Laboratory of Molecular Genetics (Frontier Research Unit)
遺伝子解析施設(フロンティア研究領域)

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The Laboratory of Molecular Genetics was established for developing various molecular genetic techniques, spreading them to IMSUT investigators and supporting security management related to experiments carried out using recombinant DNA technologies. Since 2017, this laboratory has integrated the Frontier Research Unit for supporting selected young investigators to challenge new fields of biomedical sciences for specified periods.

Frontier Research Unit

Protein phosphorylation and dephosphorylation are among the most important intracellular signaling mechanisms, and are mediated, respectively, by protein kinases and protein phosphatases. We study various aspects of cellular signal transduction with a particular emphasis on the role and regulation of protein phosphorylation and dephosphorylation in cellular stress responses, using yeast cells.

1. Activation of the Hog1 MAP kinase requires a direct osmotic priming of Hog1 itself as well as stimulation of the upstream osmosensors

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The family of protein kinases known as mitogen-activated protein kinases (MAPKs) consists of major intracellular signal transducers in eukaryotic cells. Each MAPK is activated via a three-tiered kinase cascade (MAPK cascade) composed of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. Activated MAPKKK activates a cognate MAPKK by phosphorylating two conserved phosphorylation sites in the flexible activation loop within the catalytic domain. In turn, an activated MAPKK activates a cognate MAPK by phosphorylating the conserved threonine and tyrosine in the TXY motif of the latter's activation loop. MAPK cascades are highly conserved from yeast to mammalian species, so much so that the mammalian p38 MAPK can functionally complement the yeast Hog1 MAPK. A MAPK signal transduction pathway commonly comprises, in addition to the core MAPK cascade, an upstream transmembrane receptor or sensor that detects specific extracellular stimuli, and downstream MAPK substrate molecules (effectors) both in the cytoplasm and in the nucleus. Several different MAPK pathways often co-exist within a cell. In yeast, for example, four MAPKs (Slt2/Mpk1, Kss1, Fus3, and Hog1) are expressed in a cell. If inappropriate crosstalk occurred between two MAPK cascades, a stimulus aimed at activation of only one of these cascades could incite irrelevant or even detrimental responses.

Hog1 is activated by hyperosmotic stress through the High Osmolarity Glycerol (HOG) pathway, and orchestrates osmoadaptive responses including intracellular accumulation of glycerol. The upstream portion of the HOG pathway is comprised of the functionally redundant SHO1 and SLN1 branches. In the SHO1 branch, either of the two related, but distinct, osmosensing complexes, Sho1/Opy2/Hkr1 and Sho1/Opy2/Msb2, activate the MAPKKK Ste11. In the SNL1 branch, the Sln1-Ypd1-Ssk1 phospho-

relay activates the functionally redundant MAPKKKs Ssk2 and Ssk22 (Ssk2/22). Both Ste11 and Ssk2/22 activate the MAPKK Pbs2, which then activates Hog1.

Fus3 is activated by the mating pheromones through Ste11 and the MAPKK Ste7. Although the mating pheromones activate Ste11, which is upstream of Hog1, they do not activate Hog1. Commonly, the lack of pheromone-to-Hog1 cross-talk is explained by the pathway insulation model, which posits that a scaffold protein holds several components of one pathway close together, so that signal flows only within that pathway. Indeed, the components of the Fus3 pathway (Gβγ, Ste11, Ste7, and Fus3) are scaffolded by Ste5 ((Elion, 2001), whereas the components of the Hog1 pathway (Sho1, Hkr1, Msb2, Ste11, Pbs2, and Hog1), are scaffolded by Sho1, Pbs2, Bem1, and Ahk1. To effectively prevent crosstalk, however, the scaffold proteins must hold kinases for significantly longer than the half-lives of their activities, which could be several minutes or longer. Because scaffold complexes are typically unstable, additional mechanisms other than scaffolding of signaling complexes are likely to be necessary to effectively prevent crosstalk.

In this study, we found that osmotic activation of Hog1 requires, in addition to the previously identified transmembrane osmosensors (Sho1/Opy2/Hkr1, Sho1/Opy2/Msb2 and Sln1), a fourth, cytoplasmic osmosensor OS4, which is actually Hog1 itself. The properties of a constitutively-active OS4 mutant suggest a two-step activation mechanism, in which a conformational change of Hog1 by osmostress is necessary to allow it to be activated/phosphorylated by Pbs2 that has been activated separately by upstream osmosensors. Thus, OS4 reduces noisy activation of Hog1, and especially prevents activation of Hog1 by mating factors, which also activate Ste 11. Furthermore, we found that the equivalent OS4 mutation confers constitutive activity to p38 MAPK, a mammalian homolog of Hog1. This result suggests the possibility that osmotic activation of p38 might require both osmotic stimulation of the upstream osmosensors and osmotic priming of p38 itself.

2. Interaction between the transmembrane domains of Sho1 and Opy2 enhances the signaling efficiency of the Hog1 MAP kinase cascade in *Saccharomyces cerevisiae*

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The HOG pathway employs multiple and redundant upstream osmosensing mechanisms that all lead to Hog1 activation. Specifically, upstream osmosensing signaling of the HOG pathway consists of the SLN1 branch and the SHO1 branch. The Ste 11 MAPKKK, an upstream activator of the Hog1 MAPK in the SHO1 branch, is activated by phosphorylation by the Ste20/Cla4 kinases when osmostress is applied. Overexpression of constitutively-active Ste11 mutants, such as Ste11-Q301P or Ste11-DDD, induces Hog1 activation even in the absence of osmostress. However, expressing these constitutively-active Ste11 mutant proteins at the endogenous level, *i.e.*, by using a single-copy plasmid that carries the *STE11* promoter, does not activate Hog1 in the absence of osmostress. One possible interpretation for this observation is that osmostress is still needed to activate Hog1 even when Ste11 is activated by a non-osmotic mechanism. It is possible, for example, that osmostress somehow enhances the signaling efficiency of the Ste11-Pbs2-Hog1 MAPK cascade.

In the SHO1 branch of the HOG pathway, a number of non-kinase proteins (Hkr1, Msb2, Sho1, Opy2, Ahk1, Bem1, and Ste50) are involved in activation and/or regulation of the Hog1 MAPK. The PAK-like kinase Ste20 is recruited to the membrane by the small G protein Cdc42 as well as by Hkr1 (probably through a hypothesized adaptor protein) or Msb2 through a Bem1 adaptor protein. Similarly, the MAPKK kinase (MAPKKK) Ste11 is recruited to the membrane by the Opy2-Ste50 complex. Ste50 is a cytoplasmic adaptor protein that binds both to Ste 11 and to the single-path membrane anchor protein Opy2. Finally, Pbs2 is also recruited to the membrane by Sho1. Thus, both the Ste20Ste11 reaction and the Ste11Pbs2 reaction take place on the membrane. One or both of these activation reactions are likely regulated by osmostress; however, no such mechanisms were known.

In this study, we isolated and analyzed hyperactive mutants of Sho1 and Opy2 that harbor mutations within their TM domains. Several hyperactive mutations enhanced the interaction between Sho1 and Opy2, indicating the importance of the TM-mediated interaction between Sho1 and Opy2 for facilitating effective signaling. The interaction between the TM domains of Sho1 and Opy2 will place their respective cytoplasmic binding partners Pbs2 and Ste11 in close proximity. Indeed, genetic analyses of the mutants showed that the Sho1-Opy2 interaction enhances the activation of Pbs2 by Ste11, but not Hog1 by Pbs2. Some of the hyperactive mutants had mutations at the extracellular ends of either Sho1 TM4 or Opy2 TM, and defined the Sho 1-Opy2 binding site 1 (BS1). Chemical crosslinking and mutational analyses revealed that the cytoplasmic ends of Sho1 TM1 and Opy2 TM also interact with each other, defining the Sho1-Opy2 binding site 2 (BS2). A geometric consideration constrains that one Opy2 molecule must interact with two adjacent Sho1 molecules in Sho1 oligomer. These re-

sults raise a possibility that an alteration of the conformation of the Sho1-Opy2 complex might contributes to the osmotic activation of the Hog1 MAPK cascade.

Publications

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