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. Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways

Kyoko Arimoto, Hiroyuki Fukuda[1], Shinobu Imajoh-Ohmi[1], Haruo Saito, and Mutsuhiro Takekawa: [1]Medical Proteomics Laboratory, IMSUT.

Confronted with environmental stress, cells either activate defense mechanisms to survive, or initiate apoptosis, depending on the type of the stress. Certain stresses (henceforth called type 1) such as hypoxia, heatshock, and arsenite induce cells to assemble cytoplasmic stress granules (SGs), which are multimolecular aggregates of stalled translation preinitiation complexes. In contrast, type 2 stresses such as X-rays and genotoxic drugs induce apoptosis through the stress-activated p38 and JNK MAPK (SAPK) pathways. The core of the SAPK pathways is composed of three tiers of protein kinases, namely, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. Various stress-responsive MAPKKKs are known, including MEKK1, MEKK2, MEKK3, MTK1 (MEKK4), MLKs, TAK1, and ASK1, allowing cells to respond to various stimuli. Functional relationship between the SG and SAPK responses has been unknown.

MTK1 is a major mediator of stress-induced apoptosis. In unstimulated cells, MTK1 is kept catalytically inactivate through an auto-inhibitory interaction between the auto-inhibitory domain and the kinase domain. MTK 1 activation is initiated by GADD45 family proteins whose expression is induced by stress conditions. GADD45 binds a site in MTK1 near its N-terminal auto-inhibitory domain, thereby releasing the kinase from inhibition. To study mechanisms by which MTK1 kinase activity is regulated, we identified proteins that bind to MTK1 by mass spectrometry. LC-MS-MS analyses revealed that one MTK1 associated protein is RACK1, a 36-kDa Gβ-like scaffold protein. RACK1 did not bind other MAPKKs or MAPKs.

Using a series of Myc-MTK1 deletion mutants, the RACK1 binding domain in MTK1 was defined (residues 22-371). This region partially overlaps with the GADD45-binding domain (residues 147-250) and the auto-inhibitory domain (residues 250-553). Depleting endogenous

RACK1 by shRNAi strongly inhibited MTK1 activation induced either by various stress stimuli. Thus, RACK1 is specifically required for MTK1 activation. However, we also found that RACK1 is by itself insufficient for MTK1 activation. Through a series of binding experiments, we concluded that RACK1 maintained MTK1 in a dimeric, but yet inactive, form in the absence of any stress. Preformation of such an inactive dimer facilitates the activating MTK1 dimerization induced by GADD45.

MTK1 co-localized with endogenous RACK1 in the cytoplasm of unstimulated cells. However, specific cellular stresses, such as arsenite, induced the formation of granular structures containing RACK1 within the cytoplasm, which we found to be stress granules (SGs). MTK1 remained diffuse throughout the cytoplasm. The strong activator of the stress-responsive MAPK pathways, MMS, does not induce SG formation. Thus, we could recognize two types of stresses: type 1 stresses that preferentially induce SG formation, and type 2 stresses that preferentially activate the SAPK cascades.

Because RACK1 knock-down suppressed MTK1 activation, we expected that stress-induced sequestration of RACK1 into SGs would also inhibit MTK1. Indeed, induction of SG formation by co-expression of GFP-G3BP, or by arsenite treatment, repressed MTK1 activation. These data support the idea that the formation of SGs inhibits MTK1 activation by preventing RACK1-MTK1 association. The formation of SGs also inhibited GADD45β-induced apoptosis, which is mediated, at least in part, by MTK1 activation.

In solid tumors, cancer cells rapidly outgrow their vascular supply and develop hypoxic microenvironments. Tumor hypoxia is a critical therapeutic problem as it renders tumor cells resistant to apoptotic cell death induced by radiation and chemotherapeutics. Because MTK1 mediates apoptosis as well as activation of p38 and JNK induced by anti-cancer drugs such as etoposide (Takekawa *et al*., unpublished results), we determined if inhibition of MTK1 by SG assembly might be involved in hypoxia-induced resistance to etoposide. Exposure of cells to hypoxia (0.5% O_2) induced the recruitment of RACK1 into SGs. Concurrently, hypoxia reduced etoposide-induced activation of p38 and JNK, and the resulting proteolytic cleavage of caspase 3. Altogether, our results suggested that resistance to etoposide-induced apoptosis in hypoxic cells was mediated, at least in part, by the sequestration of RACK1 into SGs.

We thus demonstrated that RACK1 is incorporated into SGs following specific stresses, and thus mediates a novel crosstalk between the SG assembly-cell survival pathway and the stress-responsive MAPK-cell death pathway. When cells are exposed to type 2 stresses, such as etoposide, MTK1 activates the p38 and JNK pathways, leading to apoptosis. In contrast, when cells are exposed to type 1 stresses, such as hypoxia, SGs are formed, thereby promoting cell survival by preventing the accumulation of misfolded proteins. When both types of stresses are simultaneously applied, sequestration of RACK1 into SGs by a type 1 stress suppresses the apoptosis induced by a type 2 stress. Cancer cells may exploit this regulatory system to acquire resistance to anticancer therapies.

2. Phosphorylated Ssk1 prevents unphosphorylated Ssk1 from activating the Ssk2 MAP kinase kinase kinase in the yeast HOG osmoregulatory pathway

Tetsuro Horie, Kazuo Tatebayashi, Rika Yamada, and Haruo Saito

The budding yeast *Saccharomyces cerevisiae* survives widely fluctuating osmotic conditions in their natural habitat. To cope with an increased external osmolarity, yeast cells synthesize, and intracellularly retain, the compatible osmolyte glycerol. Osmo-stressed cells also temporarily arrest cell cycle progression and halt protein synthesis, during which they readjust to the changed environment. These events are governed by the High Osmolarity Glycerol (HOG) signaling pathway, whose core element is the Hog1 MAPK cascade. Defects in the HOG pathway cause severe osmosensitivity in cell growth.

The Hog1 MAPK cascade is regulated by the functionally redundant, but mechanistically distinct, upstream signaling pathways, termed the SHO1 branch and the SLN1 branch. A signal emanating from either branch converges on a common MAPKK termed Pbs2, which is the specific activator of the Hog1 MAPK. In the SHO1 branch, two putative transmembrane (TM) osmosensors Msb2 and Hkr1 detect osmotic stress, and, together with the membrane anchor protein Sho1, generate an intracellular signal that leads to activation of the Ste11 MAPKKK, which activates the Pbs2 MAPKK. In the SLN1 branch, the TM histidine kinase Sln1 detects turgor changes and transmits a signal via the Sln1-Ypd1-Ssk1 multi-step phospho-relay system. Ssk1 is an activator of the redundant Ssk2 and Ssk22 MAPKKKs, which activate the Pbs2 MAPKK.

The Sln1-Ypd1-Ssk1 multi-step phospho-relay is a variation of so-called prokaryotic two-component signaling systems. In the yeast Sln1-Ypd1-Ssk1 multi-step phosphorelay, the basic

His-Asp phosphorelay reaction is duplicated so that a phosphoryl group is carried in the sequence His-Asp-His-Asp. Sln1 is structurally similar to many bacterial sensor HKs in that it has two TM domains and a cytoplasmic HK domain. It differs, however, from more simple HKs in that it also contains a C-terminal phospho-accepting receiver (Rec) domain within the molecule. The Sln1 histidine kinase activity is repressed when cells are placed under hyperosmotic stress. In unstressed cells Sln1 autophosphorylates at His576, then transfers the phosphoryl group to Asp1144 in its C-terminal Rec domain. The phosphate in Sln1-Asp1144～P is then transferred to His64 of the intermediary protein Ypd1 and finally to Asp554 of another Rec domain protein, Ssk1. Ssk1 is a molecular switch whose activity is controlled by its phosphorylation state. Specifically, it is believed that unphosphorylated Ssk1 (Ssk1-OH) is the active form, and that Ssk1～P, phosphorylated at Asp 554 by the Sln1-Ypd1-Ssk1 multistep phosphorelay mechanism, is the inactive form.

This year, we showed that constitutive activation of Ssk2 occurred when Ssk1 phosphorylation was blocked either by an Ssk1 mutation at the phosphorylation site, or by an Ssk1 mutation that inhibited its interaction with Ypd1, the donor of phosphate to Ssk1. Thus, Ssk1-OH is necessary for Ssk2 activation. However, overexpression of wild-type Ssk1, or an Ssk1 mutant that cannot bind Ssk2, prevented constitutively active Ssk1 mutants from activating Ssk2. Therefore, Ssk1 has a dual function as both an activator of Ssk2 and an inhibitor of Ssk1 itself. We also found that Ssk1 exists mostly as a dimer within cells. From mutant phenotypes, we deduce that only the Ssk1-OH/Ssk1-OH homodimer can activate Ssk2 efficiently, whereas the Ssk1-OH/Ssk1～P hetero-dimer cannot. Hence, because Ssk1～P binds to and inhibits Ssk1-OH, moderate fluctuation of the level of Ssk1-OH does not lead to nonphysiological, and potentially detrimental, activation of Hog1. In conclusion, we uncovered a novel role for Ssk1 as an inhibitor of Ssk2 activation, in addition to its role as an Ssk2 activator.

3. Two adjacent docking sites in the yeast Hog1 MAP kinase differentially interact with the Pbs2 MAP kinase kinase and the Ptp2 protein tyrosine phosphatase

Yulia Murakami, Kazuo Tatebayashi, and Haruo Saito

Five different MAPKs have been described in the budding yeast, and more than 10 in mammals. Different external stimuli, such as mitogenic growth factors, pro-inflammatory cytokines, and osmotic and oxidative stresses, activate distinct subsets of MAPKs. MAPKs are activated through a kinase cascade in which an activated MAPKKK phosphorylates, and thus activates a MAPKK. An activated MAPKK then phosphorylates and activates a MAPK. Because there are also large numbers of MAPKKs and MAPKKKs in each organism, the potential number of MAPKKK-MAPKK-MAPK combinations is enormous. However, only a small subset of the potential combinations is actually activated upon a specific stimulus, suggesting that specific MAPKKK-MAPKK-MAPK interactions are tightly regulated.

Several mechanisms exist to ensure highly selective recognition among the three tiers of kinases belonging to a single MAPK cascade. Specific substrate-enzyme interaction, *i.e.* recognition of the substrate phosphorylation site(s) by the kinase catalytic site, is obviously important. This specificity, however, is inadequate to select a unique substrate, because multiple species of MAPKs possess similar phosphorylation site sequences (the T-X-Y motif). Similarly, substrates of MAPKs have a relatively simple substrate-phosphorylation-site specificity (the S/T-P motif), which is too simple to be selective. Thus, MAPKK-MAPK specificity, as well as MAPK-substrate specificity, is enhanced by specific docking interactions. Each MAPK has a site termed the Common Docking (CD) domain, which interacts with its specific activator (MAPKK), inactivator (phosphatase), and substrates.

This year, we investigated the mechanism by which the yeast Hog1 MAPK specifically interacts with its activator, the Pbs2 MAPKK, and its major inactivator, the Ptp2 protein tyrosine phosphatase. We found, in the N-terminal noncatalytic region of Pbs2, a specific Hog1-binding domain, termed HBD-1. We also defined two adjacent Pbs2-binding sites in Hog1, namely the CD domain and the Pbs2-binding domain-2 (PBD-2). The PBD-2 docking site appears to be sterically blocked in the intact Hog1 molecule, but its affinity to Pbs2 is apparent in shorter fragments of Hog1. Both the CD and the PBD-2 docking sites are required for optimal activation of Hog1 by Pbs2, and in the absence of both sites, Hog1 cannot be activated by Pbs2. These data suggest that the initial interaction of Pbs2 with the CD site might induce a conformational change in Hog1 so that the PBD-2 site becomes accessible. The CD and PBD-2 docking sites are also involved in the specific interaction between Hog1 and Ptp2, and govern the dynamic dephosphorylation of activated Hog1.

Based on experimental data, we proposed the

following model for functional interaction among Pbs2, Hog1, and Ptp2. Initially, Hog1 binds Pbs2 via the CD domain (Step I). A conformational change in the L16 segment of Hog1 exposes the PBD-2 site for a more stable interaction with Pbs2 (Step II). Phosphorylation of Hog 1 then ensues (Step III). Phosphorylated Hog1 (P -Hog1) binds Ptp2, again via the CD domain (Step IV). A conformational change in the L16 segment allows for an additional interaction between P-Hog1 and Ptp2 (Step V). Thus, phospho -Tyr176 in P-Hog1 is productively aligned with the catalytic center (Cys666) of Ptp2. As the tyrosine residue is dephosphorylated, Ptp2 detaches itself from Hog1 (Step VI). According to this model, the Ptp2-C/S mutant is trapped at Step V, whereas wild-type Ptp2 is usually released from Hog1 in Step VI. Although this model requires further elaboration, it appears to capture the essential aspects of Pbs2-Hog1-Ptp2 interaction.

Publications

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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 have recently been neglected in Japan despite of increasing importance due to globalization and global warming. During last 10 years, we have focused on transcriptome analysis of parasites and arthropods, using full-length cDNA libraries. We have determined sequences that have been compiled in databases (http://fullmal.ims.u-tokyo.ac.jp) and utilized for genome annotations. Ultra-deep gene expression analysis is now expected to elucidate parasite-host/vector relationship, while large-scale sequencing will shed light on molecular epidemiology.

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). Entamoeba is common cause of diarrhea in tropical regions and now classifed as unikonta which includes human and fungi. Helminth are multicellular parasites including Cestodes, Trematodes and Nematodes. 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.

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

Full-Malaria/Parasites and Full-Arthropods

Hiroyuki Wakaguri[1], Yutaka Suzuki[1], Toshiaki Katayama[2], Shuichi Kawashima[2], Eri

Kibukawa[3], Kazushi Hiranuka[2], Masahide Sasaki[1], Sumio Sugano[1], Junichi Watanabe: [1]Graduate School of Frontier Sciences, [2]Institute of Medical Science, The University of Tokyo, [3]STS

Full-Malaria/Parasites is compilation of full-length cDNA databases describing the 5′-end-one-pass sequences from various parasites mapped onto their genome sequences and Full-Arthropods is that of medically important arthropods found at http://fullmal.ims.u-tokyo.ac.jp. It now contains the entire sequences of the representative clones determined using the new-generation sequencers (full-length sequence of full-length cDNA clones). It also includes millions of TSS (transcription start site) tags that represent the expressed genes.

Full-Apicomplexa

Ikuo Igarashi[4], Yoshifumi Nishikawa[4], Xuenan Xuan[4], Chihiro Sugimoto[5], Damer Blake[6], Xikun Wu[6], Fiona Tomley[6], Kiew-Lian Wan[7]: [4]Obihiro University of Agriculture and Veterinary Medicine, [5]Hokkaido University, [6]Institute for Animal Health, UK, [7]Malaysia Genome Institute, Malaysia

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 comparative analysis of transcriptomes of *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

Masahiro Kumagai[8], Kazushi Hiranuka, Toshiaki Katayama, Junichi Watanabe, Hiroyuki Wakaguri, Yutaka Suzuki, Sumio Sugano, Atsushi Toyoda[9] and Asao Makioka[8]: [8]Jikei University School of Medicine, [9]National Institute of Genetics of Japan

Full-Entamoeba, a database for full-length cDNAs from a human parasite, *Entamoeba histolytica* has been produced (http://fullent.hgc.jp/). The full-length cDNA library was produced using the oligo-capping method from axenically cultivated trophozoites. A total of 5,000 5′-end-one-pass sequences of cDNAs were mapped onto the genome contig sequences.

Full-Echinococcus

Yuzaburo Oku[5], Nariaki Nonaka[5], Jun Matsumoto[10], Masao Kamiya[11], Atsushi Toyoda, Kinpei Yagi[12], Li Shuang[2], Ryu Yamashita[2], Yuichiro Hara[5], Hidemi Watanabe[5]: [5]Hokkaido University , [10]Nihon University , [11]Rakuno Gakuen University, [12]Hokkaido Institute of Public Health, [2]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 produced a full-length cDNA library from *Echinococcus multilocularis* adult worms collected from infected dogs using the olio-capping method and determined 5′-end and 3′-end-one-pass sequences. These sequences are currently being compared with those of larva.

Full-Taenia

Raul Bobes[13], Juan Laclette[13], Masahira Hattori[1]: [13]National Autonomous University of Mexico, [1]Graduate School of Frontier Sciences, The University of Tokyo

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[4], Miho Usui[4], Sadao Nogami[10], Shinichi Noda[14], Chihiro Sugimoto, Masahira Hattori, Aksoy Serap[15], Todd Taylor[16], Shrama Vineet[16], Eri Kibukawa, Toshiaki Katayama, Kazuhisa Hiranuka and Shuichi Kawashima: [4]Obihiro University of Agriculture and Veterinary Medicine, [10]Nihon University, [14]Kagoshima University, [6]Hokkaido University, [15]Yale University, [16]Riken

We have produced full-length cDNA libraries from adult and larva of *Anopheles stephensi* that

were propagated in the lab and determined 5′-end and 3′-end-one-pass sequences, which have been compiled in a database Full-Anopheles. Full-Arthropods also includes Full-Tsetse and Full-Mite.

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 are expanding our collaborative studies on malaria with Indonesian medical doctors to study molecular epidemiology. Unique geography with numerous islands scattered in the vast ocean should provide ideal fields to study disease transmission.

Coelacanth study

Hiroshi Koie[10], Alex Masengi[17], Janny Kusen[17], Josef Tuda[17], Boetje Moningka[17], Mihoko Imada[18], Masamitsu Iwata[19], Yoshitaka Abe[19]: [10]Nihon University, [17]Sam Ratulangi University, [18]Keio University, [19]Aquamarine Fukushima

Third coelacanth was caught alive in Manado Bay near our malaria research field in 2008 and frozen after death. We also participated in dissection of African coelacanth for comparative studies. Future studies will help elucidate the process of terrestrial evolution in tetrapods.

Publications

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Database

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

Full-Entamoeba
http://fullent.hgc.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. Characteristic inhibitory modulation of synaptic plasticity in the dentate gyrus of the rat hippocampus

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

The hippocampus is essential for the formation of certain types of memory, and synaptic plasticity such as long-term potentiation (LTP) is widely accepted as a cellular and molecular basis of hippocampus-dependent memory. Although LTP in both perforant path-dentate gyrus (DG) granule cell and CA3-CA1 pyramidal cell synapses is similarly dependent on activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors, several reports suggest that modulation of LTP by γ-aminobutyric acid (GABA) receptor-mediated inhibitory inputs is stronger in perforant path-DG granule cell synapses. However, little is known about how different the mechanism and physiological relevance of the GABAergic modulation of LTP induction among different brain regions are. We confirmed that the action of $GABA_A$-receptor antagonists on LTP was more prominent in the DG, and explored the mechanism introducing such difference by examining two types of inhibition, synaptic and tonic inhibition, caused by $GABA_A$ receptors. As synaptic inhibition, we compared inhibitory versus excitatory monosynaptic responses and their summation during an LTP-inducing stimulus, and found that the balance of the summated postsynaptic currents was biased toward inhibition in the DG. As tonic inhibition, or sustained activation of extrasynaptic $GABA_A$ receptors by ambient GABA, we measured the change in holding currents of the postsynaptic cell induced by $GABA_A$-receptor antagonists, and found that the tonic inhibition was significantly stronger in the DG. Taken together, our results suggest that both the larger tonic inhibition and the larger inhibitory/excitatory summation balance during conditioning are involved in the stronger inhibition of LTP in the DG.

2. Functional contributions of synaptically localized NR2B subunits of the NMDA receptor to synaptic transmission and long-term potentiation in the adult mouse central nervous system

Hideki Miwa, Masahiro Fukaya[1], Ayako M. Watabe, Masahiko Watanabe[1] and Toshiya Manabe: [1]Department of Anatomy, Hokkaido University School of Medicine

The NMDA-type glutamate receptor is a heteromeric complex composed of the NR1 and at least one of NR2 subunits. Switching from the NR2B to NR2A subunit is thought to underlie functional alteration of the NMDA receptor during synaptic maturation, and it is generally believed that it results in preferential localization of NR2A subunits on the synaptic site and that of NR2B subunits on the extracellular site in the mature brain. It has also been proposed that activation of the NR2A and NR2B subunits results in long-term potentiation (LTP) and long-term depression (LTD), respectively. Furthermore, recent reports suggest that synaptic and extrasynaptic receptors may have distinct roles in synaptic plasticity as well as in gene expression associated with neuronal death. Here, we have investigated whether NR2B subunit-containing receptors are present and functional at mature synapses in the lateral nucleus of the amygdala (LA) and the CA1 region of the hippocampus, comparing their properties between the two brain regions. We have found, in contrast to the above hypotheses, that the NR2B subunit significantly contributes to synaptic transmission as well as LTP induction. Furthermore, its contribution is greater in the LA than in the CA1 region, and biophysical properties of NMDA receptors and the NR2B/NR2A ratio are different between the two brain regions. These results indicate that NR2B subunit-containing NMDA receptors accumulate on the synaptic site and responsible for the unique properties of synaptic function and plasticity in the amygdala.

3. Non-Hebbian synaptic plasticity physiologically induced in the hippocampus

Hiroyuki Kato, Ayako M. Watabe and Toshiya Manabe

Modern theories on memory storage have mainly focused on Hebbian long-term potentiation (LTP), which requires coincident activation of pre- and postsynaptic neurons for its induction. In addition to Hebbian LTP, the roles of non-Hebbian plasticity have also been predicted by some neuronal network models. However, still only a few pieces of evidence have been presented for the presence of such plasticity. In this study, we show in mouse hippocampal slices that LTP can be induced by postsynaptic repetitive depolarization alone in the absence of presynaptic inputs. The induction was dependent on voltage-dependent calcium channels (VDCCs) instead of N-methyl-D-aspartate receptors (NMDARs), while the expression mechanism was shared with conventional NMDAR-dependent LTP. During the potentiation, the amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) was increased, suggesting novel neuron-wide nature of this form of LTP. Furthermore, we also successfully induced LTP with trains of action potentials, which supported physiological relevance of the depolarizing pulse-induced LTP. Based on these findings, we suggest a model in which neuron-wide LTP works in concert with synapse-specific Hebbian plasticity to help information processing in memory formation.

4. Behavioral analysis of Plexin-A2 knockout mice

Yuji Kiyama, Fumikazu Suto[2], Hajime Fujisawa[3] and Toshiya Manabe: [2]Department of Developmental Neurobiology, Tohoku University Graduate School of Medicine, [3]Division of Biological Science, Nagoya University Graduate School of Science

Hippocampal mossy fibers project preferentially to the stratum lucidum, the proximal-most lamina of the suprapyramidal region of the CA3 region in the hippocampus. 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 our previous study, we found that the projection of mossy fibers was disrupted in mutant mice for semaphorin receptors, plexin-A2 and plexin-A4. Mossy fibers were projected widely within CA3 in plexin-A4 mutant animals, while failed to invade the suprapyramidal region and instead were shifted to the infrapyramidal region and the stratum pyramidalis in plexin-A2 mutant animals. We also reported that the plexin-A2 loss-of-function phenotype was genetically suppressed by Sema6 A loss-of-function. Based on these results and results of cell biological approaches, we provided a model for the lamina-restricted projection of mossy fibers, that is, mossy fibers are endowing with plexin-A4 and thereby suppressed to invade the Sema6A-expressing suprapyramidal region of CA3, but can grow into the proximal parts of the region where Sema6A activities are masked by plexin-A2. In this study, we performed behavioral experiments and found that plexin-A2 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. Use-dependent amplification of presynaptic Ca^{2+} signaling by axonal ryanodine receptors at the hippocampal mossy fiber synapse

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Presynaptic Ca^{2+} stores have been suggested to regulate Ca^{2+} dynamics within the nerve terminals at certain types of the synapse. However, little is known about their mode of activation, molecular identity, and detailed subcellular localization. Here, we show that the ryanodine-sensitive stores exist in axons and amplify presynaptic Ca^{2+} accumulation at the hippocampal mossy fiber synapses, which display robust presynaptic forms of plasticity. Caffeine, a potent drug inducing Ca^{2+} release from ryanodine-sensitive stores, causes elevation of presynaptic Ca^{2+} levels and enhancement of transmitter release from the mossy fiber terminals. The blockers of ryanodine receptors, TMB-8 or ryanodine, reduce presynaptic Ca^{2+} transients elicited by repetitive stimuli of mossy fibers, but do not affect those evoked by single shocks, suggesting that ryanodine receptors amplify presynaptic Ca^{2+} dynamics in an activity-dependent manner. Furthermore, we generated the specific antibody against the type 2 ryanodine receptor (RyR2; originally referred to as the cardiac type), and examined the cellular and subcellular localization using immunohistochemistry. RyR2 is highly expressed in the stratum lucidum of the CA3 region and mostly co-localizes with axonal marker NF160 but not with terminal marker VGLUT1. Immunoelectron microscopy revealed that RyR2 is distributed around smooth ER within the mossy fibers, but is almost excluded from their terminal portions. These results suggest that axonal localization of RyR2 at sites distant from the active zones enables use-dependent Ca^{2+} release from intracellular stores within the mossy fibers, and thereby facilitates robust presynaptic forms of plasticity at the mossy fiber-CA3 synapse.

6. Dual inhibition of SNARE complex formation by tomosyn ensures controlled neurotransmitter release

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Neurotransmitter release upon SNARE complex-mediated synaptic vesicle fusion is spatially and temporally regulated in presynaptic terminals. Tomosyn is known to bind syntaxin-1 through the C-terminal VAMP-like domain and thereby inhibits formation of the SNARE complex. Here, we found another inhibitory action of tomosyn against the SNARE function. The N-terminal WD-40 repeats domain of tomosyn had an intrinsic ability to catalyze oligomerization of the SNARE complex. Reduced oligomerization of the SNARE complex in tomosyn knockout mice increased probability of neurotransmitter release, suggesting that the oligomerized SNARE complex has an inhibitory effect on the neurotransmitter release. These results indicate that tomosyn inhibits the SNARE-dependent synaptic vesicle fusion by both the N-terminal WD-40 repeats domain-mediated oligomerization of the SNARE complex and the C-terminal VAMP-like domain-based competitive inhibition of the SNARE complex formation, resulting in potent inhibition of neurotransmitter release.

7. Ablation of NMDA receptors enhances the excitability of hippocampal CA3 neurons

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Synchronized discharges in the hippocampal CA3 recurrent network are supposed to underlie network oscillations, memory formation and seizure generation. In the hippocampal CA3 network, NMDA receptors are abundant at the re-

current synapses but scarce at the mossy fiber synapses. We generated mutant mice in which NMDA receptors were abolished in hippocampal CA3 pyramidal cells by postnatal day 14. The histological and cytological organizations of the hippocampal CA3 region were indistinguishable between control and mutant mice. We found that mutant mice lacking NMDA receptors selectively in CA3 pyramidal cells became more susceptible to kainate-induced seizures. Consistently, mutant mice showed characteristic large EEG spikes associated with multiple unit activities, suggesting enhanced synchronous firing of CA3 neurons. The electrophysiological balance between fast excitatory and inhibitory synaptic transmission was comparable between control and mutant pyramidal cells in the hippocampal CA3 region, while the NMDA receptor-slow after-hyperpolarization coupling was diminished in the mutant neurons. In the adult brain, inducible ablation of NMDA receptors in the hippocampal CA3 region by the viral expression vector for Cre recombinase also induced similar large EEG spikes. Furthermore, pharmacological blockade of CA3 NMDA receptors enhanced the susceptibility to kainate-induced seizures. These results raise an intriguing possibility that hippocampal CA3 NMDA receptors may suppress the excitability of the recurrent network as a whole *in vivo* by restricting synchronous firing of CA3 neurons.

8. Requirement of the immediate early gene vesl-1S/homer-1a for fear memory formation

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The formation of long-term memory and the late phase of long-term potentiation (L-LTP) depend on macromolecule synthesis, translation, and transcription in neurons. *vesl-1S* (*V*ASP/*E*na-related gene upregulated during seizure and *L*TP, also known as *homer-1a*) is an LTP-induced immediate early gene. The short form of Vesl (Vesl-1S) is an alternatively spliced isoform of the *vesl-1* gene, which also encodes the long form of the Vesl protein (Vesl-1L). Vesl-1L is a postsynaptic scaffolding protein that binds to and modulates the metabotropic glutamate receptor 1/5 (mGluR1/5), the IP3 receptor, and the ryanodine receptor. Vesl-1 null mutant mice show abnormal behavior, which includes anxiety- and depression-related behaviors, and an increase in cocaine-induced locomotion; however, the function of the short form of Vesl in behavior is poorly understood because of the lack of short-form-specific knockout mice. In this study, we generated short-form-specific gene targeting (KO) mice by knocking in part of *vesl-1L/homer-1c* cDNA. Homozygous KO mice exhibited a normal spine number and morphology. Using the contextual fear conditioning test, we demonstrated that memory acquisition and short-term memory were normal in homozygous KO mice. In contrast, these mice showed impairment in fear memory consolidation. Furthermore, the process from recent to remote memory was affected in homozygous KO mice. Interestingly, reactivation of previously consolidated fear memory attenuated the conditioning-induced freezing response in homozygous KO mice, which suggests that the short form plays a role in fear memory reconsolidation. General activity, emotional performance, and sensitivity to electrical footshock were normal in homozygous KO mice. These results indicate that the short form of the Vesl family of proteins plays a role in multiple steps of long-term, but not short-term, fear memory formation.

9. Critical involvement of tyrosine phosphorylation of the NR2A subunit of NMDA receptors in depression-related behavior

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Growing evidence implicates the glutamate signaling in depression, though the molecular mechanism by which the glutamate signaling regulates depression-related behavior has remained unclear. Here, we provide evidence suggesting that tyrosine phosphorylation of the NMDA receptor, an ionotropic glutamate receptor, contributes to depression-related behavior. The NR2A subunit of the NMDA receptor is tyrosine-phosphorylated, with Tyr-1325 as its major phosphorylation site. We have generated mice expressing mutant NR2A with Tyr-1325-

Phe (Y1325F) mutation. The homozygous knockin mice show significantly less immobility in the tail suspension test and the forced swim test. In the striatum of the knockin mice, DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32kD) phosphorylation at Thr-34, involved in regulation of depression-related behavior, is increased. We also show that Tyr-1325 phosphorylation site is required for Src-induced potentiation of the recombinant NMDA receptor channel. These data argue that Tyr-1325 phosphorylation regulates the NMDA receptor channel properties to modulate the NMDA receptor-mediated downstream signaling and depression-related behavior.

10. Kinase-dead knock-in mouse revealed an essential role of CaMKIIα kinase activity in dendritic spine enlargement, LTP and learning

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Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) is an essential mediator of activity-dependent synaptic plasticity that possesses multiple protein functions. So far, the autophosphorylation site-mutant mice targeted at T286 and at T305/306 have demonstrated the importance of the autonomous activity and Ca^{2+}/calmodulin-binding capacity of CaMKIIα, respectively, in the induction of long-term potentiation (LTP) and hippocampus-dependent learning. However, kinase activity of CaMKIIα, the most essential enzymatic function, has not been genetically dissected yet. Here, we generated a novel CaMKIIα knock-in mouse that completely lacks its kinase activity by introducing K42R mutation, and examined the effects on hippocampal synaptic plasticity and behavioral learning. In homozygous CaMKIIα (K42R) mice, kinase activity was reduced to the same level as in CaMKIIα null mice, while CaMKII protein expression was well preserved. Tetanic stimulation failed to induce not only LTP, but also sustained dendritic spine enlargement, a structural basis for LTP, at the Schaffer collateral-CA1 synapse, while activity-dependent postsynaptic translocation of CaMKIIα was preserved. In addition, CaMKIIα (K42R) mice showed a severe impairment in inhibitory avoidance learning, a form of memory that is dependent on the hippocampus. These results demonstrate that kinase activity of CaMKIIα is a common critical gate controlling structural, functional and behavioral expression of synaptic memory.

11. Imaging of hippocampal neuronal activity with new long-wavelength voltage-sensitive dyes

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Voltage-sensitive dye (VSD) imaging of membrane potential changes is a powerful tool for the mapping of functional circuits in brain tissues. This approach overcomes one limitation of conventional electrophysiological recordings because it can monitor the activity of many neurons at once. Improvement of instrumentation, such as sensors for detecting small changes in fluorescent intensity and high-capacity processing of large amounts of imaging data, allow optical analysis of neural activity at a millisecond time scale and with sufficient spatial resolution. Thus, VSD imaging has recently become a most promising neurobiological method. Another recent and promising method is channelrhodopsin -2 (ChR2)-assisted photostimulation, which allows excitable cells to be stimulated optically. ChR2 is a light-gated, cation-selective ion channel isolated from the green algae *Chlamydomonas reinhardtii*. Because ChR2-based photostimulation can precisely excite neurons at a single-input level, much current interest is directed towards the expression of ChR2 in brain tissues to optically control neuronal activity and, thereby, to map the distribution of synaptic inputs. The combination of VSDI and ChR-assisted optical

stimulation will enable input-output mapping in neural circuits of brain tissue. VSDs typically are organic compounds that bind to cell membranes and shift their absorption and/or fluorescence emission spectra according to the transmembrane potential. Most VSDs are excited at relatively short wavelengths, which overlap with the excitation wavelengths of light-activated proteins used to control neuronal activity, such as channelrhodopsin-2. This spectral overlap is the biggest problem to be solved to permit combination of VSD imaging and photostimulation. To overcome this problem, we have assessed the utility of five new long-wavelength fluorescent VSD for imaging the activity of populations of neurons in mouse brain slices. Although all the five were capable of detecting activity resulting from activation of the Schaffer collateral-CA1 pyramidal cell synapse, they differed significantly in their properties, most notably in the signal-to-noise ratio of the changes in dye fluorescence associated with neuronal activity. Two of these dyes, Di-2-ANBDQPQ and Di-1-APEFEQPQ, should prove particularly useful for imaging activity in brain tissue and for combining VSD imaging with the control of neuronal activity via light-activated proteins such as channelrhodopsin-2 and halorhodopsin.

12. Roles of the actin cytoskeleton in dendritic spines of neurons: morphological and synaptic maturation

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Many neurological disorders are characterized by abnormalities in the structure of dendritic spines, the postsynaptic structures that receive excitatory inputs. To study the relationship between abnormal dendritic spine structure and synaptic transmission, we focused on the development of spines of cultured hippocampal pyramidal neurons. In these neurons, pharmacological blockade of the component of glutamatergic transmission mediated by AMPA receptors (AMPARs) increases the number of headless and thin protrusions in dendrites. In contrast, blockade of NMDA receptors or metabotropic glutamate receptors did not produce such changes in spine structure. Because drebrin, a spin-rich actin-binding protein, is known to govern the critical first step of dendritic spine maturation, we examined clustering of drebrin in dendritic spines. AMPAR blockade suppressed postsynaptic clustering of drebrin without affecting presynaptic clustering of synapsin I. Furthermore, depletion of drebrin with small interfering RNA treatment caused the appearance of thin, headless spines despite the presence of normal postsynaptic AMPAR activity. These data suggest that abnormal spine morphology results from suppression of AMPAR-mediated clustering of drebrin. The dynamics of drebrin clusters was explored by photobleaching individual spines. We found that AMPAR activity increased the stable fraction of drebrin, without affecting the exchange rate or the total amount of drebrin in spines. Because an increase in the stable drebrin fraction corresponds to an increase in drebrin clustering, our findings indicate that AMPAR-mediated stabilization of drebrin plays an important role in spine maturation. It is known that the loss of drebrin is found in patients with Alzheimer's disease, mild cognitive disorders and Down's syndrome. Further, experimental reduction of drebrin expression *in vivo* causes behaviors related to schizophrenia. Given this pattern of drebrin involvement in clinical disorders, our current findings suggest that altered synapse morphology in the brains of many neurological patients could be caused by changes in synapse activity that lead to changes in drebrin and in the actin cytoskeleton. Thus, therapeutic enhancement of AMPAR-mediated drebrin stabilization may be a promising strategy for treating many neurological conditions.

13. Neurogenesis in adult brain: expression of drebrin E in migrating neuroblasts in adult rat brain

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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 pro-

tein, nestin, nor vimentin was detected in DIP cells. These results indicate that DIP cells in the RMS are migrating neuroblasts. An image subtraction method, based on using 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, detection of drebrin E＋A- signals is useful 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 at 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. The disappearance of drebrin E from the cell body may be a molecular switch for the cessation of migration in newly generated neuroblasts.

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Department of Basic Medical Sciences

Division of Biomolecular Imaging
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 macromolecules 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 three 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. 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 due to its intrinsic fluctuation and should be different from the others, and that the important information which might be lost by averaging in conventional means could be retrieved 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 among them, in a sense that it has a potential to visualize instantaneous structure of individual macromolecules. Recent trends are two ways; one, docking of crystal structures of some components or parts of supramolecular assembly to its 3-D structure reconstructed by single-particle-analysis utilizing electron cryo-microscopy; and the other, tomographic reconstruction of whole cells or tissues to obtain lower-resolution architecture of

supramolecular assemblies *in situ*, sometimes even under *in vivo* conditions. The former, higher resolution studies by cryo-EM, require numerous images to be classified and averaged to obtain final results, to compensate its very low contrast and S/N images. Though electron microscopy has a great potential to discriminate each particle's shape, current electron microscopy as above cannot be compatible with "single molecule" matter, as long as we follow the conventional means. Since motor proteins apparently involve large-scale structural changes for the function, an innovating methodology is desired that enables us to observe single molecules without any averaging, but still with a resolution enough to discriminate, at least, their 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 methodology to almost perfectly overcome problematic ghost images in 3-D tomographic reconstruction, due to "missing data-range" which is unavoidable in electron microscopy (Baba & Katayama, 2008; *ibid*. 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, this time, the internal structure of various materials, unaffected by the "ghost", which have 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 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 (Morphology-Based Detection of Structural Differences; Kimori, Baba & Katayama, 2007; Katayama et al., 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 system would be completely elucidated soon in near future. However, the essential part of the mechanism still remains unsolved even now, possibly 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 cross-bridges. 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 global 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 proposed by Prof. Yanagida's team. Though the molecular size of myosin head is too small as a subject of conventional single-particle analysis, we eagerly hoped to characterize the major intermediate species that is essential for further studies. Utilizing high contrast feature of freeze-replica images and a combination of averaging/classification procedures and structural detection method as described above, we devised a new method to reconstruct the 3-D structure of SH1-SH2 crosslinked species. Dynamics and crystallographic studies of the crosslinked and actin-attached species are also in progress in collaboration with several expertise scientists.

Since myosin crossbridges generate the force by their interaction with actin-filaments, it is natural to assume that actin monomers in the filament would receive the counteracting force and might be somewhat distorted as a consequence of actomyosin sliding. It is known that myosin-binding to actin being strongly coopera-

tive, suggesting a possibility that the local structural change by their binding might propagate pretty far from the original interaction sites. We thus started the image analysis on the structural perturbation of actin-filament during actomyosin sliding. Utilizing high contrast features of freeze-replica images, we extracted the characteristic surface pattern of actin-filaments under static and interacting conditions and compared them by a "fractal analysis" procedure. The parameter D_f, indicating the structural order, was significantly less, under sliding conditions, where myosin was bound in the presence of ATP. Though the distortion was maximal naturally at the interacting monomer, it is notable that forward actin monomers that had not yet experienced myosin binding, were also perturbed to some extent.

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

Katayama E: In collaboration with. 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 image 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 Proteins (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 their 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".

Polarized fluorescence image-capturing system and image-analysis to study the dynamic behavior of myosin heads

Kozuka J. and Katayama E.

The molecular mechanism of muscle contraction involves cyclic interaction of myosin cross-bridges with actin filaments, while myosin hydrolyzes ATP. It is generally believed that one chemical reaction as the input always generates a unit mechanical movement as the output, and that such relationship might be unaffected by the external load. However, there is no reason to assume definite one-to-one correspondence between ATP hydrolysis and the mechanical events, even if the tilting of myosin heads could occur. Oosawa and his colleagues proposed a "loose chemo-mechanical coupling" as a potential mechanism to explain the interaction between biological motors and cytoskeletal polymers, i.e. myosin and actin filaments. In that mechanism, the ratio of the number of input and output events need not to be fixed but could be automatically controlled within biomolecular machinery, depending on the environmental conditions. Actually, a modular "Feynman-type thermal ratchet model" proposed by Vale and Oosawa with two energy barriers can explain the force-velocity and heat-velocity profiles of the muscle.

Katayama previously reported that freeze-replica electron microscopic images of actomyosin complex under *in vitro* motility assay conditions exhibit various modes of crossbridge-attachment to actin, while sliding. Such results might be interpreted in a sense, as the direct showcase of loose-coupling mechanism. To study the dynamics of myosin heads as the change of attachment angles to actin filaments, we developed total internal reflection fluorescence microscope that enables simultaneous measurement of the fluorescence polarization derived from myosin heads fixed onto glass surface, and the translocation of actin filaments. To-

gether with an image analysis system to quantitatively characterize the dynamic behavior of myosin heads, we might be able to obtain the time-sequence of the events during sliding, the important information complementary to electron microscopy that can give only a static features of the structures.

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木森義隆，諸根信弘，片山栄作：Mathematical morphologyに基づくバイオイメージからの構造情報の抽出と解析：「顕微鏡」印刷中

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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 expo-

nential 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 selection and RT-PCR amplification. The selected oligo-nucleotide ligands are called 'aptamer' which has high affinity and specificity to target molecules.

a. Structural and molecular basis for hyper-specificity of RNA aptamer to human immunoglobulin G

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 Fcg 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 Fcg 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. Crystallization and preliminary X-ray diffraction studies of RNA aptamer in complex with the Fc fragment of human IgG

To clarify the structural basis for the high specificity of a RNA aptamer, we have performed a crystallographic study of RNA aptamer in complex with the Fc fragment of human IgG1. The complex has been crystallized in 20% polyethylene glycol 1000, 0.2 M calcium acetate, 100 mM Tris-HCl (pH 8.0), at 294 K by the vapor diffusion method. Preliminary X-ray diffraction studies reveal that the crystals belong to the orthorhombic space group $P2_12_12$ with unit-cell parameters $a=83.7$, $b=107.2$, $c=79.0$ Å. The data set has been collected to 2.2 Å resolution.

c. 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.

d. An aptamer-based biosensor for mammalian initiation factor eIF4A

We demonstrate that an RNA aptamer selected against eukaryotic initiation factor 4A (eIF 4A) serves as an efficient biosensor. The aptamer, when immobilized to resin, purifies eIF4 A from crude cell extracts by affinity pull-down and ^{32}P-labeled aptamer can detect some 300 ng eIF4A by dot-blot analysis. Moreover, by use of an aptamer-immobilized sensor chip, we developed a surface plasmon resonance assay to detect eIF4A at the nanogram level within whole cell lysates after optimize sample preparation, thereby showing a real-time sensor for eIF4A in cell extract solution.

e. 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 ri-

bozyme 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

Koichi Ito, Miki Wada, Kazuki Saito and Yoshikazu Nakamura

Stop codon reassignments have occurred very frequently in ciliates. In some ciliate species, the universal stop codons UAA and UAG are translated into glutamine, while in some other species, the universal stop codon UGA appears to be translated into cysteine or tryptophan. The class Litostomatea has been hypothesized to be the only group of ciliates using the universal genetic code. However, the hypothesis was based on a statistical analysis of quite small sequence dataset which was insufficient to elucidate the codon usage of the class among such highly deviated phylum. In this study, together with the updated database sequence analysis for the class, we approached the problem of stop codon usage by examining the capacity of the translation termination factor eRF1 for recognizing stop codons. Using in vivo assay systems in budding yeast, we estimated the activity of eRF1 from two litostome species Didinium nasutum and Dileptus margaritifer. The results clearly showed that Didinium and Dileptus eRF1s efficiently recognize all three stop codons. This is the first experimental evidence that strongly supports the hypothesis that litostome ciliates use universal genetic code. (in collaboration with Dr. Harumoto, Department of Biological Science, Faculty of Science, Nara Women's University)

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 G-protein γ subunit mimic is a general antagonist of prion propagation in *Saccharomyces cerevisiae*

The Gpg1 protein is a Gγ subunit mimic implicated in the G-protein glucose-signaling pathway in *Saccharomyces cerevisiae*, and its function is largely unknown. Here we report that Gpg1 blocks the maintenance of [PSI^+], an aggregated prion form of the translation termination factor Sup35. While the *GPG1* gene is normally not expressed, overexpression of *GPG1* inhibits propagation of not only [PSI^+] but also [PIN^+], [*URE 3*] prions and the toxic polyglutamine aggregate in *S. cerevisiae*. Overexpression of Gpg1 does not affect expression and activity of Hsp104, a protein-remodeling factor required for prion propagation, showing that Gpg1 does not target Hsp104 directly. Nevertheless, prion elimination by Gpg1 is weakened by overexpression of Hsp 104. Importantly, Gpg1 protein is prone to self-aggregate and transiently co-localized with Sup 35NM-prion aggregates when expressed in [PSI^+] cells. Genetic selection and characterization of loss-of-activity *gpg1* mutations revealed that multiple mutations on the hydrophobic one-side surface of predicted α-helices of the Gpg1 protein hampered the activity. Prion elimination by Gpg1 is unaffected in the *gpa2*Δ and *gpb1*Δ strains lacking the supposed physiological G-protein partners of Gpg1. These findings suggest a general inhibitory interaction of the Gpg1 protein with other transmissible and non-transmissible amyloids, resulting in prion elimination. Assuming the ability of Gpg1 to form G-protein heterotrimeric complexes, Gpg1 is likely to play a versatile function of reversing the prion state and modulating the G-protein signaling pathway.

b. 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.

c. A selfish prion of Rnq1 mutant in yeast

Rnq1Δ100 deletes the N-terminal non-prion domain of Rnq1, and eliminates [*PSI*$^+$] in [*PIN*$^+$] yeast. Here we found that [*PIN*$^+$] is transmissible to Rnq1Δ100 in the absence of full-length Rnq1, forming a novel prion variant [*RNQ1Δ100*$^+$]. [*RNQ1Δ100*$^+$] has similar [*PIN*$^+$] properties as it stimulates the *de novo* induction of [*PSI*$^+$] and is eliminated by the null *hsp104* Δmutation, but not by Hsp104 overproduction. On the other hand, [*RNQ1*Δ100$^+$] inherits the inhibitory activity and hampers the maintenance of [*PSI*$^+$] though less efficiently than [*PIN*$^+$] made of Rnq1-Rnq1Δ100 co-aggregates. Interestingly, [*RNQ1Δ100*$^+$] prion was eliminated by *de novo* [*PSI*$^+$] induction. Thus, the [*RNQ1Δ100*$^+$] prion demonstrates selfish activity to eliminate a heterologous prion in *S. cerevisiae*, showing the first instance of a selfish prion variant in living organisms.

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Our research aim is to understand various life phenomena at an atomic resolution. Our approach is to determine the tertiary structures of proteins and nucleic acids crucial for biological processes by X-ray crystallography, generate hypotheses how their functions emerge from the structures, by using computer simulation, and demonstrate the hypotheses by in vitro and in vivo functional analyses of mutants designed based on the structures. We focus on
I. Processing, chemical modification and aminoacylation of transfer RNA
II. Sensing receptors (channels) and membrane transporters
III. Structure-based cancer research.

I. Processing, chemical modification and aminoacylation of transfer RNA

Transfer RNA (tRNA) acts as an adaptor molecule to link the genetic code (in messenger RNA) to a specific amino acid. tRNA is initially transcribed by RNA polymerase as a precursor RNA with long extensions at the 5′ and 3′ terminus. Maturation of tRNA into a functional RNA requires processing of the extensional sequences, chemical modifications and specific aminoacylation. The post-transcriptional chemical modifications contribute to the structural stabilization and the specific codon recognition by tRNA. We are promoting structure determination of the tRNA-maturating enzymes in a complex with tRNA (precursor) to especially elucidate the dynamic mechanism of their highly specific chemical reactions.

1. Structural insights into RNA-dependent eukaryal and archaeal selenocysteine formation

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Recent genome analyses and subsequent biochemical analyses revealed that the genetic code has expanded through emergence of new aminoacyl-tRNA synthetases (aaRSs) that catalyze amino acid attachment to tRNA or the indirect tRNA-dependent pathways of aminoacyl-tRNA formation. The indirect tRNA-dependent pathways of aminoacyl-tRNA formation, in which a non-cognate amino acid bound to tRNA is converted to the cognate one, are widely distributed in nature; they are thought to be evolutionarily older than the direct aminoacylation route catalyzed by the aminoacyl-tRNA synthetases. Although selenocysteine occurs in or-

ganisms from all three domains of life, Sec-tRNA is synthesized solely by the indirect route; actually it is the only natural amino acid found in proteins for which a cognate aminoacyl-tRNA synthetase did not evolve. Seryl-tRNA synthetase (SerRS) forms Ser-tRNA^{Sec} in bacteria, archaea, and eukaryotes. Using the selenium donor selenophosphate bacteria convert this misacylated aminoacyl-tRNA species to Sec-tRNA^{Sec} by the action of the SelA protein, a PLP-dependent selenocysteine synthase. While methanogenic archaea harbor a gene encoding a SelA paralog (e.g., MJ0158), its product is unable to synthesize Sec-tRNA^{Sec} *in vitro*. On the other hand, eukaryotes and archaea require an additional phosphorylation step catalyzed by O-phosphoseryl-tRNASec kinase (PSTK) and convert the resulting Sep-tRNA^{Sec} to Sec-tRNA^{Sec} by Sep-tRNA:Sec-tRNA synthase (SepSecS). This reaction is reminiscent of the indirect pathway of Cys-tRNA^{Cys} synthesis in methanogens where Sep-tRNA^{Cys} is converted to Cys-tRNA^{Cys} by Sep-tRNA: Cys-tRNA synthase (SepCysS), a PLP-dependent enzyme carrying out a β-replacement on tRNA-bound Sep. The crystal structure of Archaeoglobus fuldgidus SepCysS has recently been reported.

Here, we present the crystal structure of *Methanococcus maripaludis* SepSecS conjugated with PLP at 2.5 Å resolution. SepSecS, a member of the Fold Type I PLP enzyme family, forms an $(\alpha_2)_2$ homotetramer through its N-terminal extension. The active site lies on the dimer interface with each monomer contributing essential residues. In contrast to other Fold Type I PLP enzymes, Asn247 in SepSecS replaces the conserved Asp in binding the pyridinium nitrogen of PLP. A structural comparison with *Escherichia coli* selenocysteine lyase allowed construction of a model of Sep binding to the SepSecS catalytic site. Mutations of three conserved active site arginines (Arg72, Arg94, Arg307), protruding from the neighboring subunit, led to loss of *in vivo* and *in vitro* activity. The lack of active site cysteines demonstrates that a perselenide is not involved in SepSecS-catalyzed Sec formation; instead, the conserved arginines may facilitate the selenation reaction.

2. Pyrrolysyl-tRNA synthetase:tRNA^{Pyl} structure reveals the molecular basis of orthogonality

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Pyrrolysine (Pyl), the 22nd natural amino acid to be discovered, is genetically encoded by UAG (*amber* stop codon) and inserted into proteins by the unique suppressor tRNA^{Pyl}. The Methanosarcinaceae produce Pyl and express Pyl-containing methyltransferases that allow growth on methylamines. Homologous methyltransferases and the Pyl biosynthetic and coding machinery are also found in two bacterial species. Pyl coding is maintained by pyrrolysyl-tRNA synthetase (PylRS), which catalyses the formation of Pyl-tRNA^{Pyl}. Pyl is not a recent addition to the genetic code. PylRS was already present in the last universal common ancestor; it then persisted in organisms that utilize methylamines as energy sources. Recent protein engineering efforts added non-canonical amino acids to the genetic code. This technology relies on the directed evolution of an 'orthogonal' tRNA synthetase-tRNA pair in which an engineered aminoacyl-tRNA synthetase (aaRS) specifically and exclusively acylates the orthogonal tRNA with a non-canonical amino acid. For Pyl the natural evolutionary process developed such a system some 3 billion years ago. When transformed into *Escherichia coli, Methanosarcina barkeri* PylRS and tRNA^{Pyl} function as an orthogonal pair *in vivo*. Here we show that *Desulfitobacterium hafniense* PylRS-tRNA^{Pyl} is an orthogonal pair *in vitro* and *in vivo*, and present the crystal structure of this orthogonal pair. The ancient emergence of PylRS-tRNA^{Pyl} allowed because the evolution of unique structural features in both the protein and the tRNA. These structural elements manifest an intricate, specialized aaRS-tRNA interaction surface that is highly distinct from those observed in any other known aaRS-tRNA complex; it is this general property that underlies the molecular basis of orthogonality.

3. Structure, dynamics, and function of RNA modification enzymes

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About 35% of mouse genomic DNA is transcribed into non-coding RNAs (ncRNAs) that function in the regulation of gene expression. Most ncRNAs are post-transcriptionally modified, which generally reinforces the specific tertiary structure of the RNAs to accelerate their functions. Biochemical and structural investigations of RNA modification have primarily focused on ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), the best characterized ncRNAs, which play central roles in translation of the ge-

netic code. Especially in tRNA, modifications not only stabilize the L-shaped tertiary structure, but also alter its function by improving and switching its molecular recognition. Furthermore, it has recently been proposed that the modification procedure itself contributes to the RNA (re)folding, in which the modification enzymes function as RNA chaperones. Recent genome and post-genome (proteomics and transcriptomics) analyses have identified new genes encoding enzymes responsible for ncRNA modifications. Further structural analyses of RNA-modification enzyme complexes have elucidated the structural basis by which the modification enzymes specifically recognize the target RNAs and ingeniously incorporate the chemical modifications into the precise position. This paper provides an overview of the recent progress in the structural biology of ncRNA modification enzymes.

4. Molecular basis for validation of the 3' sequence during the CCA-adding reaction by a class I CCA-adding enzyme

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CCA-adding enzyme builds an invariant 3'-end CCA of tRNA without a nucleic acid template. The molecular basis for the validation of the 3'-sequence during polymerization by CCA-adding enzyme remains elusive. Here, we present almost a dozen complex structures of class I CCA-adding enzyme and various mini-helices (mini-$D_{73}N_{74}$, mini-$D_{73}N_{74}C_{75}$ and mini-$D_{73}C_{74}N_{75}$; D_{73} is a discriminator nucleotide and N is either A, G, or U). The mini-$D_{73}N_{74}$ complexes adopt open forms, and the bases of the N_{74} nucleotides are stacked down by the β-turn of the enzyme, as in the mini-$D_{73}C_{74}$ complex. Mini-$D_{73}N_{74}$ accepts CMP to the same extent as mini-$D_{73}C_{74}$. In the complex with mini-$D_{73}U_{74}$, CTP shifts the enzyme to a closed form and induces the U_{74} flipping for CMP incorporation, as observed in the CMP incorporation into mini-$D_{73}C_{74}$. In contrast, unlike the closed form of the mini-$D_{73}C_{74}C_{75}$ complex, where the C_{74} base hydrogen bonds with the β-turn, the mini-$D_{73}N_{74}C_{75}$ and mini-$D_{73}C_{74}N_{75}$ complexes adopt open forms, and the 3'-regions of the RNA are not locked in closed forms. Only mini-$D_{73}C_{74}U_{75}$ accepts AMP to a similar extent as mini-$D_{73}C_{74}C_{75}$. In the mini-$D_{73}C_{74}U_{75}$ complex, ATP shifts the enzyme to a closed form. C_{74} and the 2-O of U_{75} hydrogen bond with the β-turn and Thr_{130} of the enzyme, respectively, and the conformation of the 3'-region of the RNA is closed for AMP incorporation. These structural and biochemical studies suggest that the validation of the 3'-region of RNA is achieved, after two nucleotide additions, by the formation of hydrogen-bonds between the nucleotides at positions 74 and 75 with the enzyme in a closed, locked form of the complex, and is prerequisite for AMP incorporation at position 76 for complete CCA synthesis.

II. Sensing receptors (channels) and membrane transporters

Five senses (touch, taste, hearing, eyesight and smell) are essential for higher eukaryotes to determine their actions in response to environmental insults. We promote structure determination of the sensing receptors complexed with ligands to elucidate the general mechanism of how external chemical and physical stimuli activate and change the conformation of the sensing receptors (channels) to form a novel interaction with the coupled G proteins or to change the cation permeability. We are further promoting X-ray crystallography of metal transporters and membrane translocon to elucidate the fundamental mechanism of the specific substance transportation through lipid bilayer membrane. Especially, we focus on the mechanism how transporters drive their transport, how transporters discriminate their specific substrate and how transporters regulate their gating.

1. Mg^{2+}-sensing mechanism of Mg^{2+} transporter MgtE probed by molecular dynamics study

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Proper regulation of the intracellular ion concentration is essential to maintain life, and is achieved by ion transporters that transport their substrates across the membrane in a strictly regulated manner. MgtE is a Mg^{2+} transporter that may function in the homeostasis of the intracellular Mg^{2+} concentration. A recent crystallographic study revealed that its cytosolic domain undergoes a Mg^{2+}-dependent structural change, which is proposed to gate the ion conducting pore passing through the transmembrane domain. However, the dynamics of Mg^{2+}-sensing, *i.e.* how MgtE responds to the change in the intracellular Mg^{2+} concentration, remained elusive. Here we performed molecular dynamics

simulations of the MgtE cytosolic domain. The simulations successfully reproduced the structural changes of the cytosolic domain upon binding or releasing Mg^{2+}, as well as the ion selectivity. These results suggested the roles of the N and CBS domains in the cytosolic domain and their respective Mg^{2+} binding sites. Combined with the current crystal structures, we propose an atomically detailed model of Mg^{2+} sensing by MgtE.

2. Conformational transition of Sec machinery inferred from bacterial SecYE structures

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Over 30% of proteins are secreted across or integrated into membranes. Their newly synthesized forms contain either cleavable signal sequences or non-cleavable membrane anchor sequences, which direct them to the evolutionarily conserved Sec translocon (SecYEG in prokaryotes and Sec61agb in eukaryotes). The translocon then functions as a protein-conducting channel. These processes of protein localization occur either co- or post-translationally. In bacteria, the SecA ATPase drives post-translational translocation. The only high-resolution structure of a translocon available to date is that for SecYEβ from an archaeon, *Methanococcus jannaschii*, which lacks SecA. Here, we present the 3.2 Å-resolution crystal structure of the SecYE translocon (ttSecYE) from a SecA-containing organism, *Thermus thermophilus*. The structure, solved as a complex with an anti-ttSecY Fab fragment, revealed a "pre-open" state of SecYE, in which several transmembrane helices are shifted, as compared to the previous SecYEβ structure, to create a hydrophobic crevasse open to the cytoplasm. Fab and SecA bind to a common site at the tip of the cytoplasmic domain of SecY. Molecular dynamics (MD) and disulfide mapping analyses suggest that the pre-open state might represent a SecYE conformational transition that is inducible by SecA-binding. Moreover, we identified a SecA-SecYE interface that comprises SecA residues originally buried inside the protein, suggesting that both the channel and the motor components of the Sec machinery undergo cooperative conformational changes upon formation of the functional complex.

III. Structure-based cancer research

We promote X-ray crystallography of various oncogenic products or signal transduction proteins in a complex form to provide the structural basis for the mechanism of how their dysfunction cause cancer and metastasis of cancer cells. We mainly focus on growth factor receptors, oncogenic mediators and transcriptional factors, which transduce the TGF-β and Wnt/β-catenine signals. We are also promoting the project on innate immunity. Our final goal is to design a novel and effective anti-cancer drugs with minimum side effects, on the basis of their atomic structures.

1. Crystallization and preliminary X-ray diffraction analysis of the full-length GCIP/HHM

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Cell growth and differentiation are complicated processes, which are well organized by cellular signaling pathways to execute the intrinsic genetic program. Especially, basic helix-loop-helix (bHLH) transcriptional factors are key regulators of cell growth, proliferation and differentiation in embryonic and adult tissues. They have been demonstrated to play critical roles in regulating gene expression, cell cycle control, cell lineage commitment, and numerous developmental processes by binding to "E box" in the promoter region of tissue-specific genes through homo- and hetero-dimerization. Based on the presence and absence of DNA-binding domain as well as other functional domains, HLH proteins can be classified into five major classes, basic HLH (bHLH) proteins, basic HLH Per-AhR-Arnt-Sim (bHLH-PAS) proteins, basic HLH leucine zipper (bHLH-LZ) proteins, and dominant-negative HLH (dnHLH) proteins. Class-A bHLH proteins, such as E2 gene products, are ubiquitously expressed in many tissues, while class-B bHLH proteins, such as MyoD, NeuroD and Hes, exhibits a tissue-specific expression and regulate tissue-specific cell growth and differentiation through heterodimerization with class-A bHLH proteins. The bHLH-LZ pro-

teins include *Myc* family proteins, which have a leucine zipper motif at the C-terminus, and regulate multiple cellular functions, e.g. proliferation, differentiation, transformation and apoptosis. The dnHLH proteins are the Id family proteins (Id1, Id2, Id3, and Id4) that lack a basic region prior to the HLH domain. Id family proteins form a complex with bHLH proteins to inhibit their functions in a dominant negative manner, acting as negative regulators of cell growth and positive regulators of cell differentiation.

GCIP is a 360-residue human HLH leucine zipper protein (*M*r of 40 kDa), which contains a putative leucine zipper motif at its N-terminus, a HLH domain in the center, and an acidic C-terminal region. GCIP was independently identified as cyclinD1-binding protein (DIP1) or human homologue of murine maternal Id-like molecule (human homologue of MAID or HHM). GCIP/HHM lacks basic DNA-binding region, like Id-family proteins, but contains leucine zipper domains similar to *Myc* family proteins, representing a new class dnHLH proteins. GCIP/HHM regulates the activity of HNF 4 hepatocyte differentiation factor, thus playing an essential role in the development and differentiation of liver. GCIP/HHM gene is localized on chromosomal 15q15, a region frequently deleted or loss of heterozygosity (LOH), in several tumors such as colorectum, breast, lung, and bladder tumors. Overexpression of GCIP/HHM in SW480 colon cancer cell line was reported to result in a significant inhibition of tumor cell colony formation, while gene silencing of GCIP/HHM by siRNA promoted cell colony formation. GCIP/HHM may function as an important regulator in tumor initiation and progression, while does not affect the proliferation of normal hepatocytes after liver injury. Recently, GCIP/HHM was suggested to interact with the human ribosomal acidic P0 protein, an essential component of eukaryotic ribosomal stalk, whose overexpression may cause tumorigenesis in breast and liver tissues. Therefore, GCIP/HHM also suppresses tumor formation by association with P0 protein. On the other hand, GCIP/HHM was also reported to interact with cyclinD1 and be involved in G1/S-phase progression of hepatocytes, which in older adult is associated with the development of liver tumor. Therefore, GCIP/HHM may be an attractive target for prevention of especially hepatocellular carcinoma.

Here we show that native and selenomethionine-derivatized (SeMet) crystals of the full length GCIP/HHM were obtained using the sitting-drop vapor-diffusion method. Crystals were much improved by adding Tris[2-carboxyethyl]phosphine (TCEP) as a reducing reagent and diffracted X-ray to 3.5 Å resolution. Preliminary phase calculation using the dataset obtained from the SeMet crystal suggested that the crystal belongs to space group $P3_221$ and contains a monomer per asymmetric unit. The structure determination by multiple-wavelength anomalous dispersion (MAD) method using the SeMet crystals is in progress. Crystal structure of GCIP/HHM may clarify the mechanism of differentiation regulation and tumorigenesis, enabling us to design new drugs to suppress hepatocellular carcinoma as well as other tumors.

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