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. We are also trying to elucidate fundamental aspects of psychiatrical and neurological disorders using model animals.

1. SNAP-25 phosphorylation at Ser187 regulates synaptic facilitation and short-term plasticity in an age-dependent manner

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Neurotransmitter release is mediated by the soluble NSF attachment protein receptor (SNARE) complex, but the role of its phosphorylation has scarcely been elucidated. Although protein kinase C (PKC) activators are known to facilitate synaptic transmission, there has been a heated debate on whether PKC mediates facilitation of neurotransmitter release through phosphorylation. One of the SNARE proteins, SNAP-25, is phosphorylated at the residue serine-187 by PKC, but its physiological significance has been unclear. To examine these issues, we analyzed mutant mice lacking the phosphorylation of SNAP-25 serine-187 and found that they exhibited reduced neurotransmitter release probability and enhanced presynaptic short-term plasticity, suggesting that not only the release process, but also the dynamics of synaptic vesicles was regulated by the phosphorylation. Furthermore, it has been known that the release probability changes with development, but the precise mechanism has been unclear, and we found that developmental changes in release probability of neurotransmitters were regulated by the phosphorylation. These results indicate that SNAP-25 phosphorylation developmentally facilitates neurotransmitter release but strongly inhibits presynaptic short-term plasticity via modification of the dynamics of synaptic vesicles in presynaptic terminals.

2. CDKL5 controls postsynaptic localization of GluN2B-containing NMDA receptors in the hippocampus and regulates seizure susceptibility

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Mutations in the *Cyclin-dependent kinase-like 5 (CDKL5)* gene cause severe neurodevelopmental disorders accompanied by intractable epilepsies, i.e. West syndrome or atypical Rett syndrome. Here, we report generation of the *Cdkl5* knockout mouse and show that CDKL5 controls postsynaptic localization of GluN2B-containing N-methyl-D-aspartate (NMDA) receptors in the hippocampus and regulates seizure susceptibility. *Cdkl5* -/Y mice showed normal sensitivity to kainic acid; however, they displayed significant hyperexcitability to NMDA. In concordance with this result, electrophysiological analysis in the hippocampal CA1 region disclosed an increased ratio of NMDA/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitatory postsynaptic currents (EPSCs) and a significantly larger decay time constant of NMDA receptor-mediated EPSCs (NMDA-EPSCs) as well as a stronger inhibition of the NMDA-EPSCs by the GluN2B-selective antagonist ifenprodil in *Cdkl5* -/Y mice. Subcellular fractionation of the hippocampus from *Cdkl5* -/Y mice revealed a significant increase of GluN2B and SAP102 in the PSD (postsynaptic density)-1T fraction, without changes in the S1 (post-nuclear) fraction or mRNA transcripts, indicating an intracellular distribution shift of these proteins to the PSD. Immunoelectron microscopic analysis of the hippocampal CA1 region further confirmed postsynaptic overaccumulation of GluN2B and SAP102 in *Cdkl5* -/Y mice. Furthermore, ifenprodil abrogated the NMDA-induced hyperexcitability in *Cdkl5* -/Y mice, suggesting that upregulation of GluN2B accounts for the enhanced seizure susceptibility. These data indicate that CDKL5 plays an important role in controlling postsynaptic localization of the GluN2B-SAP102 complex in the hippocampus and thereby regulates seizure susceptibility, and that aberrant NMDA receptor-mediated synaptic transmission underlies the pathological mechanisms of the CDKL5 loss-of-function.

Publications

Manabe, T. NMDA receptor-independent LTP in hippocampal interneurons. *J. Physiol. (Lond.)* 595. 11: 3263-3264, 2017.

Okuda, K., Kobayashi, S., Fukaya, M., Watanabe, A., Murakami, T., Hagiwara, M., Sato, T., Ueno, H., Ogonuki, N., Komano-Inoue, S., Manabe, H., Yamaguchi, M., Ogura, A., Asahara, H., Sakagami, H., Mizuguchi, M., Manabe, T. and Tanaka, T. CDKL5 controls postsynaptic localization of GluN2B-containing NMDA receptors in hippocampus and regulates seizure susceptibility. *Neurobiol. Dis.* 106: 158-170, 2017.

Katayama, N., Yamamori, S., Fukaya, M., Kobayashi, S., Watanabe, M., Takahashi, M. and Manabe, T. SNAP-25 phosphorylation at Ser187 regulates synaptic facilitation and short-term plasticity in an age-dependent manner. *Sci. Rep.* 7: 7996, 2017.

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. Identification of novel substrates and regulators of human mitogen-activated protein kinases.

Seina Oe, Moe Matsushita, Hisashi Mori-izumi, Daichi Fujikawa, Zizheng Li, Tsubasa Tokunaga, Sae Uchida, Mamiko Komori, Mari Taguchi, Takanaori Nakamura, Yuji Kubota, and Mutsuhiro Takekawa

Sequential activation of protein kinases within MAPK cascades is an evolutionary-conserved mechanism of intracellular signaling among eukaryotes. In human cells, at least three functionally distinct subfamilies of MAPKs are present, namely, ERK1/2, JNK1/2/3, and p38α/β/γ/δ. While the classical ERK MAPK is predominantly activated by mitogenic stimuli, two relatively newly identified MAPKs, p38 and JNK, are preferentially activated by various environmental stresses (e.g., ultraviolet-light and γ irradiation, oxidative stress, DNA-damaging reagents, osmotic stress, and pro-inflammatory cytokines). Therefore, p38 and JNK MAPKs are collectively referred to as stress-activated protein kinases (SAPKs). Each of these MAPK cascades can regulate several different and sometimes overlapping biological functions. In general, the ERK pathway mediates growth-promoting and anti-apoptotic signaling, while the p38 and JNK pathways play crucial roles in cellular stress responses such as growth arrest and apoptosis. In addition, the p38 and JNK pathways are involved in inflammatory responses. Dysregulation of these critical signal transduction systems is involved in the etiology of various life-threatening diseases, including cancer, autoimmune diseases, and neurodegenerative disorders. Therefore, these pathways are also of clinical importance.

Since these MAPKs exert their biological effects through the phosphorylation of their substrate proteins, the identification of which is indispensable for comprehensive understanding of the regulatory mechanisms of critical biological processes. By developing a novel screening strategy using a yeast three-hybrid system, we have isolated several new MAPK substrates from human cDNA libraries. These substrates include regulatory molecules for the expression of immediate early response genes and for assembly of the actin-cytoskeleton, and several Ser/Thr protein kinases that regulate cell proliferation and apoptosis. We confirmed that these molecules were indeed directly phosphorylated by one (or more) of the human MAPKs *in vitro* as well as *in vivo* in response to mitogenic and/or stress

stimuli. Thus, these molecules are bona fide substrates of MAPKs. Besides these substrate proteins, this year we have also identified novel regulators of MAPK signaling, including molecules involved in redox reactions and apoptotic cell death. The biological functions of these novel substrates and regulators are under investigation in our laboratory.

2. Identification of a novel inhibitor for mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)

Yuji Kubota, Masatomi Iijima[1], Ryuichi Sawa[1], Yumiko Kubota[1], Masaki Hatano[1], Masayuki Igarashi[1], Manabu Kawada[1], Isao Momose[1], Masakatsu Shibasaki[1], Mutsuhiro Takekawa: [1]Institute of Microbial Chemistry (BIKAKEN)

The RAS-ERK signaling pathway is activated by mitogenic stimuli and associates with cell proliferation. In human malignant tumors, this pathway is often hyper-activated by several oncogenes including receptor tyrosine kinases, RAS or RAF. For example, gain-of-function mutations in RAS are detected in ~30% of all human cancers and oncogenic mutations of the *BRAF* gene such as $BRAF^{V600E}$ are detected in ~66% of human melanomas, 69% of papillary thyroid tumors and a lower proportion of colorectal carcinomas. Therefore, members of the RAS-ERK pathway have been considered as attractive targets for the development of new anticancer drugs. Indeed, Vemurafenib and Dabrafenib, potent inhibitors of oncogenic BRAF, have been approved for the treatment of BRAF-mutant melanoma. Although BRAF inhibitors conferred significant survival benefits in patients with melanoma, most of those treated with vemurafenib develop resistance to it within 2-18 months. Several mechanisms of acquired resistance to RAF inhibitors have been proposed, the majority of which is the reactivation of the ERK pathway in the presence of BRAF inhibition. Accordingly, targeting MEK1/2 is a promising strategy for the treatment of cancer with hyperactivation of the RAS-ERK pathway, because inhibition of MEK1/2 can abrogate all upstream signals for ERK activation.

Microbial secondary metabolites have unlimited chemical diversity and are often used to treat human diseases. Therefore, they are considered to be a hopeful resource in drug discovery. We have screened microbial metabolites and identified a guanine derivative produced by Streptomyces sp. MK63-43F2 as a new MEK kinase inhibitor. This guanine derivative was identified to be 2-amino-4-methoxy-5-cyanopyrrolo[2,3-d]pyrimidine by spectroscopic analysis. This compound significantly and specifically inhibited MEK1 kinase activity in an ATP-dependent manner in vitro and suppressed the phosphorylation of ERK in vivo. When applied to cancer cells, this compound can inhibit the proliferation. Therefore, this compound might be a potent lead compound for the development of a new class of MEK inhibitors.

3. Development of a novel method, WGA-based lectin affinity gel electrophoresis, for the detection of O-GlcNAc-modified proteins

Yuji Kubota, Ko Fujioka, and Mutsuhiro Takekawa

Post-translational modifications (PTMs) regulate various properties of proteins such as stability, subcellular localization, and catalytic activity. Of these PTMs, O-linked β-N-acetylglucosamine modification (O-GlcNAcylation) is a divergent type of protein glycosylation in which a single-sugar, N-acetylglucosamine, is added to the hydroxyl moiety of Ser and Thr residues of cytoplasmic and nuclear proteins. Previous studies have shown that thousands of proteins in cells are modified with O-GlcNAc. Protein O-GlcNAcylation is dynamically and reversibly regulated by the paired enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). OGT catalyzes the addition of a GlcNAc moiety from a uridine diphosphate (UDP)-GlcNAc to target proteins, while OGA removes the O-GlcNAc from the modified proteins. Since both O-GlcNAcylation and phosphorylation occur at Ser and Thr residues, these two types of PTMs are mutually exclusive on the same target residue. In addition, these modifications can suppress each other within a protein even when they do not take place on the same residues. This dynamic crosstalk between phosphorylation and O-GlcNAcylation has been frequently found in various cellular proteins, and the molecular function of the modified proteins dramatically changes according to the modification state.

Recent advances in the field of mass spectrometry analysis have enabled high-throughput screening of a wide variety of PTMs including O-GlcNAc-modification. However, stoichiometric analysis of O-GlcNAcylation has been reported for only a few proteins because the conventional methods for quantifying the O-GlcNAcylation level entail laborious processes. In addition, in collision-induced dissociation (CID) for mass-spectrum analysis, O-GlcNAc is readily released from the conjugated peptide, which limits evaluation of the O-GlcNAcylation level of a specific protein. Therefore, the development of novel techniques for quantitative analysis of protein O-GlcNAcylation therefore remains an important challenge.

This year, we developed a novel method in which O-GlcNAcylated and non-O-GlcNAcylated forms of proteins are separated by lectin affinity gel electrophoresis using wheat germ agglutinin

(WGA), a lectin from *Triticum vulgaris*, which primarily binds to N-acetylglucosamine residues. Electrophoresis of cell lysates through a gel containing copolymerized WGA selectively induced retardation of the mobility of O-GlcNAcylated proteins, thereby allowing the simultaneous visualization of both the O-GlcNAcylated and the unmodified forms of proteins. Using this novel method, we successfully detected and quantified the O-GlcNAcylation status of several proteins (*i.e.*, Tab1, Nup62, and AGFG1) that regulate critical cellular functions such as signal transduction pathways and nuclear-cytoplasmic transport of mRNA and proteins. Therefore, this method enables the quantitative detection of O-GlcNAcylated proteins and is thus useful for the analysis of biological phenomena in which protein O-GlcNAcylation is involved.

4. Regulation of PLK4 activity and centrosome integrity under stress

Takanori Nakamura, Noriko Nishizumi-Tokai, Moe Matsushita, and Mutsuhiro Takekawa

Centrosomes serve as the microtubule-organizing centers (MTOCs) and regulate the assembly of mitotic spindles in animal cells. In order for cells to undergo normal bipolar cell division, the single interphase centrosome must duplicate precisely once per cell cycle. The strict control of centrosome numbers is critical for accurate chromosome segregation at cell division and for maintenance of the stability of genomes. We have previously demonstrated a direct functional interaction between the SAPK pathways and Polo-like kinase 4 (PLK4), an evolutionarily conserved main regulator of centrosome duplication. Upon stress stimuli, stress-responsive MAPKKKs directly phosphorylated and activated PLK4. Stress-induced, MAPKKK-mediated, PLK4 activation provides survival signaling and promotes centrosome duplication. At the same time, however, SAPKs and the tumor suppressor p53, both of which are also activated by various stress stimuli, cooperated to counteract PLK4 activity, thereby preventing centrosome amplification. 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 co-operation between SAPKs and p53 explains why both p53 and the MKK4 MAPKK (a SAPK activator) are frequently mutated simultaneously in cancer, in which centrosome number is often increased after stress.

This year, we investigated the molecular mechanism as to how PLK4 translocates to centrosomes. By generating a series of deletion mutants of PLK4, we mapped the region of PLK4 that is critical for its centrosomal localization. Furthermore, we have identified several molecules that selectively interact with the centrosome localization sequence of PLK4 by mass spectrometry analyses. Depletion of some of these PLK4-binding proteins by siRNAs inhibited the centrosomal localization of PLK4. Therefore, these proteins contribute to the subcellular localization of PLK4 and to the rigorous regulation of the centrosome duplication. The precise function of these molecules is under investigation in our laboratory.

5. Elucidation of the physiological functions of MCRIP1, an ERK substrate, during epithelial-to-mesenchymal transition and embryonic development.

Jane S. Weng, Takanori Nakamura, and Mutsuhiro Takekawa

Epithelial-to-mesenchymal transition (EMT) is a cellular trans-differentiation program whereby epithelial cells lose their epithelial characteristics and acquire a migratory, mesenchymal phenotype. This phenomenon is involved in a wide range of biological process, including embryonic development, tissue repair, and tissue fibrosis. Furthermore, inappropriate reactivation of the EMT program in malignant epithelial cells 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 cancer. Although TGF-β signaling is a prominent mediator of EMT, various other signaling pathways also contribute this process. In particular, it has been shown that hyper-activation of ERK signaling by certain oncogenes, 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, is involved in epigenetic gene silencing of E-cadherin during EMT. However, the functional relationship between ERK signaling and CtBP remains unclear.

We have identified a novel ERK substrate, designated MCRIP1, which mediates functional crosstalk between ERK signaling and CtBP-mediated gene silencing. CtBP is recruited to the promoter elements by interacting with the DNA-binding transcriptional repressor ZEB1. We found that MCRIP1 binds to CtBP, thereby competitively inhibiting CtBP-ZEB1 interaction. However, when phosphorylated by ERK, MCRIP1 dissociates from CtBP, allowing CtBP to interact with ZEB1. In this manner, the CtBP complex is then recruited to, and silences the E-cadherin promoter by inducing histone modifications. Expression of a constitutively-CtBP-bind-

ing MCRIP1 mutant profoundly inhibited ERK-induced EMT. These results delineate a molecular mechanism by which ERK signaling induces epigenetic silencing of tumor suppressive genes. The physiological functions of MCRIP1 in the regulation of embryonic development are currently under investigation.

6. MEK mutations associated with congenital diseases and sporadic cancers elicit distinct spatio-temporal properties of ERK signaling.

Yuji Kubota, Yusuke Takagi, Shiho Hirose, Yuta Nakano and Mutsuhiro Takekawa

The ERK pathway is frequently hyper-activated by various oncogenes, including receptor tyrosine kinases, Ras, and Raf, in human cancers. Interestingly, more than 20 different mutations in the human MEK1/2 genes have recently been identified in sporadic cancers and in congenital Ras/MAPK syndromes. The Ras/MAPK syndromes are genetic diseases that manifest symptoms of facial dysmorphisms, heart defects, mental retardation, and an increased risk of developing cancer. MEK1 mutations have also been identified in melanoma cells that are resistant to the treatment with a B-Raf inhibitor. However, the precise effects of MEK mutations on its enzymatic activity and on carcinogenesis remain elusive.

We investigated the biochemical properties of MEK mutants and found that such mutations rendered MEK constitutively active. We also identified a bunch of genes whose expressions were up-regulated when the ERK pathway is aberrantly activated by the MEK mutants. Importantly, some of these genes are indeed strongly expressed in various human cancer cell lines as well as in clinical cancer tissues, suggesting that these genes are cancer-specific antigens and are thus good targets for developing novel therapies interventions for cancer.

Publications

Kubota Y, Fujioka K and Takekawa M. WGA-based lectin affinity gel electrophoresis: a novel method for the detection of O-GlcNAc-modified proteins. PLoS ONE 12: e0180714. doi.org/10.1371/journal.pone.0180714 (2017)

Iijima M, Kubota Y, Sawa R, Kubota Y, Hatano M, Igarashi M, Kawada M, Momose I, Takekawa M and Shibasaki M. A guanine derivative as a new MEK inhibitor produced by Streptomuces sp. MK63-43F2. Journal of Antibiotics. doi.org/10.1038/ja.2017.100 (2017)

Ichimanda M, Hijiya N, Tsukamoto Y, Uchida T, Nakada, C, Akagi T, Etoh T, Iha H, Inomata M, Takekawa M and Moriyama M. Downregulation of DUSP4 enhances cell proliferation and invasiveness in colorectal carcinomas. Cancer Science. in press.

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