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Dynamic Signaling in the Hog1 MAPK Pathway Relies on High Basal Signal Transduction

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(Appropriate regulation of the Hog1 mitogen-activated protein kinase (MAPK) pathway is essential for cells to survive osmotic stress. Here, we show that the two sensing mechanisms upstream of Hog1 display different signaling properties. The Sho1 branch is an inducible nonbasal system, whereas the Sln1 branch shows high basal signaling that is restricted by a MAPK-mediated feedback mechanism. A two-dimensional mathematical model of the Sln1 branch, including high basal signaling and a Hog1-regulated negative feedback, shows that a system with basal signaling exhibits higher efficiency, with faster response times and higher sensitivity to variations in external signals, than would systems without basal signaling. Analysis of two other yeast MAPK pathways, the Fus3 and Kss1 signaling pathways, indicates that high intrinsic basal signaling may be a general property of MAPK pathways allowing rapid and sensitive responses to environmental changes.

INTRODUCTION

Appropriate regulation of signaling through mitogen-activated protein kinase (MAPK) pathways is necessary to maximize cellular responses to extracellular stimuli (1, 2). Exposure of cells to high osmolality results in activation of a conserved family of MAPKs—p38 MAPK in mammals and Hog1 in yeast. In vivo replacement of components of the Hog1 MAPK pathway by their mammalian counterparts showed that there is a strong functional preservation of these MAPK pathways from yeast to mammals (3). Exposure of yeast to high osmolality results in rapid activation of the Hog1 MAPK signaling pathway, which coordinates the adaptive program required for cell survival during periods of osmotic stress (3, 4).

Activation of the Hog1 MAPK is mediated by two independent upstream sensing mechanisms that lead to the activation of either the MAPK kinase (MAPKK) Ssk2 (or the functionally redundant Ssk22) or Ste11 (fig. S1). The Sho1 branch involves the transmembrane protein Shol and the mucin-like transmembrane proteins Hrk1 and Msh2, which are the potential osmosensors of this branch of the HOG pathway (5–7). Although additional components still need to be identified to completely understand signaling from the Sho1 branch, signaling requires the guanosine triphosphatase (GTPase) Cdc42, the adaptor protein Ste50, and the kinases Ste20 and Cla4, which are members of the PAK (p21-activated protein kinase) family (7–11). When stimulated by osmotic stress, the Sho1 branch activates the MAPKK Ste11 and, subsequently, the MAPKK Pbs2 (12). Although the exact mechanism of Pbs2 activation by the Sho1 module remains unclear, Cdc42, Ste50, and Sho1 act as adaptor proteins that control the flow of the osmotic stress signal from Ste20 and Cla4 to Ste11, and then on to Pbs2 (13, 14).

A second sensing mechanism involves the “two-component” osmosensor composed of Shl1, Ssk1, and Ypd1. The Shl1 transmembrane osmosensor has intrinsic histidine kinase activity and is a homolog of bacterial two-component signal transducers. Using a phosphorelay mechanism involving the Ypd1 and Ssk1 proteins, Sl1 inhibits the activity of Ssk1, which controls the activity of the MAPKKks Ssk2 and Ssk22 (15, 16). Therefore, under normal osmotic conditions, active Sl1 histidine kinase maintains Ssk1 in its inactive phosphorylated form, whereas, in response to osmotic stress, the kinase activity of Sl1 is inhibited and then active Ssk1 induces Ssk2 activity. Signaling from either of the two branches leads to phosphorylation of the MAPKK Pbs2 and activation of the MAPK Hog1.

Kinetic analyses of Hog1 phosphorylation using mutants in each of the two branches of the pathway revealed that they respond differently to osmotic stress (17). Band-shift analysis with a microfluidic device showed that, whereas the Sl1 branch of the pathway is capable of fast signal integration to repeated stimuli, the Sho1 branch does not, suggesting that the signaling properties of the two branches are different (18). The origin of such differences, as well as their biological relevance, is still unclear.

Signaling through the MAPK pathway is controlled not only by activity of the kinases upstream of the MAPK, but also by the activity of protein phosphatases and various feedback systems. Inactivation of the MAPK is specifically controlled by direct dephosphorylation by protein phosphatases. The type 2C serine/threonine protein phosphatases and the protein tyrosine phosphatases (Ptp2 and Ptp3) decrease Hog1 and Pbs2 activities (19). Closure of the Fps1 glycerol channel has been proposed to act as a feedback mechanism that limits sensor activation in the HOG pathway (20). In addition, phosphorylation of Sho1 upon Hog1 activation seems to be important to decrease signaling through this branch (21). Mathematical modeling and single-cell analyses have shown that inactivation of the pathway by a mechanism independent of transcription might be important to modulate acute responses; whereas transcription-dependent mechanisms might be important for proper adaptation to future stimuli (20, 22, 23). Therefore, although most of the components of the signaling pathway have been defined, the signaling properties of the pathway are still poorly understood (24, 25).

Here, we use a chemical inhibitor of the MAPK Hog1 and extensive signal quantification to show that the HOG pathway is controlled by high basal signaling counteracted by a negative feedback regulatory system. We developed a two-dimensional mathematical model that provided a
general analytic paradigm based on sensor and target architecture that can be applied to any signaling pathway and showed that a system displaying high basal signaling, such as the HOG pathway, exhibits higher efficiency than systems without basal signaling, in terms of faster response and higher sensitivity to small variations in extracellular stimuli. Similar to the HOG pathway, signaling through the MAPK pathway controlling the mating response also seems to be controlled by a similar design, indicating that high basal signaling coupled to negative feedback may be a common trend in MAPK pathways.

RESULTS

The Sln1 and Sho1 branches of the HOG pathway have different signaling properties

To understand the underlying properties of the two branches of the HOG MAPK pathway, we performed several quantitative time-course and dose-response experiments, monitoring Hog1 phosphorylation in mutants in which both or only one branch of the pathway was active wild-type yeast, ssk2/ssk22 double mutant (Sho1 branch active), and ste11 or ste50 mutant (Sln1 branch active). Cells were subjected to osmotic stress (0.07 to 0.8 M NaCl), then fixed at various times, and total and phosphorylated Hog1 were detected with specific antibodies by quantitative Western blotting (see Materials and Methods). By plotting the percentage of phosphorylated Hog1 relative to the maximum in wild type over time, it is clear that the two branches contribute differently to Hog1 phosphorylation (Fig. 1). Cells lacking the Sln1 branch (ssk2 ssk22 mutant) do not respond to low osmolarity (up to 0.1 M NaCl), display slower responses at each osmolarity, and show a lower maximum response than the responses of the wild type or ste50 mutant. In contrast, the ste50 mutant cells respond as fast as wild-type cells, with a maximum response that is similar to that of wild-type cells, but with a shorter duration (Fig. 1A). At the highest osmolarities tested, the amplitude of the response of the wild type reached a maximum and plateaued. At each higher osmolarity, the duration of the maximal response period was longer. Thus, although both branches seem important for achieving the wild-type response, the Sln1 branch is critical for setting the speed and maximum amplitude of the response. Physiologically, these differences in signaling properties result in mutants lacking signaling through the Sln1 branch that are more sensitive (reduced growth), both on solid and in liquid media, to osmotic stress than are mutants lacking signaling through the Sho1 branch (Fig. 1, B and C).

The fact that the MAPKK Pbs2 receives the signal from two upstream mechanisms, Sln1 and Sho1, with different dynamic responses to osmotic stress suggests that Pbs2 may function as a signal integrator involved in changing the response from one that is “dose encoded,” when the signal is generated by the Sln1 branch, to one that is “duration encoded,” when the signal is generated by the Sho1 branch. This would suggest that a reduction in Pbs2 activity should result in a change on both amplitude and response duration. Through orthogonal targeting, we created a yeast strain carrying a mutant allele of PBS2 (pbs2as) that is specifically inhibited by the small-molecule inhibitor 1NM-PP1 and a cell-permeable analog of 1NM-PP1, SPP86. After incubation of this pbs2as strain with different concentrations of inhibitor and exposure to 0.2 M NaCl, analysis of total and phosphorylated Hog1 in cell extracts showed that inhibition of Pbs2 reduces the maximum amplitude of the response (fig. S2); thus, Pbs2 is a limiting factor in Hog1 signaling and suggests that Pbs2 not only transmits information to Hog1 but might be critical to integrate different kinetic responses from the upstream branches.

Fig. 1. Inactivation of any of the two branches of the HOG pathway results in cells with less efficient signaling and increased sensitivity to stress. (A) Hog1 phosphorylation is specifically affected in mutants of the Sln1 branch. Different salt concentrations were added to the indicated strains and quantification of total and phosphorylated Hog1 (P-Hog1) was assessed by quantitative Western blotting. The data represent the percent of P-Hog1 relative to the maximum in wild type (wt) and are presented as the mean ± SD from three independent experiments. (B) Both branches of the HOG pathway contribute differentially to growth upon increasing osmolarity. The ssk2 ssk22 strain is more sensitive to osmotic stress than is the ste50 strain. Serial dilutions of indicated strains were spotted onto YPD and salt plates and growth was scored after 4 days. (C) Cell growth was assessed in liquid media containing different NaCl concentrations.
Inhibition of the activity of Hog1 increases its phosphorylation in both the absence and the presence of stress

In the absence of stress, catalytically inactive Hog1 shows higher basal phosphorylation than does wild-type Hog1, and stress causes a prolonged increase in its phosphorylation compared to wild-type Hog1 (26, 27). In addition, combined deletion of the phosphatases that down-regulate active Hog1 is lethal unless HOG1 or PBS2 is deleted (28, 29). Both results are compatible with constant signaling to Hog1, even in the absence of stress, which must be down-regulated for the cells to maintain viability. If Hog1 is constantly phosphorylated and its activity is required for its dephosphorylation, then specific inhibition of the kinase activity of Hog1 should result in increased phosphorylation of Hog1. To study whether Hog1 receives constant signaling that is silenced by a feedback regulatory loop that depends on Hog1 activity, we created a Hog1 mutant (hog1as) that is sensitive to the small-molecule inhibitors 1NM-PP1 and SPP86 (30, 31). Wild-type or hog1as cells carrying an empty vector or a vector containing wild-type HOG1 or a catalytically inactive hog1 (hog1kn) were grown in selective medium and the inhibitor was added at time 0. Strikingly, addition of the inhibitor to nonstressed cells resulted in rapid phosphorylation of Hog1 (Fig. 2A). The addition of the inhibitor to cells previously subjected to stress (10 min) prevented dephosphorylation of hog1as as reported before (31) (fig. S3). Addition of the inhibitor, once Hog1 was dephosphorylated after exposure to stress and adaptation, resulted in rephosphorylation of the kinase (Fig. 2B). Therefore, our results suggest that inhibition of the kinase activity of the MAPK results in rapid phosphorylation of kinase even in the absence of stress. Correspondingly, in the absence of stress, the phosphorylated form of Hog1 does not appear in wild-type cells upon addition of the inhibitor or in cells containing a wild-type allele of HOG1 together with the hog1as mutant allele (Fig. 2A). Osmotic stress triggered the accumulation of hog1as fused to green fluorescent protein (GFP) in the nucleus, whereas in the presence of the inhibitor, hog1as-GFP did not accumulate in the nucleus (fig. S4).

The Sln1 branch of the HOG pathway is responsible for high basal signaling to Hog1

Several potential regulatory feedback loops have been described for the Hog1 pathway. To test whether the control of Hog1 phosphorylation is exerted by a fast (transcription-independent) or slow (transcription-dependent) feedback loop, we analyzed phosphorylation of the kinase upon blockage of transcription or translation. Phosphorylation of Hog1 upon inhibition of its kinase activity is independent of transcription and translation, because the presence of cycloheximide (a translation inhibitor) or thiolutin (a transcription inhibitor) did not affect Hog1 phosphorylation (fig. S5A); these drugs did block the induction of Slt1 protein production (fig. S5B). The rapid production of phosphorylated Hog1 when Hog1 activity is inhibited does not depend on changes in glycerol concentration, which is known to regulate the HOG pathway. Both hog1as cells with wild-type FPS1, which encodes a glycerol channel, and hog1as cells with fps1Δ1, which encodes a constitutively open glycerol channel, show accumulation of phosphorylated Hog1 in response to inhibition of Hog1 kinase activity (Fig. 3A). Furthermore, although the amount of phosphorylated Hog1 produced in response to the inhibitor, as well as in response to osmotic

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**Fig. 2.** Inhibition of Hog1 activity results in its phosphorylation. (A) Hog1 catalytic activity is required to prevent accumulation of the phosphorylated form (P-Hog1) in the absence of stress. The indicated strains (wt and hog1as) containing a vector control or a wild type or catalytically inactive mutant were grown in SD medium and 5 μM inhibitor (SPP86) was added at time 0. Phosphorylated Hog1 (P-Hog1) was assessed as in Fig. 1A. (B) Inhibition of hog1as after adaptation to stress also induces accumulation of its phosphorylated form. Wild-type and hog1as strains were stressed with 0.4 M NaCl and inhibitor (5 μM SPP86) was added at time 40 min as indicated by the arrow. Hog1 was detected by chemoluminescence with specific antibodies.

**Fig. 3.** Phosphorylation of inactive Hog1 depends on the Sln1 branch and does not require closure of the glycerol channel. (A) hog1as cells carrying the wild-type glycerol channel (FPS1) or a constitutively active mutant (fps1Δ1) were grown in SD medium and inhibitor (5 μM SPP86) was added at time 0. Samples were collected at the indicated times and total and phosphorylated Hog1 were detected by chemoluminescence with specific antibodies. (B) The indicated strains were grown in YPD, SPP86 (5 μM) was added at time 0, and phosphorylated Hog1 (P-Hog1) was assessed as in Fig. 1A.
stress, was less in cells lacking FPS1, possibly due to increased internal glycerol content. FPS1 strains still exhibited phosphorylation of hog1as in the presence of the inhibitor (fig. S6, A and B). Thus, basal signaling through the HOG pathway is controlled by a fast feedback regulatory mechanism that is dependent on the kinase activity of Hog1.

The basal signaling to Hog1 could be produced by either or both of the upstream sensing mechanisms that converge on Pbs2. To dissect which branch of the pathway, Sln1 or Sho1 or both, is responsible for high basal signaling to Hog1, we measured Hog1 phosphorylation after addition of inhibitor to hog1as cells that also contained ssk2 ssk22 or ste50 mutations. Phosphorylation of Hog1 upon inhibition of its kinase activity in a ste50 strain showed similar kinetics to those cells in which both branches of the pathway were active (Fig. 3B). Similar results were obtained in cells containing sho1 mutation (fig. S7). Thus, loss of signal through the Sho1 branch does not affect the basal signaling to Hog1. However, Hog1 phosphorylation in response to inhibition of its kinase activity is completely absent when the Sln1 branch is inactive (Fig. 3B). Therefore, the two branches of the HOG pathway display different signaling properties, with the Sln1 branch mediating high basal signaling to Hog1 and the Sho1 branch serving as an inducible system.

A mathematical model that includes high basal signaling and a negative feedback loop describes the dynamics of the HOG pathway

Previous comprehensive mathematical models for the yeast HOG pathway included most of the components of the pathway or potential regulatory loops, but they did not include basal signaling (18, 22, 23). Therefore, we developed a new mathematical model that includes a stress-independent, basal signal for the sensor and the requirement for negative feedback regulated by the target Hog1, which involves a minimal set of nonlinear interactions (Fig. 4A and Supplementary Materials). The advantage of a model that involves just the sensor and the target components is that it is amenable to analytical mathematical analysis without requiring parameter estimation. Despite its simplicity, this model fully describes the basic mechanisms behind the observed experimental results (Fig. 4, B to D) and can be studied analytically by considering only the interactions between the sensor protein Sln1 (designated A in the model) and the target protein Hog1 (designated B in the model).

Once cells are subjected to stress, the concentration of signaling-competent Sln1 (A*) increases as does the active form of Hog1 (B*). Thus, A* and B* represent the main players immediately after stress, before cellular adaptation has begun. Assuming that the total concentrations of the proteins remains constant (22), one can reduce the pathway to a two-dimensional model, which can be described by a set of two differential equations, namely, dA*/dt = g(A*, S) (Equation 27 in Supplementary Materials) and dB*/dt = f(A*,B*) (Equation 28 in Supplementary Materials) where S represents osmotic stress and f and g are nonlinear Hill-like response functions. These equations describe the production of active Sln1 in response to stress (designated S in the model) and the variation in Hog1 phosphorylation that depends on the concentration of active Sln1 and the Hog1-mediated negative feedback loop (see Supplementary Materials for a full description of the model and equations).

Fig. 4. A mathematical model of the Sln1 branch describes the signaling properties of the pathway. (A) The minimal backbone of the model includes the sensor Sln (A, inactive state; A*, active state), the MAPK Hog1 (B, inactive state; B*, active state), osmolites (G), and a stress-regulated osmolite channel (C). Activation of A occurs spontaneously (basal activation). Deactivation of A* is stress-dependent, K_a(S), where S is osmotic stress. A* stimulates the formation of B*. B* represses its own production by either enhancing B* deactivation or by inhibiting B activation (not shown) and enhances osmolite (G) production, which exits through the stress-regulated channels (C). (B) The model describes the experimental data with the dynamics of B* [phosphorylated Hog1 (P-Hog1)] from the model matching the experimental data (colored dots) (see Supplementary Materials for parameters) in response to different S conditions (concentrations of salt). (C and D) The model describes that by breaking the B*-dependent feedback, addition of the inhibitor in the experimental condition (arrow) induces phosphorylation of B after adaptation to stress (C) and in the absence of stress (D).
With this model we explored two hypothetical scenarios—one in which the target of the feedback regulatory loop was Hog1 itself and one in which the target was a component upstream of Hog1. Both cases are biologically distinct, but the model displays the same qualitative dynamic response (see Supplementary Materials for the detailed analysis of each scenario). Thus, this new mathematical model incorporates the basal signaling and the Hog1-dependent negative feedback loop and allows analysis of the signal dynamics independently from the specific target of the feedback loop.

The two-dimensional mathematical model demonstrates why high basal signaling is essential for the stress response

To understand the properties underlying the pathway with the defined characteristics we studied the geometrical features of the so-called nullclines of the system (32), which allow one to determine the maximum and minimum states of the system. In this system, the nullclines are defined by the two curves $g(A^*, S) = f(A^*, B^*) = 0$, which allows us to determine the presence of equilibrium points and their dynamical behavior. Thus, we can analyze the relationship between active Hog1 and active Sln1 (Fig. 5A).

These curves determine how $A^*$ and $B^*$ change in time under stress. Because $g = 0$ means that $A^*$ does not change, and $f = 0$ means that $B^*$ does not change, the system will move parallel to the $A$ axis when approaching $f$ or parallel to the $B$ axis when approaching $g$. In our model, $g = 0$ is a vertical line and the location of this line depends on the values of the stress within a range $A_*^{min} < A^* < A_*^{max}$, where $A_*^{min}$ is the nonstress (basal) value and $A_*^{max}$ its upper value. Moreover, independently from the specific set of Hill factors used, the $f = 0$ nullcline exhibits, for both theoretical scenarios, generic geometrical features that determine its shape.

The $f = 0$ nullcline has a horizontal asymptote located at $B_c$, crossing the axis at $A^* = 0$, $B^* = 0$ (Fig. 5A), without local maximum or minimum values within the interval $(0, B_c)$. Therefore, when cells are adapted to the environmental osmolarity, the system is in a stable state $(A_*^{min}, B_*^{min})$. However, when cells are subjected to hyperosmotic stress, the nullcline $g = 0$ is abruptly displaced to higher values of $A^*$ (that is, $A_*^{max}$) and the previous stable state becomes unstable. After adaptation to the new osmotic conditions, the nullcline $g = 0$ moves again to lower values of $A^*$, and the system evolves toward a new stable state $(A_*^{p}, B_*^{p})$. It is worth noting that immediately after stress, cells are not adapted and the production of active Hog1 ($B^*$) is predominantly determined by the trajectory in the $A$-$B$ plane that depicts the evolution of $A^*$ versus $B^*$ comprised between $(A_*^{min}, B_*^{min})$ and $(A_*^{max}, B_*^{max})$ (Fig. 5A).

Due to the shape of the nullcline, a system starting from $A_*^{min} = B_*^{min} = 0$ (a system without basal signal) will slowly increase, because the vertical component $f$ of the field has its lowest value exactly where the horizontal component $g$ has its highest value. Therefore, the system will display a delayed response. In contrast, if the system starts with $A_*^{min} > 0$ (in the presence of basal signaling), then the response is faster and the delay is reduced or avoided. Even in cases were the value of $A_*^{min}$ is not very high (relatively low basal signaling), the response will be faster because of the position of the initial point on the region of the nullcline $f = 0$ with the steeper slope (Fig. 5B).

These results are consistent with the experimental results obtained for the basal signaling branch Sln1 and for the nonbasal branch Sho1. The Sln1 branch does not exhibit a delayed response to hyperosmotic stress, whereas the Sho1 branch does exhibit a delay (Fig. 6A). As a result of the same geometrical constraints, systems with very low or no basal signal exhibit less sensitivity to small changes in the external osmolarity. Small changes in the external osmolarity lead to small changes in the concentration of the activated sensor $A^*$.

If the initial point is at $f = 0$ or near it, changes in $A^*$ will have a small effect on $B^*$ because in this region the nullcline $f = 0$ exhibits a lower slope. However, for systems with basal signal, the initial point will be located in regions of the nullcline where the slope is steeper, and thus, small changes in $A^*$ will trigger large changes in $B^*$. Eventually, if the basal signal is too high, the initial point is located in its stable state, is performed. The curve displays an optimum at some $A^*$.

Fig. 5. Nullcline analysis reveals the need for basal signaling in the response to osmotic stress. (A) Phase space $A^*$ versus $B^*$. Concentrations are determined by the equation $\frac{dA}{dt} = g(A^*, S)$ and $\frac{dB}{dt} = f(A^*, B^*)$. Nullcline $g = 0$ defines a vertical line whose location depends on the $A^*$ value without stress (basal signal). Without basal signaling and no stress, the nullcline is located at the origin (blue line). If basal signal is present, then $A^* > 0$ (green line). The equilibrium state is determined by $g(A^*, S) = (A^*, B^*) = 0$ (circle). After stress, nullcline $g = 0$ shifts toward higher $A^*$ values (violet line), and the system moves to a new state (square).

The trajectory is confined in the gray region. The slope of $f = 0$ for small $A^*$ values introduces a slowdown in the $B^*$ response through time, but once $A^*$ is large enough ($A^* > A_*^{min}$), $B^*$ grows faster. The inset in (A) shows the ratio of the vertical and horizontal components of the field ($f/g$) when a small increase in the concentration of active sensor $A^*$, with respect to
Interestingly, addition of the inhibitor to the osmolarity and sensitivity of the pathway and its quick response to even small changes like responses, and a negative feedback provide for the high efficiency combination of basal signal, nonlinearity associated with the presence of Hill-like responses, and a negative feedback provide for the high efficiency and sensitivity of the pathway and its quick response to even small changes in osmolarity.

The Fus3 and Kss1 MAPK signaling pathways are also controlled by a high basal signal

In yeast, other MAPK signaling pathways exist that respond to external stimuli (2). Both the Fus3 and Hog1 MAPKs are activated by pheromone (33). We created strains in which only one of the two kinases was present (fus3as and kss1as strains) and strains in which the remaining kinase could be inhibited (Fus3as kss1as; Kss1as fus3as) and tested whether inhibition of the kinase activity resulted in phosphorylation of the MAPK in the absence of pheromone. As expected, the addition of pheromone stimulated the phosphorylation of wild-type and mutant fus3as, which, in turn, resulted in cell cycle arrest at G1 and induction of FUS1 expression (FUS1p:GFP) (Fig. 7).

Interestingly, addition of the inhibitor to the fus3as or kss1as cells triggered Fus1 or Kss1 phosphorylation (Fig. 7, A and D). However, inhibition of the kinase activity of the MAPK (fus3as cells) failed to trigger downstream processes, such as arrest in G1 and FUS1 expression (Fig. 7, B and C). It is worth noting that, whereas the presence of wild-type Kss1 did not affect the phosphorylation of fus3as in the presence of inhibitor, the presence of wild-type Fus3 strongly reduced the phosphorylation of kss1as in the presence of the inhibitor, indicating that the two kinases might have different input into the feedback loop(s) in response to pheromone (fig. S9). Both Fus3 and Kss1 MAPK pathways also seem to include high basal signaling. Thus, three MAPKs involved in various biological processes, from responding to osmotic stress to initiating developmental programs, seem to have high basal signaling that is inhibited by negative feedback regulatory loops controlled by kinase activity of the MAPKs. Intrinsic basal signaling may be a general property of MAPK pathways, allowing efficient response to environmental changes.

DISCUSSION

Signaling through MAPK pathways is essential for cellular response to extracellular stimuli. Yeast cells activate the Hog1 MAPK to control cellular response and adaptation to osmotic stress and, therefore, precise modulation of Hog1 activity is necessary to maximize cell survival. Activation of the MAPK is achieved by upstream sensing mechanisms with slightly different kinetics of response and sensitivity to osmotic stress [(17 and our results (Figs. 1 and 6A)], suggesting that both branches may have different signaling capacity. Partial inhibition of the MAPKK Pbs2 shows that the kinase not only integrates the signal from the two branches of the pathway but may also transform the input from a dose-encoded signal to one that is duration encoded.

The use of a kinase-inhibitable form of Hog1 showed that inactivation of its kinase activity results in its own rapid and strong phosphorylation, a region of the nullcline \( f = 0 \) where the curve is near the horizontal asymptote with a lower slope, and, as a consequence, the pathway will lose sensitivity. Based on these data, cells adapted to media with different osmolarities should display different basal signaling. Correspondingly, inhibition of hog1as in different media results in altered phosphorylation kinetics of Hog1. Thus, Hog1 is phosphorylated faster after inhibition of Hog1 activity in cells adapted to a medium with higher osmolarity (Fig. 6, B and C).

The overall behavior of the mathematical model is summarized in Fig. 6A, which explains the observed results of reduced sensitivity and slower response of ssk2 ssk22 cells (Fig. 6A and fig. S8) and reveals that a combination of basal signal, nonlinearity associated with the presence of Hill-like responses, and a negative feedback provide for the high efficiency and sensitivity of the pathway and its quick response to even small changes in osmolarity.

**Fig. 6.** Basal signaling is essential for proper dynamic response of the HOG pathway. (A) Signaling through the Sho1 branch is slower than signaling through the Sln1 branch. Indicated strains were grown in medium, subjected to 0.8 M NaCl, and phosphorylated Hog1 was assessed as in (A). (B) Reduction of the external osmolarity results in a slower phosphorylation response. The ste50 strain was grown in YPD medium, cells were centrifuged and resuspended in medium or in distilled water as indicated, then cells were subjected to 0.8 M NaCl stress and Hog1 phosphorylation was assessed as in (A). (C) The kinetics of phosphorylation of Hog1 after inhibition of Hog1 activity depends on external osmolarity. hog1as cells were grown in YPD or YPD plus the indicated salt concentrations, spun, and resuspended in YPD at the same salt concentration, YPD alone, or YPD diluted in distilled water at the indicated dilution factors. The inhibitor (5 \( \mu \)M SPP86) was added at time 0 and Hog1 phosphorylation was assessed as described in (A).
even in the absence of stress, indicating that there is a basal signal into the MAPK. This is consistent with previous studies that, upon stress, a catalytically inactive kinase remained phosphorylated longer than did the wild-type MAPK (26). The use of the inhibitable Hog1 together with specific mutations in upstream components of the pathway showed that the basal signal to Hog1 comes from only the Sln1 branch of the pathway, with the Sho1 branch acting as an inducible system without basal signaling. Thus, the Sln1 branch is possibly the key determinant of the signaling properties of the pathway. Cells with mutations in the Sln1 branch of the pathway are more sensitive to stress than are cells with mutations in the Sho1 branch. Our results are consistent with previous bandwidth analysis with a microfluidic device, which showed that only the Sln1 branch of the pathway was capable of fast signal integration (18). In some yeast species, the Sho1 branch is either absent or is not connected to Hog1 MAPK signaling but instead has a role in morphogenesis rather than osmosensing (34).

Thus, in contrast to the typical assumption that the signal originates in response to the presence of a stimuli, our experimental studies indicate that the signaling through several yeast MAPK pathways depends on high basal signal transduction that must be constantly counteracted by a fast-acting feedback mechanism that is controlled by the kinase activity of the MAPK. Although we do not know the exact nature of such a negative feedback, it does not involve transcription or translation and is independent of glycerol accumulation. Cells with a constitutively open glycerol channel responded in the same manner as the wild type. Protein phosphatases are good candidates for mediating the negative feedback. Elimination of phosphatases results in hyperactivation of the Hog1, and studies on cells exposed to stress pulses followed by frequency determination showed the presence of a fast-acting negative feedback regulatory loop and suggested that it could involve protein phosphatases (23). For cells grown in control or osmotic stress conditions, we can estimate the ratio of the phosphorylated and dephosphorylated states of Hog1 in the presence of the inhibitor by assuming that because the catalytic activity of Hog1 is inhibited and the external osmolarity is maintained constant, the rates of phosphorylation and dephosphorylation are constant. Under basal conditions, the ratio $K_{\text{phosphorylation}}/K_{\text{dephosphorylation}} \approx 1.1$, whereas under 0.4 M salt stress conditions, $K_{\text{phosphorylation}}/K_{\text{dephosphorylation}} \approx 13.3$. These ratios have been calculated such that they reproduce the different slopes and the relative difference of phosphorylated Hog1 in the presence of inhibitor (Fig. 6C). We have also analyzed the model to estimate which of the two components of the HOG pathway is the target of the negative feedback. The model shows that regardless of whether the target of the feedback regulatory loop is Hog1 or is upstream of Hog1, the qualitative results are similar. However, the model also indicates that if the target of the feedback loop is located upstream of Hog1, the system seems to be more efficient (see the Mathematical Analysis section of the Supplementary Materials).

Feedback regulatory loops have profound implications and, typically, systems with negative feedback show higher robustness against external and stochastic noise (35, 36), thereby increasing the efficiency in signal transmission. However, the presence of negative feedbacks introduces several constraints on the dynamics of the systems because they introduce delays in the response and reduce the sensitivity. Thus, the high basal signal may function to counteract the constraints created by the negative feedback loops.

To explore the properties of a system with high basal signal restriction by a negative feedback loop, we developed a two-dimensional mathematical model of the HOG pathway based on the minimal backbone of interactions that reproduces the experimental results and allows analytical analysis of the dynamic properties of the pathway. The analysis of this model was simplified by considering that just after stress only the sensor-target protein interaction and the negative feedback are relevant to explain the basic dynamical behavior. The analysis of the main interactions showed that the dynamics of the pathway immediately after stress depend on the balance between the negative feedback and the basal signaling from the sensor. The feedback tends to delay Hog1 phosphorylation; whereas the basal signal tends to enhance its phosphorylation. Therefore, the sensitivity to small changes in the external osmolarity depends on the amount of basal
signaling, such that this system is much more sensitive than one without basal signaling. Correspondingly, cells deficient on the Sln1 branch show reduced sensitivity to small variations in osmolarity, responded more slowly to osmotic stress, and were more osmosensitive. Because high basal signaling seems to be a key factor that counteracts the lack of response and delay caused by the negative feedback, the dynamics of the response should change upon variations in the basal signal. We showed that cells that were adapted to a reduced osmolarity exhibited reduced basal signaling, reduced capacity to cope with small variations in stress conditions, and responded more slowly to osmotic stress (Fig. 6B). Therefore, in this system with a negative feedback loop, a high basal signal is critical for determining the signaling capacity.

To explore how common basal signaling with negative feedback is, we extended our studies to the pheromone-responsive Fus3 and Kss1 MAPK pathways. Both kinases became phosphorylated upon inhibition of their kinase activity, even in the absence of pheromone, suggesting that these MAPK signaling pathways are also controlled by high basal signal together with a MAPK-dependent negative feedback. This is consistent with a study that identified Sst2 as the target of the feedback loop controlled by Fus3 (37). Thus, three MAPK pathways appear to have developed high basal signaling repressed by MAPK-dependent negative feedback loops, which implies that high intrinsic basal signaling could be a general property of MAPK pathways, allowing efficient response to environmental changes.

MATERIALS AND METHODS

Quantitative Western blotting

Samples were taken in mid-exponential growth phase and fixed in 20% trichloroacetic acid for SDS–polyarylamide gel electrophoresis and immunoblotting. Hog1, Fus3, and Pbs2 were detected with antibodies specific for these proteins (Santa Cruz), phosphorylated Hog1 with an antibody against phospho p38 (Cell Signaling), and both P-Kss1 and P-Fus3 with an antibody against phospho p44/42 (Cell Signaling). Quantification analysis was performed by fluorescence detection with the IRDye 800CW donkey antibody against goat immunoglobulin G (IgG) and the IRDye 680 donkey antibody against rabbit IgG (LI-COR Biosciences) and the ODISSEY application software 2.1 (LI-COR Biosciences). All phosphorylated Hog1 values were normalized against the 10-min sample taken from the wild type stressed with 0.4 M salt.

Flow cytometry

The pheromone pathway was activated with α-factor (2 μg/ml) and DNA content was assessed by staining with propidium iodide.

Inhibitors

1NM-PP1 was used at 5 μM to inhibit analog-sensitive mutants (38). SPP86, a cell-permeable adenine analog similar to 1NM-PP1, was used at 5 μM.

Strains and plasmids

W303-1A (wild type) and derivatives ssk2 ssk22, ste50, ste11, sho1, hog11 TIOG (hog1as), ssk2 ssk22 hog1as, ste50 hog1as, fps1 hog1as, bar1 Fus3::GFP, bar1 kss1 Fus3::GFP, P(FUS3)::fus3 38 N3 (fus3as), bar1 kss1 Fus3::GFP P(KSS1)::kss1 2944 (kss1as), bar1 fus3 Fus3::GFP P(FUS3):fus3as, bar1 kss1 Fus3::GFP P(KSS1):kss1as, and psi2 322 53 538 (psi2as) were used in this study. YCPlac11 with HOG1-3HA or hog1as 3HA (hog1-1KN), YEPlac195 with FPS1 or fps1ΔA13-230 (fps1ΔA1) and pRS416 with HOG1-GFP or hog1as-GFP plasmids were used in this study.

Growth conditions

 Cultures were maintained on YPD (1% yeast extract, 2% Baco Peptone, 2% glucose) or on the appropriate synthetic dropout (SD) medium [0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose, Complete Supplement Mixture quadruple dropout (Qbiogene) (0.6 g/lter), and histidine, tryptophan, uracil, or leucine combined (40 mg/liter)] to maintain the appropriate selection for maintenance of the plasmids. Cells were grown at 30°C and NaCl was added at various concentrations as indicated. Solid culture was performed on 1.5% agar plates of YPD or YPD with NaCl at the indicated concentration. Plates were incubated at 30°C.

REFERENCES AND NOTES

39. We thank E. de Nadal for helpful discussions and support, M. Morillas and A. Vendrell for strains, and M. Grötzl (Sweden) for inhibitor design and supply. This work was supported by an FPU fellowship to S.R.; grant CSD2007-0015 from Ministerio de Ciencia y Tecnología, Consolider Ingenio 2010 program; through the European Commission Directorate General Research, FP6 contract no. ERAS-CT-2003-980409 EURYI (European Young Investigator Awards) award (www.esf.org/euryi) and QUASI to F.P; and FP6-2005-NEST-PATH CELLCOMPUT project to F.P. and R.S. The FP laboratory also receives support from the Fundación Marcelino Botín and Institució Catalana de Recerca i Estudis Avançats (ICREA) Academia (Generalitat de Catalunya). Submitted 23 September 2008 Accepted 6 March 2009 Final Publication 24 March 2009 10.1126/scisignal.2000056 Citation: J. Macia, S. Regot, T. Peeters, N. Conde, R. Solé, F. Posas, Dynamic signaling in the Hog1 MAPK pathway relies on high basal signal transduction. *Sci. Signal.* **2**, ra13 (2009).