Department of Basic Medical Sciences

Division of Molecular Cell Signaling (1)
分子細胞情報分野(1)

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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. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans-autophosphorylation of the MTK1 kinase domain.

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Mammalian cells are frequently exposed to cellular stresses, which are defined as diverse environmental conditions that are detrimental to the normal growth and survival of the cells. Typical cellular stresses include ultraviolet (UV), ionizing radiation (IR), genotoxins, hyperosmolarity, oxidative stress, low oxygen supply (hypoxia), and inhibition of protein synthesis by antibiotics and plant toxins. In coping with the barrage of these and other cellular stresses, multi-cellular eukaryotic organisms have developed a strategy as to how damaged cells will respond to stresses. In general, if the intensity of damage is moderate, the affected cell will seek to repair the damage. If, however, the damage to a cell is too severe for a complete repair, the affected cells are eliminated by apoptosis. This reduces the risk to the organism as a whole, such as development of a cancer. Such a crucial decision-making between repair or death is, at least in part, mediated by the Stress-activated MAP kinase (SAPK) pathways.

As the name implies, the SAPK pathways are homologous to and share many characteristics with the classic (ERK1/2) MAPK pathway. Eukaryotic MAPK pathways are conserved signaling modules that serve to transmit signals from the cell surface to the nucleus. The core of any MAPK pathway is composed of three tiers of sequentially activating protein kinases, namely, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. Activation of MAPKs is achieved by phosphorylation of a threonine and a tyrosine residues within a conserved Thr-Xaa-Tyr motif in the activation loop (also called the T-loop) catalyzed by MAPKKs. MAPKKs, in turn, are activated by any of several MAPKKKs, via phosphorylation of serine and/or threonine residues within their activation loop.

All eukaryotic cells possess multiple MAPK pathways, each of which is activated by distinct

sets of stimuli. In the budding yeast *Saccharomyces cerevisiae*, for example, hyperosmotic stress activates the Hog1 MAPK pathway, whereas mating pheromones activate the Fus3/Kss1 MAPK pathway (13, 18). In mammalian cells, four different subfamilies of MAPKs are present, namely, ERK1/2, JNK1/2/3, p38α/β/γ/δ, and ERK5. The ERK1/2 MAPK pathway is preferentially activated in response to mitogenic stimuli, such as growth factors and phorbol esters, and plays a role in cell growth and cell survival. The ERK1/2 pathway is mainly regulated by the monomeric GTPase Ras, which recruits MAPKKKs of the Raf family to activate the two downstream MAPKKs: MEK 1 / 2. These MAPKKs, in turn, activate the ERK 1/2 MAPKs. The JNK and p38 MAPKs (collectively called SAPKs), in contrast, preferentially respond to various cellular stresses, and are thus called SAPK pathways. Besides cell stresses, the SAPK pathways are also activated by cytokines such as IL-1, TNFα, and TGF-β. The JNK subfamily of MAPKs are activated mainly by the MKK4 and MKK7 MAPKKs, while the p38 subfamily MAPKs are activated by the MKK3 and MKK6 MAPKKs. In clear contrast to this limited number of MAPKKs in the SAPK pathways, there are numerous MAPKKKs that function upstream of the JNK and p38 MAPKs. These include MEKK1/2/3, MTK1 (also known as MEKK4), TAK1, ASK1/2, TAO1/2/3, MLKs, and perhaps others. This multiplicity at the level of MAPKKK reflects the vastly diverse stress stimuli that can recruit these SAPK pathways.

MTK1 is one of the human MAPKKKs belonging to the SAPK pathways, and the mouse ortholog is called MEKK4. The kinase domain of MTK1 (MEKK4) is homologous to other MAPKKKs, especially similar to mammalian MEKK1/2/3 and ASK1/2 and yeast SSK2/SSK 22, but its N-terminal non-catalytic domain (regulatory domain) is unique. Analyses using MEKK4-deficient mice have shown that the MEKK4 signaling pathway integrates signals from both the T cell antigen receptor and IL-12/STAT4 in developing Th1 cells, and promotes STAT4-independent IFNγ production, and that MEKK4 is essential for normal neural and skeletal development.

In a yeast two-hybrid screening aimed to identify MTK1 activator(s), we found three Growth-Arrest and DNA Damage-inducible 45 (GADD45) family proteins to be strong binding partners of MTK1. The GADD45 gene was originally identified as a UV-inducible gene in Chinese hamster cells. The human genome encodes three GADD45-like proteins, GADD45α (the original GADD45), GADD45β, and GADD45γ. These will be referred to collectively as the GADD45 proteins. The three GADD45 genes are all inducible by cellular stresses, although optimal stimuli for each gene appear to be different. The GADD45 proteins interact with various intracellular molecules, such as proliferating cell nuclear antigen (PCNA), Cdc2-CyclinB1 complex, $p21^{Waf1/Cip1}$, and core histones, and play important roles in stress-adaptive processes including growth control, maintenance of genomic stability, DNA repair, and apoptosis. In other words, the GADD45 proteins are emergency calls in damaged cells.

Expression of transfected GADD45 genes strongly activates co-expressed MTK1 kinase and induces apoptosis in mammalian cells. TGF-β-induced GADD45β expression also activates p38 MAPK through MTK1 activation. MEKK4-deficient mice have lost GADD45-induced IFNγ production. Activation of the SAPK pathway by MTK1 and GADD45 is temporally a slow process, because it requires induction of GADD45 gene expression prior to activation of MTK1. Thus, the activation of MTK1 by GADD45 differs from other modes of SAPK activation that occur within minutes. GADD45/MTK1 mediated SAPK activation may therefore serve as a more long-term adaptive mechanism for stressed cells.

We previously proposed that binding of GADD45 to the N-terminal region of MTK1 counters the autoinhibitory effect of the MTK1 N-terminal segment on the kinase domain, and at the same time enables the MTK1 kinase domain to bind its cognate MAPKKs (MKK3 and MKK6) via the latter's DVD docking sites. The details of MTK1 activation by GADD45, however, remained obscure. This year, we investigated the molecular mechanism by which GADD45 regulates MTK1 kinase activity.

The data from this study lead to the following model of the GADD45 mediated activation of the MTK1 MAPKKK. Activation of MTK1 by GADD45 occurs through a series of molecular steps (I through VI). In brief, each step is as follows. (I) In unstimulated cells, MTK1 is in a closed (inhibited) conformation in which the N-terminal autoinhibitory domain (AID) blocks the C-terminal kinase catalytic domain (KD). (II) Extracellular stimuli, such as MMS exposure, induce the expression of stress-inducible GADD45 proteins, which bind to the MTK1 N-terminal GADD45-binding domain (BD). (III) GADD45-binding to MTK1 dissociates the latter's AID from the C-terminal kinase catalytic domain. (IV) At the same time, GADD45-binding unmasks the MTK1 dimerization domain, inducing homo-dimer formation. At this stage, the MTK1 kinase domain is in open conformation (i.e., not actively inhibited), but not yet fully active as a

kinase. (V) Dimerized MTK1 becomes fully activated when Thr-1493 is trans-autophosphorylated. (VI) GADD45 binding also unmasks a site in the MTK1 kinase domain that interacts with the MAPKK DVD docking sites, allowing MTK1 to interact with, and phosphorylate, the cognate MAPKKs, namely MKK3 and MKK6.

Thus, full activation of MTK1 by GADD45 entails four different molecular mechanisms: removal of the autoinhibitory domain; dimerization; phosphorylation of the activation loop; and unmasking of the docking site for MAPKKs. Individually, these mechanisms are used by other MAPKKKs. However, the details are different for each MAPKKK, reflecting their different physiological roles. In this study, we revealed how the binding of one protein (GADD45) orchestrated these mechanisms, thereby converting an inert enzyme (MTK1) to a fully active one.

2. Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway.

Kazuo Tatebayashi, Keiichiro Tanaka, Hui-Yu Yang, Katsuyoshi Yamamoto, Yusaku Matsushita, Taichiro Tomida, Midori Imai, and Haruo Saito

The budding yeast *Saccharomyces cerevisiae* survive widely fluctuating osmotic conditions in their natural habitat, such as the surface of ripening grapes. To cope with an increased external osmolarity, yeast synthesize, and intracellularly retain, the compatible osmolyte glycerol. There is also a temporary arrest in cell cycle progression and inhibition of protein translation, during which cells readjust to the changed environment. These events are governed by the High Osmolarity Glycerol (HOG) signaling pathway, whose core element is the Hog1 MAP kinase (MAPK) cascade. As a result, defects in the HOG pathway cause severe osmosensitivity in cell growth. The upstream part of the HOG pathway is composed of the functionally redundant, but mechanistically distinct, SLN1 and SHO1 branches. A signal emanating from either branch converges on a common MAPK kinase (MAPKK), Pbs2, which is the specific activator of the Hog1 MAPK. For yeast to survive on high osmolarity media, either the SLN1 or the SHO1 branch alone is sufficient.

For each branch, there must be an osmosensor that generates an intracellular signal in response to extracellular osmolarity variations. It is believed that the osmosensor for the SLN1 branch is Sln1, a transmembrane (TM) histidine kinase. Sln1 detects turgor changes and transmits a signal via the Sln1-Ypd1-Ssk1 phospho-relay system. Unphosphorylated Ssk1 binds and activates the functionally redundant Ssk2/Ssk22 MAPKK kinases (MAPKKK) that activate the Pbs2 MAPKK. In contrast, the osmosensor in the SHO1 branch has been elusive. There are three candidates, but none has been convincingly shown to be an osmosensor. The first candidate is the branch's namesake, Sho1, which is, to date, the most upstream known component of the pathway. Sho1 has four TM domains, TM1~TM4, separated by short loops (Loop-1～Loop-3) of 5 to 8 amino acids each. The Sho1 C-terminal cytoplasmic region contains an SH3 domain and binds both the Pbs2 MAPKK and the complex of the Ste11 MAPKKK and the Ste50 adaptor protein . Thus, Sho1 serves as an obligatory adaptor between the Ste11 MAPKKK and its substrate Pbs2. It has not, however, been experimentally determined if Sho1 serves an osmosensor function as originally postulated. A second candidate for the osmosensor in the SHO1 branch is Msb2. The *MSB2* gene was originally identified as a multicopy suppressor of the budding defect of *cdc24-ts*, and its product is a member of the highly glycosylated mucin family. More recently, it was shown that Msb2 is at the head of the filamentous growth (FG) MAPK signal pathway, which under certain conditions can be activated by osmostress. A physiological role for Msb2 in the HOG MAPK pathway, however, has been dismissed, because *msb2*Δ mutants (in a host strain that is defective in the SLN1 branch) are osmoresistant, with robust Hog1 phosphorylation and HOG-dependent gene expression upon osmostress stimulation. Finally, a third candidate for the osmosensor in the SHO1 branch is Opy2. Opy2 is a type 1 TM protein, recently shown to have an essential role in the SHO1 branch. However, there is no evidence that Opy2 participates in an osmosensing process. Thus, despite much speculation, the identity of the osmosensor in the SHO1 branch has been elusive.

This year, to investigate how osmostress activates the SHO1 branch of the Hog1 MAP cascade, we screened for mutants that disable the SHO1 branch in the absence of the Msb2 glycoprotein. We thus found that another mucin-like transmembrane proteins Hkr1 functions redundantly with Msb2 as potential osmosensors for the SHO1 branch. Hyperactive forms of either Hkr1 or Msb2 can activate the HOG pathway only in the presence of Sho1, while a hyperactive Sho1 mutant activates the HOG pathway in the absence of both Hkr1 and Msb2, indicating that Hkr1 and Msb2 are the most upstream elements known so far in the SHO1 branch. Hkr1 or Msb2 can individually form a

complex with Sho1, and, upon high external osmolarity stress, induces Sho1 to generate an intracellular signal (mode 1 activation). Furthermore, Msb2, but not Hkr1, can generate an intracellular signal in a Sho1-independent manner (mode 2 activation). We also found that the role of the Opy2 membrane protein is to recruit the Ste50/Ste11 complex to the cytoplasmic membrane, where activation of the Ste11 MAPKKK by the Ste20 PAK-like kinase takes place. Thus, we have uncover ed a complex interplay among two mucin-like glycoproteins, Hkr1 and Msb2, and two other transmembrane proteins Sho1 and Opy2, in the yeast osmosensing reaction.

Publications

Miyake, Z., Takekawa, M., Ge, Q., and Saito, H. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated *trans*-autophosphorylation of the MTK1 kinase domain. *Mol. Cell. Biol*., 27: 2765-2776, 2007.

Tatebayashi, K., Tanaka, K., Yang, H.-Y., Yamamoto, K., Matsushita, Y., Tomida, T., Imai, M., and Saito, H. Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J*., 26: 3521-3533, 2007.

Department of Basic Medical Sciences

Division of Molecular Cell Signaling (2)
分子細胞情報分野(2)

Assistant Professor Junichi Watanabe, M.D., Ph.D. 助 教 医学博士 渡 邉 純 一

Parasitology and medical entomology are closely related areas of research in which our institute has had a long history since its establishment in Meiji Era. During last 10 years, we have produced twenty full-length cDNA libraries from apicomplexan protozoan parasites including those causing malaria, helminthes and medically important arthropods. We have determined sequences that have been compiled in databases (http://fullmal.ims.u-tokyo.ac.jp). Parallel bioinformatic analysis will elucidate parasite-vector relationship. We are expanding our global collaborations as our sequence data combined with other data is much more informative than either analyzed separately.

Overview

Eukaryotic pathogens represent some of the most important and complex disease causing agents on a global scale. Apicomplexan protozoa are the most successful parasites in terms of morbidity and mortality; malaria kills over a million people every year and toxoplasma chronically infects one third of the human population causing high mortality rates amongst the immunocompromised (e.g. AIDS patients). The genetic complexity of these species, once a major hurdle for detailed elucidation, can now be exploited with the advent of genome and cDNA sequencing. The generation and assemblage of genome sequence provides a plethora of new information for biological investigation. During the past ten years we have added a new dimension to the study of these organisms through the generation of full-length cDNA libraries. The sequence data from these libraries have been invaluable because they provide key information on gene structure, notably the start site of transcription and identification of the first exon. This is particularly important for the parasites and vectors that we study because computational methods to identify genes from genomic sequence are poorly developed for these organisms. We have produced two databases to hold these data: Full-Parasites that contain the numerous 5′ end-one-pass sequences of full-length cDNA libraries of various parasites and Comparasite that contains the homologous Reffull sequences of apicomplexa protozoa.

Arthropods are evolutionarily advanced and sophisticated organisms, including some that are of medical importance because they can transmit pathogens (including eukaryotic parasites) and cause allergy. Driven by their role in the spread of deadly disease and threats of bioterrorism, many of their genome sequences have been determined recently. However, the annotation of these genome sequences is more challenging because of the sequence complexity and poor understanding of gene structure. Full-length cDNA analysis allows us to define transcription units and gene structures that would otherwise be poorly inferred by bioinformatics as computational methods are notoriously unreliable for finding the 5′ exons of genes, let alone the start site of transcription. We have extended our interest to arthropod vectors, including mosquitoes, mites and ticks where co-evolution and complex ecology are to be addressed. On the ba-

sis of sequence data of several full-length cDNA libraries from arthropods we have started construction of database, Full-Arthropods.

Full-Parasites

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Full-Parasites is compilation of full-length cDNA database describing the 5′ end-one-pass sequences from various parasites mapped onto their genome sequences and found at http://fullmal.ims.u-tokyo.ac.jp.

Full-Apicomplexa

Ikuo Igarashi[2], Yoshifumi Nishikawa[2], Xuenan Xuan[2], Damer Blake[3], Xikun Wu[3], Fiona Tomley[3]: [2]Obihiro University of Agriculture and Veterinary Medicine, [3]Institute for Animal Health, UK

The phylum Apicomplexa is comprised of obligatory parasitic protozoa characterized by a unique organelle, the apical complex. The Apicomplexa consists of four groups; 1) gregarines which are extracellular parasites of arthropods and may includes *Cryptosporidium*, 2) coccidia which includes *Toxoplasma* and *Neospora*, 3) hemosporidia which includes *Plasmodium* (malaria), and 4) piroplasma which includes *Babesia* and *Theileria*.

Progress in this area was the production of full-length cDNA libraries and determination of one-pass sequences for: *Babesia bovis, B. equi*, and *B. cabalii* that are tick-borne parasites of cows and horses; and *Neospora caninum* that causes infertility in cows.

Full-Entamoeba

Asao Makioka[4], Masahiro Kumagai[4], Takahiro Terakawa[5]: [4]Jikei Medical University, [5]Effector Cell Institute

We have produced full-length cDNA libraries from trophozoite stage parasites of *Entamoeba histolytica* and *E. invadens* and determined one-pass sequences. *E. histolytica* that causes diarrhea is the second commonest parasitic disease in the world next to malaria. We also started proteome analysis using LC/MS/MS.

Full-Echinococcus

Yuzaburo Oku[6], Nariaki Nonaka[6], Jun Matsumoto[6], Masao Kamiya[7], Atsushi Toyoda[8], Yoshiyuki Sakaki[8], Kinpei Yagi[9], Li Shuang[10], Ryu Yamashita[10]: [6]Hokkaido University, [7]Rakuno Gakuen University, [8]Riken, [9]Hokkaido Institute of Public Health, [10]Institute of Medical Science, The University of Tokyo

Echinococcus is a small tapeworm of which life cycle is maintained by rodent and wild foxes. Humans are infected by ingestion of eggs through contact with fox or dog feces. Slow but progressive growth of hydatid cysts cause serious and fatal disease. It is endemic in Hokkaido and increasing in number. We have determined 5′-end and 3′-end-one-pass sequences from the full-length cDNA library produced from hydatid cysts developed in cotton rats that were infected with *Echinococcus multilocularis*. These sequences are currently being analyzed.

Full-Taenia

Raul Bobes[11] and Juan Laclette[11]: [11]National Autonomous University of Mexico

Taenia solium is a tapeworm of which infection is caused by ingestion of undercooked pork and migrating larva causes serious neurological symptoms. The genome project is underway by the researchers of National Autonomous University of Mexico (UNAM) and a full-length cDNA library was produced as a part of an ongoing collaboration.

Full-Arthropods

Ryuichiro Maeda[2], Sadao Nogami[12], Shinichi Noda[13], Chihiro Sugimoto[6], Masahira Hattori[1], Aksoy Serap[14], Todd Taylor[8], Shrama Vineet[8], Eri Kibukawa[15], Toshiaki Katayama[10], Kazuhisa Hiranuka[10] and Shuichi Kawashima[10]: [2]Obihiro University of Agriculture and Veterinary Medicine, [12]Nihon University, [13]Kagoshima University, [6]Hokkaido University, [1]Graduate School of Frontier Sciences, The University of Tokyo, [10]Institute of Medical Science, The University of Tokyo, [14]Yale University, [8]Riken, [15]STS.

Construction of a novel database, Full-Arthropods is underway utilizing the InterMine program in collaboration with Dr. Gos Micklem of Cambridge University.

We have produced full-length cDNA libraries from larva and pupa of *Glossina morsitans* (tsetse flies) that were propagated in the lab and determined 5′-end- and 3′-end-one-pass sequences, which have been deposited at International

Glossina Genome Initiative (IGGI) database for communal annotation.

Global collaborations

Department of Parasitology has had a long history of international academic collaborations because many of the diseases exist in tropical and remote areas. Now it is apparent that global collaboration is important beyond simply collecting samples and is quite productive science-wise. We started collaborative studies on malaria with Indonesian medical doctors because *Plasmodium vivax* malaria parasite is difficult to culture and needs to be obtained from the patients in the endemic area. Successful production of a full-length cDNA library after four years' effort has attracted researchers from abroad, leading to the expansion of field studies.

Coelacanth study

Hiroshi Koie[12], Alex Masengi[16], Janny Kusen[16], Josef Tuda[16], Boetje Moningka[16], Mihoko Imada[17], Masamitsu Iwata[18], Yoshitaka Abe[18]: [12]Nihon University, [16]Sam Ratulangi University, [17]Keio University, [18]Aquamarine Fukushima

A coelacanth was caught alive in Manado Bay near our malaria research field and frozen after death. We performed CT scan imaging at the local clinic and collected tissue samples for genome analysis. Future studies will help elucidate the process of terrestrial evolution in tetrapods.

References

Josef Tuda, Mihoko Imada, Junichi Watanabe and Boetje Moningka. Expert Commentary: Novel Scheme for International Academic Collaboration in Malaria Research Trends. Ed. Devin A. Flanigan. Nova Publishers. 2007.

Database

Full-Malaria/Comparasite
http://fullmal.ims.u-tokyo.ac.jp

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. Inhibitory modulation of synaptic plasticity is stronger in the dentate gyrus than in the CA1 region of the hippocampus.

Fumiko Arima-Yoshida, Ayako M. Watabe and Toshiya Manabe

Long-term potentiation (LTP) is a phenomenon that the efficacy of synaptic transmission is enhanced after high-frequency activation of the synapse. It was first discovered in the hippocampus, and it has been widely accepted as a cellular basis of certain forms of memory. In the medial perforant pathway-dentate gyrus granule cell synapse and in the CA3-CA1 pyramidal cell synapse, LTP is induced by a similar mechanism (postsynaptic N-methyl-D-aspartate receptor dependent), while several reports suggested that the modulation of LTP by γ-aminobutyric acid type A ($GABA_A$) receptor-mediated inhibitory inputs is stronger in the medial perforant pathway-dentate gyrus granule cell synapse. To explore the underlying molecular mechanism that makes the difference between the two regions, we compared LTP in the presence of the $GABA_A$ receptor antagonist picrotoxin with LTP in its absence in the CA1 region and in the dentate gyrus using acute slices of the rat hippocampus. We then compared the inhibitory monosynaptic responses with excitatory monosynaptic responses, and also compared their summation during an LTP-inducing stimulus between the two regions. Our results suggest that the stronger inhibitory modulation of LTP in the dentate gyrus may be due to the balance biased towards inhibition between the summated inhibitory and excitatory postsynaptic currents during conditioning in the dentate gyrus. Besides these examinations of synaptic inhibitory inputs, several reports suggested that continuous activation of extrasynaptic $GABA_A$ receptors by ambient GABA is different in several aspects between the two regions, which could also contribute to the finding about LTP modulation as well. For example, it is reported that continuous activation of extrasynaptic $GABA_A$ receptors is mediated by the receptor with different subunit compositions between the two regions, which may result in different properties of the inhibition. Thus, we are currently

examining whether this kind of inhibition is associated with the stronger inhibitory modulation of LTP in the dentate gyrus using the whole-cell patch-clamp technique.

2. Functional properties of the NMDA receptor in the lateral amygdala: a comparison with those in the hippocampal CA1 region.

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The amygdala is a crucial brain structure for the acquisition and expression of fear memory. The N-methyl-D-aspartate (NMDA)-type glutamate receptor channel, composed of the NR1 (GluRζ) and NR2 (GluRε) subunits, plays a key role in synaptic plasticity in the central nervous system. NR2 subunits (NR2A-NR2D) are differentially expressed, depending on developmental stages and brain regions, but their functional roles in the amygdala are still largely unknown. In this study, we have investigated the properties of synaptic NMDA receptors in the lateral nucleus of the amygdala (LA), comparing them with those in the hippocampal CA1 region. We find that the biophysical properties of NMDA receptors and the NR2A/NR2B ratio in the LA are distinct from those in the CA1 region and that the NR2B subunit contributes to synaptic transmission and LTP induction to a greater extent in the LA than in the CA1 region. Our data suggest that these properties of NMDA receptors in the LA are responsible for unique properties of amygdaloid synaptic function and plasticity.

3. GABAergic interneurons facilitate mossy fiber excitability in the developing hippocampus.

Michiko Nakamura, Yuko Sekino and Toshiya Manabe

Profound activity-dependent synaptic facilitation at hippocampal mossy fiber synapses is a unique and functionally important property. Although presynaptic ionotropic receptors, such as kainate receptors, contribute partially to the facilitation in the hippocampus, the precise mechanisms of presynaptic regulation by endogenous neurotransmitters remain unclear. In this study, we report that axonal $GABA_A$ receptors on mossy fibers are involved in the activity-dependent facilitation during development. In immature mouse hippocampal slices, short-train stimulation (5 pulses at 25 Hz) caused frequency-dependent facilitation of not only postsynaptic responses but also presynaptic fiber volleys that represent presynaptic activities. This fiber volley facilitation was inhibited by selective $GABA_A$ receptor antagonists, or by enkephalin that selectively suppresses excitability of interneurons. Furthermore, we directly demonstrated that this facilitation resulted from depolarization of mossy fibers in imaging experiments using a voltage-sensitive dye. This increased mossy fiber excitability caused by depolarizing action of GABA gradually decreased with development and eventually disappeared at around postnatal day 30. These results suggested that GABA released from interneurons acted on axonal $GABA_A$ receptors on mossy fibers and contributed at least partially to the activity- and age-dependent facilitation in the hippocampus.

4. Physiological and behavioral analysis of Plexin-A2 knockout mice.

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Hippocampal mossy fibers project preferentially to the stratum lucidum, the proximal-most lamina of the suprapyramidal region of the CA3 region in the hippocampus. The molecular mechanisms that govern this lamina-restricted projection are still unknown. Type A plexins can directly show repulsive activities, and all type A plexins (plexin-A1, -A2, -A3, and -A4) are expressed in the developing hippocampal system, suggesting their involvement in neuronal wiring in the hippocampus. In this study, we generated Plexin-A2-null mutant mice and found that Plexin-A2 deficiency caused a shift of mossy fibers from the suprapyramidal region to the infra- and intrapyramidal regions. However, all of the electrophysiological properties examined, including paired-pulse facilitation, sensitivity to group II mGluR agonists and LTP, were normal. In the behavioral analysis, mutant mice exhibited enhanced hippocampus-dependent spatial reference memory and spatial pattern separation tested by the 8-arm radial maze task. These results suggest that the pattern of synaptic inputs in the CA3 region determines the spatial learning ability.

5. Involvement of protein-tyrosine phosphatase PTPMEG in motor learning and cerebellar long-term depression.

Shinji Kusakawa, Yuji Kiyama, Shin-ichiro Kina[6], Tohru Tezuka[6], Yasushi Kishimoto[7], Sho Kakizawa[8], Koichi Hashimoto[7], Miho Ohsugi[6], Reiko Horai[9], Katsuko Sudo[9], Shigeru Kakuta[9], Yoichiro Iwakura[9], Masamitsu Iino[8], Masanobu Kano[7], Tadashi Yamamoto[6] and Toshiya Manabe: [6]Division of Oncology, Department of Cancer Biology, [7]Department of Cellular Neuroscience, Graduate School of Medicine, Osaka University, [8]Department of Cellular and Molecular Pharmacology, Graduate School of Medicine, University of Tokyo, [9]Division of Cell Biology, Center for Experimental Medicine.

Although protein-tyrosine phosphorylation is important for hippocampus-dependent learning, its role in cerebellum-dependent learning remains unclear. We previously found that PTPMEG, a cytoplasmic protein-tyrosine phosphatase expressed in Purkinje cells (PCs), bound to the carboxyl-terminus of the glutamate receptor δ2 via the postsynaptic density-95/discs-large/ZO-1 domain of PTPMEG. In the present study, we generated PTPMEG-knockout (KO) mice, and addressed whether PTPMEG is involved in cerebellar plasticity and cerebellum-dependent learning. The structure of the cerebellum in PTPMEG-KO mice appeared grossly normal. However, we found that PTPMEG-KO mice showed severe impairment in the accelerated rotarod test. These mice also exhibited impairment in rapid acquisition of the cerebellum-dependent delay eyeblink conditioning, in which conditioned stimulus (450-ms tone) and unconditioned stimulus (100-ms periorbital electrical shock) were co-terminated. Moreover, long -term depression at parallel fiber-PC synapses was significantly attenuated in these mice. Developmental elimination of surplus climbing fibers and the physiological properties of excitatory synaptic inputs to PCs appeared normal in PTPMEG-KO mice. These results suggest that tyrosine dephosphorylation events regulated by PTPMEG are important for both motor learning and cerebellar synaptic plasticity.

6. The neuropeptide nociceptin is a synaptically released endogenous inhibitor of hippocampal long-term potentiation.

Saknan Bongsebandhu-phubhakdi and Toshiya Manabe

Hippocampal long-term potentiation (LTP) of excitatory synaptic transmission has been regarded as a cellular model of learning and memory. Its induction is regulated by many functional molecules at synapses, including the neuropeptide nociceptin identified as an endogenous ligand for the orphan opioid receptor. Mutant mice lacking the receptor exhibit enhanced LTP and hippocampus-dependent memory formation; however, the precise molecular and cellular mechanism is largely unknown. In this study, we show that LTP in the hippocampal CA1 region is inhibited by nociceptin synaptically released from interneurons by tetanic stimulation. This endogenous nociceptin downregulates the excitability of pyramidal cells by the hyperpolarization induced by the activation of K^+ channels, which are the common target shared with γ-aminobutyric acid type B ($GABA_B$) receptors although the mode of action is considerably different. Interestingly, the modulation of LTP by endogenous nociceptin is not observed when theta-burst stimulation is used in stead of tetanic stimulation, suggesting that relatively longer high-frequency synaptic activation is required for the release of endogenous nociceptin. These results indicate that, in addition to GABA, nociceptin released from interneurons by their high-frequency activation is a novel endogenous neuromodulator that negatively regulates LTP induction in the hippocampus through direct modulation of pyramidal cells.

7. Roles of the actin cytoskeleton in synaptogenesis, synaptic plasticity and adult neurogenesis.

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Excitatory synapses in the central nervous system generally have specialized postsynaptic structures called dendritic spines, and their morphological plasticity is believed to play a pivotal role in higher brain functions, such as learning and memory. The spine morphology is dynamically regulated by the actin cytoskeleton, which is highly concentrated in dendritic spines. Many studies suggest that actin remodeling is the molecular mechanism underlying activity-dependent morphological changes. Mechanical properties of actin filaments are generally regulated by their side-binding proteins. This project aims to elucidate a role of reorganization of the

actin cytoskeleton in the spine during the maturation of synaptic functions and plasticity.

Drebrin, one of the actin side-binding proteins, is highly enriched in dendritic spines of the mature brain. By immunoelectron microscopy using a newly-developed antibody against drebrin A, a neuron-specific isoform of drebrin, we have shown that drebrin A localizes in sites of prospective excitatory synapses in the immature brain, and suggested that the drebrin content in a dendritic spine might be closely related to its synaptic function. Interestingly, some recent studies on neurological disorders accompanied by cognitive deficits suggested that the loss of drebrin in dendritic spines is a common pathognomonic feature of synaptic dysfunction. We have also found that the translocation of drebrin from the dendritic spines is induced by glutamate, which might be related to morphological plasticity. We are now investigating the ATPase-dependent mechanism of the drebrin translocation.

Down-regulation of the drebrin-A isoform caused by antisense oligonucleotides in developing cultured hippocampal neurons prevents spine formation and PSD-95 accumulation in dendritic spines. Filopodia, which are thin and headless protrusions, are thought to be precursors of dendritic spines. Drebrin is responsible for recruiting F-actin and PSD-95 into filopodia, and is suggested to govern spine morphogenesis. We are now interested in a role of drebrin in trafficking of glutamate receptors during synaptogenesis.

"Synaptic scaling" has been reported as scaling up of AMPA receptor (AMPAR)-mediated miniature excitatory postsynaptic currents (mEPSCs) induced by blockade of action potentials or AMPARs. In this study, we showed a novel type of synaptic scaling induced by the N-methyl-D-aspartate receptor (NMDAR) blockade. We analyzed AMPAR-mediated mEPSCs in hippocampal neurons (16 days in vitro) treated with the NMDAR antagonist D-(-)-2-amino-5-phosphonopentanoic acid (AP5) for 48 h in low-density cultures, using a whole-cell patch-clamp technique. The mEPSC amplitudes recorded from neurons chronically treated with AP5 were significantly larger than those from control neurons, whereas the frequency of mEPSCs was not changed. Immunocytochemistry showed that the number of synapsin I clusters of AP5-treated neurons was not different from that of control neurons, suggesting that the number of synapses was unchanged. Cumulative amplitude histograms revealed that the amplitude of mEPSCs was scaled multiplicatively after the AP 5 treatment. We are currently examining whether knockdown of drebrin expression by RNAi affects the synaptic scaling observed in developing neurons induced by NMDA receptor blockade.

Migrating neuroblasts in the adult brain form the rostral migratory stream (RMS) from the lateral ventricle to the olfactory bulb (OB) and then differentiate in the OB. In this study, we immunohistochemically analyzed drebrin expression in the RMS of the adult rat brain. Although drebrin is concentrated in dendritic spines of mature neurons , drebrin-immunopositive (DIP) cell bodies were observed in the RMS. The polysialated form of a neural cell adhesion molecule (PSA-NCAM) was detected in DIP cells. Ki-67, a marker of proliferating cells, was also detected in a subset of DIP cells; however, neither glial fibrillary acidic protein, nestin nor vimentin was detected in DIP cells. These results indicate that DIP cells in the RMS are migrating neuroblasts. An image subtraction method with anti-pan-drebrin and anti-drebrin A antibodies demonstrated that DIP migrating neuroblasts are immunopositive for drebrin E but not for drebrin A (E+A-). Furthermore, olfactory bulbectomy increased the number of cells with drebrin E+A- signals in the RMS, indicating that these cells migrate along the RMS. Drebrin E+A- cells were also found in the subgranular layer of the dentate gyrus and in the piriform cortex. Thus, the detection of drebrin E+A- signals can be used for identifying migrating neuroblasts in the adult brain. In the OB, drebrin E+A- signals were observed in the cell bodies of migrating neuroblasts in the core region; however, only fibrous and punctate drebrin E+A- signals were observed in postmigratory neuroblasts in the outer layers. These data demonstrate that the disappearance of drebrin E+A- signals from the cell body coincides with the cessation of neuronal migration. Thus, the disappearance of drebrin E from the cell body may be a molecular switch for the cessation of migration in newly generated neuroblasts.

8. Physiological roles of adenosine A_1 receptors in modulation of neuronal activity.

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ment of Physiology and Pharmacology, Karolinska Institute, Sweden.

Adenosine promotes cytoprotection under conditions of infection, ischemic preconditioning and oxidative stress. Our previous studies indicate that the expression of the adenosine A_1 receptor (A_1AR) is induced by oxidative stress via activation of nuclear factor NF-κB. To determine the role of NF-κB in the regulation of the A_1AR in vivo, we compared the A_1AR RNA and protein levels in the brains of mice lacking the p50 subunit of NF-κB (p50-/- mice) and age-matched B6129PF2/J (F2) controls. Radioligand binding assays in the cortex revealed a significantly lower number of A_1AR in the cortex of p 50-/- mice than in the F2, but no change in the equilibrium dissociation constant. Similar reductions in A_1AR were detected in the hippocampus, brain stem and hypothalamus and in peripheral tissues, such as the adrenal gland, kidney and spleen. Estimation of the A_1AR following purification by antibody affinity columns also indicated reduced A_1AR in the p50-/- mice cortex, as compared with the F2 mice. A_1AR immunocytochemistry indicated distinct neuronal labeling in the F2 cortex, which was substantially reduced in similar sections obtained from p50-/- mice. The p50-/- mice expressed lower levels of A_1AR mRNA than F2 mice, as determined by real time PCR. Quantification of the A_1AR-transducing G proteins by Western blotting showed significantly less $G_{\alpha i3}$, no change in $G_{\alpha i1}$, but higher levels of $G_{\alpha o}$ and G_{β} in the cortices of p50-/-, as compared with F2 mice. Administration of bacterial lipopolysaccharide (LPS), an activator of NF-κB, increased A_1AR expression in the cortices of F2 mice but not p50-/- mice. Cortical neuron cultures prepared from p 50-/- mice showed a greater degree of apoptosis, compared with neurons from F2 mice. Activation of the A_1AR reduced apoptosis with greater efficacy in cultures from F2 than p50-/- mice. Taken together, these data support a role for NF-κB in determining both the basal and LPS-stimulated A_1AR expression in vivo, which could contribute to neuronal survival.

We also explored the possibility of intimate functional interplay between $G_{i/o}$ protein-coupled A_1AR and type-1 mGluR (mGluR1) naturally occurring in cerebellar Purkinje cells. Using a perforated-patch voltage-clamp technique, we found that both synthetic and endogenous agonists for A_1AR induced continuous depression of a mGluR1-coupled inward current. A_1AR agonists also depressed mGluR1-coupled intracellular Ca^{2+} mobilization monitored by fluorometry. A_1AR indeed mediated this depression because genetic depletion of A_1AR abolished it. Surprisingly, A_1AR agonist-induced depression persisted after blockade of $G_{i/o}$ protein. The depression appeared to involve neither the cAMP-protein kinase A cascade downstream of the a subunits of $G_{i/o}$ and G_s proteins, nor cytoplasmic Ca^{2+} that is suggested to be regulated by the β-γ subunit complex of $G_{i/o}$ protein. Moreover, A_1AR did not appear to affect G_q protein that mediates the mGluR1-coupled responses. These findings suggest that A_1AR modulates the mGluR1 signaling without the aid of the major G proteins. In this respect, the A_1AR-mediated depression of the mGluR1 signaling shown here is clearly distinguished from the A_1AR-mediated neuronal responses described so far. These findings demonstrate a novel neuromodulatory action of adenosine in central neurons.

9. Tonic enhancement of endocannabinoid-mediated retrograde suppression of inhibition by cholinergic interneuron activity in the striatum.

Madoka Narushima[7], Motokazu Uchigashima[1], Masahiro Fukaya[1], Minoru Matsui, Kouichi Hashimoto[7], Masahiko Watanabe[1], Masanobu Kano[7] and Toshiya Manabe

Tonically active cholinergic interneurons in the striatum modulate activities of striatal outputs from medium spiny (MS) neurons and significantly influence overall functions of the basal ganglia. Cellular mechanisms of this modulation are not fully understood. In this study, we show that ambient acetylcholine (ACh) derived from tonically active cholinergic interneurons constitutively upregulates depolarization-induced release of endocannabinoids from MS neurons. The released endocannabinoids cause transient suppression of inhibitory synaptic inputs to MS neurons through acting retrogradely onto presynaptic CB_1 cannabinoid receptors. The effects were mediated by postsynaptic M_1 subtype of muscarinic ACh receptors, because the action of a muscarinic agonist to release endocannabinoids and the enhancement of depolarization-induced endocannabinoid release by ambient ACh were both deficient in M_1 knock-out mice and were blocked by postsynaptic infusion of guanosine-5′-O-(2-thiodiphosphate). Suppression of spontaneous firings of cholinergic interneurons by inhibiting I_h current reduced the depolarization-induced release of endocannabinoids. Conversely, elevation of ambient ACh concentration by inhibiting choline esterase significantly enhanced the endocannabinoid release. Paired recording from a cholinergic interneuron and an MS neuron revealed that the

activity of single cholinergic neuron could influence endocannabinoid-mediated signaling in neighboring MS neurons. These results clearly indicate that striatal endocannabinoid-mediated modulation is under the control of cholinergic interneuron activity. By immunofluorescent and immunoelectron microscopic examinations, we demonstrated that M_1 receptor was densely distributed in perikarya and dendrites of dopamine D_1 or D_2 receptor-positive MS neurons. Thus, we have disclosed a novel mechanism by which the muscarinic system regulates striatal output and may contribute to motor control.

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分子構造解析分野

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Electron microscopy provides a unique means to investigate the structure of biological materials including cells/tissues and supuramolecular assemblies. Instantaneous structure of functioning molecules not only in solution but also in live cells can be visualized with high contrast after adequate processing. Though the spatial resolution remains less than crystallography, the real superior feature of the method is its almost unlimited applicability to the materials whose structure cannot otherwise be pursued. For two years, we have been involved in a new project to develop several innovative techniques to characterize three-dimensional structure of individual macromolecules under functional states in solution or in live cells in situ. Our research activities are dedicated in two ways; one, development of new experimental tools and the other, their application, both to realize "Structural Biology of Single Molecules".

Visualization of structural features of functioning protein molecules during various molecular events related to cell motility

Katayama, E., Kimori, Y. and Shiraishi, T.: In collaboration with Prof. N. Baba's team, Kogakuin Univ.

We have been investigating the three-dimensional (3-D) architecture of various macromolecular assemblies that might play crucial roles in a number of cell motility and intracellular signal-transduction systems. Since a unique experimental approach, "single molecule physiology" was introduced to the field of molecular motor research about 20 years ago, it completely renewed the conventional knowledge on the intrinsic properties of various motor proteins and their interactions. The most important message from this new concept is that the behavior of individual protein molecule is stochastic and should be different from the others, and that the important information which might be lost by averaging in conventional means could be retrieved only by observation and separately analyzing unaveraged behavior of each single molecule. Various experimental techniques to deal single molecules have already been established and used as the most powerful and indispensable tools in current biophysical sciences.

On the other hand, conventional means of structural biology; *i.e*. X-ray crystallography or multi-dimensional NMR analysis, collects the data from a vast number of particles to be averaged both in time and space. Electron microscopy is quite unique, in a sense that it has a potential to visualize the instantaneous structure of individual macromolecules. Recent trend is the docking of crystal structures to the results of electron cryo-microscopy utilizing the capability of electron microscopy to visualize the structure of individual macromolecules. Structural analysis by Cryo-EM, however, requires numerous images to be classified and averaged to obtain the final results, because of its very low contrast and S/N. Thus, all these techniques cannot be compatible to "single molecule" matter. An innovating methodology is desired especially for

the study of motor proteins, that enables us to observe single molecules without any averaging, but still with a resolution enough to discriminate subdomain arrangement,

Quick-freeze deep-etch replica electron microscopy is exactly the right one for such purpose, and probably the sole technique to give high contrast snapshots of individual protein assemblies within cells as well as in solution, keeping approximately 1.5 nm resolution. We have actually shown the surface profiles of various protein assemblies such as molecular motors and intracellular receptor molecules *in situ*, utilizing that technique. Several years ago, we introduced a new method to perfectly overcome problematic ghost images in 3-D tomographic reconstruction, due to "missing data-range" which is unavoidable in electron microscopy (Katayama & Baba; Pat. 2002). We successfully applied it to obtain 3-D image of single protein molecules in the replica image, and from much less number of tilt-series images than usual. Recently, we further improved the reconstruction procedure based on completely different principle from the previous ones, and soon would be able to reconstruct even more precisely the internal structure of various materials, unaffected by the "ghost", which has been the biggest obstacle that hampered high-resolution 3-D electron tomography. As a complementary approach to characterize the 3-D structure of the target particles utilizing single but very high-resolution images, we devised a computer program to automatically extract both outline shape and characteristic surface pattern of the protein particles from replica images (Katayama, Kimori & Baba; Pat. Pend., 2006). By quantitative cross-correlation of those features with a comprehensive set of artificial images of the atomic-models in various configurations, viewed from all 3-D angles, we could objectively and reliably determine the best-matched configuration and the orientation of given particles picked up from the replica images.

Fifteen years have passed since the crystal structures of actin and myosin head were solved. Together with the powerful achievements by single molecule physiology, many people in the field expected at that time, that the operation mechanism of actomyosin motor would be completely elucidated soon in a near future. However, the essential part of the mechanism still remains vague even now, because of the lack of appropriate means to directly visualize the scene of the phenomena *in situ* with high enough resolution. We have utilized above new methodologies to visualize high-resolution and high-contrast snapshots of sliding actomyosin and revealed the instantaneous 3-D structure of functioning crossbridges. We found that the structure of the majority of crossbridges during sliding is not the ADP-Vi structure as postulated in widely-believed conventional "tilting lever-arm hypothesis", but actually is a new reversely-kinked configuration whose crystal structure has not yet been reported. The novel structure closely resembled that of putative intermediate analogue in which SH1 and SH2 are chemically crosslinked in the presence of ADP. We also noticed the presence of the crossbridges in the same total configuration but with different attachment angle to actin filament. Postulating that the crossbridges translocates actin by "Brownian ratchet" while in "weakly actin-bound state", and that they occasionally pivots into "primed configuration" to generate tension by stretching its lever-arm moiety, we might be able to comprehensively explain not only our own observations but also almost all the experimental results by others, especially even mysterious "chemo-mechanical loose-coupling" as found by Prof. Yanagida's team but remains unexplained even now. Dynamics and crystallographic studies of the crosslinked and actin-attached species are now in progress in collaboration with several expertise scientists.

Development and application of new marker probes for electron and fluorescence microscopy

Katayama, E.: In collaboration with Dr. T. Murayama's team Juntendo Univ. and Dr. T. QP Uyeda's team in Nat'l Inst. Adv. Ind. Sci. Tech.

In order to facilitate the recognition of the specific targets or the sites in the replica images keeping its high resolution, we have been developing a rod-shaped protein module that enables us high-resolution protein surface mapping by putting landmarks in electron microscopic images of the targets, together with simultaneous fluorescence detection of the same molecules within cells or solutions (Katayama; Pat. Pend., 2005). The marker probe was designed to include Fluorescent Protein (i.e. GFP) on one end and fusible by the other end to any position along the amino-acid sequence of the given protein. Interpretation of low-resolution images by conventional rotary-shadowing or high-speed atomic force microscopy might get much easier, only by supplementary information on the location of the landmark.

Preliminary examination with several examples (myosin-II and -V, clathrin, calmodulin etc) proved that the probe could work as a good

landmark at least in isolated protein assemblies, without much hampering host proteins' functional properties. We are now improving the visibility of the marker probes so that we might recognize their location more easily within cells *in situ*.

Exploiting such novel methodologies as above; i.e. EM observation, 3-D image analyses and the use of high-resolution marker probes, efficiently combined with conventional crystallography, we aim to capture the instantaneous structure of certain protein assembly at a moment during its function, and analyze the 3-D structure of each component. Together with the information on the dynamics obtained by optical or atomic force microscopy, our final goal is to produce the movie to show the time-sequence of certain biological events as animated atomic models, based on real experimental data. Along that way, we should be able to fully understand the operation mechanisms of the molecular machinery involved in various biological events. Our ultimate aim, "Structural Biology of Single Molecule" could also be the attempt to realize "the Dream of Life Scientists".

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◎分子モジュール

片山栄作，村山尚，樫山拓，小林琢也 出願番号：PCT/JP2007/073482 2007年12月5日

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Division of Molecular Biology
遺伝子動態分野

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RNA no longer stands behind DNA or protein but stands in front of DNA and protein. Recent achievements and discovery in biological science clearly emphasized the importance of RNA in life; the discovery of RNA interference, molecular mimicry between protein and RNA, and ribosome structure at atomic resolution. Moreover, the completed human genome project revealed, to our great surprise, the existence of a large amount of protein-noncoding RNAs (ncRNAs). These ncRNAs can be classified into two types: one, like antisense and microRNA, those function with the sequence complementarity to the target mRNA or DNA, while the other, like aptamer, those function independent of the sequence complementarity. In our laboratory, we aim to: 1) uncover the natural aptamers encoded in human genome; and 2) create artificial aptamers to target proteins of therapeutic interest. By studying these natural and artificial RNA aptamers, we hope to clarify superior potential of RNA, which would be highly beneficial to the development of RNA medicine and the comprehensive understanding of human genome RNA function. In addition to these RNA oriented study, two lines of translation orientated studies are in progress: 1) the molecular mechanism of translation termination and the molecular basis of mimicry between translation factors and tRNA; and 2) the 'prion' nature associated with yeast translation factor Sup35.

1. RNA Aptamers and Therapeutic Applications

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The systematic evolution of ligands by exponential enrichment (SELEX) method is based on the *in vitro* selection of oligo-nucleotide ligands from large random-sequence libraries by repeated reactions of DNA transcription, RNA se-

lection and RT-PCR amplification. The selected oligo-nucleotide ligands are called 'aptamer' which has high affinity and specificity to target molecules. We have initiated SELEX experiments using mammalian translation initiation factors including eIF4E, eIF4G, eIF1A, eIF4AI and eIF4AIII in collaboration with Dr. Nahum Sonenberg (McGill University, Canada) and Dr. John Hershey (UC-Davis, USA). eIF4G and eIF4 AI proteins are known to be crucial for catalyzing the initiation of protein synthesis by playing as a multipurpose ribosome adapter bridging eIF4E (cap-binding protein), eIF3 (40S subunit binding protein), eIF4AI and Pab1p (poly-A binding protein), and an RNA unwinding helicase, respectively. Importantly, the abnormality in the protein level or the activity of either initiation factor is known to cause cell proliferation. We aim to test the possibility of developing anti-eIF RNA aptamers for novel diagnostic and therapeutic tools.

a. Structural and molecular basis for hyperspecificity of RNA aptamer to human immunoglobulin G

Potential applications for functional RNAs are rapidly expanding, not only to address functions based on primary nucleotide sequences, but also by RNA aptamer, which can suppress the activity of any target molecule. Aptamers are short DNA or RNA folded molecules that can be selected *in vitro* on the basis of their high affinity for a target molecule. Here, we demonstrate the ability of RNA aptamers to recognize-and bind to-human IgG with high specificity and affinity. An optimized 23-nucleotide aptamer, apt8-2, was prepared, and was shown to bind to the Fc domain of human IgG, but not to other IgG's, with high affinity. Apt8-2 was observed to compete with protein A, but not with the Fcγ receptor, for IgG binding. NMR chemical shift analyses localized the aptamer-binding sites on the Fc sub-domain, which partially overlaps the protein A binding site but not the Fcγ receptor binding site. The tertiary structures of the predicted recognition sites on the Fc domain differ significantly between human IgG and other species of IgGs; this in part accounts for the high specificity of the selected aptamer. Apt8-2 can therefore be used as a protein A alternative for affinity purification of human IgG and therapeutic antibodies. Using Apt8-2 would have several potential advantages, raising the possibility of developing new applications based on aptamer design.

b. Inhibition of midkine protects against experimental autoimmune encephalomyelitis through the expansion of the $CD4^+CD25^+$ regulatory T cell population

$CD4^+CD25^+$ regulatory T (Treg) cells are crucial mediators of autoimmune tolerance. The factors that regulate Treg cells, however, are largely unknown. Here, we show that mice deficient for midkine (MK), a heparin-binding growth factor involved in oncogenesis, inflammation, and tissue repair, were resistant to experimental autoimmune encephalomyelitis (EAE) due to an expansion of the Treg cell population in peripheral lymph nodes and decreased numbers of autoreactive T-helper type 1 and T-helper-17 cells. Additionally, MK administration to MK-deficient mice abrogated the resistance to EAE and the Treg cell expansion. MK also dose-dependently decreased the Treg cell population ex vivo. Moreover, administration of anti-MK RNA aptamers significantly expanded the Treg cell population and alleviated the symptoms of EAE. These observations indicate that MK serves as a critical suppressor of Treg cells, and inhibition of MK using RNA aptamers may provide an effective therapeutic strategy against autoimmune diseases, including multiple sclerosis.

c. Selection of a novel class of RNA-RNA interaction motifs based on the ligase ribozyme with defined modular architecture

To develop molecular tools for the detection and control of RNA molecules whose functions rely on their three-dimensional (3D) structures, we have devised a selection system to isolate novel RNA motifs that interact with a target RNA structure within a given structural context. In this system, a GAAA tetraloop and its specific receptor motif (11-ntR) from an artificial RNA ligase ribozyme with modular architecture (the DSL ribozyme) were replaced with a target structure and random sequence, respectively. Motifs recognizing the target structure can be identified by *in vitro* selection based on ribozyme activity. A model selection targeting GAAA-loop successfully identified motifs previously known as GAAA-loop receptors. In addition, a new selection targeting a C-loop motif also generated novel motifs that interact with this structure. Biochemical analysis of one of the C-loop receptor motifs revealed that it can also function as an indepenent structural unit.

2. Translation Termination

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Termination of protein synthesis takes place on the ribosomes as a response to a stop, rather than a sense, codon in the'decoding' site (A site). Translation termination requires two classes of polypeptide release factors (RFs): a class-I factor, codon-specific RFs (RF1 and RF2 in prokaryotes; eRF1 in eukaryotes), and a class-II factor, non-specific RFs (RF3 in prokaryotes; eRF3 in eukaryotes) that bind guanine nucleotides and stimulate class-I RF activity. The underlying mechanism for translation termination represents a long-standing coding problem of considerable interest since it entails protein-RNA recognition instead of the well-understood codon-anticodon pairing during the mRNA-tRNA interaction.

a. The role of N-terminal domain of translational release factor eRF3 for the control of functionality and stability in *S. cerevisiae*

Eukaryotic release factor eRF1 recognizes all three stop codons and induces polypeptide release, while eRF3 binds to eRF1 and participates in translation termination. However, the regulatory role of eRF3 is still unknown. Importantly, eRF3 interacts with various proteins of distinct biological functions. Here, we investigated the effect of these binding factors on functionality and stability of eRF3 using a temperature-sensitive mutant eRF3ts, which is susceptible to factor binding to change the growth phenotype or cellular protein level. Of factors tested, Itt1 over-expression and Sla1 knockout severely impaired viability of eRF3ts cell and its protein abundance in permissive and semi-permissive conditions. Sla1 over-expression reversed the phenotype. Itt1 and Sla1 bind to the N-terminal extension domain (NED) of eRF3, unlike the other no-effect factors that bind to the C-terminal domain. Although NED itself is dispensable, NED-less eRF3 altered in the stability and functionality. Moreover, Itt1-induced eRF3ts lethality was significantly restored by *pep4*, *prb1*, and *prc1* knockouts that are defective in vacuolar proteolysis. These findings suggest that NED functions to switch the functional mode of eRF3 depending on the nature of binding factors.

3. Yeast Prion

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The Sup35 protein of the budding yeast *Saccharomyces cerevisiae* is a subunit of the eukaryotic polypeptide-release factor (eRF3) and is essential for terminating protein synthesis at stop codons. Sup35p also exists as a stable amyloid fibril, termed [PSI^+], that propagates its aberrant fold in the cytoplasm in a manner analogous to the "protein only" transmission of mammalian prion protein (PrP). [PSI^+] cells are marked by an altered protein conformation of Sup35p whereby the protein is converted from a soluble, active state to an aggregated inactive state. In the aggregated state, ribosomes often fail to release polypeptides at stop codons, causing a non-Mendelian trait easily detected by the suppression of nonsense mutations. Thus, the conversion of soluble Sup35p [psi^-] to the aggregated form [PSI^+] serves as a useful model for studying the formation of amyloid deposits and the prion-like transmission of an altered protein conformation.

a. A Regulatory role of the Rnq1 non-prion domain for prion propagation and polyglutamine aggregates

Rnq1 is required for the yeast prion [PIN^+], which is necessary for the de novo induction of a second prion, [PSI^+]. Here we isolate a [PSI^+]-eliminating mutant, Rnq1Δ100, that deletes the non-prion domain of Rnq1. Rnq1Δ100 inhibits not only [PSI^+] prion propagation but also [*URE 3*] prion and huntingtin's polyglutamine aggregate propagation in a [PIN^+] background, but not in a [pin^-] background. Rnq1Δ100, however, does not eliminate [PIN^+]. These findings are interpreted as showing a possible involvement of Rnq1 prion in the maintenance of heterologous prions and polyQ aggregates. Rnq1 and Rnq1Δ 100 form a SDS-stable and Sis1 (a Hsp40 chaperone protein)-containing co-aggregate in [PIN^+] cells. Importantly, Rnq1Δ100 is highly QN-rich and prone to self-aggregate or co-aggregate with Rnq1 when co-expressed in [pin^-] cells. However, the [pin^-] Rnq1-Rnq1Δ100 co-aggregate does not represent a prion-like aggregate. These findings suggest that [PIN^+] Rnq1-Rnq1Δ100 aggregates interact with other transmissible and non-transmissible amyloids to destabilize them, and that the non-prion domain of Rnq1 plays a crucial role to self-regulate the highly reactive QN-rich prion domain of Rnq1.

b. Genetic mutation study of Hsp104 for thermotolerance and prion-specific propagation

We constructed an auxotrophic chromosomal marker, *ura3-197* (nonsense allele), applicable to

selection for loss of [*PSI*$^+$] to [*psi*$^-$]. Unlike [*psi*$^-$] yeast strains, [*PSI*$^+$] yeast strains exhibit nonsense suppression of the *ura3-197* allele and are not viable in the presence of 5-fluoroorotic acid (5-FOA) that is converted to a toxic material by the readthrough product of Ura3. We selected twenty 5-FOA resistant, loss-of-[*PSI*$^+$], mutants spontaneously or by transposon-mediated mutagenesis from *ura3-197* [*PSI*$^+$] cells. All of the 20 [*psi*$^-$] isolates were affected in Hsp104, a protein-remodeling factor. Although most of them were disabled in a normal Hsp104 function for thermotolerance, three single mutants, L 462R, P557L and D704N, remained thermotolerant. Importantly, L462R and D704N also eliminate other yeast prions [*URE3*] and [*PIN*$^+$], while P557L does not, suggesting that Hsp104 harbors a unique activity to prion propagation independent of its function in thermotolerance. The mutations that are specific to prion propagation are clustered around the lateral channel of the Hsp104 hexamer, suggesting a crucial and specific role of this channel for prion propagation.

To gain further insight into the structure-and-function relationship of Hsp104 for [*PSI*$^+$], we engineered 58 chromosomal *hsp104* mutants that affect residues considered structurally or functionally relevant to Hsp104 remodeling activity, yet most remain to be examined for their significance to [*PSI*$^+$] in the same genetic background. Many of these *hsp104* mutants were affected both in thermotolerance and [*PSI*$^+$] propagation. However, nine mutants were impaired exclusively for [*PSI*$^+$], while two mutants were impaired exclusively for thermotolerance. Mutations exclusively affecting [*PSI*$^+$] are clustered around the lateral channel of the Hsp104 hexamer. These findings suggest that Hsp104 possesses shared as well as distinct remodeling activities for stress-induced protein aggregates and [*PSI*$^+$] prion aggregates and that the lateral channel plays a role specific to [*PSI*$^+$] prion propagation.

4. Human and mouse protein non-coding snoRNA host genes with dissimilar nucleotide sequences show chromosomal synteny

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snoRNAs are small protein non-coding RNAs essential for pre-rRNA processing and ribosome biogenesis, and are encoded intronically in host genes (HGs) that are either protein-coding or non-coding. mRNAs of protein non-coding HGs differ in their nucleotide sequences among species. Although the reason for such sequential divergence has not been well explained, we have evidence here that such structurally different HGs have evolved from a common ancestral gene. We first identified two novel protein non-coding HGs (mU50HG-a and -b) that intronically encode a mouse orthologue of a human snoRNA, hU50. The sequences of mU50HG mRNA differed from that of hU50HG. However, a chromosome mapping study revealed that mU 50HGs is located at 9E3-1, the murine segment synteneic to human 6q15 where hU50HG is located. Synteny is a phenomenon whereby gene orthologues are arranged in the same order at equivalent chromosomal loci in different species; synteny between two species means it is highly likely that the genes have evolved from a common ancestral gene. We then extended this mapping study to other protein non-coding snoRNA HGs, and found again that they are synteneic, implying that they have evolved from genes of common ancestral species. Furthermore, on these synteneic segments, exons of adjacent protein-coding genes were found to be far better conserved than those of non-coding HGs, suggesting that the exons of protein non-coding snoRNA-HGs have been much more fragile during evolution.

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