Stress-specific Activation Mechanisms for the “Cell Integrity” MAPK Pathway*

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Many environmental stresses trigger cellular responses by activating mitogen-activated protein kinase (MAPK) pathways. Once activated, these highly conserved protein kinase cascades can elicit cellular responses such as transcriptional activation of response genes, cytoskeletal rearrangement, and cell cycle arrest. The mechanism of pathway activation by environmental stresses is in most cases unknown. We have analyzed the activation of the budding yeast “cell integrity” MAPK pathway by heat shock, hypoosmotic shock, and actin perturbation, and we report that different stresses regulate this pathway at different steps. In no case can MAPK activation be explained by the prevailing view that stresses simply induce GTP loading of the Rho1p GTPase at the “top” of the pathway. Instead, our findings suggest that the stresses can modulate at least three distinct kinases acting between Rho1p and the MAPK. These findings suggest that stresses provide “lateral” inputs into this regulatory pathway, rather than operating in a linear “top-down” manner.

Both extracellular signaling molecules and intracellular stresses frequently elicit their specific cellular responses through highly conserved signal transduction modules consisting of protein kinase cascades culminating in the activation of mitogen-activated protein kinases (MAPKs)1 (1). MAPKs are a family of serine/threonine protein kinases that are activated by an unusual mechanism involving dual threonine and tyrosine phosphorylation, which is catalyzed by a family of MAPK kinases (MAPKKs). These in turn are phosphorylated and activated by a family of MAPKK kinases (MAPKKKs), and the three kinases of a particular MAPK module are frequently physically associated with each other (sometimes with the help of noncatalytic scaffold proteins) in multimeric complexes (2). In contrast to the highly conserved molecular architecture of the MAPK cascade, the mechanisms whereby various stimuli activate MAPK activity appear to be quite diverse.

Perhaps the most extensively investigated instance of MAPK activation involves signal transduction by receptor tyrosine kinases, which activate a MAPK cascade in response to extra- cellular growth factors (3). Activation of the receptor upon growth factor binding promotes the recruitment of the guanine nucleotide exchange factor Sos to the plasma membrane, where Sos encounters its target GTPase, Ras, leading to GTP loading of Ras. GTP-bound Ras then stimulates a MAPK cascade involving the kinases Raf or Raf-B (MAPKKK), MEK (MAPKK), and ERK (MAPK) (4).

In contrast to the detailed understanding of MAPK cascade activation by growth factors, much less is known about how MAPK pathways are stimulated by cellular stresses. One stress-responsive MAPK that has been extensively studied in Saccharomyces cerevisiae is the Slt2p/Mpk1p MAPK, which is activated by the related (and redundant) MAPKKs Mkk1p and Mkk2p, which are in turn activated by the MAPKKK Bck1p (5). This signaling module has been called the “cell integrity” MAPK cascade, as it is required for proper construction of the cell wall in order to prevent cell lysis (6, 7). Mpk1p is activated in response to various stresses, and Mpk1p activation serves to protect yeast cells from stress by inducing transcription of genes that promote cell wall remodeling and by contributing to the cell cycle arrest triggered by the morphogenesis checkpoint, which delays mitosis until cells have successfully built a bud (5, 8, 9).

Genetic studies established that Pck1p, the sole protein kinase C homologue in S. cerevisiae, is absolutely required for any activity in the cell integrity MAPK pathway, and acts upstream of the MAPKKK Bck1p (10, 11). Several additional functions, independent of the MAPK pathway, have also been ascribed to Pck1p (12). Pck1p activity in turn requires the binding of GTP-loaded Rho1p (13, 14), and GTP loading of Rho1p is mediated by the partially redundant guanine nucleotide exchange factors Rom1p and Rom2p (15). By analogy to the Ras-Raf-MEK-ERK pathway, it has been suggested that stresses activate the cell integrity pathway by stimulating GTP loading of Rho1p, thereby activating Pck1p to phosphorylate Bck1p, initiating activation of the kinase cascade (5, 9, 16, 17). A multiplicity of stimuli have been shown to promote Mpk1p activation. These include heat shock, hyposmototic shock, actin depolymerization, and treatment with chlorpromazine, caffeine, vanadate, zymolase, Congo red, calcofluor, rapamycin, and mating pheromone (8, 10, 18–21). A simplifying hypothesis to accommodate these observations is that there is a common stressful consequence of all of these treatments that causes cells to activate Mpk1p (10). A number of plasma membrane glycoproteins (Wsc1p-3p, Mid2p) that influence Mpk1p activity have been identified, and it is thought that these proteins might act as “stress sensors” that somehow detect cellular stress and transduce a signal to activate Rom1p/Rom2p (16, 22–24). However, these putative stress sensors appear to be dispensable for Mpk1p activation in response to actin depolymerization (8) or rapamycin (18), raising the possibility that different stresses might employ distinct pathways to activate.

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The abbreviations used are: MAPK, mitogen-activated protein kinase; GAP, GTPase-activating protein.
Mpk1. Precedent for stress-specific regulation of MAPK pathways is provided by findings on the Schizosaccharomyces pombe Sty1p pathway, in which it appears that although other stresses activate Sty1p through regulation of its upstream kinases, heat shock activates Sty1p primarily through regulation of Sty1p-directed phosphatases (25-27).

In this report, we have dissected the requirements for activation of the cell integrity pathway by different stresses. Our findings are consistent with the view that all stresses activate the pathway in a "top-down" manner by stimulating Rho1p GTP loading, resulting in the activation of a linear Pkc1p/Bck1p–MKK1–Mpk1p kinase cascade. We find that different stresses can activate the pathway by different mechanisms, in one case regulating the core kinases Mkk1,2p/Mpk1p kinase cascade. We find that different stresses activate Mpk1p through signaling pathways that impact the MAPK cascade at different levels.

**Materials and Methods**

**Strains, Media, and Growth Conditions**

| Table 1 |
|---|
| **Strains used in this study** |
| --- |
| DLY1 | a bar1 ade1 his2 ura3 leu2 trp1 | 55 |
| DLY455 | a bck1ΔLEU2 BCK1–20:URA3 | This study |
| DLY456 | a plc1ΔLEU2 galPKC1:TRP1 | This study |
| DLY3962 | a plc1ΔLEU2 galPKC1:TRP1 | 8 |
| DLY3994 | a bck1:LEU2 | 8 |
| DLY3996 | a bar1 msg5:LEU2 | This study |
| DLY4351 | a mkk1:LEU2 mkk2:URA3 | 8 |
| DLY4497 | a bar1 rho2:kan8 | 8 |
| DLY4509 | a bar1 RHOD1:RHO1*::LEU2 | This study |
| DLY6473 | a bar1 RHOD1:RHO1*::LEU2 | This study |
| DLY6474 | a ptpr2::hyg | This study |
| DLY6475 | a bar1 sdp1:kan8 msg5:LEU2 | This study |
| DLY6506 | a ptpr2::URA3 | This study |
| DLY6508 | a sdp1:kan8 msg5:LEU2 ptpr2::hyg8 | This study |
| DLY6509 | a msg5:LEU2 ptpr2::hyg8 | This study |
| DLY6510 | a sdp1:kan8 ptpr2::hyg8 | This study |
| DLY6519 | a msg5:LEU2 ptpr2::URA3 | This study |
| DLY6520 | a sdp1:kan8 ptpr3:URA3 | This study |
| DLY6521 | a sdp1:kan8 ptpr3:URA3 | This study |
| DLY6522 | a sdp1:kan8 msg5:LEU2 ptpr3:URA3 | This study |
| DLY6523 | a sdp1:kan8 msg5:LEU2 ptpr3:hyg8 | This study |
| DLY6524 | a ptpr3:URA3 ptpr2::hyg8 | This study |
| DLY6526 | a bar1 sdp1:kan8 | This study |
| DLY6536 | a msg5:LEU2 ptpr3:URA3 ptpr2::hyg8 | This study |

*All strains are in the BF284–15D (55) strain background (ade1 his2 ura3 leu2 trp1).*

Mpk1p. Precedent for stress-specific regulation of MAPK pathways is provided by findings on the Schizosaccharomyces pombe Sty1p pathway, in which it appears that although other stresses activate Sty1p through regulation of its upstream kinases, heat shock activates Sty1p primarily through regulation of Sty1p-directed phosphatases (25–27).

In this report, we have dissected the requirements for activation of the cell integrity pathway by different stresses. Our findings are consistent with the view that all stresses activate the pathway in a “top-down” manner by stimulating Rho1p GTP loading, resulting in the activation of a linear Pkc1p/Bck1p–MKK1–Mpk1p kinase cascade. We find that different stresses can activate the pathway by different mechanisms, in one case regulating the core kinases Mkk1,2p/Mpk1p kinase cascade. We find that different stresses activate Mpk1p through signaling pathways that impact the MAPK cascade at different levels.

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**Biochemical Procedures**—Procedures for cell lysis, SDS-PAGE, and Western blotting were as described (8).

**RESULTS**

**Effect of RHO1*::LEU2 on Mpk1p Activity**—As mentioned in the Introduction, the activity of Pkc1p and subsequent kinases in the cell integrity pathway requires GTP-bound Rho1p. Although it is thought that stresses trigger an increase in GTP loading of Rho1p and in Pkc1p activity, technical limitations have thus far precluded direct measurement of the amount of Rho1p-GTP or of the activity of Pkc1p in vivo. In the case of the mitogenic growth factors that promote GTP loading of Ras, one of the major findings that established the paradigm was that oncogenic mutants of Ras that lock the protein in its GTP-bound state mimic constitutive signaling by the receptors, leading to constitutive ERK activation and uncontrolled proliferation (28). By analogy, we reasoned that expression of a GTP-
locked mutant of RHO1 should lead to constitutive Mpk1p activation. Mpk1p activity can be monitored using a phosphospecific antibody that recognizes only the doubly phosphorylated (threonine and tyrosine) form of Mpk1p. Using this reagent, we compared Mpk1p activity in wild-type cells to that in cells that expressed the GTP-locked RHO1Q68H allele (29), analogous to the GTP-locked oncogenic Ras mutant RasQ61H (28).

Previous studies showed that overexpression of RHO1Q68H from the strong GAL1 promoter triggered the accumulation of multiple transcripts known to be responsive to Mpk1p activity (30), indicating that Mpk1p had indeed been activated by excess GTP-Rho1p. However, GAL1-RHO1Q68H also caused dramatic actin depolarization, through a pathway that required Pkc1p but not Mpk1p (31). This result raised the possibility that Mpk1p activation in GAL1-RHO1Q68H strains was the result of actin stress induced by overexpression. To assess whether more physiological (and therefore less proactive) activation of Rho1p would activate Mpk1p, we introduced a single integrated copy of RHO1Q68H expressed from the RHO1 promoter into a wild-type strain (in addition to wild-type RHO1). Single-copy RHO1Q68H caused detectable but much less severe actin depolarization than GAL1-RHO1Q68H (data not shown), suggesting that limited hyperactivation of Pkc1p took place, but the cells were viable and proliferated normally. However, the activity of Mpk1p in cells with a single integrated copy of RHO1Q68H was indistinguishable from that in wild-type cells (Fig. 1A). The simplest interpretation of this result is that additional GTP loading of Rho1p (without overexpression) does not automatically activate Mpk1p activity. Alternatively, constitutive activation of Rho1p might provoke adaptive desensitization of the pathway, returning Mpk1p activation to basal levels. If these cells were desensitized, then we would expect that they would no longer activate Mpk1p in response to stress. However, activation of Mpk1p in response to stress from heat shock, hypoosmotic shock, or actin depolymerization was completely unaffected by RHO1Q68H (Fig. 1B). This stress responsiveness was not because of Rho2p (which has some functional overlap with Rho1p (15, 32)), because similar results were obtained in cells lacking Rho2p (Fig. 1B). These results are inconsistent with the hypothesis that Mpk1p regulation occurs simply by modulating Rho1p GTP loading, and imply that significant regulation of the cell integrity pathway must occur downstream from Rho1p, presumably by regulating the activity of one or more of the downstream kinases.

Regulatory or Permissive Roles for the Cell Integrity Kinases?—For each of the kinases upstream of Mpk1p in the cell integrity pathway, one can ask whether regulation of that kinase is important for the activation of Mpk1p in response to a given stress. Previous studies demonstrated that these kinases are each absolutely required for Mpk1p activity (10, 11, 13, 14, 33), but that requirement does not discriminate whether a given kinase plays an active role in stress-mediated Mpk1p activation or a passive role providing a constitutive pathway input that is then modulated at a level downstream of that kinase by stress-responsive signaling pathways. In the latter scenario, it should be possible to dispense with a particular kinase entirely yet retain stress-responsive Mpk1p activity if an alternative source for the constitutive pathway input could be found. In the studies reported below, we have employed mutant alleles of BCK1 and MKK1 to provide a constitutive pathway input in the absence of the normally essential upstream kinases (Pkc1p and Bck1p, respectively).

To provide a constitutive pathway input in the absence of Pck1p, we employed the previously described BCK1–20 allele (11). The lethality of the PKC1 deletion is suppressed by the dominant BCK1–20 allele, a result that we confirmed in our strain background (Fig. 2A). Bck1p is a large 1478-amino acid kinase with a C-terminal catalytic domain (residues 1175 to 1440), and Bck1–20p contains a missense mutation immediately upstream of the kinase domain (Ala-1174 to Pro) (11). Bck1–20p is thought to encode a constitutively activated, Pck1p-independent form of Bck1p that mimics Pck1p-phosphorylated Bck1p, although the Pck1p-targeted phosphorylation sites on Bck1p have not yet been mapped (11).

To provide a constitutive pathway input in the absence of Bck1p, we constructed the MKK1DD allele. In other MAPK cascades, MAPKKK activation by the MAPKKK involves the phosphorylation of two conserved serine residues on the MAPKK (34, 35). Mutation of these residues to aspartic acid mimics phosphorylation, yielding constitutively active MAPKKs (26, 34). By analogy to these earlier studies we generated a mutant, MKK1DD, predicted to encode an activated version of Mkk1p. Mkk1pDD was expressed at levels comparable with those of wild-type Mkk1p (data not shown), and it was functional by the criterion that it could rescue the caffeine sensitivity of mkk1Δ mkk2Δ cells (Fig. 2C) (yeast cells lacking components of the cell integrity pathway are unable to grow on medium containing 10 mM caffeine, presumably because of their weakened cell wall (36). Mkk1pDD, but not wild-type Mkk1p, also rescued the caffeine sensitivity of bck1Δ cells (Fig.
containing 10 mM caffeine (streaked onto Stress caffeine suggesting that Mkk1p DD is activated and does not require MKK1 active was monitored by Western blot.

of galactose to the culture for the indicated time, and Mpk1p activity (DLY455) cells carrying pDL242 (CEN24) were monitored by Western blot. The basal level in absence of Bck1p, wild type Mkk1p is inactive and cannot support Pkc1p/Mpk1p pathway cannot grow on this media (see text). In the absence of Bck1p, wild type (DLY1), BCK1–20 (pDLB823), or Mkk1/2p DD (pDLB824) plasmids as indicated were transformed into S. pombe (Bck1 ΔH9004) strain. Mkk1pDD as their only source of Mkk1p/Mkk2p displayed a lower basal level of Mpk1p activity that was not affected by the presence of Bck1p. The activity of Mpk1p was not efficiently stimulated by either hypoosmotic shock (Fig. 3E) or actin depolymerization (Fig. 3F), in the presence or absence of Bck1p. These results suggest that Mkk1pDD is no longer responsive to the signal triggered by hypoosmotic shock. The simplest interpretation of this result is that hypoosmotic shock acts by modulating Bck1p activity in a manner that is different from Bck1p control by Pkc1p (see “Discussion” for other possible interpretations).

We next examined whether any of the cellular stresses could regulate Mpk1p in the absence of Bck1p. Cells expressing Mkk1pDD as their only source of Mkk1p/Mkk2p displayed a basal level of Mpk1p activity that was not affected by the presence of Bck1p. The activity of Mpk1p was not efficiently stimulated by either hypoosmotic shock (Fig. 3E) or actin depolymerization (Fig. 3F), in the presence or absence of Bck1p. These results suggest that Mkk1pDD is no longer responsive either to Bck1p or to regulation by these two stresses, consistent with our conclusions (above) that both of these stresses act via Bck1p. In contrast to these results, heat shock robustly activated Mpk1p in cells carrying Mkk1pDD, either in the presence or absence of Bck1p (Fig. 3D). This result indicates that heat shock can promote pathway activation at the level of Mkk1/2p and/or Mpk1p.

One possible scenario is that heat shock inhibits the phosphatases that dephosphorylate Mpk1p (Msg5p, Sdp1p, Ptp2p, and Ptp3p), so that constitutive signaling through the kinase cascade is allowed to activate Mpk1p. Indeed, a similar scheme has been shown to apply for heat shock-induced activation of the MAPK Sty1p in S. pombe (27). Analyses of strains deleted for individual phosphatases have shown that deletion of the phosphatase caused elevation of both the basal and the heat shock-induced level of Mpk1p activity (19, 37–40). However, there remained the possibility that the phosphatases were coordinately regulated by heat shock so that inhibition of the phosphatases still present could mediate the response. To address this issue we generated a panel of strains deleted for every combination of the four phosphatases. As shown in Fig. 4A, Mpk1p basal activity increased as progressively more phosphatases were deleted, reaching very high levels in the sdp1 Δmsg5 Δptp2 Δptp3 quadruple mutant. Nevertheless, Mpk1p could still be stimulated further by heat shock treatment of these strains (Fig. 4B). These findings suggest that heat shock could still induce Mpk1p independent of phosphatase regulation, perhaps by activating the kinases Mkk1/2p that act on...
Mpk1p (Fig. 5). In aggregate, these data provide strong evidence that regulation of the cell integrity pathway is mediated by distinct, stress-specific mechanisms.

**DISCUSSION**

The most striking conclusion from our results is that the prevailing top-down view of MAPK signaling, wherein regulatory signals act to stimulate guanine nucleotide exchange factor-mediated GTP loading of the G protein at the top of the cascade and activation is then transmitted automatically through sequential activation of the various kinases, cannot account for stress-induced regulation of the cell integrity pathway in yeast. Instead, our results suggest that various steps downstream of Rho1p GTP loading can be modulated by separate inputs that act in response to specific stresses (Fig. 5).

We found that expression of a constitutively GTP-loaded allele of Rho1p, Rho1pGTP63G, from its own promoter was insufficient to stimulate Mpk1p activity. Moreover, cells containing this excess GTP-Rho1p were still responsive to the stresses we examined. This result indicates that cells must have “buffering” mechanisms to protect themselves from excess GTP-Rho1p. One likely layer of protection is provided by the multiple GTPase activating proteins (GAPs) that are thought to act on Rho1p-GTP including Bem2p, Sac7p, Lrg1p, and Bag7p (32, 41–44). It has been suggested that the different GAPs may regulate distinct pools of Rho1p that control different downstream pathways (44, 45). It seems plausible that the GAPs may sequester much of the Rho1pGTP63G, and that stresses trigger release of the sequestered Rho1pGTP63G from specific GAPs. In the context of wild-type Rho1p, such GAP down-regulation by stress would “open the gate” for signaling by GTP-Rho1p, and could act together with up-regulation of Rho1p GTP loading to achieve appropriate signaling.

Downstream of Rho1p, we found that different stresses exhibited different genetic requirements for signaling. Previous studies have shown that Mpk1p activity is absolutely dependent on the upstream kinases Pkc1p and Bck1p (10), but they did not distinguish whether those kinases were regulated in response to stress or were merely required to provide a basal pathway input that could be regulated downstream of those kinases. Our data on Mpk1p activation by heat shock strongly support the latter hypothesis. In particular, we found that strains lacking Pkc1p (and providing pathway activity through Bck1–20p) and strains lacking Bck1p (and providing pathway activity through Mkk1pDD) were still able to activate Mpk1p in response to heat shock. The simplest interpretation of these results is that heat shock triggers a signaling pathway that regulates either Mkk1/2p or Mpk1p itself (Fig. 5).

In contrast to heat shock, actin depolymerization and hypoosmotic shock no longer promoted Mpk1p activation if Bck1p was absent, suggesting that these stresses regulate the pathway at or above the level of Bck1p. Interestingly, actin stress was capable of activating Mpk1p in cells containing Bck1–20p as the sole source of Bck1p activity, whereas hypoosmotic shock was not. This result indicates that the two stresses must have different effects on the cell integrity pathway. Elucidating the basis for that difference is complicated by the poorly understood nature of the Bck1–20p protein. Previous studies, confirmed here, indicated that Bck1–20p could suppress the need for Pkc1p in Mpk1p activation, and suggested that Bck1–20p might encode an activated, Pkc1p-independent form of Bck1p (11). However, although Bck1–20p certainly displays a Pck1p-independent basal activity, we found that overexpression of an activated allel of Pkc1p dramatically activated Mpk1p in cells containing Bck1–20p. This result suggests that Bck1–20p is still responsive to Pkc1p, although it is no longer absolutely dependent on Pkc1p.

Actin stress could activate Mpk1p in a strain containing both Pkc1p and Bck1–20p, but could not activate Mpk1p in a strain...
containing Bck1–20p but lacking Pck1p. This result suggests that actin stress-mediated signaling is transmitted through Pck1p, and we suggest that this stress acts either by modulating the Rho1p GAPs (allowing Pck1p activation by Rho1p-GTP) or through a separate input to modulate Pck1p activity (Fig. 5). Pck1p is subject to several modes of regulation including Rho1p binding, interaction with membrane lipids, localization, and phosphorylation (13, 14, 46, 47). Whether any of these modes is responsible for Mpk1p regulation in response to actin perturbation is unknown.

Unexpectedly, we found that hypoosmotic shock was unable to activate Mpk1p in cells containing Bck1–20p as the only source of Bck1p, even if they contained Pck1p. This defect was recessive, in that cells containing both wild-type Bck1p and Bck1–20p were able to activate Mpk1p following hypoosmotic shock (data not shown). These findings suggest that Bck1–20p is specifically unable to mediate signaling by hypoosmotic shock, perhaps suggesting that this stress normally acts on the pathway at the level of Bck1p (Fig. 5). Alternatively, the BCK1–20 mutation may affect scaffolding functions of Bck1p important for allowing effective input at other levels of the pathway. Given the marked difference in the time course of Mpk1p activation by actin stress (gradual sustained increase over 2 h) and hypoosmotic shock (rapid but transient induction peaking at ~1 min), it is also possible that both stresses act through Pck1p, but that Bck1–20p may be a “slow” form of Bck1p, unable to respond to Pck1p rapidly enough to activate Mpk1p following hypoosmotic shock.

Many MAPK pathways have been identified that respond to various forms of “stress” rather than to specific extracellular signaling molecules. These include the Sty1p pathway in S. pombe, the cell integrity and Hog1p pathways in S. cerevisiae, and the p38 and c-Jun N-terminal kinase pathways in animal cells. In the p38 and c-Jun N-terminal kinase pathways it is believed that regulation of upstream kinases underlies stress-mediated activation of the MAPKKK and hence the MAPK cascade (48). However, we are not aware of any comparable experiments to those performed here that would allow one to discriminate whether the signaling elements in those pathways are regulatory or simply necessary for basal pathway activity. It may be that the use of multiple “lateral” inputs into the different steps in these pathways, as described here for the cell integrity pathway, endows them with the flexibility to integrate information from several different stress sensors to determine the appropriate pathway output under different conditions.

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