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Perspective

In vivo measurement of signaling cascade dynamics

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Genetic and biochemical studies yield information about the component proteins and interactions involved in a cellular signaling pathway. However this parts inventory often does not immediately reveal the in vivo signal processing capabilities and function of the pathway. Signaling pathways are complex systems with dynamic behavior and a systems level approach is needed to understand the physiological roles they play within the cell. We recently used such an approach to measure the signal processing behavior of the budding yeast HOG MAP kinase pathway in response to precisely varied temporal stimuli controlled with a microfluidic device. Despite being a well-studied pathway with well-known components the signaling dynamics and biochemical parameters of this pathway were not known. Our approach allowed us to characterize the pathway's in vivo signal processing and put bounds on all of the in vivo reaction rates. The experimental and theoretical techniques used in our study are general and can be applied to understanding other signaling pathways in a range of biological systems.

Introduction

Cellular responses to external changes in the environment must be tightly regulated in order for cells to maintain their normal physiology (pH, nutrient abundance, etc.,). Cells use signaling pathways to effectuate this regulation. Signaling pathways convert input stimuli from the environment into the outputs inside of the cell that are used to regulate cellular response. To understand the physiological behavior of a signaling pathway we wish to understand the pathway's output over a range of fluctuating environmental inputs. This problem is commonly approached from a bottom-up methodology in which the molecular components of the signaling pathway are analyzed piece-by-piece. Genes are knocked-out or knocked-down and the output of the pathway in these mutant strains is analyzed. Binding affinities and reaction rates are measured by combining pathway components in vitro. Unfortunately, in vitro data and data from knockouts may not accurately predict how the system functions as a whole in vivo. Bottom-up approaches are often incorporated into detailed models that attempt to incorporate knowledge of all possible interactions within the pathway in order to predict pathway behavior, sometimes with moderate success.[1] However the exhaustive modeling approach suffers from missing information in the form of unknown components and reaction parameters. Parameters are usually fit from existing data, and it is not always certain how new information will perturb or invalidate the model.

It is clear that signaling pathways cannot always be viewed as mere collections of proteins and protein interactions. They are complicated systems whose properties emerge from architectural complexity and the dynamics of biochemical reactions. A complementary approach to understanding signaling pathways that embraces this inherent complexity is to study the input-output characteristics of a signaling pathway in response to well-defined inputs and use this information to infer properties of the signaling pathway. This technique is common in reverse systems-engineering, but has not been widely applied to biological signaling networks until recently.[2-4] A notable exception is the application of such techniques to the study of the bacterial chemotaxis response.[5-7] A common input used in these kinds of studies is an oscillatory signal. Oscillatory input signals offer a number of advantages, including improved signal-to-noise in measurements as well as control over various input parameters.[8] The response of a biological signaling pathway to an oscillatory signal depends on pathway architecture and the biochemical reaction rates in the pathway. Thus, the dynamical properties of a signaling pathway can be extracted by considering the output of the pathway in response to oscillatory input varying over a range of frequencies.

We recently developed a versatile technique that allows us to stimulate the *Saccharomyces cerevisiae* high-osmolarity glycerol (HOG) response mitogen-activated protein (MAP) kinase cascade with oscillatory stimuli. This technique utilizes a microfluidic device coupled with fluorescence microscopy to study the in vivo response of the pathway under these conditions. By measuring the input-output properties of this pathway in response to oscillatory stimuli we were able to uncover previously unknown in vivo properties of the pathway. This technique is general and can be extended to other signaling pathways.

A Canonical Signaling Pathway: The HOG MAP Kinase Cascade

The HOG pathway (Fig. 1) is one of the best-studied MAP kinase pathways in *Saccharomyces cerevisiae*[9,10] and presents an ideal

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system in which to employ a systems-approach to studying signaling. The HOG pathway allows yeast cells to respond to changes in external osmolarity. Yeast cells use glycerol to regulate their intracellular osmolarity and it is the HOG pathway that controls increased glycerol production in response to increased external osmolarity. Despite being well-known with well defined components, its in vivo dynamics and reaction kinetics are not well understood. The core of the HOG signaling pathway consists of the MAP kinase kinase Pbs2 which dual phosphorylates Hog1 on conserved threonine and tyrosine residues. This phosphorylation activates the Hog1 protein and actived Hog1 accumulates in the nucleus where it interacts with a number of transcription factors. Approximately 600 genes are differentially regulated in response to osmotic stress, although only about 20% rely on Hog1 for their transcriptional response.[10] Several transcription factors are involved in Hog1-dependent response including Hot1, Sko1, Msn2/Msn4, Msn1 and Smp1. Hog1 is recruited to promoters by these proteins however it seems to be the Hog1 kinase itself that is the activating transcription factor for its targets.[9,11-15] The gene-expression of key enzymes Gpd1 (glycerol-3-phosphate dehyrogenase) and Gpp2 (glycerol-3-phosphatase) involved in glycerol production is stimulated by osmotic stress in a Hot1-dependent manner.

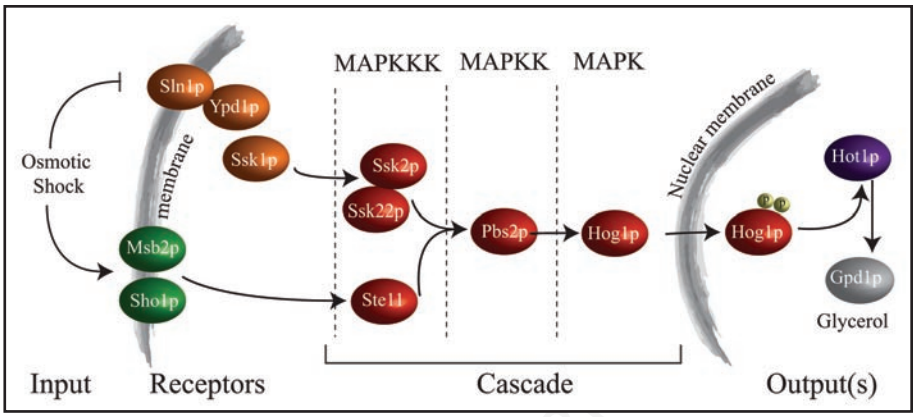

Figure 1. The HOG pathway consists of two input branches. The SHO1 branch activates Pbs2 through the MAPKKK Ste11 under conditions of hyperosmotic stress. In the SLN1 branch, the protein Ssk1 is repressed by the SLN1 phosphorelay under osmotically neutral conditions. Hyperosmotic stress inhibits Sln1 and the subsequent derepression of Ssk1 allows it to activate the MAPKKKs Ssk2 and Ssk22, which in turn phosphorylate Pbs2. Phosphorylation of Hog1 by Pbs2 leads to its nuclear localization. In the nucleus Hog1 activates the transcriptional response to hyperosmotic stress through interaction with a number of transcription factors. Gpd1 is a protein involved in glycerol synthesis which is upregulated in response to osmotic stress.

Two input branches, the SLN1,[16,17] and SHO1[18-20] branch, converge on the core MAP kinase kinase Pbs2. These two branches are redundant for cell survival under conditions of moderate osmotic stress. The SLN1 branch consists of a two-component phospho-relay more commonly found in prokaryotes.[21] Sln1 is thought to sense changes in turgor pressure,[22] although this is not well understood. Deletion of SLN1 is known to be lethal due to a hyperactive HOG pathway, thus the SLN1 branch is a negative regulator of the HOG map kinase cascade under iso-osmotic conditions. In response to increased osmolarity, the Sln1 protein is deactivated, and Ssk1 becomes dephosphorylated. In its dephosphorylated form Ssk1 is capable of phosphorylating the downstream MAP kinase kinase kinase proteins Ssk2 and Ssk22. Ssk2/Ssk22 phosphorylate Pbs2 which in turn phosphorylates Hog1.

The Sho1 branch includes the membrane proteins Sho1, Msb2, Opy2. Activation of the Sho1 branch results in the activation of the MAP kinase kinase kinase Ste11 which phosphorylates the core protein Pbs2. What is being sensed by the Sho1 branch of the pathway remains unclear. Proteins in the Sho1 branch are exclusively shared with the signaling pathways in *S. cerevisiae* that sense mating pheromone or lead to pseudohyphal growth. Furthermore, the Sho1 branch in many fungi does not seem to be connected to Pbs2 homologues nor involved in responding to osmotic stress.[23-25]

Methodology

This brief description of the HOG signaling pathway illustrates the significant amount of knowledge available describing pathway components and their interactions. The HOG pathway represents a particularly ideal system for our study because the input (high osmolarity) and output (phosphorylated Hog1) of the HOG pathway are easily controlled and measured, In a laboratory setting, yeast cells are exposed to well-defined increases in osmolarity by adding sorbitol or sodium chloride to their growth media. The output of the pathway, phosphorylated Hog1, can be measured by using fluorescence microscopy to monitor the amount of Hog1 protein tagged with GFP localized to the nucleus of the cell.

To study the response of the HOG pathway to oscillatory stimuli varying over a range of frequencies we needed to be able to precisely change the cells' growth media between hyperosmotic and iso-osmotic media. Microfluidic devices can be used to grow cells in channels that allow the rapid switching of media, while simultaneously monitoring cell response under a fluorescent microscope.[3,4,26] We designed a microfluidic device that takes advantage of the property of laminar flow for small liquid volumes to allow rapid media switching. Our flow-cell is a Y-shaped device with media entering a channel through two inlets. One inlet flows iso-osmotic media while the other flows hyperosmotic media (1 M sorbitol). Because flow is laminar near the inlets mixing is minimal. By changing the pressure of the iso-osmotic media using a computer-controlled switch we are able to rapidly fill the channel with either low or high-osmolarity media. Cells growing in the channel are exposed to a square-wave of oscillatory input-stimulus. Our device allows media switching as rapidly as twice per second with minimal degradation of the input signal. Though we designed this device to study the HOG pathway it could be easily modified to study other signaling pathways in other organisms.

Results

The use of this microfluidic device was the key to understanding the in vivo signal processing of the HOG MAP kinase pathway. We measured the Hog1 localization response of the pathway to hyperosmotic input varying over a range of frequencies. Up to input varying as rapidly as once every 220 s (or ~0.0046 s^{-1}) the Hog pathway's response faithfully follows the input (Fig. 2) with Hog1 localizing and delocalizing faithfully as the media is changed. However, for

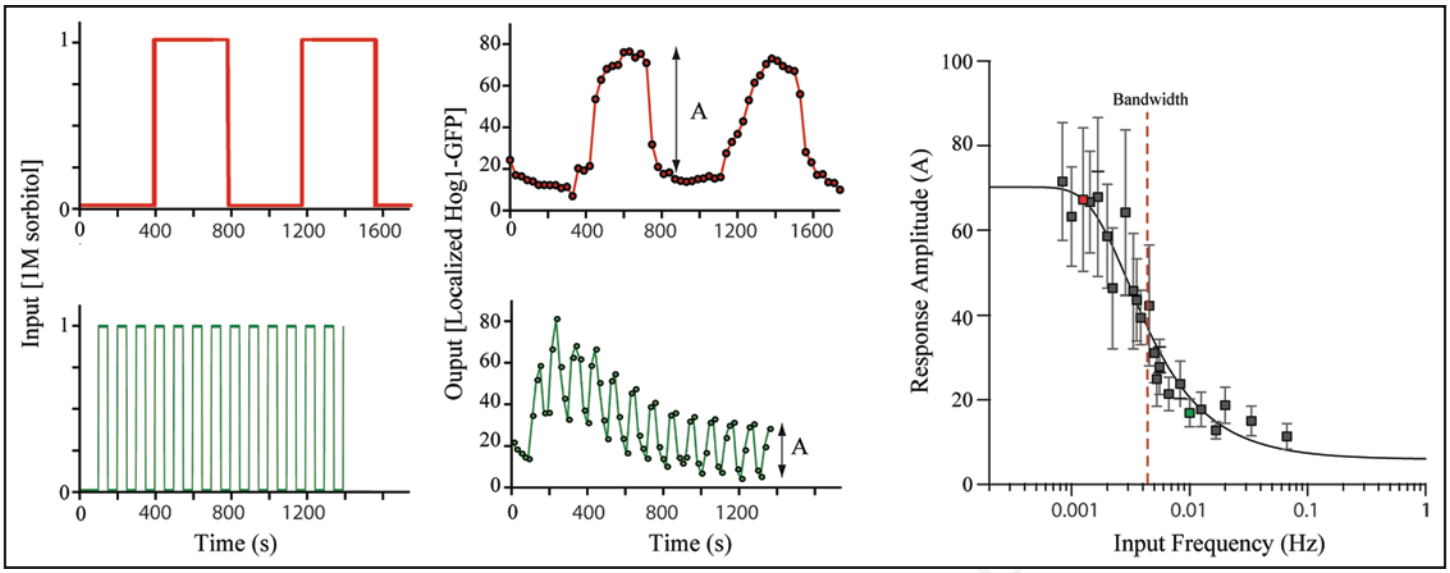

Figure 2. For slow inputs (1 M sorbitol in our experiments), pathway output measured as the localization of GFP-tagged Hog1 to the nucleus is able to faithfully follow the input (top). For rapidly varying inputs, the pathway output is unable to faithfully follow the input (bottom). In this example the pathway output transiently integrates over the incoming signal before reaching an oscillatory state with attenuated amplitude. The pathway response, measured as the amplitude of Hog1-GFP localization to the nucleus once oscillations have reached steady state, is measured in response to input varying over a range of frequencies (right). The crossover point between input frequencies, at which the output responds faithfully and input frequencies at with the pathway does not, defines the pathway's bandwidth or critical frequency.

input varying more rapidly than ~0.0046 s^{-1} the pathway output no longer follows the input faithfully, but instead integrates over the incoming input. Thus there is a bandwidth or critical frequency of input, ~0.0046 s^{-1}, defined by where the pathway switches from a faithfully following to a not-faithful regime (Fig. 2). The response for fast frequency input behaves like the pathway is responding to a step input of half the amplitude of the actual incoming square wave, suggesting that the HOG pathway at high frequencies actually performs an average over the incoming stimuli.

We also measured the mechanical response due to water entering and leaving cells as the external osmolarity was changed. The mechanical response faithfully follows signals that change less frequently than once every 30 s (or ~0.033 s^{-1}). Thus the mechanical response responds much more quickly than the Hog1 localization response. This indicates that signaling through the cascade is not limited by the mechanical response of the cell but rather by the biochemical reactions occurring in the HOG cascade. The critical frequency, at which the HOG pathway can no longer respond faithfully to the incoming input, must be determined by the slowest biochemical timescale in the pathway cascade. Thus, the activation and deactivation rates of all components in the HOG pathway must be greater than ~0.0046 s^{-1}.

In order to understand the contribution of each input branch to the pathway's signal processing we measured the response of the pathway to oscillatory input in which only one input branch was present. We found that the SHO1-branch alone can respond faithfully to signals varying as rapidly as ~0.0026 s^{-1}, while the SSK1 branch alone has the same response as the wild-type pathway. Thus the SHO1 branch is slower than the wild-type and SSK1 branch by almost two-fold. This is consistent with previous biochemical evidence.[27]

To understand whether it was the activation or deactivation time of the pathway that was limiting its ability to faithfully following incoming input signals, we analyzed the pathway's response to very rapidly varying input signals. For rapidly varying input signals, the HOG pathway transiently integrates the input signal. Transient integration can only occur if the pathway is not fully deactivated between input pulses. Therefore, it must be that the deactivation of the HOG pathway is limiting for pathway bandwidth. The rapid switching available in our microfluidic device allowed us to see this previously undiscovered behavior in the HOG pathway. By using asymmetric input pulses, in which the amount of time cells spent in 1 M sorbitol was kept constant, but the "OFF" time of the signal was varied we were able to measure the deactivation time of the HOG pathway. This time was found to be ~0.0041 s^{-1}, in excellent agreement with the critical frequency measured for the wild-type pathway.

Perspectives

Our experiments illustrate the filtering properties of the HOG pathway. The HOG pathway faithfully transmits information about signals that vary more slowly than once every 220 s, while it transiently integrates over signals that vary more rapidly. It is interesting to speculate that this behavior has been tuned throughout evolution to an optimal value for the critical frequency at which the pathway switches between these two types of behaviors. Faithfully following an input signal may give the cell a fitness advantage as the input varies slowly, while integration may allow it to avoid constant computation while still relaying information about rapidly incoming signals. It would be interesting to compare the critical frequency of the HOG pathway between yeast species that live in different environments. One can imagine that the critical frequency might be tuned differently depending on the spectrum of input frequencies the cell expects to see in the environment. Furthermore, one would expect this frequency to vary significantly between different signaling pathways and even different MAP kinase pathways as the timescale of desired cellular response might be very different for different inputs.

We found that the slowest rate in the HOG pathway was in the deactivation of the pathway. To understand how pathway behavior can be tuned throughout evolution it is important to understand what reaction rate or protein concentration is setting the critical frequency. In our previous work we showed that the amount of Pbs2 protein in the pathway is not limiting for pathway bandwidth: severe Pbs2 dilution is needed to observe a slow-down of the pathway dynamics and overexpression of Pbs2 does not increase the pathway's ability to faithfully follow the input stimuli.[3] Preliminary work in our lab suggests that deletion of two key phosphatases responsible for deactivation of the HOG pathway, PTP2 and PTP3, and overexpression of the SLN1 branch protein Ssk1 does not affect pathway critical frequency. Future work will investigate which key reaction or reactions are setting the critical frequency. It will be very interesting to see if it is sensitive to the level of a particular protein. One could then speculate that throughout evolution or indeed, throughout development, changing of the level of one protein would allow continuous tuning of pathway critical frequency.

Improving our knowledge of pathway dynamics is very relevant if one wants to synthetically engineer new function with controlled dynamics. One possible direction is to alter the structure of key proteins, in order to increase the biochemical rates, or to change the way scaffolding proteins behave. Bashor et al.[28] have recently shown that reengineering the scaffold protein Ste5 in the yeast mating pathway enables them to alter the dynamics of the mating response. This is an exciting avenue of research, since scaffolding is also a key player in setting the specificity of pathway dynamics. By studying quantitatively the dynamics of signaling processes in such synthetically engineered pathways, one may start to shed light on evolution processes that have led to actual signaling pathways while developing the tools that synthetic biology will need to design new functions and control system dynamics.

Conclusion

In this review we have highlighted the use of approaches from systems engineering coupled to microfluidics and fluorescence microscopy to quantitatively measure the in vivo dynamics of a biological signaling pathway, the HOG MAP kinase pathway. Studying the input-output relationship of the pathway allows measurement of pathway bandwidth which yields insight into pathway architecture as well as biochemical signaling kinetics. Other properties of cascades, such as sensitivity, efficiency, energy consumption and cross talk can be analyzed using similar methods. Such approaches may be generalized to other signaling pathways in other organisms, and in particular to the important family of MAPK cascades which are preserved throughout higher eukaryotes.

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References

1. Klipp E, Nordlander B, Kruger R, Gennemark P, Hohmann S. Integrative model of the response of yeast to osmotic shock. Nat Biotechnol 2005; 23:975-82.
2. Bennett MR, Pang WL, Ostroff NA, Baumgartner BL, Nayak S, Tsimring LS, et al. Metabolic gene regulation in a dynamically changing environment. Nature 2008; 454:1119-22.
3. Hersen P, McClean MN, Mahadevan L, Ramanathan S. Signal processing by the HOG MAP kinase pathway. Proc Natl Acad Sci USA 2008; 105:7165-70.
4. Mettetal JT, Muzzey D, Gomez-Uribe C, van Oudenaarden A. The frequency dependence of osmo-adaptation in *Saccharomyces cerevisiae*. Science 2008; 319:482-4.
5. Block SM, Segall JE, Berg HC. Impulse responses in bacterial chemotaxis. Cell 1982; 31:215-26.
6. Block SM, Segall JE, Berg HC. Adaptation kinetics in bacterial chemotaxis. J Bacteriol 1983; 154:312-23.
7. Segall JE, Block SM, Berg HC. Temporal comparisons in bacterial chemotaxis. Proc Natl Acad Sci USA 1986; 83:8987-91.
8. Lipan O, Wong WH. The use of oscillatory signals in the study of genetic networks. Proc Natl Acad Sci USA 2005; 102:7063-8.
9. Hohmann S. Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 2002; 66:300-72.
10. Hohmann S, Krantz M, Nordlander B. Yeast osmoregulation. Methods Enzymol 2007; 428:29-45.
11. Alepuz PM, de Nadal E, Zapater M, Ammerer G, Posas F. Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. EMBO J 2003; 22:2433-42.
12. Alepuz PM, Jovanovic A, Reiser V, Ammerer G. Stress-induced map kinase Hog1 is part of transcription activation complexes. Mol Cell 2001; 7:767-77.
13. De Nadal E, Zapater M, Alepuz PM, Sumoy L, Mas G, Posas F. The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. Nature 2004; 427:370-4.
14. Pokholok DK, Zeitlinger J, Hannett NM, Reynolds DB, Young RA. Activated signal transduction kinases frequently occupy target genes. Science 2006; 313:533-6.
15. Proft M, Struhl K. Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol Cell 2002; 9:1307-17.
16. Posas F, Saito H. Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. EMBO J 1998; 17:1385-94.
17. Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell 1996; 86:865-75.
18. Posas F, Witten EA, Saito H. Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. Mol Cell Bio 1998; 18:5788-96.
19. Tatebayashi K, Tanaka K, Yang HY, Yamamoto K, Matsushita Y, Tomida T, et al. Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. EMBO J 2007; 26:3521-33.
20. Tatebayashi K, Yamamoto K, Tanaka K, Tomida T, Maruoka T, Kasukawa E, et al. Adaptor functions of Cdc42, Ste50 and Sho1 in the yeast osmoregulatory HOG MAPK pathway. EMBO J 2006; 25:3033-44.
21. Catlett NL, Yoder OC, Turgeon BG. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Euk Cell 2003; 2:1151-61.
22. Reiser V, Raitt DC, Saito H. Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure. J Cell Biol 2003; 161:1035-40.
23. Bahn YS, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME. Sensing the environment: lessons from fungi. Nat Rev Micro 2007; 5:57-69.
24. Furukawa K, Hoshi Y, Maeda T, Nakajima T, Abe K. Aspergillus nidulans HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. Mol Microbiol 2005; 56:1246-61.
25. Krantz M, Becit E, Hohmann S. Comparative genomics of the HOG-signalling system in fungi. Curr Genet 2006; 49:137-51.
26. Beebe DJ, Mensing GA, Walker GM. Physics and applications of microfluidics in biology. Ann Rev Biomed Eng 2002; 4:261-86.
27. Maeda T, Takekawa M, Saito H. Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science 1995; 269:554-8.