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 investigators to challenge new fields of bio-medical sciences.

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. Negative regulation of Pbs2 MAP2K by PP2C phosphatases in the yeast HOG pathway.

Kazuo Tatebayashi

The budding yeast *Saccharomyces cerevisiae* survive greatly fluctuating osmotic conditions in natural environment. To cope with an increased external osmolarity, yeast cells elicit a coordinated adaptive response. These adaptive responses are governed by the Hog1 MAP kinase (MAPK), which is activated via the High Osmolarity Glycerol (HOG) signaling pathway. The HOG pathway consists of a core module of three tiers of protein kinases termed a MAP kinase (MAPK), a MAPK kinase (MAPKK, MAP2K), and a MAPKK kinase (MAPKKK, MAP3K). In addition, the upstream part of the HOG pathway comprises the functionally redundant, but mechanistically distinct, SHO1 and SLN1 branches. When yeast cells are exposed to extracellular high osmolarity, the osmosensors in the SHO1 and SLN1 branches independently detect osmostress to activate cognate MAP3Ks. In the SHO1 branch, osmosensing complexes composed of Sho1, Opy2, Hkr1, and Msb2 activate the MAP3K Ste11. In the SLN1 branch, the Sln1-Ypd1-Ssk1 phospho-relay system activates the functionally redundant MAP3Ks Ssk2 and Ssk22 (Ssk2/22). Activated Ste11 and Ssk2/22 phosphorylate and activate the MAP2K Pbs2. Activated Pbs2, in turn, phosphorylates the MAPK Hog1 at T174 and Y176 in its activation loop for its activation.

Unregulated continuous activation of the HOG pathway is deleterious to cell growth, probably by preventing cell cycle progression. Therefore, a mechanism is needed that appropriately inactivates the HOG pathway. Two groups of the protein phosphatases are involved in the HOG pathway inactivation. The first group contains the members of the protein tyrosine phosphatases (PTP), namely, Ptp2 and Ptp3, which dephosphorylate Hog1 at Y176. The second group contains the members of the serine/threonine protein phosphatase type 2 (PP2C), namely, Ptc1, Ptc2, Ptc3, and Ptc4. Of these, Ptc1, Ptc2, and Ptc3 had been proposed as negative regulators of the HOG pathway, because their overexpression rescued the lethality of the *sln1*Δ cell by inhibiting the constitutive activation of the HOG pathway. Furthermore, overexpression of either Ptc1 or Ptc2 inactivated Hog1 *in vivo*, and purified Ptc1 and Ptc2 dephosphorylated T174 *in vitro*. In contrast, it was concluded that these phosphatases did not inhibit Pbs2 in vivo, because

overexpression of either Ptc1 or Ptc2 did not reduce Hog1 phosphorylation at Y176, which served as an indicator of the Pbs2 activity. Thus, it had been proposed that these type 2C phosphatases inactivate Hog1, but not Pbs2. The phosphatases that dephosphorylate Pbs2 had not been identified yet.

The results of overexpression experiments must be regarded cautiously, because at high level of phosphatase expression, non-physiological substrates might be sufficiently dephosphorylated. For that reason, we consider the gene inactivation experiments more reliable. Furthermore, estimating Pbs2 activity indirectly from the extent of Hog1 phosphorylation might be difficult, as the efficiencies of Pbs2 phosphorylating at Hog1 T174 and Y176 may be significantly different.

This year, we examined the effects of inactivating the genes of type 2C phosphatases upon the phosphorylation status of Pbs2 at the activating phosphorylation sites Ser-514 and Thr-518 (S514 and T518) by directly measuring the levels of Pbs2 phosphorylation at S514 and T518 using the assay method we developed recently. We found that S514 and T518 are differentially dephosphorylated by different type 2C serine/threonine phosphatases. Ptc1-Ptc4 collectively regulate Pbs2 negatively, but each Ptc acts differently to the two phosphorylation sites in Pbs2. T518 is predominantly dephosphorylated by Ptc1, whereas the effect of Ptc2-Ptc4 could be seen only when Ptc1 is absent. On the other hand, S514 is more evenly dephosphorylated by Ptc1, Ptc2, Ptc3, and Ptc4. We also show that Pbs2 dephosphorylation by Ptc1 requires the adaptor protein Nbp2 that recruits Ptc1 to Pbs2.

2. Osmostress enhances Pbs2 MAP2K phosphorylation by Ste11 MAP3K in the yeast osmo-regulatory HOG pathway

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The yeast MAPK Hog1 is activated by hyperosmotic stress through the High Osmolarity Glycerol (HOG) pathway, and orchestrates an array of osmo-adaptive changes in transcription, translation, cell cycle, and metabolism. The current widely held model of the HOG pathway is as follows. The upstream portion of the HOG pathway is comprised of the functionally redundant SHO1 and SLN1 branches. In the SHO1 branch, osmosensing complexes composed of Sho1, Opy2, Hkr1, and Msb2 activate the MAP3K Ste11. In the SLN1 branch, the Sln1-Ypd1-Ssk1 phospho-relay mechanism is involved in activation of the functionally redundant MAP3Ks Ssk2 and Ssk22 (Ssk2/22). Ste11 and Ssk2/22 phosphorylate the MAP2K Pbs2 at Ser-514 and/or Thr-518. Phosphorylated Pbs2 then activates Hog1. Recently, we found that osmostress not only activates membrane-associated osmosensors, but also enhances Hog1 phosphorylation by mono-phosphorylated Pbs2. The lack of the osmotic enhancement of the Pbs2-Hog1 reaction suppresses Hog1 activation by basal MAP3K activities and prevents pheromone-to-Hog1 crosstalk in the absence of osmostress, which ensures the appropriate Hog1 activation only under high osmolarity. It has been unclear if osmostress acts on other phosphorylation steps in the MAP kinase cascade of the HOG pathway.

This year, we examined if osmostress enhances Pbs2 MAP2K phosphorylation by Ste11 MAP3K as well as Hog1 phosphorylation. Ste11 is phosphorylated and activated by the Ste20/Cla4 kinases upon high osmolarity. The constitutively-active Ste11-DDD mutant carries the substitution mutations of all activating phosphorylation sites for Ste20/Cla4 to phosphomimic Asp (S281D, S285D, and T286D), which circumvent the need for Ste20/Cla4 for Ste11 activation. The endogenous-level expression of Ste11-DDD did not induce the phosphorylation of Pbs2 under unstimulated condition, but induced it upon high osmolarity. These results strongly suggest that Pbs2 phosphorylation by activated Ste11 is osmotically-enhanced as Hog1 is. The underlying mechanism is under study.

Publication

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