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. The temporal pattern of external stimulation determines the extent and duration of MAPK activation in a *C. elegans* sensory neuron

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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 in higher eukaryotes, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Of these MAPKs, the ERK MAPK family is prominently involved in the control of cell growth, including cellular responses to various growth factors. However, the ERK MAPK family also has other functions, such as a role in differentiation and the regulation of long-term potentiation and memory in the nervous system.

Both the intensity and the duration of MAPK activity are factors that determine the outcome of a specific stimulation. For example, treatment of the neuroendocrine cell line PC12 with epidermal growth factor (EGF) transiently activates ERK and induces cell proliferation, whereas treatment with nerve growth factor (NGF) causes persistent activation of ERK and differentiation into neuron-like cells. Thus, the kinetics of MAPK activation is as important as the peak extent of MAPK activity in determining cellular responses to a given stimulus.

Mechanisms of MAPK activation and regulation have been elucidated in such detail that it is becoming possible to computationally predict the dynamics of a MAPK signaling pathway. However, it is still difficult to actually monitor the dynamics of MAPK activation in single cells in a living organ-

ism. The conventional methods used to detect MAPK activity, such as immunostaining of fixed cells or immunoblotting of cell extracts using antibodies specific for phosphorylated MAPK can show only static snapshots or population averages of MAPK activation. Furthermore, most experimental analyses of MAPK pathways have been limited to measurement of responses to continuous or stepwise stimulation. A more sophisticated approach to measurement of MAPK pathway activation is a control-systems-engineering type of analysis that consists of application of a set of defined oscillatory inputs followed by measurement of output responses, from which system properties are deduced. Pioneering applications of this approach to analysis of the osmoregulatory Hog1 MAPK pathway in yeast have been reported. These analyses were made possible by the very rapid nuclear translocation of Hog1 that followed its activation. However, there have been few, if any, applications of similar strategies to monitor MAPK pathway activation in higher eukaryotes because of the difficulty of rapidly changing the effective concentration of a stimulant around a cell.

To overcome these limitations, we developed a fluorescence resonance energy transfer (FRET)-based probe specific for ERK activity and optimized for use in living animals, such as the transparent nematode *Caenorhabditis elegans*. We expressed the probe in the ASER neuron, which responds to changes in NaCl concentrations and then monitored activity of the MAPK pathway as the intensity and duration of the ERK FRET probe. Sensory neurons of this nematode are ideal model cells for systems analysis, because they can naturally respond to rapidly changing external stimuli and, the ASER neuron is particularly useful because the NaCl concentration in the environment can be changed rapidly.

Our data demonstrated that temporal stimulation patterns, rather than merely duration of stimulation or stimulation frequencies determine the intensity and the duration of MPK-1 activity in the nematode ASER neuron. In particular, we found that both constant environmental stimulation and a very rapidly alternating stimulation and resting cycles only transiently activated MPK-1. In contrast, cyclic stimulation at moderate frequency, with a sufficiently long inter-stimulation resting period, led to robust and persistent MPK-1 activation. Because the ERK-family MAPKs are involved in long-term potentiation and memory, both the intensity and the duration of MAPK activation are likely to affect the behavior of *C. elegans*. It is possible that, in interpreting environmental changes, a neuron ignores an unchanging environment, as well as noisy changes in the environment, and remembers only behaviorally relevant environmental changes.

We also found that complex dynamics of MPK-1 activity can be explained by the activation properties of the stimulus-induced Ca^{2+} transient that is dependent on the TAX-4 Ca^{2+} channel. In conclusion, a systems-engineering approach combined with in vivo imaging in intact animals provided insight into the complexity of MAPK dynamics under environmental fluctuations, as well as the signaling mechanism that underlies such complexity.

Publications

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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. Functional coupling of the metabotropic glutamate receptor, inositol triphosphate receptor and L-type Ca^{2+} channel in mouse CA1 pyramidal cells

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Activity-dependent regulation of calcium dynamics in neuronal cells can play significant roles in the modulation of many cellular processes such as intracellular signaling, neuronal activity and synaptic plasticity. Among many calcium influx pathways into neurons, the voltage-dependent calcium channel (VDCC) is the major source of calcium influx, but its modulation by synaptic activity has still been under debate. While the metabotropic glutamate receptor (mGluR) is supposed to modulate L-type VDCCs (L-VDCCs), its reported actions include both facilitation and suppression, probably reflecting the uncertainty of both the molecular targets of the mGluR agonists and the source of the recorded calcium signal in previous reports. In this study, using subtype-specific knockout mice, we have shown that mGluR5 induces facilitation of the depolarization-evoked calcium current. This facilitation was not accompanied by the change in single-channel properties of the VDCC itself; instead, it required the activation of calcium-induced calcium release (CICR) that was triggered by VDCC opening, suggesting that the opening of CICR-coupled cation channels was essential for the facilitation. This facilitation was blocked or reduced by the inhibitors of both L-VDCCs and inositol triphosphate receptors (IP_3Rs). Furthermore, L-VDCCs and mGluR5 were shown to form a complex by coimmunoprecipitation, suggesting that the specific functional coupling between mGluR5, IP_3Rs and L-VDCCs played a pivotal role in the calcium-current facilitation. Finally, we showed that mGluR5 enhanced VDCC-dependent long-term potentiation of synaptic transmission. Our study has identified a novel mechanism of the interaction between the mGluR and calcium signaling, and suggested contribution of mGluR5 to synaptic plasticity.

2. Dysfunction of the RAR/RXR signaling pathway in the forebrain impairs hippocampal memory and synaptic plasticity

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Retinoid signaling pathways mediated by retinoic acid receptor (RAR)/retinoid X receptor (RXR)-mediated transcription play critical roles in hippocampal synaptic plasticity. Furthermore, recent studies have shown that treatment with retinoic acid alleviates age-related deficits in hippocampal long-term potentiation (LTP) and memory performance and, furthermore, memory deficits in a transgenic mouse model of Alzheimer's disease. However, the roles of the RAR/RXR signaling pathway in learning and memory at the behavioral level have still not been well characterized in the adult brain. We here show essential roles for RAR/RXR in hippocampus-dependent learning and memory. In the current study, we generated transgenic mice in which the expression of dominant-negative RAR (dnRAR) could be induced in the mature brain using a tetracycline-dependent transcription factor and examined the effects of RAR/RXR loss. The expression of dnRAR in the forebrain down-regulated the expression of RARβ, a target gene of RAR/RXR, indicating that dnRAR mice exhibit dysfunction of the RAR/RXR signaling pathway. Similar with previous findings, dnRAR mice displayed impaired LTP and AMPA receptor-mediated synaptic transmission in the hippocampus. More importantly, these mutant mice displayed impaired hippocampus-dependent social recognition and spatial memory. However, these deficits of LTP and memory performance were rescued by stronger conditioning stimulation and spaced training, respectively. Finally, we found that pharmacological blockade of RARα in the hippocampus impairs social recognition memory. From these observations, we concluded that the RAR/RXR signaling pathway greatly contributes to learning and memory, and LTP in the hippocampus in the adult brain.

3. NMDAR2B tyrosine phosphorylation is involved in thermal nociception

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Previous studies found that the NMDA receptor-mediated signaling regulates thermal nociception, though the underlying molecular mechanism remains unclear. The GluN2B subunit of the NMDA receptor is tyrosine-phosphorylated, Tyr-1472 being the major phosphorylation site. In this study, we have found that homozygous knock-in mice that express a Tyr-1472-Phe mutant of GluN2B display defects in the nociceptive response in the hot plate test. Expression of the neurotensin receptor subtype 2 (NTSR2), which is relevant to the regulation of thermal nociception, is decreased in the amygdala of GluN2B Tyr-1472-Phe knock-in mice. In addition, NTSR2-mediated c-fos induction is impaired in the amygdala of these mice. These data suggest that Tyr-1472 phosphorylation on GluN2B is involved in thermal nociception through regulating the NTSR2 mRNA expression in the amygdala.

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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. Centrosome integrity under stress is maintained by a network of PLK4, p53 and SAPK pathways

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Centrosomes serve as microtubule-organizing centers that are a prerequisite 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 by causing erroneous, merotelic, kinetochore-microtubule attachment, thereby promoting chromosome missegregation. Since chromosome missegregation results in both numerical (aneuploidy) and structural (translocations) 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.

2. Identification of a novel ERK substrate that mediates ERK-induced gene silencing during epithelial-to-mesenchymal transition.

Kenji Ichikawa, Yuji Kubota, Taichiro Tomida[1], Takanori Nakamura, Jane Weng, Haruo Saito[1] and Mutsuhiro Takekawa: [1]Division of Molecular Cell Signaling, IMSUT.

The ERK pathway not only upregulates growth-related genes such as Jun, Fos, and cyclin D, but also down-regulates several anti-proliferative and tumor suppressive genes, such as E-cadherin, in various cellular processes including the epithelial-to-mesenchymal transition. Epithelial-to-mesenchymal transition 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. Importantly, epithelial-to-mesenchymal transition is considered to be a major mechanism for the induction of tumor invasion and metastasis. Therefore, comprehensive understanding of the molecular basis of epithelial-to-mesenchymal transition 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 epithelial-to-mesenchymal transition, accumulating evidence has revealed that various other signaling pathways also play a role in this process. In particular, it has recently been reported that hyperactivation of the ERK pathway, by constitutively active Ras or by over-expression of ERK2, is sufficient to induce epithelial-to-mesenchymal transition 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 epithelial-to-mesenchymal transition. However, the functional relationship, if any, between the ERK pathway and CtBP remains obscure.

This year, we developed a novel cloning strategy for the screening of human cDNA expression libraries for ERK substrate proteins using *Saccharomyces cerevisiae*. Using this screening system, 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 epithelial-to-mesenchymal transition, and delineated a molecular mechanism by which ERK signaling induces epigenetic silencing of specific genes.

3. Feedback phosphorylation of MAPKKs by MAPKs and its relationship to tumor development

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Both post-translational modifications and protein-protein interactions are keys for understanding the regulation of the dynamics of intracellular signaling and the resulting cellular outcomes. MAPK cascades are pivotal signaling modules that control a wide range of cellular functions in eukaryotes. Four 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. In contrast, the p38 and JNK pathways are activated by environmental stresses and contribute to cell growth arrest and/or apoptosis. Previous studies have extensively documented that the activity and the signaling fidelity of MAPK cascades are regulated mainly by protein phosphorylation and by docking interactions between the component kinases. Regarding the docking interactions, we have recently showed that mammalian MAPKKs bind their upstream MAPKKKs through the DVD site, a docking site of about 24 amino acids that is located at their C termini.

This year, we identified a novel mechanism for regulation of the ERK pathway 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 MEK negatively regulates the ERK pathway, thereby inhibiting cell proliferation and tumorigenesis.

4. Molecular basis of constitutively active MEK mutants in congenital Ras/MAPK syndromes and sporadic cancers

Yuji Kubota, Seina Oe, and Mutsuhiro Takekawa

The RAS-ERK MAPK cascade (Raf-MEK-ERK) is activated by mitogenic stimuli and is generally associated with cell proliferation and malignant transformation. This pathway is frequently hyper-activated by various oncogenes such as Ras, and Raf in human cancers. We previously reported that the MEK MAPKKs are modified with Sumo1 in cells. MEK sumoylation blocks ERK activation by disrupting the specific docking interaction between MEK and ERK. Expression of an unsumoylatable MEK mutant enhances ERK activation, cell proliferation, and malignant transformation. Interestingly, MEK sumoylation is abrogated in cancer cells that harbor Ras mutations. We found that oncogenic Ras inhibits MEK sumoylation by impairing the MEK-specific Sumo E3 ligase activity of MEKK1. Furthermore, forced enhancement of MEK sumoylation suppresses Ras-induced cell transformation. Thus, dysregulation of post-translational modifications (such as phosphorylation and sumoylation) of ERK signaling components contributes to carcinogenesis.

Recently, more than 20 different mutations in the MEK1/2 genes have been identified in sporadic cancers and the cancer-prone congenital syndromes (the Ras-MAPK syndromes). The 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. This year, we investigated the biochemical properties of such MEK mutants, and identified that such mutations perturbed post-transcriptional modifications (phosphorylation and sumoylation) of MEK, thereby rendering MEK mutants constitutively active. 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 hyperactive MEK mutants in cancer cells.

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