Department of Basic Medical Sciences

Division of Molecular Cell Signaling
分子細胞情報分野

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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 both mammalian and yeast cells.

1. Yeast osmosensors Hkr1 and Msb2 activate the Hog1 MAPK cascade by different mechanisms

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Signaling by the conserved mitogen-activated protein kinase (MAPK) family is a major cellular mechanism through which eukaryotic cells respond to various extracellular stimuli. All MAPKs are activated through a three-tier kinase cascade, composed of a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Distinct MAPKKKs activated by a specific stimulus phosphorylate and thus activate a cognate MAPKK, which then phosphorylates and activates a downstream MAPK. Activated MAPKs regulate pertinent adaptive responses, such as gene expression, cell cycle progression, and apoptosis. There are several subfamilies of MAPKs both in higher and lower eukaryotes.

In response to environmental high osmolarity, the budding yeast *Saccharomyces cerevisiae* induces an array of adaptive responses, including the synthesis and accumulation of the osmolyte glycerol, changes in the global pattern of gene expression, and a temporary arrest of cell cycle progression. These responses are all controlled by the MAPK Hog1, which is activated by the High Osmolarity Glycerol (HOG) signaling pathway. Upstream in the HOG pathway are two independent signaling branches called, respectively, the Sln1 branch and the Sho1 branch. The osmosensor for the Sln1 branch is the sensor histidine kinase Sln1, which transmits the signal through a two-component phosphorelay mechanism to the MAPKKKs Ssk2 and Ssk22 (collectively referred to as Ssk2/22). In contrast, the Sho1 branch involves two functionally redundant osmosensors, Hkr1 and Msb2, which are transmembrane mucin-like glycoproteins with no known enzymatic activity. It is intriguing, therefore, that yeast has three osmosensors, when any one is sufficient to respond and adapt to the external high osmolarity. There is evidence that the stimulus recognized by the Sln1 branch is qualitatively different from that recognized by the Sho1 branch. For example, the Sln1 branch responds to changes in tur-

gor pressure, whereas the Sho1 branch does not. However, it is unclear whether there is any functional difference between Hkr1 and Msb2.

The extracellular domains of Hkr1 and Msb2 are structurally similar to each other in that both contain a long (> 700 aa) Ser/Thr-rich (STR) region, which is highly O-glycosylated, and a ~200 aa-long region termed the Hkr1-Msb2 homology (HMH) domain. For both osmosensors, an inhibitory role of the STR region and an activating role of the HMH domain are suggested by the phenotypes of mutants. In contrast, the cytoplasmic regions of Hkr1 and Msb2 share no structural similarity to each other, even though they both activate the Ste11-Pbs2-Hog1 MAPK cascade. Either of the p21-activated kinase (PAK)-family kinases Ste20 and Cla4 (collectively referred to as Ste20/Cla4) can activate the MAPKKK Ste11. Ste11 localizes to the membrane through an adaptor protein Ste50 that binds both Ste11 and the transmembrane protein Opy2; whereas Pbs2 localizes to the membrane by binding to the SH3 domain of the tetraspanning (i.e., with four TM domains) membrane protein Sho1. Their membrane localization is essential for an efficient activation of Pbs2 by Ste11.

This year, we found that the signaling mechanism by which Hkr1 or Msb2 stimulated the Hog1 cascade was specific to each osmosensor. In particular, activation of Hog1 by Msb2 required the scaffold protein Bem1 and the actin cytoskeleton. Bem1 is involved in cell polarity establishment and bud site selection, and interacts with many proteins, including Ste20, Cla4, the guanosine triphosphatase (GTPase) Cdc42, Cdc24 [a guanine nucleotide exchange factor (GEF) for Cdc42], and the actin cytoskeleton. We found that Bem1 bound to the cytoplasmic domain of Msb2 and thus recruited the kinases Ste20 and Cla4 to the membrane where either of them can activate the kinase Ste11. The cytoplasmic domain of Hkr1 also contributed to the activation of Ste11 by Ste20, but through a mechanism that involved neither Bem1 nor the actin cytoskeleton. Furthermore, we found a PXXP motif in Ste20 that specifically bound to the Sho1 SH3 domain. This interaction between Ste20 and Sho1 contributed to the activation of Hog1 by Hkr1, but not by Msb2. These differences between Hkr1 and Msb2 may enable differential regulation of these two proteins and provide a mechanism through Msb2 to integrate the signals from the external osmotic conditions with those from the internal cytoskeletal conditions.

Publications

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2. Tanaka K, Tatebayashi K, Nishimura A, Yamamoto K, Yang HY, and Saito H. (2014) Yeast osmosensors Hkr1 and Msb2 activate the Hog1 MAPK cascade by different mechanisms. *Science Signal.*, 7: ra21.

Department of Basic Medical Sciences

Division of Neuronal Network
神経ネットワーク分野

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Our major research interest is the molecular mechanisms of higher brain functions in mammals such as emotion, and learning and memory. We are especially focusing on the roles of functional molecules localized in synapses, for instance, neurotransmitter receptors, signal transduction molecules and adhesion molecules, in neuronal information processing. We are examining receptor functions, synaptic transmission and plasticity, and their roles in the whole animal with electrophysiological, biochemical, molecular genetic and behavioral approaches.

1. Point mutation in syntaxin-1A causes abnormal vesicle recycling, behaviors, and short-term plasticity

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Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is an important modulator of neural plasticity. CaMKII is a major protein component not only of postsynaptic densities but also of presynaptic terminals; therefore, CaMKII is likely to mediate presynaptic plasticity via regulation of the exocytotic machinery. Previously, we found that autophosphorylated CaMKII interacts with syntaxin-1A to regulate exocytosis and that a syntaxin missense mutation [R151G] severely attenuated exocytosis. To more precisely analyze the physiological importance of this interaction, we generated mice with a knock-in (KI) syntaxin-1A [R151G] mutation. These KI mice exhibited abnormal presynaptic short-term plasticity in electrophysiological examinations. Biochemically, these mice exhibited reduced recruitment of complexin, which is known to modulate exocytosis, to the SNARE complex via the CaMKII-syntaxin interaction. These results indicate that presynaptic CaMKII plays an important role in the expression of short-term plasticity through the interaction with syntaxin-1A, which regulates the exocytotic mechanisms directly.

2. LMTK3 deficiency causes pronounced locomotor hyperactivity and impairs endocytic trafficking

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LMTK3 belongs to the LMTK family of protein kinases that are predominantly expressed in the brain. Physiological functions of LMTK3 and other members of the LMTK family in the central nervous system remain unknown. In this study, we performed a battery of behavioral analyses using *Lmtk3*$^{-/-}$ mice and showed that these mice exhibited abnormal behaviors, including pronounced locomotor hyperactivity, reduced anxiety behavior, and decreased depression-like behavior. Concurrently, the dopamine metabolite levels and dopamine turnover rate were increased in the striata of *Lmtk3*$^{-/-}$ mice compared with wild-type controls. In addition, using cultured primary neurons from *Lmtk3*$^{-/-}$ mice, we found that LMTK3 was involved in the endocytic trafficking of *N*-methyl-D-aspartate receptors, a type of ionotropic glutamate receptor. Altered membrane traffic of the receptor in *Lmtk3*$^{-/-}$ neurons may underlie behavioral abnormalities in the mutant animals. Taken together, our data suggest that LMTK3 plays an important role in regulating locomotor behavior in mice.

3. The glutamate receptor GluN2 subunit regulates synaptic trafficking of AMPA receptors in the neonatal mouse brain

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The N-methyl-D-aspartate receptor (NMDAR) plays various physiological and pathological roles in neural development, synaptic plasticity and neuronal cell death. It is composed of two GluN1 and two GluN2 subunits, and in the neonatal hippocampus, most synaptic NMDARs are GluN2B-containing receptors, which are gradually replaced with GluN2A-containing receptors during development. Here, we examined whether GluN2A could be substituted for GluN2B in neural development and functions by analyzing knock-in (KI) mice in which GluN2B is replaced with GluN2A. The KI mutation was neonatally lethal, although GluN2A-containing receptors were transported to the postsynaptic membrane even without GluN2B and functional at synapses of acute hippocampal slices of postnatal day 0 (P0), indicating that GluN2A-containing NMDARs could not be substituted for GluN2B-containing NMDARs. Importantly, the synaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) subunit GluA1 was increased, and the transmembrane AMPAR regulatory protein (TARP) and synaptic Ras-GTPase activating protein (SynGAP), which are both involved in AMPAR synaptic trafficking, were increased and decreased, respectively, in KI mice, whereas calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) was not involved in the increase of GluA1. Although the regulation of AMPARs by GluN2B has been reported in cultured neurons, we showed here that AMPAR-mediated synaptic responses were increased in acute KI slices, suggesting differential roles of GluN2A and GluN2B in AMPAR expression and trafficking *in vivo*. Taken together, our results suggest that GluN2B is essential for the survival of animals and that the GluN2B-GluN2A switching plays a critical role in synaptic integration of AMPARs through regulation of GluA1 in the whole animal.

Publications

Watanabe, Y., Katayama, N., Takeuchi, K., Togano, T., Itoh, R., Sato, M., Yamazaki, M., Abe, M., Sato, T., Oda, K., Yokoyama, M., Takao, K., Fukaya, M., Miyakawa, T., Watanabe, M., Sakimura, K., Manabe, T. and Igarashi, M. Point mutation in syntaxin-1A causes abnormal vesicle recycling, behaviors, and short-term plasticity. *J. Biol. Chem.* 288: 34906-34919, 2013.

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第２編　神経と筋　第６章　pp. 154－166,

Department of Basic Medical Sciences

Division of Cell Signaling and Molecular Medicine
分子シグナル制御分野

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The aims of the ongoing research projects in our laboratory are to elucidate the regulatory mechanisms of intracellular signal transduction systems responsible for cell-fate decisions, such as MAP kinase cascades and Stress granules. Perturbation of these signaling systems is involved in a variety of life-threatening diseases, including cancer, autoimmune diseases, neurodegenerative disorders and type 2 diabetes. Our laboratory also aims to develop new diagnostic or therapeutic tools for currently intractable disorders in which these pathways are involved.

1. MCRIP1, a novel ERK substrate, mediates ERK-induced gene silencing during epithelial-to-mesenchymal transition.

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Epithelial-to-mesenchymal transition (EMT) is a critical biological process during embryonic development, tissue repair, and tissue fibrosis, in which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced cell-cell contact and increased motility. More importantly, EMT is considered to be a major mechanism for the induction of tumor invasion and metastasis. Therefore, comprehensive understanding of the molecular basis of EMT is crucial for the development of novel therapeutic interventions for human cancer. Although transforming growth factor β (TGF-β) signaling is a prominent mediator of EMT, several lines of evidence have revealed that various other signaling pathways also play a role in this process. In particular, it has recently been reported that hyper-activation of the ERK pathway, by constitutively active Ras or by over-expression of ERK2, is sufficient to induce EMT in many, if not all, types of cells. Besides ERK signaling, C-terminal binding protein (CtBP), which is a core component of the transcriptional co-repressor complex that contains histone modifying enzymes (e.g., histone deacetylases, and methyltransferases), is also involved in epigenetic gene silencing of E-cadherin during EMT. However, the functional relationship, if any, between the ERK pathway and CtBP remains obscure.

By developing a novel technique to isolate ERK substrate proteins, we identified a previously uncharacterized protein of unknown function, designated MCRIP1, as a novel and specific substrate of ERK. MCRIP1 is conserved in all vertebrates from fish to man, and is expressed in all tissues examined. Interestingly, we found that MCRIP1 directly binds to CtBP, thereby inhibiting the formation of the CtBP-containing transcriptional co-repressor complex. However, when cells are stimulated with growth factors or mitogens, activated ERK efficiently phosphorylates MCRIP1 and induces dissociation of MCRIP1 from CtBP, allowing CtBP to form the co-repressor complex. As a result, the CtBP complex, which contains chromatin-remodeling enzymes, is recruited to, and silences the E-cadherin promoter by regulating post-translational modifications of histone proteins. Overexpression of

an MCRIP1 mutant that constitutively binds to CtBP profoundly inhibited ERK-induced epithelial-to-mesenchymal transition. Our findings demonstrated that MCRIP1 mediates the functional interaction between ERK signaling and CtBP during EMT, and delineated a molecular mechanism by which ERK signaling induces epigenetic silencing of specific genes.

2. SAPK pathways and p53 cooperatively regulate PLK4 activity and centrosome integrity under stress

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Centrosomes, which consist of a pair of centrioles surrounded by an amorphous pericentriolar material, serve as the microtubule-organizing centers that are essential for the formation of mitotic spindles in animal cells. In order for cells to undergo normal bipolar cell division, the single interphase centrosome must duplicate precisely once before mitosis. The rigorous control of centrosome numbers directs accurate chromosome segregation at cell division and is thus crucial for maintenance of the stability of genomes. The presence of more than two centrosomes (centrosome amplification) leads to the formation of multipolar mitotic spindles and consequentially to chromosome segregation errors. Centrosome amplification significantly increases the frequency of lagging chromosomes during anaphase, thereby promoting chromosome missegregation. Since chromosome missegregation results in both numerical and structural abnormalities of chromosomes, ablation of the numeral integrity of centrosomes induces chromosomal instability, and thus is considered to be a major cause of cancer development and progression. Indeed, centrosome amplification and the resulting chromosomal instability are common features of various cancers, and correlate with poor clinical outcomes. Interestingly, centrosome number is often increased in cancer cells after various stress stimuli, although the mechanism by which centrosome amplification arises after stress in cancer cells remains obscure.

Polo-like kinase 4 (PLK4), a divergent member of the polo-like kinase family, is an evolutionarily conserved key regulator of centrosome duplication. PLK4 localizes to centrosomes and is essential for centriole biogenesis. Depletion of PLK4 in cells leads to centrosome duplication arrest, while overexpression of PLK4 induces centrosome amplification by production of multiple procentrioles. Previous reports have demonstrated that dysregulation (both hyperactivation and deactivation) of PLK4 predisposes cells to the development of cancer. PLK4 is expressed aberrantly (either over- or under-expression) in human colorectal and liver cancers. Therefore, PLK4 activity must be adequately controlled to maintain centrosome integrity and to prevent carcinogenesis. Regulatory mechanisms of PLK4 activity, however, remain to be elucidated. In particular, although PLK4 has been suggested to be involved in cellular stress responses, it is unclear if PLK4 activity is regulated after stress, which frequently provokes centrosome amplification in cancer cells.

Stress-activated p38 and JNK MAPK (SAPK) signaling pathways are composed of three tiers of protein kinases, namely MAPKKK, MAPKK, and MAPK. This year, we demonstrated a direct functional link between the SAPK pathways and PLK4. We found that, upon stress stimuli, stress-responsive MAPKKKs such as MTK1 and TAK1 directly phosphorylated and activated PLK4, a master regulator of centrosome duplication. Stress-induced, MAPKKK-mediated, PLK4 activation provides survival signaling and promoted centrosome duplication. At the same time, however, the p38 and JNK SAPKs and the tumor suppressor p53 protein, which are also activated by various stress stimuli, cooperated to counteract PLK4 activity, thereby preventing centrosome amplification. Importantly, we demonstrated that simultaneous inactivation of SAPKs and p53 in cells exposed to stress allowed unchecked activation of PLK4, leading to centrosome overduplication and chromosomal instability, both of which are hallmarks of cancer cells. This cooperation between SAPKs and p53 explains why both p53 and the MKK4 MAPKK (a SAPK activator) are frequently mutated simultaneously in human cancer cells, in which centrosome number is often increased after stress.

Mutational inactivation of the MKK4 MAPKK has been shown to drive carcinogenesis (known as "driver" mutations) and is indeed frequently observed in a variety of human cancers. However, the mechanism by which MKK4 prevents carcinogenesis remains to be elucidated. Our results provide the first evidence that centrosome integrity is synergistically regulated by the two important tumor suppressors, MKK4 and p53, and reveal a molecular mechanism that underlies centrosome amplification in cancer cells. Based on these findings, we proposed that MKK4 is a novel type of tumor suppressor whose function is manifested particularly when p53 is also inactivated.

3. Biochemical analyses of MEK mutants associated with Ras/MAPK syndromes and sporadic cancers, and their implications for resistance to anti-cancer reagents

Yuji Kubota, Seina Oe, and Mutsuhiro Takekawa

The RAS-ERK MAPK signaling pathway is a key

transducer of mitogenic signals and is frequently hyper-activated by various oncogenes, such as Ras and Raf, in human cancers. Recently, more than 20 different mutations in the MEK1/2 genes have been identified in sporadic cancers (e.g., melanoma, lung and colon cancers) and in congenital Ras-MAPK syndromes (i.e., Noonan, Costello, Cardio-facio-cutaneous, and LEOPARD syndromes). These Ras-MAPK syndromes are genetic disorders that include symptoms such as mental retardation, facial dysmorphisms, heart defects, developmental delay, and an increased risk of developing cancer. MEK1 mutations have also been identified in melanoma cells that are resistant to a B-Raf inhibitor PLX4032. However, the precise effects of MEK mutations on MEK enzymatic activity and on carcinogenesis are still ill-defined.

We investigated the biochemical properties of such MEK mutants, and identified that such mutations rendered MEK constitutively active by perturbing post-transcriptional modifications (phosphorylation and sumoylation) of MEK. The MEK mutants can be classified into two groups based on their activities: 1) hyper-active, oncogenic mutants, and 2) moderately active, non-oncogenic mutants. We found that these two classes of MEK mutations lead to distinct gene expression profiles by differentially altering spatio-temporal patterns of ERK signaling, thereby generating different clinical manifestations. We also found that cancer cells harboring such MEK mutants are resistant not only to the B-Raf inhibitor but also to several MEK-specific inhibitors. These findings alert us to the need for the development of novel drugs that can inhibit hyper-active MEK mutants in cancer cells.

4. Phosphorylation of MAPKKs by activated MAPKs and its relationship to tumor development

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MAPK cascades are critical signaling modules that regulate a wide array of biological phenomena in eukaryotes. At least four distinct MAPK pathways (ERK, p38, JNK, and ERK5) co-exist in mammalian cells. Of these pathways, the ERK pathway is activated by mitogens and is associated with proliferative responses, whereas the p38 and JNK pathways are activated by environmental stresses and contribute to cell growth arrest and/or apoptosis. Several lines of evidence revealed that the activity and the fidelity of MAPK signaling are regulated mainly by post-translational modifications and by docking interactions between the component kinases. Regarding the docking interactions, we have shown that mammalian MAPKKs bind their upstream MAPKKKs through the DVD site, a docking site of about 20 amino acids that is located at their C termini, thereby ensuring the fidelity and efficiency of MAPK signaling.

We identified a novel regulatory mechanism for regulation of the ERK pathways through feedback phosphorylation of MEK by its downstream kinase ERK. A Thr residue adjacent to the MEK DVD docking sites is phosphorylated by ERK upon growth factor stimulation. Phosphorylation at this site by a constitutively active ERK strongly inhibits MEK activation by mitogens, such as EGF and TPA. In contrast, inhibition of phosphorylation of this residue, either by mutation or by the chemical inhibitor U0126, induces prolonged MEK activation. In MEF cells expressing a MEK1 mutant that lacks this feedback phosphorylation site, mitogen-stimulated activation of ERK is greatly prolonged and proliferation of the cells is enhanced. Furthermore, knock-in mice that express the MEK mutant are highly susceptible to skin tumor development induced by TPA. Interestingly, the MEK DVD sites can be also phosphorylated by SAPK in response to stress stimuli, resulting in inhibition of MEK activation. Therefore, feedback phosphorylation by ERK or crosstalk phosphorylation by SAPK of MEK1 negatively regulates the ERK pathway, thereby inhibiting cell proliferation and tumorigenesis.

Publications

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