sho1Δ* cells showed similar actin organization to wild-type cells during the incubation (Fig. 2A and C). These findings suggest that activation of Hog1p occurs mainly via the Sln1 signalling pathway branch under conditions of low pH. Deletion of the Sln1p is lethal, because the ensuing accumulation of non-phosphorylated Ssk1p constitutively activates the HOG pathway (*20*). Therefore, we used the *ssk1Δ sln1Δ* deletion mutant.

The cell-wall status in yeast is monitored by a family of five cell-surface proteins that includes Wsc1-3p, Mid2p and Mtl1p. Wsc1p is required for the Slt2 MAPK pathway activation at high pH (*31*), while Mid2p regulates activation of the protein kinase C pathway at low pH (*32*). To determine if these stress sensors are directly involved in the actin reorganization that occurs at low pH, *wsc1Δ*, *wsc2Δ*, *wsc3Δ*, *mid2Δ* and *mtl1Δ* mutants were incubated at pH 3.0 for 2 h. The actin distribution in all of these mutants during the incubation was similar to that in wild-type cells (BY4741; data not shown), suggesting that these stress sensors play no part in reorganizing the actin cytoskeleton at low pH.

Subcellular Localization of Hog1p—To determine the subcellular localization of Hog1p, we examined cells expressing Hog1p-GFP by fluorescence microscopy after incubation at pH 3.0. Hog1p-GFP was distributed throughout the cytosol at pH 6.5 (Fig. 3A), whereas it maintained a predominantly nuclear location after 5 min of pH shift from pH 6.5 to 3.0 (Fig. 3B and C). It was distributed throughout the cytosol again after 20 min of the pH shift (Fig. 3D). These findings indicate that Hog1p rapidly translocates to the nucleus in response to a low pH.

Hog1p Directly Influences the Actin Cytoskeleton—Consistent with the nuclear localization of Hog1p under low pH, DNA microarray studies showed the transcriptional induction of 36 genes in *S. cerevisiae* exposed to pH 3.5 (*1*). Most of the low-pH-induced genes were inducible by hyperosmotic shock and dependent on Hog1 signalling. Hog1p regulates gene expression through several transcription factors: Hot1p, Sko1p, Smp1p, Msn1p, Msn2p and Msn4p (*27–30*). We showed previously that Msn2p and Msn4p are not involved in the reorganization of actin cytoskeleton (*2*). To determine whether the other transcription factors play any role in actin reorganization, several deletion mutants were incubated at pH 3.0. The actin distribution in *hot1Δ*, *sko1Δ*, *smp1Δ* and *msn1Δ* cells was similar to that in wild-type cells (data not shown), suggesting that these factors are not required to reorganize the actin cytoskeleton at low pH.

To examine if protein synthesis is required for the depolarization, wild-type and *hog1Δ* cells were incubated

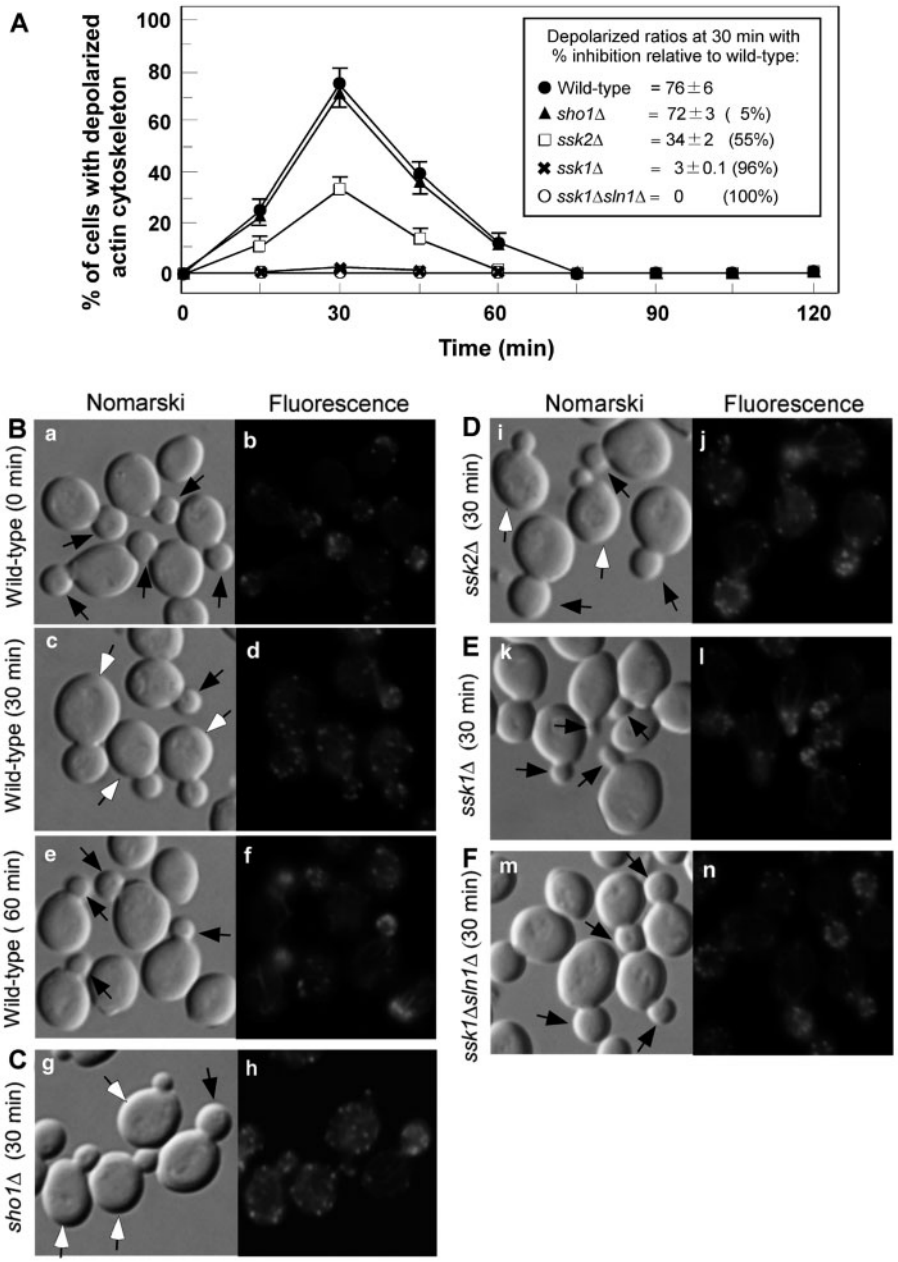

Fig. 2. **Distribution of actin in wild-type, *sho1*Δ, *ssk2*Δ, *ssk1*Δ and *ssk1*Δ *sln1*Δ cells during incubation in YPD at pH 3.0 and 30°C.** Wild-type (BY22286) (A and B), *sho1Δ* (A and C), *ssk2Δ* (A and D), *ssk1Δ* (A and E) and *ssk1Δ sln1Δ* (A and F) cells were grown in YPD (pH 6.5) to exponential phase, and then shifted to pH 3.0 for the indicated period. The cells were fixed and stained with rhodamine-conjugated phalloidin to visualize the actin cytoskeleton. (A) Wild-type or mutant cells were incubated for the indicated times. The percentage of cells ($n > 150$) exhibiting depolarized actin patches was determined. Data are the mean ± SD values of three independent experiments. (B) Wild-type cells were incubated for 0, 30 or 60 min. The *sho1Δ* (C), *ssk2Δ* (D), *ssk1Δ* (E) or *ssk1Δ sln1Δ* (F) cells were incubated for 30 min. Nomarski (a, c, e, g, i, k and m) and fluorescence (b, d, f, h, j, l and n) images are shown. Black arrows indicate buds with polarized actin (a, c, e, g, i, k and m). White arrows indicate mother cells with a depolarized distribution of actin (c, g and i).

at pH 3.0 with or without cycloheximide, which rapidly induces inhibition of protein synthesis in yeast (*33*). The non-treated cells grew 1.7-fold over a 2-h incubation (Fig. 4A). Cycloheximide completely inhibited cell proliferation while retaining the CFA in >90% of the cells (Fig. 4A), suggesting that inhibition of protein synthesis did not affect cell viability over the duration of this experiment. The cycloheximide-treated cells showed a similar rate of actin depolarization to the untreated, control cells (Fig. 4B–D). The depolarization observed within 30 min in the wild-type cells treated with cycloheximide was therefore due to the low pH conditions, and not dependent on protein synthesis, because the *hog1Δ* cells retained the polarized cytoskeleton at least for

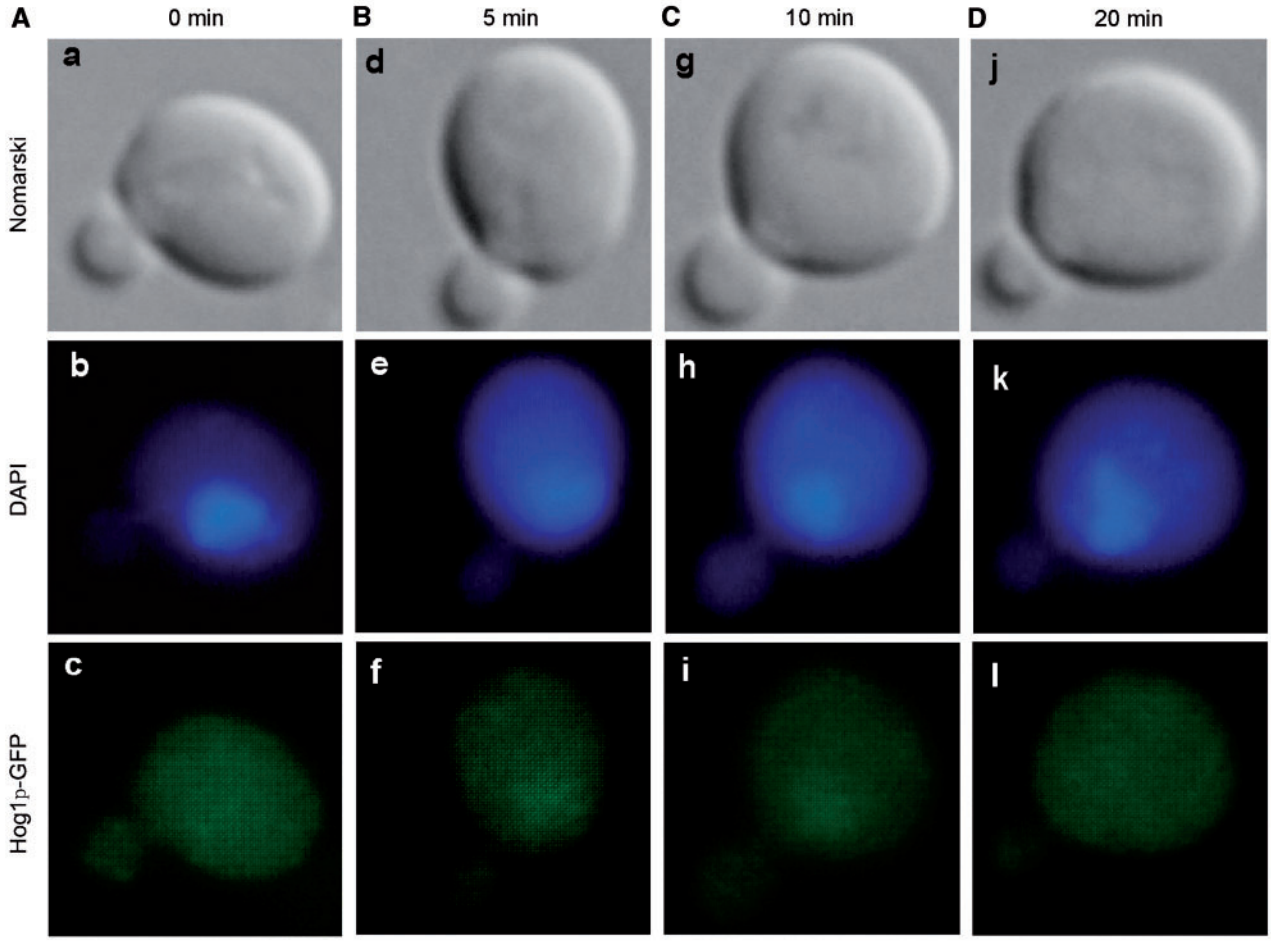

Fig. 3. **Subcellular localization of Hog1p.** Cells expressing Hog1p-GFP were incubated in YPD at pH 3.0 for 0 min (A), 5 min (B), 10 min (C) or 20 min (D), and then examined by fluorescence microscopy. Cells shown are representative of >100 cells observed. Nomarski (a, d, g and j), DAPI staining (b, e, h and k), and GFP (c, f, i and l) images are shown.

30 min in the presence of cycloheximide (Fig. 4B and F). These findings suggest that the actin depolarization in response to low pH is independent of *de novo* protein synthesis, and is a direct effect by Hog1p. However, protein synthesis is required for the formation of the polarized cytoskeleton, because cycloheximide inhibited the actin repolarization in the wild-type cells after 30 min at pH 3.0 (Fig. 4B).

Involvement of Polarisome Proteins in Reorganizing the Actin Cytoskeleton—Osmotic stress also induces a transient and reversible depolarization of the yeast actin cytoskeleton (*34*). Although the mechanism underlying this phenomenon is unknown, Ssk2p and the polarisome proteins, such as Bni1p, Spa2p, Pea2p and Bud6p, are important players in recovery of the normal actin organization following osmotic stress (*35*). Thus, in this study, we examined the requirement of Ssk2p in actin reorganization at low pH. Deletion of Ssk2p partially inhibited the actin depolarization, but did not affect the actin repolarization (Fig. 2A and D), suggesting that Ssk2p functions as a member of the Sln1 branch to disassemble polymerized actin cytoskeleton *via* Hog1p activation at low pH.

Deletion mutants of the polarisome proteins were also incubated at pH 3.0 to assess their effect on actin organization. While 85% of the wild-type cells had a depolarized actin cytoskeleton after 30 min at pH 3.0, only 39% of *pea2Δ* and 26% of *spa2Δ* cells were depolarized (Fig. 5, Supplementary Fig. S1). The actin distribution of *bud6Δ* was very similar to that of the wild-type cells (Fig. 5, Supplementary Fig. S1). These results suggest that Spa2p and Pea2p play important roles at least in the actin depolarization observed at low pH.

Bni1p and Bnr1p are nucleators in yeast for actin cable formation (*8*, *9*). Deletion mutants of these nucleators were incubated at pH 3.0 and then analyzed for actin distribution. The *bnr1Δ* cells showed similar actin organization to wild-type cells during the incubation (data not shown), whereas the deletion of Bni1p notably delayed the actin repolarization after 30 min at pH 3.0 (Fig. 5). These results suggest that Bni1p is the main player in nucleating actin repolarization in small- and medium-budded cells at low pH. This conclusion is consistent with the findings that Bni1p nucleates actin cables that originate in the bud and that Bnr1p polymerizes actin cables at the bud neck, although Bni1p

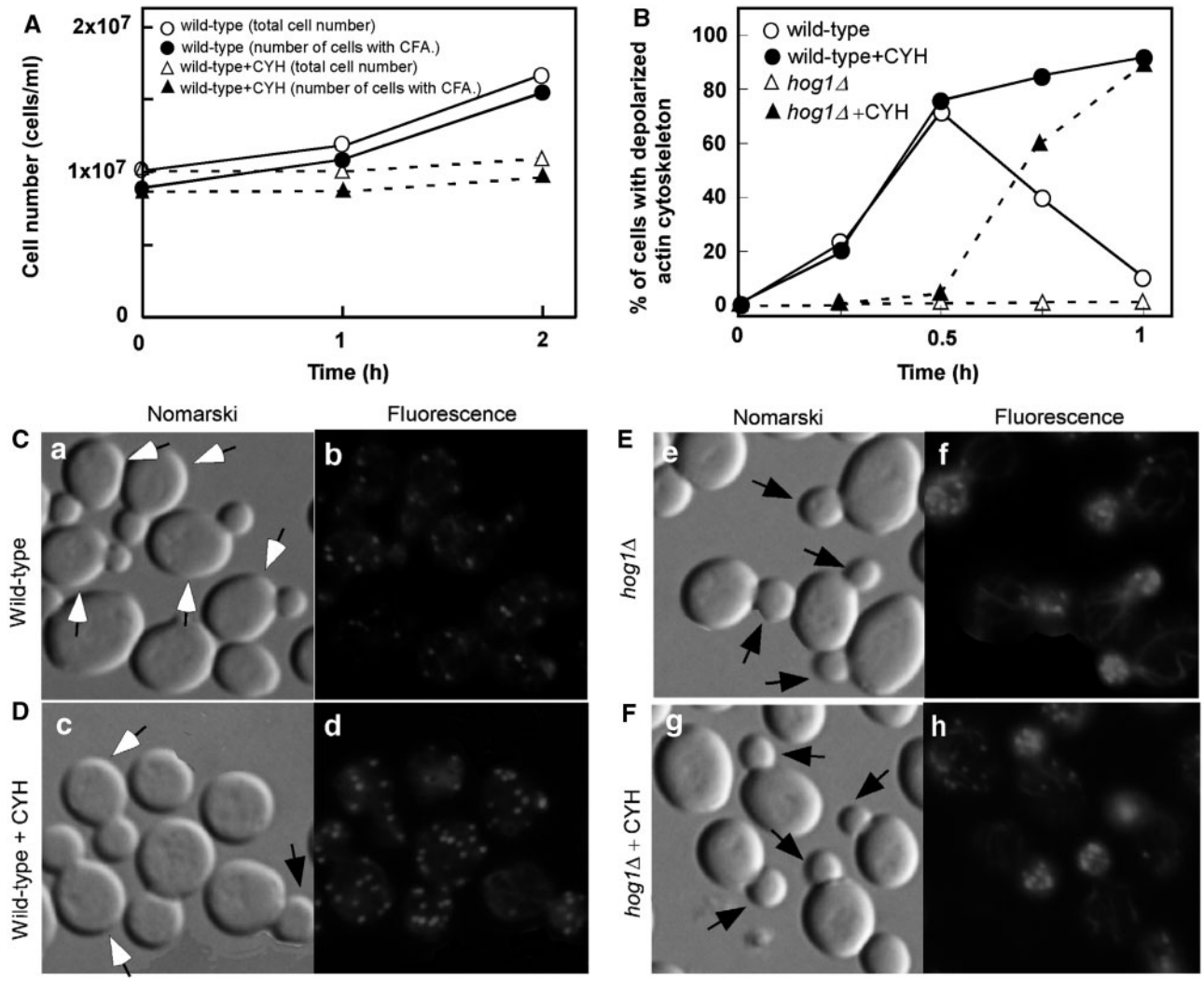

Fig. 4. **Total cell numbers, cells with CFA (A), and actin distribution (B) in wild-type (BY4741) and *hog1*Δ cells exposed to cycloheximide (CYH).** The cells were incubated in YPD at pH 3.0 and 30°C with or without CYH at 10 μg/ml. The number of CFA-positive cells was calculated from the percentage of active cells in the samples. The distribution of actin was determined as described in Fig. 2. Untreated (C) or CYH-treated wild-type cells (D), and untreated (E) or CYH-treated *hog1Δ* cells (F) were incubated for 30 min. Black arrows: buds with polarized actin (c, e and g), white arrows: mother cells with a depolarized distribution of actin (a and c). The results shown are representative of two independent experiments.

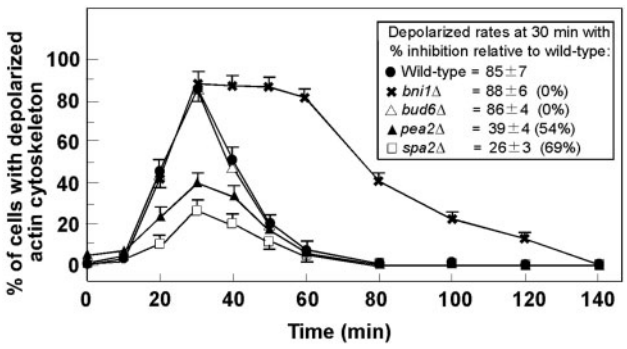

Fig. 5. **Actin distribution in wild-type (BY4741), *bni1*Δ *bud6*Δ, *pea2*Δ and *spa2*Δ cells during incubation in YPD at pH 3.0 and 30°C.** The distribution of actin was determined as described in Fig. 2. Data are mean ± SD values of three independent experiments.

and Bnr1p show some ability to compensate for the loss of the other isoform (*36*).

DISCUSSION

The present study sought to identify more components of the yeast response to low pH by assessing actin reorganization in wild-type and mutant cells at pH 3.0. The results showed that Hog1p activity was stimulated predominantly *via* the Sln1 branch of the HOG pathway to directly mediate actin depolarization. Furthermore, the polarisome proteins Spa2p, Pea2p and Bni1p were required for reorganization of actin cytoskeleton.

The findings here indicated Sln1-stimulated signal transduction to be important for Hog1p activation in response to low pH. The Sho1 and Sln1 branches of the HOG pathway are redundant for promoting growth

on high-osmolarity medium (*20*), but differ in their responses to other external stress signals. For example, Sho1p is required for activating Hog1p in response to heat stress (*37*), while Sln1 signalling has no effect. However, Sln1p is specifically required for Hog1p activation in response to citric acid stress (*38*) and to defects in GPI anchor synthesis (*39*).

The time course of actin depolarization in the present study was unaffected by the deletion of transcription factors, Hot1p, Sko1p, Smp1p or Msn1p, or by the presence of protein-synthesis inhibitor cycloheximide. Together, these findings suggest that actin depolarization induced by low pH is directly mediated *via* Hog1p.

Many of the components are required for cell polarity in *S. cerevisiae*. Such factors include cytoskeletal elements, motor proteins, G proteins and polarisome. Polarisome is a protein complex that alters polarized growth in *S. cerevisiae*, but the underlying mechanisms are not clear. It consists of Spa2p, Bni1p, Bud6p, Pea2p and perhaps other proteins (*14*). Spa2p interacts with all the known components through distinct domains and is thus considered the scaffold protein of the polarisome (*14*, *19*). The Bni1p binds directly to the C-terminal region of Spa2p (*15*). Bud6p is an actin monomer-binding protein that promotes Bni1p-stimulated actin assembly *in vitro* (*40*, *41*). The polarized localization of Spa2p is dependent on its binding partner Pea2p (*42*).

Regulation of Bni1p and Bnr1p is considered to involve activation through a conformational change induced by Cdc42p and other Rho GTPases (*36*, *43*, *44*). The binding of the Rho-GTPases to Bni1p is thought to relieve an autoinhibitory loop formed between the N-terminal and C-terminal portions of Bni1p such that the C-terminal fragment including the FH2 domain can nucleate actin cable formation. Furthermore, conditional mutants of *BNI1* in a *bnr1* background exhibit a rapid and reversible loss of actin cables after shifting to the restrictive temperature, without any immediate effect on cortical patches (*8*, *9*). Therefore, Bni1p appears to be an important player in the regulation for the assembly of polarized actin cables in the bud. When yeast cells are exposed to low pH, inactivation of Bni1p may be involved in Hog1p-mediated depolarization of the actin cytoskeleton. Probably, Spa2p and Pea2p contribute to Bni1p localization in the bud.

In summary, this is the first demonstration of the involvement of yeast polarisome proteins Spa2p, Pea2p and Bni1p in Hog1p-mediated actin reorganization. These results enhance our understanding of HOG signalling in the adaptation of yeast cells to low pH.

SUPPLEMENTARY DATA

Supplementary Data are available at *JB* online.

CONFLICT OF INTEREST

None declared.

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