Cell Wall Stress Depolarizes Cell Growth Via Hyperactivation of RHO1

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Abstract. Cells sense and physiologically respond to environmental stress via signaling pathways. *Saccharomyces cerevisiae* cells respond to cell wall stress by transiently depolarizing the actin cytoskeleton. We report that cell wall stress also induces a transient depolarized distribution of the cell wall biosynthetic enzyme glucan synthase FKS1 and its regulatory subunit RHO1, possibly as a mechanism to repair general cell wall damage. The redistribution of FKS1 is dependent on the actin cytoskeleton. Depolarization of the actin cytoskeleton and FKS1 is mediated by the plasma membrane protein WSC1, the RHO1 GTPase switch, PKC1, and a yet-to-be defined PKC1 effector branch. WSC1 behaves like a signal transducer or a stress-specific actin landmark that both controls and responds to the actin cytoskeleton, similar to the bidirectional signaling between integrin receptors and the actin cytoskeleton in mammalian cells. The PKC1-activated mitogen-activated protein kinase cascade is not required for depolarization, but rather for repolarization of the actin cytoskeleton and FKS1. Thus, activated RHO1 can mediate both polarized and depolarized cell growth via the same effector, PKC1, suggesting that RHO1 may function as a rheostat rather than as a simple on-off switch.

Key words: actin cytoskeleton • yeast • PKC1 • integrins • WSC1

ROWTH of a *Saccharomyces cerevisiae* cell is polarized, occurring at a defined position on the cell surface. This oriented growth is mediated by a polarized actin cytoskeleton that directs secretory vesicles to the growth or bud site (Chant, 1996; Li et al., 1995; Madden and Snyder, 1998). The actin cytoskeleton is composed of two morphologically distinct pools of actin polymers, patches and cables. Patches are foci of actin and actin-binding proteins that are associated with plasma membrane invaginations (Mulholland et al., 1994), and move rapidly over long distances (Waddle et al., 1996). Cables are bundles of overlapping actin filaments (Karpova et al., 1998). Recent studies suggest that patches and cables associate and form an integrated system (Amberg, 1998; Karpova et al., 1998). The distribution of actin patches and cables is polarized in a cell cycle–dependent manner (Adams and Pringle, 1984; Schmidt and Hall, 1998). In G0 cells, actin patches and cables are randomly distributed. As a yeast cell initiates growth, patches concentrate at a preselected bud site, forming a ring structure, and cables orient toward this site. Bud emergence begins and subsequently actin patches migrate into the bud. During bud maturation, patches are found exclusively in the bud, and cables are aligned longitudinally from the mother cell into the bud. As the daughter cell reaches a critical size, the asymmetric distribution of actin is lost. Late in the cell cycle, before cytokinesis, the actin cytoskeleton repolarizes as patches accumulate in and cables orient toward the mother–bud neck region. The cell cycle–dependent organization of the actin cytoskeleton is controlled by Rho-type guanosine triphosphatases (GTPases)[1] (Schmidt and Hall, 1998).

Five Rho-like GTPases have been identified in budding yeast (CDC42 and RHO1–4), and all have been implicated in the organization of the actin cytoskeleton (Leberer et al., 1997; Helliwell et al., 1998b; Schmidt and Hall, 1998; Tanaka and Takai, 1998). CDC42 is required for establishing the polarity of the actin cytoskeleton and is, thus, required for emergence of a bud at a preselected site. RHO1–4 further mediate the polarized organization of the actin cytoskeleton, and are required for maintenance of bud growth.

RHO1 mediates bud growth by controlling polarization of the actin cytoskeleton and cell wall synthesis (Cabib et al., 1998). RHO1 is activated by at least two separate inputs, the phosphatidylinositol kinase homologue TOR2 and cell wall defects, both of which activate RHO1 via the exchange factor ROM2 (and possibly also via the related ex-

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change factor ROM1) (Ozaki et al., 1996; Schmidt et al., 1997; Bickle et al., 1998). Cell wall defects, which hyperactivate ROM2 (Bickle et al., 1998), might be sensed and signaled to ROM2 by the plasma membrane protein WSC1/SLG1/HCS77 (see below) (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998). RHO1 has four downstream effectors. It binds and activates PKC1, which in turn controls the actin cytoskeleton and transcription of cell wall biosynthesis genes via a mitogen-activated protein (MAP) kinase cascade (Nonaka et al., 1995; Igual et al., 1996; Kamada et al., 1996; Helliwell et al., 1998b; Zhao et al., 1998; see below). RHO1 also binds and activates the integral plasma membrane protein FKS1 (β-1,3-glucan synthase) and, thereby, controls cell wall synthesis directly (Drgonova et al., 1996; Qadota et al., 1996). Finally, RHO1 interacts with BNI1, a formin family member, and SKN7, a two component signaling protein (Kohno et al., 1996; Alberts et al., 1998). RHO1 may also control the actin cytoskeleton via BNI1 that binds profilin (Evangelista et al., 1997). RHO1 and ROM2 are localized at growth sites (Yamochi et al., 1994; Manning et al., 1997), and RHO1 may activate only a subset of its effectors in response to a particular input (Helliwell et al., 1998b).

The RHO1 effector PKC1 activates a MAP kinase cascade composed of BCK1/SLK1 (MAPKKK), MKK1 and MKK2 (MAPKK), and MPK1/SLT2 (MAPK) (Levin et al., 1994; Banuett, 1998; Gustin et al., 1998). This MAP kinase cascade is activated by hypotonic or heat shock, and also during periods of polarized cell growth (Davenport et al., 1995; Kamada et al., 1995; Zarzov et al., 1996), and controls actin organization and transcription of cell wall biosynthesis genes (Igual et al., 1996; Helliwell et al., 1998b; Zhao et al., 1998). Heat shock is thought to induce a weakness in the cell wall that leads to activation of the MAP kinase cascade via a putative cell wall sensor (Kamada et al., 1995; Bickle et al., 1998). Several observations suggest that the cell wall sensor is the type I transmembrane protein WSC1/SLG1/HCS77 (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998; Lodder et al., 1999). WSC1, a member of the WSC family of plasma membrane proteins that includes WSC1–4 and the related MID2 protein, is required for heat stress activation of the MAP kinase cascade (Gray et al., 1997; Verna et al., 1997; Ketela et al., 1999; Rajavel et al., 1999). Furthermore, a WSC1 deficiency is suppressed by overexpression of ROM2, RHO1, or PKC1 (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998).

The yeast cell wall is a dynamic structure that comprises 15–30% of the dry weight of the cell (Orlean, 1997; Kapteyn et al., 1999). It is composed of three major components, mannoproteins, β-1,6-glucan, and β-1,3-glucan, all cross-linked to each other and to chitin, an N-acetylglucosamine polymer (Kollar et al., 1997; Kapteyn et al., 1999). β-1,3-glucan is synthesized by the integral plasma membrane protein FKS1. The cell wall determines cell shape, provides rigidity that counteracts the outward turgor pressure on the plasma membrane, and acts as a diffusion barrier delimiting the periplasmic space. Cell wall synthesis, a poorly understood process, is temporally and spatially controlled in response to growth, stress, and mating signals (Orlean, 1997; Kapteyn et al., 1999).

The yeast actin cytoskeleton is also reorganized in response to mating pheromone or environmental stress.

Mating pheromone induces polarization of the actin cytoskeleton and a characteristic mating projection (Read et al., 1992). These changes are mediated by the well characterized pheromone signaling pathway and the Rho-type GTPase CDC42 (Leberer et al., 1997; Schmidt and Hall, 1998; Tanaka and Takai, 1998). Hypertonic or heat shock induces a transient depolarization of the actin cytoskeleton (Chowdhury et al., 1992; Lillie and Brown, 1994). However, the physiological significance of this response, and the factors or signaling pathways that mediate it are unknown. In mammalian cells, the induction of actin stress fibers is controlled by integrins and Rho GTPases (Hall, 1998; Howe et al., 1998; Schmidt and Hall, 1998).

Here, we investigate the transient depolarization of the actin cytoskeleton in response to environmental stress. We show that actin depolarization in response to cell wall damage is necessary to depolarize glucan synthase FKS1. The transient depolarization of the actin cytoskeleton and FKS1 may be a homeostasis mechanism to repair general cell wall damage. This response is controlled by a putative signaling pathway consisting of WSC1, ROM2, RHO1, and PKC1. The PKC1-activated MAP kinase pathway is not required for this depolarization process, but rather for repolarization, suggesting that a yet-to-be defined PKC1 effector pathway mediates depolarization.

Material and Methods

Strains, Plasmids, and Media

The *S. cerevisiae* strains and plasmids used in this study are listed in Table I and II, respectively. All strains are isogenic derivatives of JK9-3d. Rich media (YPD) or synthetic minimal media (SD, SRAFF, and SGAL) complemented with the appropriate nutrients for plasmid maintenance were as described (Guthrie and Fink, 1991). Latrunculin-A (200 μM) was added to cultures from a 200-mM stock in DMSO. A culture of cells was treated with Zymolyase 20T (7.5 U/ml; Seikagaku Corp.) added from a 750-U/ml stock in YPD at 30°C for 20 min. A culture of cells was treated with SDS (0.02%) added from a 10% stock in water at 24 or 30°C for 10 min. For all heat shock experiments, 2-ml aliquots were removed from a

Table I. Yeast Strains Used in This Study

Strain	Genotype
JK9-3da/α	*MAT***a**/α *leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1/trp1 his4/his4 rme1/rme1 HML***a**/*HML***a**
JK9-3da	*MAT***a** *leu2-3,112 ura3-52 trp1 his4 rme1 HML***a**
TB50a	*MAT***a** *leu2-3,112 ura3-52 trp1 his3 rme1 HML***a**
MH272-1da	*MAT***a** *leu2-3,112 ura3-52 trp1 his3 rme1 HML***a**
AS135-1a	JK9-3da *rho2::kanMX4*
AS138-1b	JK9-3da *rom2::URA3*
AS167-1d	MH272-1da *bni1::URA3*
IH1-5a	TB50a *wsc4::HIS3MX6*
MB146-3d	MH272-1da *fks1::HIS3*
NB75-1a	JK9-3da *skn7::LEU2*
PA39-1b	JK9-3da *wsc1::kanMX4*
PA62-2d	JK9-3da *wsc3::kanMX4*
PA88-1a	JK9-3da *wsc2::kanMX4*
PA96	JK9-3da/α *wsc1::kanMX4/wsc1::kanMX4*
PA109-1c	TB50a *bck1::HIS3MX6*
PA120-3b	JK9-3da *rho1::kanMX4* / pHA-RHO1
PE3-2a	JK9-3da *mid2::kanMX4*
SD4-2c	JK9-3da *rom1::kanMX4*
TS45-1a	TB50a *mpk1::TRP1*

Table II. Plasmids Used in This Study

Plasmid	Characteristic and source
pGAL-BCK1*	pPAD91, *BCK1-20* allele under the control of the *GAL1* promoter
pGAL-MKK1*	pNV7-MKK1(P386), expresses activated-MKK1 under the control of the *GAL1* promoter (Watanabe et al., 1995)
pGAL-PKC1*	pBM743, expresses activated-PKC1 under the control of the *GAL1* promoter (D.E. Levin)
pGAL-RHO1	pTB230, expresses the functional NH_2 terminally 2×HA-tagged RHO1 under the control of the *GAL1* promoter (Schmidt et al., 1997)
pGAL-RHO1*	pAS106, expresses functional NH_2 terminally HA-tagged activated RHO1 (H68) under the control of the *GAL1* promoter
pGAL-RHO2	pAS35, expresses functional NH_2 terminally HA-tagged RHO2 under the control of the *GAL1* promoter
pGAL-ROM2	pAS48, expresses functional NH_2 terminally HA-tagged ROM2 under the control of the *GAL1* promoter
pGAL-WSC1	pPAD62, *WSC1* under the control of the *GAL1* promoter
pGAL-WSC2	pPAD45, expresses functional COOH terminally cmyc-tagged WSC2 under the control of the *GAL1* promoter
pGAL-WSC3	pPAD48, expresses functional COOH terminally cmyc-tagged WSC3 under the control of the *GAL1* promoter
pHA-RHO1	pAS97, expresses functional NH_2 terminally HA-tagged RHO1 from its own promoter (*CEN URA3*)
pHAs	pPAD80/81/82/83, 3×HA tagging vector (see Materials and Methods), 2µ*LEU2, CEN LEU2, CEN URA3*, 2µ*URA3*, respectively
pWSC1-HA	pPAD86, expresses functional COOH terminally 3×HA-tagged WSC1 from its own promoter (see Materials and Methods)

logarithmic phase culture grown at 24°C and incubated at 37°C for the indicated time.

Genetic Techniques

Plasmid DNA was isolated as described (Sambrook et al., 1989). Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). *Escherichia coli* strain MH1 was used for propagation and isolation of plasmids (Sambrook et al., 1989). The entire open reading frames of *WSC1*, *WSC2*, *WSC3*, *BCK1*, and *MKK1* were replaced by PCR-generated *kanMX4* or *HIS3MX6* cassettes, as described (Wach et al., 1994). Disruptions were verified by PCR.

Construction of 3×HA-tagged WSC1

A 0.45-kb fragment encoding a triple hemagglutinin (HA) tag followed by the *CYC1* transcription terminator was generated by PCR using the plasmid pYADE4 (Brunelli and Pall, 1993), containing the *CYC1* terminator sequence, as a template and the following two oligonucleotides: 5′-ACAT**GCATGC**TACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCGGACTATGCAGGATATCCATATGACGTTCCAGATTACGCTTAACCAAGATGGCCTTTGGTGGGTTGAAGAAG-3′ (SphI site in bold and HA epitope coding sequence underlined) and 5′-ATAGCAAAGATTGAATAAGGC-3′. The 0.45-kb fragment was cut with SphI and HindIII, and the resulting fragment was cloned into YEplac181 (2µ*LEU2*), YCplac111 (*CEN LEU2*), YCplac33 (*CEN URA3*), and YEplac195 (2µ*URA3*) (Gietz and Sugino, 1988), creating plasmids pPAD80, pPAD81, pPAD82, and pPAD83, respectively. These plasmids, containing a 3×HA coding sequence preceded by a multiple cloning site and followed by a termination codon and the *CYC1* terminator sequence, are useful for constructing COOH terminally 3×HA–tagged proteins. pPAD86 (pPAD81::*WSC1*) (*CEN LEU2*) contains *WSC1* as a 1.6-kb SacI-SmaI PCR fragment. The SacI site was introduced 460 nucleotides upstream of the *WSC1* initiation codon, and the SmaI site replaced the termination codon.

Indirect Immunofluorescence

Logarithmically growing cells were fixed for 2 h in the growth medium supplemented with formaldehyde (3.7%) and potassium phosphate buffer (100 mM, pH 6.5). Cells were washed and resuspended in sorbitol buffer (1.2 M sorbitol, 100 mM potassium phosphate, pH 6.5). Cell walls were digested for 60–120 min at 37°C in sorbitol buffer supplemented with β-mercaptoethanol (20 mM) and Zymolyase 20T (0.1 mg/ml; Seigagaku Corp.) or lyticase (gift from P. Ernst, Biozentrum Basel). Spheroblasts were attached on poly-L-lysine–coated glass slides and permeabilized in PBT (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl, 0.1% Triton X-100). Immunofluorescent detection of FKS1 was performed with the T2B8 anti–FKS1 mAb (Qadota et al., 1996). HA-tagged WSC1 and RHO1 were detected with a high affinity monoclonal anti–HA antibody (clone 16B12;

BAbCO) at a final dilution of 1:1,000 in PBT. The samples were treated with primary antibody for 2 h and subsequently with Cy3-conjugated rabbit anti–mouse IgG (Molecular Probes, Inc.), diluted 1:1,000 in PBT for 90 min. Washed cells were examined with a Zeiss Axiophot microscope (100× objective) and a video imaging system (MWG Biotech).

Actin Staining

Logarithmically growing cells were fixed in formaldehyde (3.7%) and potassium phosphate buffer (100 mM, pH 6.5), and stained with TRITC-phalloidin (Sigma Chemical Co.) to visualize actin, as described previously (Benedetti et al., 1994).

Results

Cell Wall Stress Induces Actin Cytoskeleton Depolarization

The actin cytoskeleton is transiently depolarized upon heat shock (Lillie and Brown, 1994; Desrivieres et al., 1998). To investigate this effect further, we examined the actin cytoskeleton in wild-type cells shifted from 24°C to 37°C for different time intervals (see Materials and Methods). The percentage of small- and medium-budded cells (cells that normally exhibit a polarized actin cytoskeleton) containing a depolarized actin cytoskeleton was determined. Cells containing <50% of their actin patches in the bud were considered to have a depolarized actin cytoskeleton. The maximum percentage of depolarized cells (~95%) was reached after ~30 min of heat shock, with complete repolarization occurring after ~120 min (Fig. 1). The maximally depolarized state persisted for ~15 min. Thus, as seen previously, heat shock induces a rapid and severe, but transient, depolarization of the actin cytoskeleton. Interestingly, heat shock did not depolarize actin patches at the mother–bud neck in large-budded cells.

Heat shock is thought to cause a cell wall weakening (Kamada et al., 1995). To determine if heat-induced actin depolarization is a consequence of a cell wall defect, we examined if osmotic stabilization of the cell wall by 1 M sorbitol blocked the actin depolarization response. The response was partly blocked in cells shifted to a high temperature in the presence of 1 M sorbitol (data not shown). To
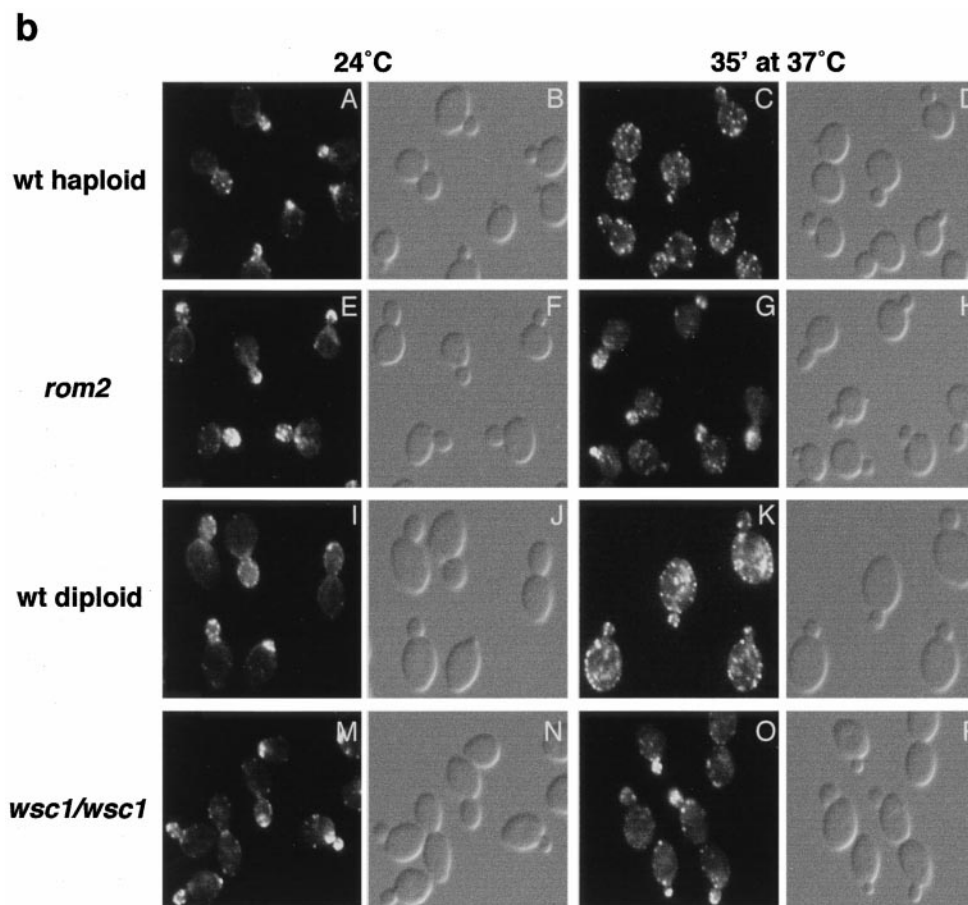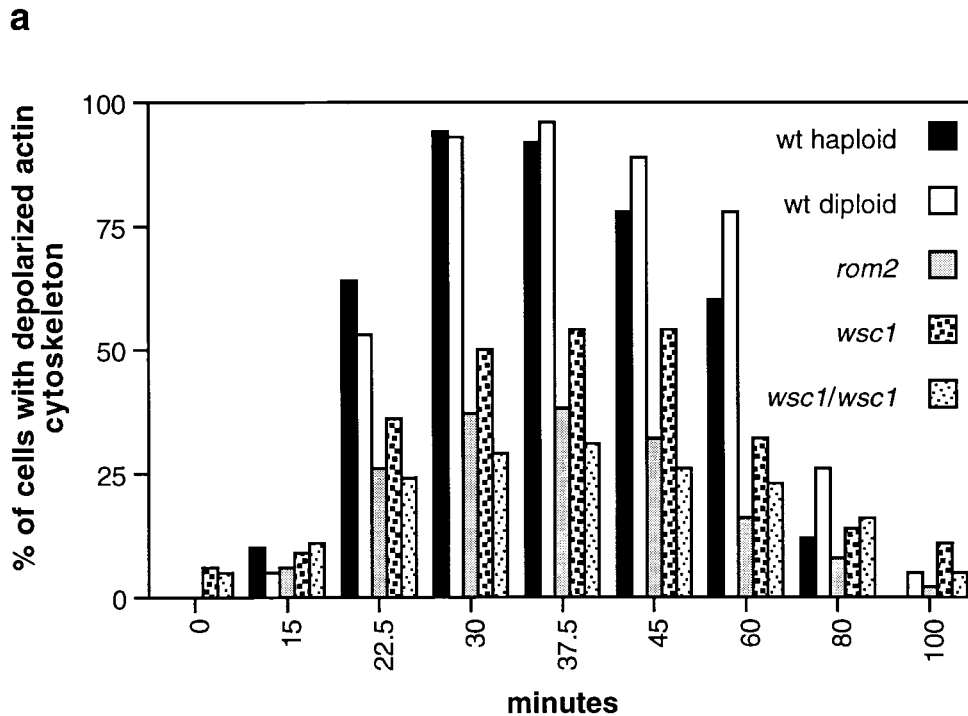
a

b

Figure 1. WSC1 and ROM2 are required for heat-induced depolarization of the actin cytoskeleton. (a) Wild-type (wt) (JK9-3d**a**), *rom2* (AS138-1b), and *wsc1* (PA39-1b) haploid cells, and wild-type (JK9-3d**a**/α) and *wsc1*/*wsc1* (PA96) diploid cells were grown in rich medium at 24°C, shifted to 37°C for the indicated time (in minutes), fixed, and processed for visualization of the actin cytoskeleton. The percentage of cells ($n > 200$) exhibiting depolarized actin patches was determined (see Materials and Methods). A representative of three experiments is shown. (b) Wild-type (JK9-3d**a**) (A–D) and *rom2* (AS138-1b) (E–H) haploid cells, and wild-type (JK9-3d**a**/α) (I–L) and *wsc1*/*wsc1* (PA96) (M-P) diploid cells were grown in rich medium at 24°C, shifted to 37°C for 35 min, fixed, stained with TRITC-phalloidin, and observed by fluorescence (A, C, E, G, I, K, M, and O) and Nomarski (B, D, F, H, J, L, N, and P) microscopy to visualize the actin cytoskeleton and whole cells, respectively.

determine further if actin depolarization is a consequence of a cell wall defect, the actin cytoskeleton was examined in cells in which a cell wall defect was induced either by mild treatment with Zymolyase (β-1,3-glucanase), which digests the cell wall, or by treatment with the anionic detergent SDS (see Materials and Methods) (Kitamura et al., 1974; Igual et al., 1996; Bickle et al., 1998). Essentially all cells treated with 7.5 U/ml Zymolyase or with 0.02% SDS

exhibited a depolarized actin cytoskeleton (data not shown). However, unlike heat-induced depolarization, the Zymolyase- and SDS-induced depolarization persisted for the duration of the experiment (120 min), possibly because cells cannot adapt to these treatments as they can adapt to heat shock. These results suggest that depolarization of the actin cytoskeleton is a response to cell wall stress.

Cell Wall Stress Depolarizes the Cellular Distribution of Glucan Synthase

The above findings suggested that cells might delocalize the actin cytoskeleton as a homeostasis mechanism to re-pair cell wall damage. To investigate this possibility, we first examined the cellular localization of FKS1 in heat shocked cells by immunofluorescence (see Materials and Methods). FKS1, the presumed catalytic subunit of the cell wall biosynthetic enzyme β-1,3-glucan synthase, normally localizes with its regulatory subunit, RHO1, and actin patches at sites of cell growth (Drgonova et al., 1996; Qadota et al., 1996). As expected, FKS1 displayed a polarized distribution in cells growing at 24°C, found associated with the plasma membrane exclusively in the bud. Upon heat shock, the distribution of FKS1 was transiently depolarized, with a time course similar to that of the actin cytoskeleton (Fig. 2 a). After 35 min at 37°C, the FKS1 signal
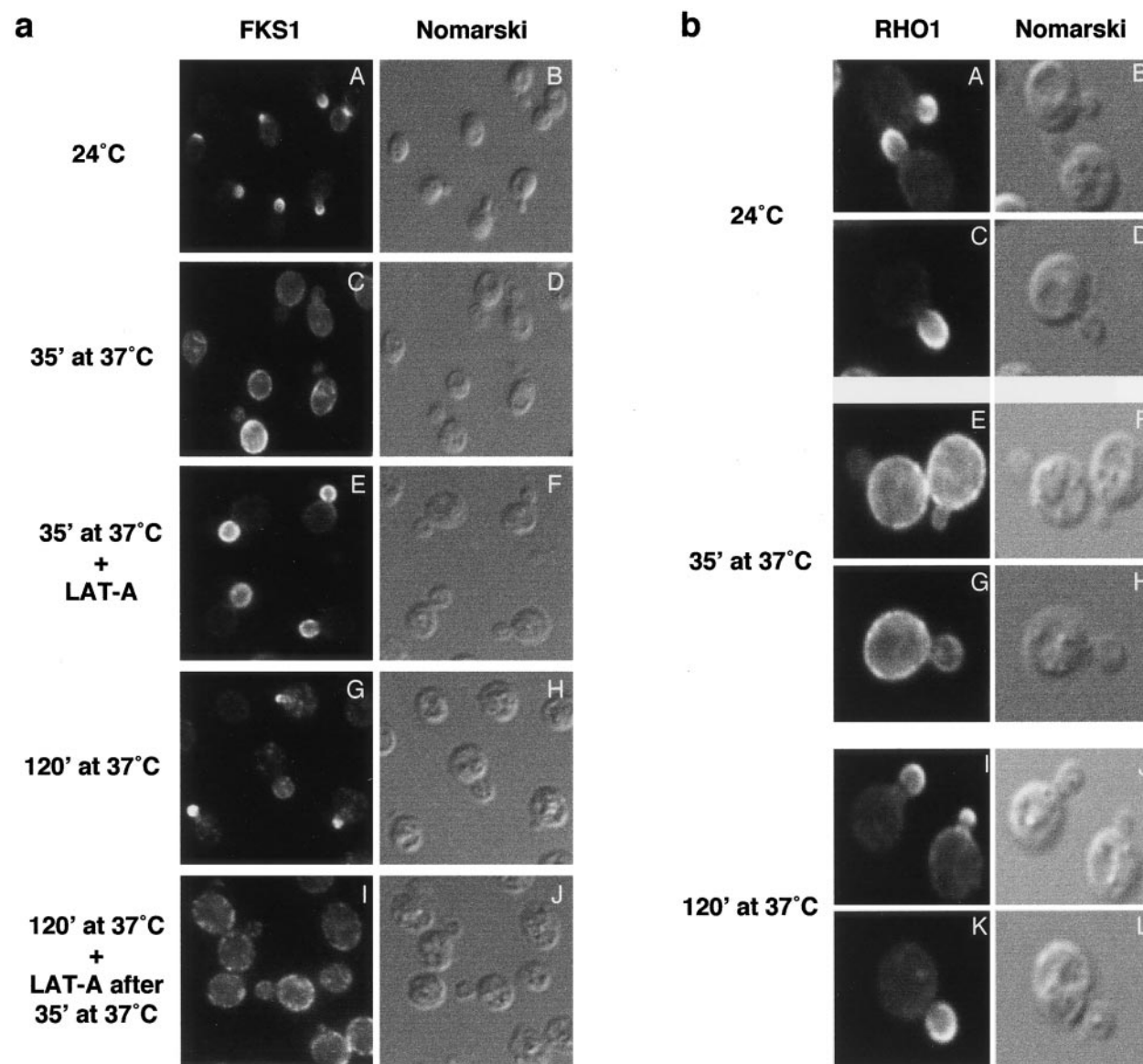

Figure 2. The actin cytoskeleton is required for de- and repolarization of glucan synthase upon heat shock. (a) Wild-type cells (JK9-3d**a**) were grown at 24°C, shifted at 37°C for the indicated time, fixed, and processed for detection of FKS1 by immunofluorescence (see Materials and Methods). LAT-A was added at time of shift to 37°C (E–F) and after 35 min at 37°C (I–J). Fluorescence (A, C, E, G, and I, FKS1) and Nomarski (B, D, F, H, and J) images are shown. (b) Cells expressing HA-RHO1 (PA120-3b) were grown in rich medium at 24°C, shifted to 37°C for the indicated time, fixed, and processed for detection of HA-RHO1 by immunofluorescence (see Materials and Methods). Fluorescence (A, C, E, G, I, and K, RHO1) and Nomarski (B, D, F, H, J, and L) images are shown.

decreased in the bud and appeared uniformly at the periphery of the entire cell. After 120 min at 37°C, FKS1 was relocalized exclusively to the bud. A depolarization of FKS1 was also observed in SDS-treated cells (data not shown). We also examined the cellular localization of the glucan synthase regulatory subunit RHO1 in heat shocked cells. Cells expressing HA-tagged RHO1 (PA120-3b) were shifted from 24 to 37°C and further incubated for 35 or 120 min. Visualization of RHO1 by indirect immunofluorescence revealed a transient depolarization similar to that observed for FKS1 (Fig. 2 b). Thus, glucan synthase is redistributed evenly throughout the cell periphery in response to cell wall stress. This depolarization of cell growth may be to repair general cell wall damage.

FKS1 Requires the Actin Cytoskeleton for De- and Repolarization

To investigate further the possibility that actin delocalization is part of a homeostasis mechanism to depolarize cell growth and repair general cell wall damage, we examined if FKS1 de- and repolarization are dependent on the actin cytoskeleton. To determine if the actin cytoskeleton is required for FKS1 depolarization, FKS1 localization was examined in cells treated simultaneously with heat and latrunculin-A (LAT-A), a toxin that disrupts the actin cytoskeleton by sequestering actin monomers (Coue et al., 1987). LAT-A prevented the heat-induced depolarization of FKS1, indicating that the actin cytoskeleton is required for FKS1 depolarization (Fig. 2 a). To determine if the actin cytoskeleton is also required for FKS1 repolarization, we examined the effect of LAT-A on depolarized FKS1. Cells previously incubated at a high temperature (37°C) for 35 min were treated with LAT-A and examined for localization of FKS1. After 120 min of heat shock and 85 min of LAT-A treatment, FKS1 remained depolarized, whereas FKS1 repolarized in mock-treated cells (Fig. 2 a). Thus, the actin cytoskeleton is required for both FKS1 de- and repolarization, suggesting that cells depolarize their actin cytoskeleton in response to cell wall damage as a mechanism to depolarize cell growth and repair general cell wall damage.

ROM2 and WSC1, But Not the PKC1-activated MAP Kinase Cascade, Are Required for Stress-induced Actin Cytoskeleton and FKS1 Depolarization

RHO1 and the PKC1-activated MAP kinase cascade are activated by cell wall damage (Kamada et al., 1995; Bickle et al., 1998). To determine if RHO1 or the PKC1-activated MAP kinase cascade mediates the stress-induced depolarization of the actin cytoskeleton, we examined this response in null mutants individually lacking a WSC family member (WSC1, WSC2, WSC3, WSC4, or MID2), a RHO1 exchange factor (ROM1 or ROM2), the nonessential GTPase RHO2, a RHO1 effector (BNI1, SKN7, or FKS1), or a member of the PKC1-activated MAP kinase pathway (BCK1 or MPK1). *rho1* and *pkc1* null mutants were not examined because RHO1 and PKC1 are essential for growth. The *wsc1*, *wsc2*, *wsc3*, *wsc4*, *mid2*, *rom1*, *rom2*, *rho2*, *bni1*, *skn7*, *fks1*, *bck1*, and *mpk1* null mutants were grown at 24°C, shifted to 37°C for different time intervals, and processed for visualization of the actin cytoskeleton. The *wsc1* and *rom2* mutants were severely, but

not completely, defective in heat-induced depolarization of the actin cytoskeleton (Fig. 1). A maximum of ~55% and ~40% of the *wsc1* and *rom2* cells (versus ~95% for wild-type cells), respectively, exhibited a depolarized actin cytoskeleton after 30 min at 37°C. This defect was not simply a delay in depolarization of the actin cytoskeleton, as a higher percentage of depolarized cells was not observed at later time points. Furthermore, *wsc1* and *rom2* mutants were sensitive to SDS for growth, and exhibited a defect in FKS1 depolarization that correlated with these mutants' defect in actin depolarization (data not shown). Because a *wsc1/wsc1* homozygous diploid strain has a more severe growth defect than a *wsc1* haploid (Gray et al., 1997), we also examined the actin cytoskeleton in a heat shocked *wsc1/wsc1* diploid. A maximum of ~30% of the *wsc1/wsc1* cells (versus ~95% for wild-type diploid cells) exhibited a depolarized actin cytoskeleton, after 30 min at 37°C, indicating that the block in depolarization of the actin cytoskeleton was indeed more pronounced in the homozygous diploid mutant. Surprisingly, all of the other mutants listed above exhibited a wild-type–like depolarization response. These observations suggest that stress-induced actin and growth depolarization requires WSC1 and ROM2, but not the PKC1-activated MAPK kinase pathway or any RHO1 effector other than PKC1.

Hyperactivated RHO1 or PKC1, But Not BCK1 or MKK1, Is Sufficient to Induce Actin and FKS1 Depolarization

The previous observation that cell wall defects increase ROM2 exchange activity toward RHO1 (Bickle et al., 1998) and the above finding that a *wsc1* or *rom2* mutation reduces stress-induced depolarization suggest that the depolarization process requires RHO1 hyperactivation. To examine this further, we investigated the effect of overexpressing constitutively activated RHO1 (RHO1*) on the actin cytoskeleton and FKS1 localization. As RHO1* is toxic, we constructed a plasmid-borne *RHO1** allele under control of the strong and galactose-inducible *GAL1* promoter (pGAL-RHO1*). This construct was not toxic in cells grown on a carbon source other than galactose (data not shown). Cells containing pGAL-RHO1* were grown to logarithmic phase in medium containing raffinose as a carbon source, and galactose was added to a final concentration of 2%. Aliquots of cells were removed at different time points after addition of galactose and processed for visualization of the actin cytoskeleton. In raffinose-containing medium, cells displayed a normal vegetative distribution of actin patches. After 1.5 h in galactose, the number of patches in the mother cell increased (data not shown). After 2.5 h in galactose, the actin cytoskeleton was depolarized in essentially all cells (Fig. 3). FKS1 was also depolarized, with similar kinetics and severity as observed for the actin cytoskeleton (data not shown). Expression of wild-type RHO1 from the *GAL1* promoter (pGAL-RHO1) had no depolarizing effect (data not shown). Thus, RHO1 hyperactivation is sufficient to induce actin and FKS1 depolarization. This finding, combined with the previous findings that cell wall stress hyperactivates ROM2 exchange activity toward RHO1 (Bickle et al., 1998) and that a *rom2* mutation blocks depolariza-
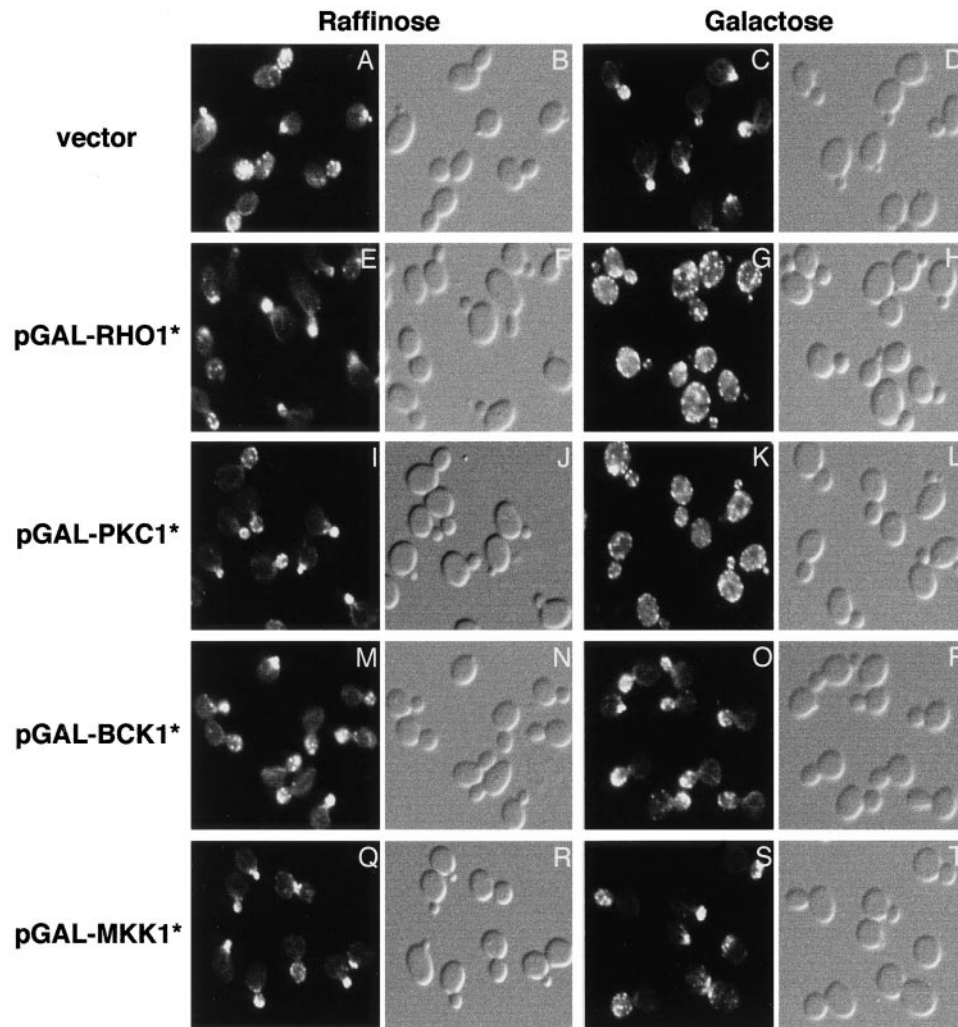
Raffinose **Galactose**

vector

pGAL-RHO1*

pGAL-PKC1*

pGAL-BCK1*

pGAL-MKK1*

Figure 3. Hyperactivated RHO1 or PKC1 is sufficient to induce actin depolarization. Wild-type (JK9-3d**a**) cells carrying an empty vector (A–D), pGAL-RHO1* (E–H), pGAL-PKC1* (I–L), pGAL-BCK1* (M–P), or pGAL-MKK1* (Q–T) were grown at 30°C in minimal medium containing raffinose. Galactose was added to a final concentration of 2% and strains were further incubated for 2.5 h (empty vector, pGAL-RHO1* or pGAL-PKC1*) or 5 h (pGAL-BCK1* or pGAL-MKK1*). Cells containing pGAL-BCK1* or pGAL-MKK1* gave the same result when incubated in galactose for 2.5 or 5 h. Samples were fixed, stained with TRITC-phalloidin, and observed by fluorescence (A, C, E, G, I, K, M, O, Q, and S) and Nomarski (B, D, F, H, J, L, N, P, R, and T) microscopy to visualize the actin cytoskeleton and whole cells, respectively.

tion, suggests that depolarization results from RHO1 hyperactivation.

We examined the effect of overexpressed and constitutively activated PKC1 (PKC1*), BCK1 (BCK1*), and MKK1 (MKK1*) on the actin cytoskeleton and FKS1 distribution. Like pGAL-RHO1*, pGAL-PKC1*, pGAL-BCK1*, and pGAL-MKK1* were also toxic uniquely in cells grown on galactose-containing medium (data not shown). The effect of pGAL-PKC1* on the actin cytoskeleton and FKS1 distribution was indistinguishable from that of pGAL-RHO1*, indicating that hyperactivation of PKC1 is also sufficient to elicit the depolarization response (Fig. 3 and data not shown). Hyperactivation of BCK1 or MKK1 did not induce a depolarization of the actin cytoskeleton or FKS1 in cells shifted to galactose for 2.5 (as performed above for RHO1* and PKC1*) or 5 h (Fig. 3 and data not shown). These results further indicate that the PKC1-activated MAP kinase cascade is not involved in stress-induced depolarization of growth, and suggest that a yet-to-be defined PKC1 effector pathway (Lee and Levin, 1992; Helliwell et al., 1998b) mediates the depolarization response. Expression of wild-type WSC1, WSC2, WSC3, RHO2, or ROM2 from the GAL1 promoter had no effect on the actin cytoskeleton (data not shown).

PKC1-activated MAP Kinase Cascade Is Required for Repolarization of the Actin Cytoskeleton and FKS1

The previous findings that mutants defective in MPK1/SLT2 exhibit a depolarized actin cytoskeleton (Mazzoni et al., 1993) and overexpression of MPK1 suppresses the actin polarization defect of a *rho1* mutant (Helliwell et al., 1998b) suggested that the PKC1-activated MAP kinase pathway might be required for repolarization. To investigate this further, we examined repolarization of the actin cytoskeleton and FKS1 in *bck1* and *mpk1* null mutants. As described above, the actin cytoskeleton and FKS1 were normally depolarized in *bck1* and *mpk1* cells shifted to 37°C for 35 min (Fig. 4). However, after 120 min and longer, when the actin cytoskeleton is repolarized in wild-type cells, depolarization of the actin cytoskeleton persisted in most *bck1* and *mpk1* cells (Fig. 4). A similar effect was obtained when examining FKS1 distribution (data not shown). It is important to note that *bck1* and *mpk1* null mutations in our strain background do not confer a severe
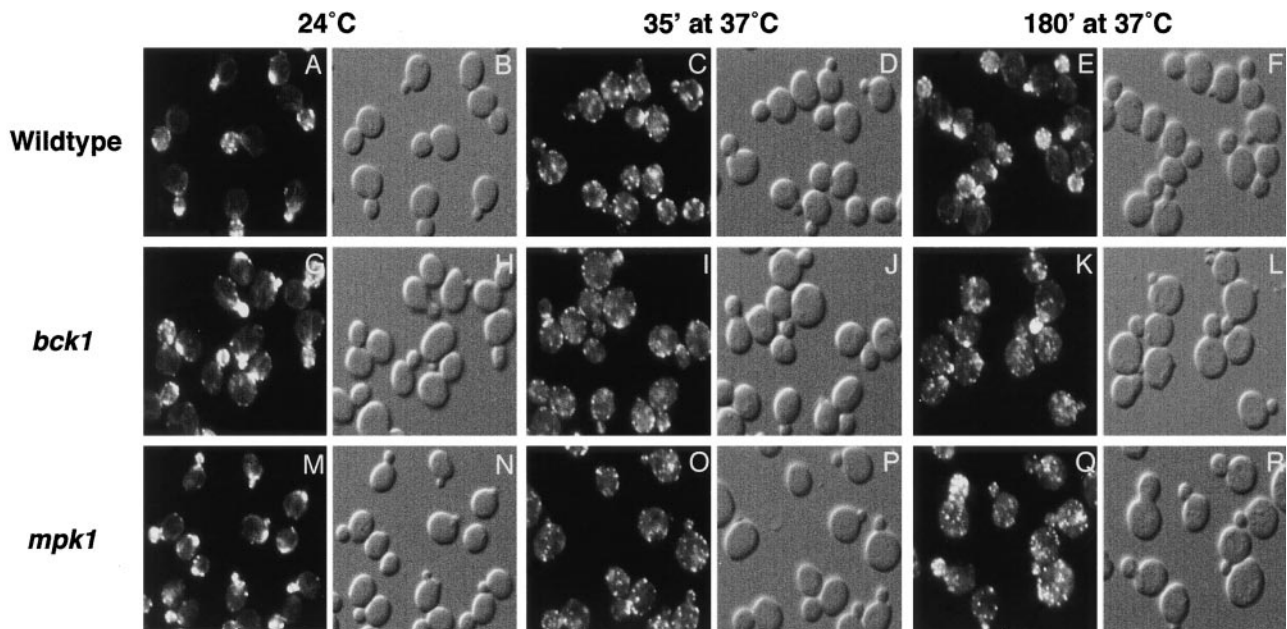
Figure 4. BCK1 and MPK1 are required for repolarization of the actin cytoskeleton upon heat shock. Wild-type (A–F, JK9-3d**a**), *bck1* (G–L, PA109-1c), and *mpk1* (M–R, TS45-1a) cells were grown in rich medium at 24°C and shifted to 37°C for 35 and 180 min. Samples were fixed, stained with TRITC-phalloidin, and observed by fluorescence (A, C, E, G, I, K, M, O, and Q) and Nomarski (B, D, F, H, J, L, N, P, and R) microscopy to visualize the actin cytoskeleton and whole cells, respectively.

temperature sensitive growth defect, as reported for other strain backgrounds, and therefore the persistent depolarization of the actin cytoskeleton and FKS1 cannot be attributed to cell death. Thus, the PKC1-activated MAP kinase cascade is required for repolarization rather than depolarization of cell growth.

Stress-induced Depolarization of WSC1 Distribution Is Independent of a Depolarized Actin Cytoskeleton yet WSC1 Localization Is Controlled by the Actin Cytoskeleton

To determine if cell wall stress affects localization of the plasma membrane protein WSC1, we constructed a COOH terminally, 3×HA-tagged, functional WSC1 (pWSC1-HA) (see Materials and Methods). Visualization of WSC1 by indirect immunofluorescence revealed a polarized distribution similar to that of FKS1 and other proteins found at a growth (bud) site (Fig. 5). Surprisingly, WSC1 had previously been reported to be evenly distributed throughout the cell periphery (Verna et al., 1997). This discrepancy may be because of the earlier study using overexpressed WSC1, whereas we examined WSC1 expressed from its own promoter on a centromeric plasmid. To examine WSC1 localization in response to stress, growing wild-type cells containing pWSC1-HA were shifted from 24 to 37°C, incubated at the higher temperature for 35 and 120 min, and processed for visualization of WSC1 (WSC1-HA). After 35 min, WSC1 was dispersed throughout the cell periphery (Fig. 5). After 120 min at 37°C, WSC1 was repolarized (data not shown). Thus, like the actin cytoskeleton and FKS1, WSC1 displayed a transient depolarization upon heat shock. Interestingly, WSC1 was

also found in internal compartments at both low and high temperatures.

We asked if, like for FKS1, stress-induced depolarization of WSC1 is LAT-A sensitive and, thus, dependent on the actin cytoskeleton. Interestingly, WSC1 was depolarized in LAT-A–treated cells grown at both low and high temperatures, indicating that LAT-A treatment induces WSC1 depolarization independently of stress (Fig. 5). Thus, LAT-A could not be used to determine if stress-induced WSC1 depolarization requires the actin cytoskeleton. However, this experiment demonstrates that WSC1 responds to a change in the actin cytoskeleton in addition to controlling the actin cytoskeleton, as shown above.

To determine if the stress-induced depolarization of WSC1 is dependent on a depolarized actin cytoskeleton, we investigated WSC1 distribution in heat shocked *rom2* cells. As reported above, a *rom2* mutation blocks stress-induced depolarization of the actin cytoskeleton and FKS1. WSC1 distribution was still depolarized in a *rom2* mutant shifted to a high temperature for 35 min (Fig. 5). Thus, WSC1 depolarization is independent of depolarization of the actin cytoskeleton, suggesting that WSC1 may be a stress-specific actin landmark in addition to a signal transducer (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998). The above results taken together suggest that WSC1, like mammalian integrins, both controls and responds to the actin cytoskeleton (see Discussion).

Discussion

We have shown that the actin cytoskeleton and the distribution of the cell wall synthesis enzyme glucan synthase
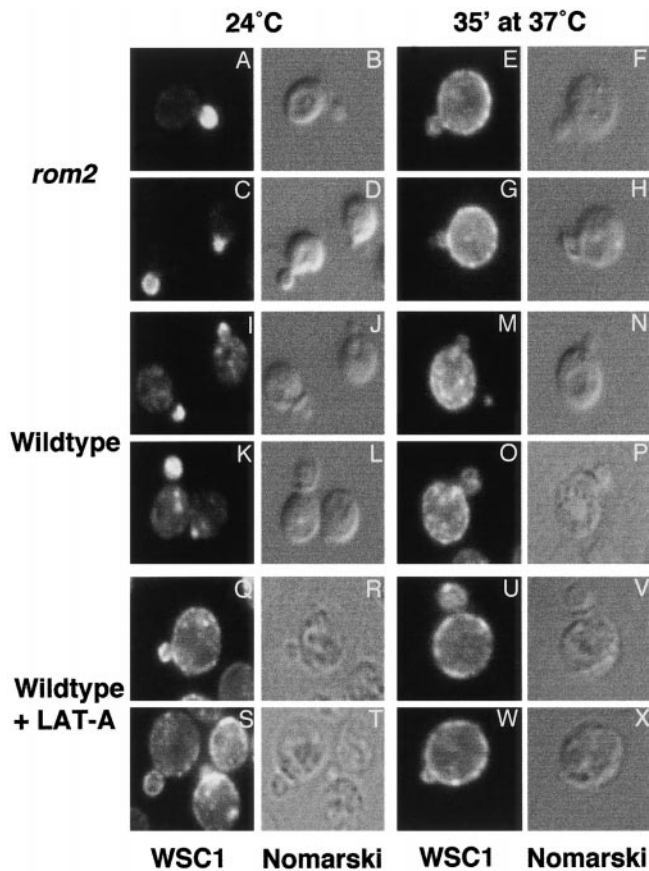
Figure 5. Stress-induced depolarization of WSC1 distribution is independent of a depolarized actin cytoskeleton, yet WSC1 localization is controlled by the actin cytoskeleton. Wild-type (I–X, JK9-3d**a**) or *rom2* (A–H, AS138-1b) cells expressing WSC1-HA (pWSC1-HA) were grown in minimal medium at 24°C, shifted to 37°C for 35 min or maintained at 24°C, fixed, and processed for detection of WSC1-HA by immunofluorescence (see Materials and Methods). In cells grown continuously at 24°C, LAT-A was added 35 min before fixation (Q–T). In cells shifted to 37°C, LAT-A was added at the time of shift (U–X). Fluorescence (A, C, E, G, I, K, M, O, Q, S, U, and W) and Nomarski (B, D, F, H, J, L, N, P, R, T, V, and X) images are shown.

(FKS1 and its regulatory subunit RHO1) are transiently depolarized in response to cell wall stress. In heat shocked or SDS-treated cells, actin patches and glucan synthase are redistributed from a polarized growth site (bud) to the periphery of the entire cell. This stress-induced depolarization of cell growth, possibly as a mechanism to repair general cell wall damage, is mediated by a putative signaling pathway consisting of the plasma membrane protein WSC1, the RHO1 exchange factor ROM2, RHO1, PKC1, and a yet-to-be identified PKC1 effector branch. The PKC1-activated MAP kinase pathway mediates repolarization of the actin cytoskeleton and FKS1. The de- and repolarization of FKS1 is dependent on the actin cytoskeleton. A model summarizing our results is shown in Fig. 6.

The type I transmembrane protein WSC1 is possibly a signal transducer that senses and signals the integrity of the cell wall. A *wsc1* mutant is defective in stress-induced depolarization and in activation of MPK1, sensitive to cell

wall stress (SDS and heat), and is suppressed by overexpression of ROM2, RHO1, or PKC1 (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998). The mechanisms by which WSC1 senses and signals cell wall integrity remain to be determined. WSC1 might sense and respond to a turgor pressure–induced outward stretching of the plasma membrane that results from a cell wall defect. Consistent with this possibility, several treatments that stretch the plasma membrane induce a depolarization response. Chlorpromazine, a cationic amphipath that inserts into and stretches the plasma membrane, induces depolarization of the actin cytoskeleton (Delley, P.-A., and M.N. Hall, unpublished results) and MPK1 activation (Kamada et al., 1995). Hyperosmotic shock also induces depolarization of the actin cytoskeleton (Chowdhury et al., 1992). Depolarization of the actin cytoskeleton in response to chlorpromazine or hyperosmotic shock is partly blocked in a *wsc1* or *rom2* mutant (Delley, P.-A., and M.N. Hall, unpublished results). Alternatively, WSC1 might interact with and directly sense the cell wall (Lodder et al., 1999). It also remains to be determined if WSC1 indeed controls ROM2 activity and, if so, how this might be achieved.

In addition to its role as a putative sensor and signal transducer, WSC1 might also serve as a stress-specific actin landmark. WSC1 also depolarizes upon heat shock and this depolarization is independent of a depolarization of
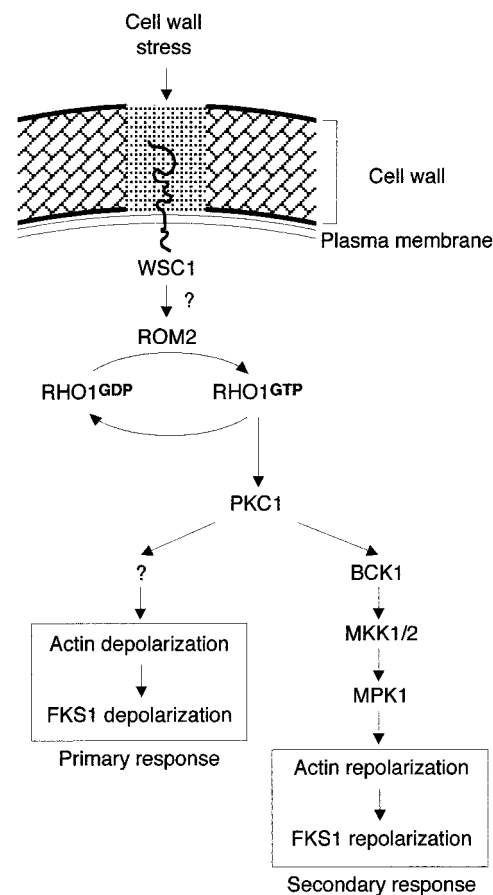

Figure 6. Model for the regulation of actin cytoskeleton and FKS1 distribution upon cell wall stress (see Discussion for further details).

the actin cytoskeleton. Interestingly, although WSC1 may control the actin cytoskeleton both as a signal transducer and a landmark, it also responds to the actin cytoskeleton as LAT-A–induced depolymerization of the actin cytoskeleton also causes WSC1 depolarization. Thus, there appears to be a bidirectional signaling between WSC1 and the actin cytoskeleton. A similar bidirectional signaling between integrins and the actin cytoskeleton has been observed in mammalian cells (Schoenwaelder and Burridge, 1999).

The RHO1 GTPase switch and the RHO1 effector PKC1 mediate the transient depolarization response. ROM2 is necessary and hyperactivated RHO1 or PKC1 is sufficient to induce depolarization of the actin cytoskeleton and FKS1. Interestingly, RHO1 and PKC1 are also required for polarization of the actin cytoskeleton. First, loss-of-function *rho1* mutants are defective in polarization of the actin cytoskeleton (Helliwell et al., 1998b). Second, overexpression of PKC1 restores actin organization in a *rho1* mutant (Helliwell et al., 1998b). Third, overexpression of RHO1 or PKC1 rescues a *tor2* mutant defective in polarized organization of the actin cytoskeleton (Schmidt et al., 1997; Helliwell et al., 1998a). The finding that activated RHO1 and its effector PKC1 control both polarization and depolarization of the actin cytoskeleton suggest that RHO1 may be a more finely-tuned switch than previously thought. RHO1 in a hyperactive state, as suggested by our findings, may cause depolarization, whereas RHO1 in a normally active state mediates polarization. Thus, RHO1 may function as a molecular rheostat, with more than one on position, rather than as a simple on-off switch. A second on position could require another RHO1 regulator in addition to ROM2. Alternatively, RHO1 may still function as a simple on-off switch, and depolarization may result from activation of a larger or a specific pool of RHO1.

PKC1, like RHO1, also appears to mediate both depolarization and repolarization of the actin cytoskeleton and FKS1. However, unlike RHO1, PKC1 may mediate these two different responses via different effectors. First, mutants defective in MPK1/SLT2 exhibit a depolarized actin cytoskeleton (Mazzoni et al., 1993). Second, overexpression of MPK1 suppresses the actin polarization defect of an *rho1* mutant (Helliwell et al., 1998b). Third, *bck1* and *mpk1* mutants are not defective in stress-induced actin and FKS1 depolarization (Fig. 4). Fourth, BCK1 and MPK1 are necessary to restore polarization of the actin cytoskeleton and FKS1 after cell wall stress (Fig. 4). Furthermore, hyperactivated BCK1 or MKK1 does not induce actin and FKS1 depolarization (Fig. 3). These findings suggest that the PKC1-activated MAP kinase cascade mediates repolarization, and that a PKC1 effector branch other than this well characterized MAP kinase cascade mediates depolarization. Although the components of a second PKC1 effector branch have not been identified, the existence of a signaling bifurcation below PKC1 has been proposed previously (Lee and Levin, 1992; Helliwell et al., 1998b).

RHO1 and PKC1 may control both depolarization and repolarization by activating the two PKC1 effector pathways at different times. Activation of the yet-to-be defined PKC1 effector branch, which causes depolarization (primary response), may occur rapidly and only upon stress-induced hyperactivation of RHO1. Activation of the PKC1-activated MAP kinase cascade may be a delayed response that results in repolarization (secondary response). Interestingly, Kamada et al. (1995) reported that the MPK1 MAP kinase is maximally activated after 30 min of heat shock, which is significantly delayed compared with the rapid induction (1–2 min) of MPK1 or the HOG1 MAP kinase by hypotonic or hypertonic stress, respectively (Chowdhury et al., 1992; Brewster et al., 1993). When we use the same regimen to heat shock cells as that used by Kamada et al. (1995), we find that the maximum percentage of depolarized cells is reached within ~15 min (Delley, P.-A., and M.N. Hall, unpublished results), suggesting that the actin depolarization response precedes activation of MPK1.

The actin-dependent depolarization of glucan synthase (FKS1 and RHO1) in response to cell wall stress may be a homeostasis mechanism to repair general cell wall damage. Lillie and Brown (1994) reported that MYO2, a yeast class V myosin, and SMY1, a MYO2-interacting protein, disappear from and reappear at the bud upon heat shock, within a time course similar to that of actin de- and repolarization. MYO2 plays a role in the transport of secretory vesicles along actin cables to the growth site, and is, thus, involved in polarized growth and secretion. It will be of interest to determine if depolarization of the integral membrane protein FKS1 involves depolarized secretion of newly made FKS1 or dispersal of previously polarized FKS1. Attempts to address this issue by examining FKS1 localization in heat-stressed and cycloheximide-treated cells were inconclusive because cycloheximide alone affects actin organization. Furthermore, the mechanism by which the peripheral membrane protein RHO1 is redistributed may be different from that of FKS1. Unlike depolarization of FKS1, depolarization of RHO1 was not prevented by LAT-A treatment or by a *rom2* or *wsc1* mutation (Delley, P.-A., and M.N. Hall, unpublished results).

The regulation of FKS1 de- and repolarization by RHO1 is the third level on which RHO1 controls FKS1. In addition to controlling FKS1 localization, RHO1 also controls transcription of glucan synthase encoding genes (Igual et al., 1996; Zhao et al., 1998), and is a regulatory subunit of the glucan synthase complex (Drgonova et al., 1996; Qadota et al., 1996; Cabib et al., 1998).

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