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. Stimulus-specific distinctions in spatial and temporal dynamics of stress-activated protein kinase kinase kinases (SAP3Ks) revealed by a FRET biosensor

Taichiro Tomida, Mutsuhiro Takekawa, Pauline O'Grady, and Haruo Saito

MAP kinases (MAPKs) are important intracellular signaling molecules that are activated through kinase cascades composed of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. In mammalian cells, several MAPK cascades co-exist: the growth promoting ERK MAPK cascade, and the growth suppressing stress-activated protein kinase (SAPK) cascades. Together, these kinase cascades make the critical choice between growth and apoptosis under various environmental conditions. Two families of SAPKs, namely JNK and p38, are activated through kinase cascades composed of a SAPK kinase kinase (SAP3K) and a SAPK kinase (SAP2K), by a variety of environmental stresses such as UV, gamma-rays, translation inhibitors, osmostress, and oxidative stress, as well as by physiological mediators such as TGF-β, IL-1β, and TNF-α. The SAPK cascades are also important in embryonic development and in immune responses in the adult organism. The p38 SAPK is activated mainly by the MKK3 and MKK6 SAP2Ks, whereas the JNK SAPK is activated by the MKK4 and MKK7 SAP2Ks. These SAP2Ks are, in turn, activated by diverse SAP3Ks including MEKK1, MEKK2, MEKK3, and MTK1 (MEKK4), TAK1, ASKs, MLKs, and TAOs.

This large array of SAP3Ks undoubtedly reflects the diversity of the stress stimuli that can activate the SAPK cascades. In spite of extensive investigation, however, the molecular mechanisms by which stresses such as UV-C activate SAP3Ks are still unclear. UV-C can be absorbed by, and affect the chemistry of, proteins, RNA, and DNA. Potentially, therefore, UV-C may initiate the activation of the SAPK pathway on the cell membrane, in the cytoplasm, or in the nucleus. One approach to distinguish these possibilities is to identify the subcellular location where SAP3K activation primarily takes place.

The activity of SAP3Ks has been convention-

ally monitored either by measuring their kinase catalytic activities *in vitro*, or, more frequently, by detecting the phosphorylation status of their specific substrates such as MKK3 and MKK6 by immunoblotting or immunofluorescence staining with phospho-specific antibodies. However, these methods can only measure the average activity of SAP3Ks in a cell population. Furthermore, they cannot easily distinguish the different subcellular locations where SAP3K activation might take place. As a consequence, the intracellular dynamics of SAP3K activity are currently largely unknown.

This year, we developed a reporter of SAP3K activity that can uncover the dynamic behavior of SAP3K activation in cells based on fluorescence resonance energy transfer (FRET). In this FRET reporter (L225), SAP3K-specific phosphorylation of the specific substrate sequence (MKK6 Thr-211) leads to the binding of the phosphorylated threonine to the FHA1 domain in the same reporter, which modulates intramolecular YFP-CFP interaction. The L225 SAP3K reporter has, in addition to the MKK6 phosphorylation site, the DVD docking site of MKK6 that specifically interact with MAP3Ks that activates MKK6. Thus, the L225 reporter is highly specific to the group of stress-activated SAP3Ks that interact with, and activate, MKK6. L225 does not respond to the Raf family MAP3Ks.

Using the L225 SAP3K reporter, we demonstrated that SAP3K activation occurs either synchronously or asynchronously among a cell population depending on the type of stress applied. Furthermore, SAP3K activation occurs in different cellular compartments in single cells depending on the type of stress. In particular, SAP3Ks are activated by EGF and osmostress on the plasma membrane, by anisomycin and UV in the cytoplasm, and by etoposide in the nucleus. These observations revealed previously unknown heterogeneity in SAPK responses, and supplied answers to the question of which cellular location various stresses induce stimulus-specific SAPK responses.

2. Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK

Hui-Yu Yang, Kazuo Tatebayashi, Katsuyoshi Yamamoto[1], and Haruo Saito: [1]1Frontier Research Initiative, IMSUT.

Under optimal growth conditions the budding yeast *Saccharomyces cerevisiae* grows as round-shaped, single cells. Upon nitrogen starvation or in the presence of poor carbon sources, however, diploid cells of certain strains (such as Σ1278) initiate a fungus-like filamentous growth (FG). Haploid cells also undergo a related phenomenon termed invasive growth (IG), whereby the elongated cells invade solid media. These responses, which are collectively referred to as FG/IG, are an adaptive mechanism that allows normally sessile yeast to forage for scarce nutrients and to evade harmful waste products. An FG/IG response requires a specific MAPK pathway, whose core is a three-kinase cascade composed of the Ste11 MAPKKK, the Ste7 MAPKK, and the Kss1 MAPK.

Another MAPK cascade, termed the High Osmolarity Glycerol (HOG) MAPK pathway, is activated by, and required for adaptation to, an increased external osmolarity. The HOG MAPK pathway is composed of two functionally redundant upstream signaling branches that regulate the common Hog1 MAPK. Of these branches, the SHO1 branch is closely related to the FG/IG MAPK pathway. The SHO1 branch is initiated by an interplay between the membrane anchor protein Sho1 and the putative osmosensor membrane proteins Msb2 and Hkr1, leading to activation of Ste11. Thus, the same Ste11 MAPKKK is involved in both the HOG and the FG/IG MAPK pathways. In the HOG pathway, however, Ste11 activates the Pbs2 MAPKK, which then activates the Hog1 MAPK. Activated Hog1 governs a series of adaptive responses to high osmolarity, including temporarily arrest of the cell cycle progression, readjustment of the transcription and translation patterns, and synthesis and intracellular retention of the compatible osmolyte glycerol.

Although both the FG/IG and the HOG MAPK pathways involve Ste11, each controls completely different, and mutually incompatible responses. Normally, osmostress activates only the Hog1 MAPK. However, osmostress can activate the FG/IG MAPK pathway in mutant cells, such as *pbs2*Δ or *hog1*Δ, in which the Hog1 MAPK cannot be activated. In other words, activated Hog1 inhibits undesirable cross-talk activation of the FG/IG MAPK pathway.

This year, we found that the FG/IG-specific Kss1 MAPK was activated by a combination of an O-glycosylation defect caused by disruption of the protein O-mannosyltransferase gene *PMT4*, and an N-glycosylation defect induced by tunicamycin. We also found that defective Msb2 glycosylation activated the FG/IG MAPK pathway. Although the osmoregulatory HOG MAPK pathway and the FG/IG MAPK pathway share almost the entire upstream signaling machinery, osmostress activated only the HOG-specific Hog1 MAPK. Conversely, we showed that glycosylation defects activated only Kss1. Furthermore, activated Kss1 inhibited Hog1.

When activation of Kss1 was blocked by mutations, such as *ste7*Δ or *kss1*Δ, the Hog1 MAPK was activated by glycosylation defects. Kss1 inhibited Hog1 indirectly through the Ptp2 protein phosphatase. Thus, the reciprocal inhibitory loop between Kss1 and Hog1 allows only one or the other of these MAPKs to be stably activated under various stress conditions.

Publications

Tomida T, Takekawa M, O'Grady P, and Saito H. Stimulus-specific distinctions in spatial and temporal dynamics of stress-activated protein kinase kinase kinases revealed by a fluorescence resonance energy transfer biosensor. *Mol. Cell. Biol*., 29: 6117-6127, 2009.

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Department of Basic Medical Sciences

Department of Parasitology
分子細胞情報分野(2)

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Genome research has made a remarkable progress in the areas of parasitology and medical entomology. 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/Apicomplexa and annotation

Hiroyuki Wakaguri[1], Yutaka Suzuki[1], Masahide Sasaki[1], Sumio Sugano[1], Junichi Watanabe: [1]Graduate School of Frontier Sciences, The University of Tokyo

We used a total of 61,056 5′-end-single-pass

cDNA sequences from *Plasmodium falciparum, P. vivax, P. yoelii, P. berghei, Cryptosporidium parvum*, and *Toxoplasma gondii*. We compared these partially sequenced cDNA sequences with the currently annotated gene models and observed significant inconsistencies between the two datasets. 16% of the current gene models contain at least one mis-annotation and should be re-evaluated. We also identified a large number of transcripts that had been previously unidentified. We then compared 732 *T. gondii* cDNAs of which entire sequences were determined. We found that 41% of the *T. gondii* gene models contained at least one inconsistency. We also identified and confirmed by RTPCR 140 previously unidentified transcripts found in the intergenic regions of the current gene annotations. We show that the majority of these discrepancies are due to questionable predictions of one or two extra exons in the upstream or downstream regions of the genes. Our unique full-length cDNA information is especially useful for further refinement of the annotations for the genomes of apicomplexa parasites.

Transcription start site analysis of *Toxoplasma gondii*

Junya Yamagishi[2], Hiroyuki Wakaguri, Akio Ueno[2], Youn-Kyoung Goo[2], Makoto Igarashi[2], Yoshifumi Nishikawa[2], Chihiro Sugimoto[3], Sumio Sugano, Yutaka Suzuki, Junichi Watanabe, and Xuenan Xuan[2]: [2]Obihiro University of Agriculture and Veterinary Medicine, [3]Hokkaido University

Ultra-deep gene expression analysis using the novel method, TSS-seq was applied to tachyzoites of *T. gondii* for detailed characterization of the core promoters. TSS-seq that determines numerous oligo-capped transcription start sites using the Solexa sequencers will refine the previous analysis using the oligo-capped full-length cDNA libraries.

Transcription start site analysis of *Giardia lamblia* and *Entamoeba invadens*

Mohammed Tolba, Seiki Kobayashi[4], Tsutomu Takeuchi[4], Asao Makioka[5], Masahiro Kumagai[5], Kazushi Hiranuka, Hiroyuki Wakaguri, Sumio Sugano, Yutaka Suzuki, Junichi Watanabe: [4]Keio University, [5]Jikei University of School of Medicine

Giardia and Entamoeba are most important pathogenic enteric protozoa that parasitize upper (duedenum) and lower intestine (colon and cecum), respectively, causing diarrhea. Induction of resistant cysts from trophozoites is critical for infection. They also have extremely short 5′UTR (untranslated region) in spite of totally different phylogeny. To elucidate cyst induction mechanism and analyze their extremely short 5′UTR (untranslated region) we have started comparative TSS-seq analysis.

CD4+ T cell response in early infection of mouse malaria

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Plasmodium berghei ANKA causes lethal malaria in mice; C57BL/6 mice die early with fulminant symptoms including convulsion, whereas BALB/c mice survive this phase and die later of anemia and prostration. To elucidate the CD4+ T cell responses in early death due to severe malaria, the kinetics of CD4+ T cells were compared by analyzing cell surface markers and the production of cytokines and transcription factors. The results revealed that cytokine production by CD4+ T cells was induced as early as 5 days after infection and the maintenance of higher levels of IL-4 and IL-10 may be associated with the protection of BALB/c mice from early death. These results suggest that parasite control in the early phase of infection may be important for the development of an effective vaccine.

Full-Echinococcus and tetraspanin vaccine

Zhisheng Dang[3], Kinpei Yagi[7], Yuzaburo Oku[3], Hirokazu Kouguchi[3], Kiichi Kajino[3], Junichi Watanabe, Jun Matsumoto[8], Ryo Nakao[3], Hiroyuki Wakaguri, Atsushi Toyoda[9], Chihiro Sugimoto[3]: [3]Hokkaido University, [7]Hokkaido Institute of Public Health, [8]Nihon University, [9]National Institute of Genetics

Echinococcus multilocularis causes an important zoonotic cestode disease. The metacestode stage proliferates in the liver of intermediate hosts including human and rodents and forms multiple cysts. Recently, members of a transmembrane protein tetraspanin (TSP) family have been used as vaccines against schistosomosis, or as diagnostic antigens for cysticercosis. In this study, seven tetraspanins of *E. multilocularis*, designated as TSP1 to TSP7, were evaluated for their protective potential against primary alveolar echinococcosis. The large extracellular loop (LEL) region of these tetraspanins was cloned

from a full-length enriched cDNA library of *E. multilocularis* metacestodes and expressed in Escherichia coli as a fusion protein with thioredoxin. Recombinant TSPs were applied as vaccines against an *E. multilocularis* primary experimental infection in BALB/c mice. Cyst lesions in the livers of vaccinated and non-vaccinated mice were counted. The cyst lesion reduction rates induced by the seven tetraspanins in vaccinated vis-a`vis non-vaccinated mice were: 87.9%, 65.8%, 85.1%, 66.9%, 73.7%, 72.9% and 37.6%. Vaccination conferred protective rates to mice ranging from 0% (TSP5, 6, 7) to maximally 33% (TSP1, 3). The results indicated that recombinant tetraspanins have varying protective effects against primary alveolar echinococcosis and could be used in vaccine development.

Global collaborations

Ryuichiro Maeda[2], Yuki Eshita[10], Mihoko Imada[4], Chihiro Sugimoto: [10]Oita University

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.

Publications

Hiroyuki Wakaguri, Yutaka Suzuki, Masahide Sasaki, Sumio Sugano and Junichi Watanabe. Inconsistencies of genome annotations in apicomplexan parasites revealed by 5'-end-one-pass and full-length sequences of oligo-capped cDNAs *BMC Genomics* 2009, 10: 312 doi: 10.1186/1471-2164-10-312

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Hiroshi Bannnai, Yoshifumi Nishikawa1, Hany Mohamed Ibrahim, Kyoko Yamada, Osamu Kawase, Jun-ichi Watanabe, Chihiro Sugimoto and Xuenan Xuan. Overproduction of the Pro-Apoptotic Molecule, Programmed Cell Death 5, in *Toxoplasma gondii* Leads to Increased Apoptosis of Host Macrophages. *J. Vet. Med. Sci*. 71 (9): 1183-1189, 2009

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Zhisheng Dang, Kinpei Yagi, Yuzaburo Oku, Hirokazu Kouguchi, Kiichi Kajino, Junichi Watanabe, Jun Matsumoto, Ryo Nakao, Hiroyuki Wakaguri, Atsushi Toyoda, Chihiro Sugimoto. Evaluation of *Echinococcus multilocularis* tetraspanins as vaccine candidates against primary alveolar echinococcosis. Vaccine 27 (2009) 7339-7345

Database

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

Department of Basic Medical Sciences

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

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

1. Non-Hebbian synaptic plasticity induced by repetitive postsynaptic action potentials

Hiroyuki K. 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 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 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 the possible existence of depolarizing pulse-induced LTP in vivo. 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.

2. Plexin-A2 regulates spatial memory and pattern separation through structural modification of mossy fiber projection in the CA3 region of the mouse hippocampus

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The hippocampus has been implicated in certain types of memory, including spatial memory. It has been known that the distribution of

mossy fibers, axons of dentate gyrus (DG) granule cells, is modified dynamically by spatial learning in living animals. However, the precise mechanism of the regulation of mossy fiber distribution during memory formation is not well understood. We have previously reported that plexin-A2 (PLA2), one of the type A plexins that show repulsive activities to the class 6 semaphorins, regulates the distribution of mossy fiber terminals in the CA3 region and that the mutant mice lacking PLA2 ($PLA2^{-/-}$ mice) exhibit a shift of mossy fibers from the suprapyramidal to the infra- and intrapyramidal regions. In order to test whether the difference in the distribution of mossy fiber terminals affects abilities of learning and memory, we have performed extensive behavioral analyses of $PLA2^{-/-}$ mice. We found that sensorimotor functions and emotional behaviors of $PLA2^{-/-}$ mice were normal, although motor learning was markedly impaired presumably through aberrant distribution of cerebellar granule cells, and that contextual and auditory fear conditioning, which is at least partially dependent on the hippocampus, was also intact. In contrast, $PLA2^{-/-}$ mice exhibited enhanced hippocampus-dependent spatial reference memory and spatial pattern separation, which is the ability to discriminate fine differences in external environments, tested by the 8-arm radial maze task. These results suggest that the projection of mossy fibers regulated by PLA2 may be a specific determinant of the ability of spatial reference memory and pattern separation.

3. 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, NMDARs are abundant at the recurrent synapses but scarce at the mossy fiber synapses. We generated mutant mice in which NMDARs 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 NMDARs 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 NMDAR-slow after-hyperpolarization coupling was diminished in the mutant neurons. In the adult brain, inducible ablation of NMDARs in the hippocampal CA3 region by the viral expression vector for Cre recombinase also induced similar large EEG spikes. Furthermore, pharmacological blockade of CA3 NMDARs enhanced the susceptibility to kainate-induced seizures. These results raise an intriguing possibility that hippocampal CA3 NMDARs may suppress the excitability of the recurrent network as a whole *in vivo* by restricting synchronous firing of CA3 neurons.

4. 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 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 un-

derstood 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.

5. Involvement of tyrosine phosphorylation of the NMDAR GluN2A subunit in depression-related behavior

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Major depressive and bipolar disorders are serious illnesses that affect millions of people. Growing evidence implicates glutamate signaling in depression, though the molecular mechanism by which glutamate signaling regulates depression-related behavior remains unknown. In this study, we provide evidence suggesting that the tyrosine phosphorylation of the NMDAR, an ionotropic glutamate receptor, contributes to depression-related behavior. The GluN2A subunit of the NMDAR is tyrosine-phosphorylated, with Tyr-1325 as its one of the major phosphorylation site. We have generated mice expressing mutant GluN2A with a Tyr-1325-Phe mutation to prevent phosphorylation of this site in vivo. The homozygous knock-in mice show antidepression-like behavior in the tail suspension test and in the forced swim test. In the striatum of the knock-in mice, DARPP-32 phosphorylation at Thr-34, which is important for the regulation of depression-related behaviors, is increased. We also show that the Tyr-1325 phosphorylation site is required for Src-mediated potentiation of the recombinant NMDAR channel. These data argue that the Tyr-1325 phosphorylation regulates NMDAR channel properties and the NMDAR-mediated downstream signaling to modulate depression-related behavior.

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

Shizuka Kobayashi, Yoko Yamagata[13], Tatsuya Umeda[14], Akihiro Inoue[14], Hiroyuki Sakagami[15], Masahiro Fukaya[4], Masahiko Watanabe[4], Nobuhiko Hatanaka[16], Masako Totsuka[9], Takeshi Yagi[17], Kunihiko Obata[18], Keiji Imoto[13], Yuchio Yanagawa[19], Shigeo Okabe[20] and Toshiya Manabe: [13]Department of Information Physiology, National Institute for Physiological Sciences, [14]Department of Cell Biology, Tokyo Medical and Dental University, [15]Department of Anatomy, Kitasato University School of Medicine, [16]Division of System Neurophysiology, National Institute for Physiological Sciences, [17]Graduate School of Frontier Biosciences, Osaka University, [18]Laboratory of Neurochemistry, National Institute for Physiological Sciences, [19]Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, [20]Department of Cellular Neurobiology, Graduate School of Medicine, University of Tokyo

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 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.

7. Roles of the actin cytoskeleton in dendritic spine morphogenesis of hippocampal neurons

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Spine morphogenesis mainly occurs during development as a morphological shift from filopodia-like thin protrusions to bulbous ones. We have previously reported that synaptic clustering of the actin-binding protein drebrin in dendritic filopodia governs spine morphogenesis and synaptic PSD-95 clustering. Here, we report the activity-dependent cellular mechanisms for spine morphogenesis, in which the activity of AMPA receptors (AMPARs) regulates drebrin clustering in spines by promoting drebrin stabilization. In cultured developing hippocampal neurons, pharmacological blockade of AMPARs, but not of other glutamate receptors, suppressed postsynaptic drebrin clustering without affecting presynaptic clustering of synapsin I (synapsin-1). Conversely, the enhancement of the action of AMPARs promoted drebrin clustering in spines. When we explored drebrin dynamics by photobleaching individual spines, we found that AMPAR activity increased the fraction of stable drebrin without affecting the time constant of drebrin turnover. An increase in the fraction of stable drebrin corresponded with increased drebrin clustering. AMPAR blockade also suppressed normal morphological maturation of spines and synaptic PSD-95 clustering in spines. Together, these data suggest that AMPAR-mediated stabilization of drebrin in spines is an activity-dependent cellular mechanism for spine morphogenesis.

8. Fos-positive neurons in the supramammillary nucleus of the rat exposed to novel environment

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The supramammillary nucleus (SuM) in the hypothalamus is proposed to regulate the function of the hippocampus through distinct fiber connection. Several investigations suggest that the SuM is relevant to anxiety and defensive behavior. Function of the SuM, however, is not known exactly. In order to demonstrate the spatial activation of the SuM in physiologically behaving rats, we investigated Fos induction in the SuM by exposure to novel environment. To correct uneven background in microscopic preparations, we applied a convolution filter, resulting in reliable automatic counting of Fos-positive neurons and analyzed the distribution of Fos-positive neurons in the whole region of SuM. A large number of Fos-positive neurons were observed throughout the entire SuM after rats exposed to a novel open field. A three-dimensional density map revealed that density of the Fos-positive neurons was highest in the medial SuM, especially in its core regions. Based on these results we suggest that the medial SuM modulates defensive behavior and that the lateral SuM modulates emotional and memory functions of the hippocampus.

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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 molecules not only in solution but also in live cells can be visualized with high contrast after adequate processing. Though the spatial resolution remains less than crystallography, the real superior feature of the method is its almost unlimited applicability to the materials whose structure cannot otherwise be pursued. For 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., (In collaboration with Dr. Y. Kimori; Nat'l Inst. of Neurol. & Psychiat, and 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 more than 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 low S/N images. Though electron microscopy has a great potential to discriminate each particle's shape, current electron microscopy as above is be compatible to "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 choice 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, though various factors should be considered for the final interpretation. We have actually shown the surface profiles of various protein assemblies such as molecular motors and intracellular receptor molecules *in situ*, utilizing that technique. To characterize the 3-D structure of the target particles captured as above in 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 a sufficient resolution. We have utilized above new methodologies to visualize meso-resolution but extremely high-contrast snapshots of sliding actomyosin and revealed the instantaneous 3-D structure of functioning crossbridges. There, we found that the structure of the majority of crossbridges during sliding is not the ADP-Vi-type structure as postulated in widely believed conventional "tilting lever-arm hypothesis", but actually is a new oppositely kinked configuration whose crystal structure has not yet been solved. The novel structure closely resembled that of putative intermediate analogue in which two reactive thiols along SH-helix are chemically crosslinked in the presence of ADP (hereafter called SH-corsslinked species). 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 translocate actin by "Brownian ratchet" while in "weakly actin-bound state" through one-site attachment, and that they occasionally pivots into "primed configuration" with two-site attachment., 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 Yanagida's team. We eagerly hoped to characterize the structure of major intermediate species that should be essential for further studies, but the molecular size of myosin head is too small as an object of conventional single-particle analysis. Utilizing very contrasty nature of metal-replicated images, conventional classification/averaging procedure, together with a new structural detection method as above, we attempted to reconstruct the 3-D structure of SH-crosslinked species by a special version of Single-Particle-Analysis devised to adapt small-sized asymmetrical particles. With a new procedure to manage a small number of replica images, we could finally obtain 3-D envelope of the myosin head analogous to the intermediate structure under sliding conditions, in which the leverarm kinked oppositely to that of ADP/Vi-bound form. We cut original atomic model of pPDM-treated-ADP-structure (1L20) into motor-domain and the leverarm, and relocated each module at best-matching position and the orientation, to generate a tentative model that best-fits to the reconstructed envelope. The envelope of the candidate fit well to 3-D reconstructed tomogram of tension-developing insect flight-muscle (Taylor et al., 1999) and the image of myosin-V walking along actin filament (Burgess et al., 2002), both of which are very few examples to show the myosin crossbridge structure under actually functional states. We then examined whether that model of SH-crosslinked configuration can universally explain all the observed images of actin-sliding crossbriges. The

images of actin-attached crossbridges during *in vitro* motility were classified by their 2-D appearances and each class-average was compared with 2-D projections of the SH-crosslinked species as reference. By analyzing the orientation of motor-domain and leverarm separately, we found that there could be several subpopulations, some matched to, but some deviated from the standard SH-crosslinked structure. Ten to fifteen percent of actin-attached particles showed a configuration whose leverarm significantly twisted to anti-clockwise direction from the standard. The presence of such configuration in our quick-freeze replica images might suggest that we could have captured another intermediate structural-state during actin-sliding movement. We aligned the motor-domains of our new structures, together with those of the comprehensive crystal structures of the common molecular species (scallop), so that two actin-binding sites face to the counterpart sites on F-actin, both determined by quenched chemical-crosslinking study (Andreev & Reshetnyak, 2007). Remarkably, we realized that the leverarm's distal end draws a trajectory to indicate smooth anticlockwise rotation that possibly follows the reaction process. We previously reported that the leverarm of SH-crosslinked structure is kinked to the opposite side of ADP/Vi-bound structure, strongly suggesting that the leverarm should be twisted in our new primed structure immediately before the power-stroke. Since all the structures actually exist and would be realized somewhere during myosin's chemical-reaction cycle, the present finding suggests that, during the "power-stroke", such twist might be reversed to the original position probably by anti-clockwise rotation, to recover nucleotide-free rigor-structure.

Hence, we elucidated the whole process of structural changes during crossbridge-cycle at mesoscopic level, by a direct electron-microscopic observation of actin-sliding heavy-meromyosin heads during *in vitro* actin-sliding phenomena.

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 location of specific sites on the targets 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 actual observation data. Along that way, we should be able to deeply 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".

Development of novel stain reagents for transmission electron microscopy

Nakakoshi, M., Sato, M. and Katayama, E.

Negative-staining is a popular conventional technique of transmission electron microscopy to visualize fine structure of biological macromolecules or supramolecular assembly with an inversed contrast, by embedding the materials in heavy-metal salts. Though uranyl acetate gives the best performance and has been most extensively used for the purpose, it gets more and more difficult to obtain, especially in Japan and several countries, because of its high toxicity that may lead to environmental problems, so that the waste fluids and contaminated apparatus should be permanently kept at service sites,

strictly controlled by the government. Hence, development of substituting safer stain reagents has been strongly desired by the people working in cutting-edge research fields. The requested properties of the reagents as a useful negative stain might be as follows. First, since the contrast in electron microscopy arises from electron scattering by the observed materials, atomic radius of the stain-constituting metal must be large enough to screen electron beam and the one whose radius is larger than uranium atom is preferable to get high-contrast images. Second, metal salts should be easily soluble and kept amorphous when dried in the vacuum, while the solution should be close to neutral pH to reduce the stress to the biological materials. Third, a certain degree of fixative effect is needed to maintain the targets' structures under electron-beam. Though uranyl acetate shows acidic pH in itself, it is usable as unbuffered solution that instantaneously fixes the materials. Fourth, the atom with least toxicity is a mandatory requirement considering the environmental respect. Samarium and gadolinium ions are good candidates to form acetic acid salts that are easily soluble to water and give neutral pH. They have excellent environmental compatibility with very little toxicity that enables safe and easy handling.? We used them to stain amyloid-β1-42 fiber and T4 phage as examples, and found that they give negatively stained images with good contrast almost comparable to that with uranyl acetate. Both salts could also be useful as good electron-dense stains for plastic-embedded thin sections of tissues and cells. We have a view that the ionic bond or the chelate uniting might be related to the dyeing effect of gadolinium ion.

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電子染色剤，染色液および電子染色方法

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Department of Basic Medical Sciences

Division of Molecular Biology
遺伝子動態分野

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RNA no longer stands behind DNA or protein but stands in front of DNA and protein. Recent achievements and discovery in biological science clearly emphasize 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

Shoji P Ohuchi, Akira Ishiguro, Yasuko Yamamura, Takahiro Negishi, Yuki Fujimoto, Toshiro Iwagawa, Yusuke Mori, Kei Endo, Hironori Adachi, Kazuyoshi Murakami, Yoichiro Sugimoto, Maiko Sakamoto, Hideya Arimura, Makie Oshimi, Ryo Ishikawa, Taishin Akiyama[1], Jun-ichiro Inoue[1], Sumiko Watanabe[2], and Yoshikazu Nakamura: [1]Division of Cellular and Molecular Biology, and [2]Division of Molecular and Developmental Biology, IMSUT.

The systematic evolution of ligands by exponential enrichment (SELEX) method is based on the *in vitro* selection of oligo-nucleotide ligands from large random-sequence libraries by repeated reactions of DNA transcription, RNA selection and RT-PCR amplification. The selected oligo-nucleotide ligands are called "aptamer" which has high affinity and specificity to target molecules.

a. An RNA aptamer against Interleukin-17 inhibits development of autoimmunity in mouse models

Interleukin-17 is a pro-inflammatory cytokine produced primarily by a subset of CD4+ T cells, called Th17 cells, and is involved in host defense, inflammatory and autoimmune disorders, including rheumatoid arthritis and multiple sclerosis. We tested whether an RNA aptamer to IL-17 would inhibit IL-17 action and the development of autoimmunity in mouse models. Aptamers are short single-stranded DNA or RNA sequences that are selected in vitro based on their high affinity to a target molecule. By selection and manipulation of RNA aptamers to human IL-17 (hIL-17), Apt3-4 and Apt21-2 were defined to inhibit the binding of hIL-17 to the human receptor IL-17R. Importantly, Apt21-2, but not Apt3-4, inhibited the binding of mouse IL-17 to mouse IL-17R, and hampered the IL-17 bioactivity of triggering phosphorylation of downstream signaling factors and producing interleukin-6 in human (NHDF) and mouse (MEF) cells. We examined the efficacy of Apt21-2 (a 33-mer PEGylated form) to neutralize IL-17 in two sets of Th17 cell-mediated autoimmune mouse models; EAE (experimental autoimmune encephalomyelitis) and GPI (glucose-6-phosphateisomerase) induced arthritis. When Apt21-2 was injected immediately after immunization with MOG (EAE-inducing agent) and GPI, inhibited the development of symptoms, as evaluated with a visual scoring system, in a dose-dependent manner. Interestingly, even after the development of arthritis, injection of Apt21-2 significantly suppressed the progression of arthritis, showing some therapeutic effects on GPI induced arthritis. These results demonstrate for the first time that the anti-IL-17 aptamer inhibits IL-17 action and the development of autoimmunity in mouse models, offering a potential therapeutic approach with an RNA molecule to the treatment of the autoimmune disorder.

b. RNA aptamers targeting mouse embryonic stem cells

Embryonic stem cells (ESCs) are capable of unlimited self-renewal and differentiation into multiple cell types. Cell surface molecules are useful markers for classification and isolation of desired cell populations, and various surface molecules have been identified as ESC markers. However, detailed analysis of individual markers and characterization of cellular phenotypes associated with each marker remain largely unknown. In this study, to develop mouse ESC (mESC)-specific surface probes, we used intact, live mESCs as a target for Systematic Evolution of Ligands by EXponential enrichment (SELEX). Aptamers are short single-stranded nucleic acid sequences that are selected *in vitro* based on their high affinity to a target molecule. The initial trial provided us with several mESC-binding aptamers with completely distinct primary sequences. However, all the aptamers examined competed with one another for the binding, indicating that they bind to a same target on mESCs. Therefore, to isolate the aptamers for the various markers, we used one of the aptamers isolated in the initial SELEX as a competitor in the subsequent SELEX procedure. Consequently, we successfully isolated several mESC-binding aptamers non-competing with one another. These aptamers would be utilized for the molecular probes and the manipulation tools for mESCs. Selection and characterization of the aptamers will be presented, showing a promising approach with an RNA aptamer to the identification and characterization of mESC populations.

c. A binary Cy3 aptamer probe composed of folded modules

Aptamers are short single-stranded DNA or RNA sequences that are selected *in vitro* based on their high affinity to a target molecule. Dye-binding aptamers are promising tools for real-time detection of not only DNA or RNA sequences but also proteins of interest both *in vitro* and *in vivo*. In this study, we aimed to isolate an RNA aptamer to Cy3, a widely-used and membrane-permeant and non-toxic fluorescent cyanine dye. Extensive selection of affinity RNA molecules to Cy3 yielded a unique sequence aptamer named Cy3_apt. The selected Cy3_apt was 83 nucleotides long and successfully shortened to 49 nucleotides long with increased affinity to Cy3 by multiple base changes. The shortest Cy3_apt is composed of two separate hairpin modules that are required for the affinity to Cy3 as monitored by the surface plasmon resonance (SPR) assay. Also, the fluorescence of Cy3 increased upon binding to Cy3_apt. The two modules of Cy3_apt, when detached from each other functioned as a binary aptamer probe. We demonstrate that the binary Cy3_apt probe is applicable to the detection of target oligonucleotides or RNA-RNA interaction by tagging with target sequences. This binary probe consists of two folded modules, hence referred to as a folded binary probe.

2. Structural Biology of RNA Aptamers: Crystallization and X-ray diffraction studies of RNA aptamer in complex with the Fc fragment of human IgG

Yusuke Nomura[1], Shigeru Sugiyama[2], Taiichi

Sakamoto[1], Shin Miyakawa[3], Hiroaki Adachi[2], Kazufumi Takano[2], Satoshi Murakami[4], Tsuyoshi Inoue[2], Yusuke Mori[2], Hiroyoshi Matsumura[2], and Yoshikazu Nakamura: [1]Faculty of Engineering, Chiba Institute of Technology, [2]Graduate School of Engineering, Osaka University, [3]Ribomic Inc., [4]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology.

To clarify the structural basis for the high specificity of RNA aptamer targeting human IgG-Fc, 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. 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. We have determined the 2.15 Å crystal structure of the Fc fragment of human IgG1 (hFc1) complexed with the aptamer. The aptamer adopts a distorted structure, which are stabilized by a calcium ion, explaining why the calcium ions are required for binding activity. The crystal structure demonstrates that the aptamer-hFc1 interaction involves mainly van der Waals contacts and hydrogen bonds rather than electrostatic forces. The structure also sheds light on the question of how the aptamer exhibits the high specificity to the human IgGs, not to the IgGs from other sources.

3. Translation Termination

Koichi Ito, Miki Wada, Kazuki Saito, and Yoshikazu Nakamura

a. Structural insights into eRF3 and stop codon recognition by eRF1.

Eukaryotic translation termination is mediated by two interacting release factors, eRF1 and eRF3, which act cooperatively to ensure efficient stop codon recognition and fast polypeptide release. The crystal structures of human and Schizosaccharomyces pombe full-length eRF1 in complex with eRF3 lacking the GTPase domain revealed details of the interaction between these two factors and marked conformational changes in eRF1 that occur upon binding to eRF3, leading eRF1 to resemble a tRNA molecule. Small-angle X-ray scattering analysis of the eRF1/eRF3/GTP complex suggested that eRF1's M domain contacts eRF3's GTPase domain. Consistently, mutation of Arg192, which is predicted to come in close contact with the switch regions of eRF3, revealed its important role for eRF1's stimulatory effect on eRF3's GTPase activity. An ATP molecule used as a crystallization additive was bound in eRF1's putative decoding area. Mutational analysis of the ATP-binding site shed light on the mechanism of stop codon recognition by eRF1.

b. A novel class of bacterial translation factor RF3 mutations suggests specific structural domains for premature peptidyl-tRNA drop-off

The bacterial translation factor RF3 promotes translation termination by recycling the tRNA-mimicking release factors, RF1 and RF2, after mature polypeptide release. RF3 also enhances the premature peptidyl-tRNA drop-off reaction in the presence of RRF and EF-G. Despite the recently resolved X-ray crystal structure of RF3, the molecular details of the bimodal functionality of RF3 remain obscure. In this report, we demonstrate a novel class of RF3 mutations specifically defective in the tRNA drop-off reaction. These mutations suggest differential molecular pathways closely related to the guanine nucleotide modes of RF3.

4. Yeast Prion

Hiroshi Kurahashi, Masao Ishiwata, Shoichiro Shibata, Chie Arai, Keita Oishi, and Yoshikazu Nakamura

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. Localization of prion-destabilizing mutations in the N-terminal non-prion domain of Rnq1 in *Saccharomyces cerevisiae*

[*PIN*$^+$] is the prion form of Rnq1 in *Saccharomyces cerevisiae* and is necessary for the *de novo* induction of a second prion, [*PSI*$^+$]. The function of Rnq1, however, is little understood. The limited availability of defective *rnq1* alleles impedes the study of its structure-function relationship by genetic analysis. In this study, we isolated *rnq1* mutants that are defective in the stable maintenance of the [*PIN*$^+$] prion. Since there is no *rnq1* phenotype available that is applicable to a direct selection or screening for loss-of-function *rnq1* mutants, we took advantage of a prion inhibitory agent, Rnq1Δ100, to develop a color-based genetic screen. Rnq1Δ100 eliminates the [*PSI*$^+$] prion in the [*PIN*$^+$] state but not in the [*pin*$^-$] state. This allows us to find loss-of-[*PIN*$^+$] *rnq1* mutants as white [*PSI*$^+$] colonies. Nine *rnq1* mutants with single-amino-acid substitutions were defined. These mutations impaired the stable maintenance of [*PIN*$^+$] and, as a consequence, were also partially defective in the *de novo* induction of [*PSI*$^+$]. Interestingly, eight of the nine alleles were mapped to the N-terminal region of Rnq1, which is known as the non-prion domain preceding the asparagine and glutamine rich prion domain of Rnq1. Notably, over-expression of these *rnq1* mutant proteins restored [*PIN*$^+$] prion activity, suggesting that each of the *rnq1* mutants was not completely inactive. These findings indicate that the N-terminal non-prion domain of Rnq1 harbors a potent activity to regulate the maintenance of the [*PIN*$^+$] prion.

b. Alterations of predicted α-helices in non-prion domains of Rnq1 are inhibitory to heterologous prion interactions in yeast

[*PIN*$^+$] is the prion form of the Rnq1 protein that has unknown function in *Saccharomyces cerevisiae*. While [*PIN*$^+$] is required for the *de novo* induction of a second prion, [*PSI*$^+$], it is not required for [*PSI*$^+$] propagation. A glutamine (Q), asparagine (N) rich C-terminal domain (amino acids 153-405) is know to be necessary for propagation of [*PIN*$^+$], while the N-terminal region (amino acids 1-152) is non-Q/N rich and considered the non-prion domain. However, over-expression of an N-terminal deletion of Rnq1, Rnq1Δ100, inhibits [*PSI*$^+$] propagation in the [*PIN*$^+$] state, but not in the [*pin*$^-$] state. Here, we isolated numerous single amino acid mutations in Rnq1, whose over-expression inhibits [*PSI*$^+$] propagation similarly to Rnq1Δ100. Surprisingly all the 28 *rnq1* alleles thus isolated are mapped within the N-terminal non-prion domain of Rnq1. These [*PSI*$^+$]-inhibitory *rnq1* mutations did not affect [*PIN*$^+$] propagation when over-expressed from a strong promoter, but instead destabilized [*PIN*$^+$] when expressed from the weak authentic *RNQ1* promoter. Importantly, 22 of 28 *rnq1* mutations are mapped to the surface, and on one-side, of five contiguous a-helices. These findings suggest that the contiguous a-helices in the non-prion domain of Rnq1 play a crucial role for heterologous prion interactions.

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Department of Basic Medical Sciences

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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. Editing Mechanism of Aminoacyl-tRNA Synthetases Operates by a Hybrid Ribozyme/Protein Catalyst

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Aminoacyl-tRNA synthetases (aaRSs) are critical for the translational process, catalyzing the attachment of specific amino acids to their cognate tRNAs. To ensure formation of the correct aminoacyl-tRNA, and thereby enhance the reliability of translation, several aaRSs have an editing capability that hinders formation of misaminoacylated tRNAs. We investigated theoretically the mechanism of the editing reaction for a class I enzyme, leucyl-tRNA synthetase (LeuRS), complexed with a misaminoacylated tRNA(Leu), employing ab initio hybrid quantum mechani-

cal/molecular mechanical potentials in conjunction with molecular dynamics simulations. It is shown that the water molecule that acts as the nucleophile in the editing reaction is activated by a 3′-hydroxyl group at the 3′-end of tRNA (Leu) and that the O2′ atom of the leaving group of the substrate is capped by one of the water's hydrogen atoms. Thus, it is shown that editing is a self-cleavage reaction of the tRNA and so it is the tRNA, and not the protein, that drives the reaction. The protein does, however, have an important stabilizing effect on some high-energy intermediates along the reaction path, which is more efficient than the ribozyme would be alone. This indicates that editing is achieved by a novel "hybrid ribozyme/protein catalyst". Analysis of existing experimental data and additional modeling shows that this ribozymal mechanism appears to be widespread, occurring in the ribosome as well as in other aaRSs. It also suggests transitional forms that could have played an important role in the RNA world hypothesis for the origin of life.

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. Structural basis for translational fidelity ensured by transfer RNA lysidine synthetase

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Maturation of precursor transfer RNA (pre-tRNA) includes excision of the 5′ leader and 3′ trailer sequences, removal of introns and addition of the CCA terminus. Nucleotide modifications are incorporated at different stages of tRNA processing, after the RNA molecule adopts the proper conformation. In bacteria, tRNA(Ile2) lysidine synthetase (TilS) modifies cytidine into lysidine (L; 2-lysyl-cytidine) at the first anticodon of tRNA(Ile2) (refs 4-9). This modification switches tRNA(Ile2) from a methionine-specific to an isoleucine-specific tRNA. However, the aminoacylation of tRNA (Ile2) by methionyl-tRNA synthetase (MetRS), before the modification by TilS, might lead to the misincorporation of methionine in response to isoleucine codons. The mechanism used by bacteria to avoid this pitfall is unknown. Here we show that the TilS enzyme specifically recognizes and modifies tRNA(Ile2) in its precursor form, thereby avoiding translation errors. We identified the lysidine modification in pre-tRNA (Ile2) isolated from RNase-E-deficient Escherichia coli and did not detect mature tRNA (Ile2) lacking this modification. Our kinetic analyses revealed that TilS can modify both types of RNA molecule with comparable efficiencies. X-ray crystallography and mutational analyses revealed that TilS specifically recognizes the entire L-shape structure in pre-tRNA (Ile2) through extensive interactions coupled with sequential domain movements. Our results demonstrate how TilS prevents the recognition of tRNA(Ile2) by MetRS and achieves high

specificity for its substrate. These two key points form the basis for maintaining the fidelity of isoleucine codon translation in bacteria. Our findings also provide a rationale for the necessity of incorporating specific modifications at the precursor level during tRNA biogenesis.

4. Structure of a tRNA-dependent kinase essential for selenocysteine decoding

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Compared to bacteria, archaea and eukaryotes employ an additional enzyme for the biosynthesis of selenocysteine (Sec), the 21(st) natural amino acid (aa). An essential RNA-dependent kinase , O-phosphoseryl-tRNA (Sec) kinase (PSTK), converts seryl-tRNA(Sec) to O-phosphoseryl-tRNA(Sec), the immediate precursor of selenocysteinyl-tRNA(Sec). The sequence of Methanocaldococcus jannaschii PSTK (MjPSTK) suggests an N-terminal kinase domain (177 aa) followed by a presumed tRNA binding region (75 aa). The structures of MjPSTK complexed with ADP and AMPPNP revealed that this enzyme belongs to the P-loop kinase class, and that the kinase domain is closely related to gluconate kinase and adenylate kinase. ATP is bound by the P-loop domain (residues 11-18). Formed by antiparallel dimerization of two PSTK monomers, the enzyme structure shows a deep groove with positive electrostatic potential. Located in this groove is the enzyme's active site, which biochemical and genetic data suggest is composed of Asp-41, Arg-44, Glu-55, Tyr-82, Tyr-83, Met-86, and Met-132. Based on structural comparison with *Escherichia coli* adenylate kinase a docking model was generated that assigns these amino acids to the recognition of the terminal A76-Ser moieties of Ser-tRNA(Sec). The geometry and electrostatic environment of the groove in MjPSTK are perfectly complementary to the unusually long acceptor helix of tRNA (Sec).

5. Mechanism for the definition of elongation and termination by the class II CCA-adding enzyme

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The CCA-adding enzyme synthesizes the CCA sequence at the 3′ end of tRNA without a nucleic acid template. The crystal structures of class II Thermotoga maritima CCA-adding enzyme and its complexes with CTP or ATP were determined. The structure-based replacement of both the catalytic heads and nucleobase-interacting neck domains of the phylogenetically closely related Aquifex aeolicus A-adding enzyme by the corresponding domains of the T. maritima CCA-adding enzyme allowed the A-adding enzyme to add CCA in vivo and in vitro. However, the replacement of only the catalytic head domain did not allow the A-adding enzyme to add CCA, and the enzyme exhibited (A, C)-adding activity. We identified the region in the neck domain that prevents (A, C)-adding activity and defines the number of nucleotide incorporations and the specificity for correct CCA addition. We also identified the region in the head domain that defines the terminal A addition after CC addition. The results collectively suggest that, in the class II CCA-adding enzyme, the head and neck domains collaboratively and dynamically define the number of nucleotide additions and the specificity of nucleotide selection.

6. Structural basis of AdoMet-dependent aminocarboxypropyl transfer reaction catalyzed by tRNA-wybutosine synthesizing enzyme, TYW2.

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S-adenosylmethionine (AdoMet) is a methyl donor used by a wide variety of methyltransferases, and it is also used as the source of an alpha-amino-alpha-carboxypropyl ("acp") group by several enzymes. tRNA-yW synthesizing enzyme-2 (TYW2) is involved in the biogenesis of a hypermodified nucleotide, wybutosine (yW), and it catalyzes the transfer of the "acp" group from AdoMet to the C7 position of the imG-14 base, a yW precursor. This modified nucleoside yW is exclusively located at position 37 of eukaryotic tRNA(Phe), and it ensures the anticodon-codon pairing on the ribosomal decoding site. Although this "acp" group has a significant role in preventing decoding frame shifts, the mechanism of the "acp" group transfer by TYW2 remains unresolved. Here we report the crystal structures and functional analyses of two archaeal homologs of TYW2 from Pyrococcus horikoshii and Methanococcus jannaschii. The in vitro mass spectrometric and

radioisotope-labeling analyses confirmed that these archaeal TYW2 homologues have the same activity as yeast TYW2. The crystal structures verified that the archaeal TYW2 contains a canonical class-I methyltransferase (MTase) fold. However, their AdoMet-bound structures revealed distinctive AdoMet-binding modes, in which the "acp" group, instead of the methyl group, of AdoMet is directed to the substrate binding pocket. Our findings, which were confirmed by extensive mutagenesis studies, explain why TYW2 transfers the "acp" group, and not the methyl group, from AdoMet to the nucleobase.

7. Structural modelling of the complex of leucyl-tRNA synthetase and mis-aminoacylated tRNA(Leu).

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To assure fidelity of translation, class Ia aminoacyl-tRNA synthetases (aaRSs) edit mis-aminoacylated tRNAs. Mis-attached amino acids and structural water molecules are not included simultaneously in the current crystal structures of the aaRSctRNA complexes, where the 3′-ends (adenine 76; A76) are bound to the editing sites. A structural model of the completely solvated leucyl-tRNA synthetase complexed with valyl-tRNA(Leu) was constructed by exploiting molecular dynamics simulations modified for the present modelling. The results showed that the ribose conformation of A76 is distinct from those observed in the above-mentioned crystal structures, which could be derived from structural constraints in a sandwiched manner induced by the mis-attached valine and tRNA (Leu).

8. The structure of alanyl-tRNA synthetase with editing domain

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Alanyl-tRNA synthetase (AlaRS) catalyzes synthesis of Ala-tRNA(Ala) and hydrolysis of mis-acylated Ser- and Gly-tRNA(Ala) at 2 different catalytic sites. Here, we describe the monomer structures of C-terminal truncated archaeal AlaRS, with both activation and editing domains in the apo form, in complex with an Ala-AMP analog, and in a high-resolution lysine-methylated form. The structures show docking of the editing domain to the activation domain opposite from the predicted tRNA-binding surface. Thus, the editing site is positioned >35 A from the activation site, prompting us to model 2 different tRNA complexes: one binding tRNA at the activation site, and the other binding tRNA at the editing site. Interestingly, a gel-shift assay also implies the presence of 2 types of tRNA complex with different mobility. These results suggest that tRNA translocation via a canonical CCA flipping is unlikely to occur in AlaRS. The structure also demonstrated the binding of zinc in the editing site, in which the specific coordination of zinc would be facilitated by a conserved GGQ motif, implying that the editing mechanism may not be the same as in ThrRS. As Asn-194 in eubacterial AlaRS important for Ser misactivation is replaced by Thr-213 in archaeal AlaRS, a different Ser accommodation mechanism is proposed.

9. Identification of the nucleophilic factors and the productive complex for the editing reaction by leucyl-tRNA synthetase

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To ensure fidelity of translation, several aminoacyl-tRNA synthetases (aaRSs) possess editing capability to hydrolyse mis-aminoacylated tRNAs. In this report, based on our previously-modelled structure of leucyl-tRNA synthetase (LeuRS) complexed with valyl-tRNA(Leu), further structural modelling has been performed along with molecular dynamics simulations. This enabled the identification of the nucleophile, which is different from that suggested by the crystal structure of the LeuRS * Nva2AA complex. Our results revealed that the 3′ hydroxyl group of A76 acts as a "gate" to regulate the accessibility of the nucleophile; thus, the opening of the gate leads to the productive complex for the reaction.

10. Conserved cysteine residues of GidA are essential for biogenesis of 5-carboxymethylaminomethyluridine at tRNA anticodon

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The 5-carboxymethylaminomethyl modification of uridine (cmnm(5)U) at the anticodon first position occurs in tRNAs that read split codon boxes ending with purine. This modification is crucial for correct translation, by restricting codon-anticodon wobbling. Two conserved enzymes, GidA and MnmE, participate in the cmnm(5)U modification process. Here we determined the crystal structure of Aquifex aeolicus GidA at 2.3 A resolution. The structure revealed the tight interaction of GidA with FAD. Structure-based mutation analyses allowed us to identify two conserved Cys residues in the vicinity of the FAD-binding site that are essential for the cmnm(5)U modification in vivo. Together with mutational analysis of MnmE, we propose a mechanism for the cmnm(5)U modification process where GidA, but not MnmE, attacks the C6 atom of uridine by a mechanism analogous to that of thymidylate synthase. We also present a tRNA-docking model that provides structural insights into the tRNA recognition mechanism for efficient modification.

11. Atomic structure of a folate/FAD-dependent tRNA T54 methyltransferase

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tRNAs from all 3 phylogenetic domains have a 5-methyluridine at position 54 (T54) in the T-loop. The methyl group is transferred from S-adenosylmethionine by TrmA methyltransferase in most Gram-negative bacteria and some archaea and eukaryotes, whereas it is transferred from 5,10-methylenetetrahydrofolate (MTHF) by TrmFO, a folate/FAD-dependent methyltransferase, in most Gram-positive bacteria and some Gram-negative bacteria. However, the catalytic mechanism remains unclear, because the crystal structure of TrmFO has not been solved. Here, we report the crystal structures of Thermus thermophilus TrmFO in its free form, tetrahydrofolate (THF)-bound form, and glutathione-bound form at 2.1-, 1.6-, and 1.05-A resolutions, respectively. TrmFO consists of an FAD-binding domain and an insertion domain, which both share structural similarity with those of GidA, an enzyme involved in the 5-carboxymethylaminomethylation of U34 of some tRNAs. However, the overall structures of TrmFO and GidA are basically different because of their distinct domain orientations, which are consistent with their respective functional specificities. In the THF complex, the pteridin ring of THF is sandwiched between the flavin ring of FAD and the imidazole ring of a His residue. This structure provides a snapshot of the folate/FAD-dependent methyl transfer, suggesting that the transferring methylene group of MTHF is located close to the redox-active N5 atom of FAD. Furthermore, we established an in vitro system to measure the methylation activity. Our TrmFO-tRNA docking model, in combination with mutational analyses, suggests a catalytic mechanism, in which the methylene of MTHF is directly transferred onto U54, and then the exocyclic methylene of U54 is reduced by FADH(2).

12. Structural basis of tRNA modification with CO2 fixation and methylation by wybutosine synthesizing enzyme TYW4

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Wybutosine (yW), one of the most complicated modified nucleosides, is found in the anticodon loop of eukaryotic phenylalanine tRNA. This hypermodified nucleoside ensures correct codon recognition by stabilizing codon-anticodon pairings during the decoding process in the ribosome. TYW4 is an S-adenosylmethionine (SAM)-dependent enzyme that catalyzes the final step of yW biosynthesis, methylation and methoxycarbonylation. However, the structural basis for the catalytic mechanism by TYW4, and especially that for the methoxycarbonylation, have remained elusive. Here we report the apo and cofactor-bound crystal structures of yeast TYW4. The structures revealed that the C-terminal domain folds into a beta-propeller structure, forming part of the binding pocket for the target nucleoside. A comparison of the apo, SAM-bound, and S-adenosylhomocysteine-bound structures of TYW4 revealed a drastic structural change upon cofactor binding, which may sequester solvent from the catalytic site during the reaction and facilitate product release after the reaction. In conjunction with the functional analysis, our results suggest that TYW4 catalyzes both methylation and methoxycarbonylation at a single catalytic site, and in the latter reaction, the methoxycarbonyl

group is formed through the fixation of carbon dioxide.

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.

13. Mg(2+)-dependent gating of bacterial MgtE channel underlies Mg(2+) homeostasis.

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The MgtE family of Mg(2+) transporters is ubiquitously distributed in all phylogenetic domains. Recent crystal structures of the full-length MgtE and of its cytosolic domain in the presence and absence of Mg(2+) suggested a Mg(2+)-homeostasis mechanism, in which the MgtE cytosolic domain acts as a 'Mg(2+) sensor' to regulate the gating of the ion-conducting pore in response to the intracellular Mg(2+) concentration. However, complementary functional analyses to confirm the proposed model have been lacking. Moreover, the limited resolution of the full-length structure precluded an unambiguous characterization of these regulatory divalent-cation-binding sites. Here, we showed that MgtE is a highly Mg(2+)-selective channel gated by Mg(2+) and elucidated the Mg(2+)-dependent gating mechanism of MgtE, using X-ray crystallographic, genetic, biochemical, and electrophysiological analyses. These structural and functional results have clarified the control of Mg(2+) homeostasis through cooperative Mg(2+) binding to the MgtE cytosolic domain.

14. Structural basis of novel interactions between the small-GTPase and GDI-like domains in prokaryotic FeoB iron transporter

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The FeoB family proteins are widely distributed prokaryotic membrane proteins involved in Fe(2+) uptake. FeoB consists of N-terminal cytosolic and C-terminal transmembrane domains. The N-terminal region of the cytosolic domain is homologous to small GTPase (G) proteins and is considered to regulate Fe(2+) uptake. The spacer region connecting the G and TM domains reportedly functions as a GDP dissociation inhibitor (GDI)-like domain that stabilizes the GDP-binding state. However, the function of the G and GDI-like domains in iron uptake remains unclear. Here, we report the structural and functional analyses of the FeoB cytosolic domain from Thermotoga maritima. The structure-based mutational analysis indicated that the interaction between the G and GDI-like domains is important for both the GDI and Fe(2+) uptake activities. On the basis of these results, we propose a regulatory mechanism of Fe(2+) uptake.

15. Crystallization and preliminary X-ray diffraction analysis of the truncated cytosolic domain of the iron transporter FeoB

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FeoB-family proteins are widely distributed in bacteria and archaea and are involved in high-affinity Fe(2+) uptake through the plasma membrane. FeoB consists of an N-terminal cytosolic region followed by a C-terminal transmembrane region. The cytosolic region contains small GTPase and GDP dissociation inhibitor-like domains, which serve a regulatory function. The truncated cytosolic region of the iron transporter FeoB from Thermotoga maritima was overexpressed, purified and crystallized. Four native or SeMet crystal forms in a nucleotide-free state or in complex with either GDP or GMPPNP diffracted to resolutions of between

1.5 and 2.1 A.

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.

16. 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 proteins 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 HNF4 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.

1. Tuberous sclerosis tumor suppressor complex-like complexes act as GTPase-activating proteins for Ral GTPases

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The small GTPases RalA and RalB are multifunctional proteins regulating a variety of cellular processes. Like other GTPases, the activity of Ral is regulated by the opposing effects of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Although several RalGEFs have been identified and characterized, the molecular identity of RalGAP remains unknown. Here, we report the first molecular identification of RalGAPs, which we have named RalGAP1 and RalGAP2. They are large heterodimeric complexes, each consisting of a catalytic alpha1 or alpha2 subunit and a common beta subunit. These RalGAP complexes share structural and catalytic similarities with the tuberous sclerosis tumor suppressor complex, which acts as a GAP for Rheb. In vitro GTPase assays revealed that recombinant RalGAP1 accelerates the GTP hydrolysis rate of RalA by 280,000-fold. Heterodimerization was required for this GAP activity. In PC12 cells, knockdown of the beta subunit led to sustained Ral activation upon epidermal growth factor stimulation, indicating that the RalGAPs identified here are critical for efficient termination of Ral activation induced by extracellular stimuli. Our identification of RalGAPs will enable further understanding of Ral signaling in many biological and pathological processes.

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