Department of Basic Medical Sciences

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

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We study various aspects of cellular signal transduction with a particular emphasis on the roles of protein phosphorylation and dephosphorylation. Protein phosphorylation and dephosphorylation are among the most important intracellular signaling mechanisms, and are mediated respectively by protein kinases and protein phosphatases. This year, we have studied the activation mechanism of a yeast osmosensor histidine kinase, a novel MAPKKK-MAPKK docking interaction in the yeast HOG MAP kinase cascade, and the role of the protein tyrosine phosphatase Dlar in fly development.

1. Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure

Vladimír Reiser[1], Desmond C. Raitt[1], and Haruo Saito: [1]Harvard Medical School

Cells have developed elaborate and sensitive protection systems that enable them to rapidly signal, respond, and properly adapt to osmotic changes. The HOG (High Osmolarity Glycerol) MAP kinase signaling pathway in yeast and homologous p38 pathways in more complex eukaryotes play a central role in such osmoprotection systems. In budding yeast (*Saccharomyces cerevisiae*), exposure to high osmolarity environment leads to rapid phosphorylation and activation of the MAP kinase Hog1. Hog1 is activated through phosphorylation by MAP kinase kinase Pbs2, which is itself activated by upstream kinases, either through SLN1 or SHO1 branch of the HOG pathway. Activated Hog1 rapidly, but transiently, accumulates in nuclear compartment, where it participates in a modification of transcriptional program in response to stress.

Osmosensors are proteins whose primary role is to monitor fluctuations in external osmolarity and initiate an activation of signaling pathways for osmo-adaptation. Although a wealth of information is available on regulation of signaling pathways controlled by osmosensors in both prokaryotes and eukaryotes, our understanding of how osmosensors actually respond to osmotic changes is limited.

The two upstream branches (henceforth called the SLN1 and SHO1 branches) in the HOG pathway respond independently to osmotic status of the environment and are apparently redundant. However, the unique compositions of the SLN1 and SHO1 branches suggest they have distinct cellular functions. In the SLN1 branch, a transmembrane histidine kinase Sln1 serves as an osmosensor, and transmits the signal through the Sln1-Ypd1-Ssk1 multistep phosphorelay to the redundant pair of kinases Ssk2 and Ssk22. In contrast, another transmembrane protein Sho1 serves as a facilitator of signaling module assembly that includes Pbs2, Ste11, Ste20 and Cdc 42.

High osmolarity stress causes a rapid reduction of turgor pressure with associated reduction in cell volume. We thus tested if a turgor pres-

sure manipulation, without any application of osmotic stress, could activate the HOG pathway. For this purpose, we used a membrane permeabilizing antifungal drug nystatin. Leakage of low molecular weight cytosolic components imitates a reduction in turgor caused by water efflux during high osmolarity stress. We found that nystatin was indeed an effective inducer of cell volume shrinkage, an indication of reduced turgor, in yeast. More important, a treatment of the cells with nystatin strongly activated the HOG pathway. We also observed that the nystatin activates Hog1 in both wild-type and *ste11* mutant cells, but not in the *ssk2 ssk22* double mutant or the *ssk1* mutant, suggesting that nystatin selectively stimulates the SLN1 branch.

Reduction in turgor, induced by high osmolarity, leads concomitantly to shrinkage of cytosolic volume and an increase in the distance between plasma membrane and cell-wall. Thus, monitoring of the turgor pressure by the Sln1 osmosensor might be effected through the contact between plasma membrane and cell-wall. To simulate the conditions of membrane detached from cell-wall, we enzymatically removed the yeast cell-wall using zymolyase. Removal of the cell-wall gave rise to the Hog1 activation in spheroplasts, and this activation was through the SLN1 pathway, because it is abrogated in *ssk2 ssk22* double mutant, but not in *ste 11* mutant. Taken together, these observations indicate that there is a causal link between the pressure of the plasma membrane against the cell-wall and the stimulation of Hog1 through the SLN1 branch.

Cre1 (cytokinin-response 1) is a plant (*Arabidopsis thaliana*) hybrid histidine kinase, which has been identified as a receptor for the plant hormone cytokinin and shown to be involved in plant vascular morphogenesis. Cre1 and Sln1 have similar domain organization. When expressed in yeast in the presence of a cytokinin (*e.g.*, zeatin), Cre1 functionally interacts with the yeast Ypd1-Ssk1 phospho-relay system and suppresses the Hog1 hyperactivation in the *sln1* deletion mutant. Surprisingly, high osmolarity stress rapidly activated the HOG pathway in *CRE1* expressing cells in the presence of zeatin, indicating that the active form of Cre1 (*i.e.*, bound to zeatin) has transformed to inactive in response to sudden increase of external osmolarity. Cre1 activity could be also modulated by turgor pressure. The *sln1*Δ mutants expressing *CRE1*, or *SLN1* as a control, were treated either with nystatin or with zymolyase. The both treatments induced Hog1 activation in *sln1*Δ *CRE1* cells. These results indicate that cytokinins and hyperosmotic stress (or reduction of turgor) regulate antagonistically the activity of Cre1 in yeast. The remarkable similarities between Sln1 and Cre1, and the fact that plant cells are also surrounded by cell-wall, suggest that Cre1 could have a dual sensor function as a cytokinin receptor and an osmosensor in plants.

2. A docking site determining specificity of Pbs2 MAPKK for Ssk2/Ssk22 MAPKKKs in the yeast HOG pathway

Kazuo Tatebayashi, Mutsuhiro Takekawa, and Haruo Saito

Mitogen Activated Protein Kinases (MAPKs) are a conserved family of protein kinases that serve major roles in intracellular signal transduction in eukaryotic cells. MAPK is activated through a cascade of three sequentially activated kinases: MAPK is phosphorylated and thus activated by a MAPK kinase (MAPKK), and MAPKK is activated by a MAPKK kinase (MAPKKK). Once activated in the cytoplasm, MAPK translocates to the nucleus where it regulates the expression of various effector genes by phosphorylating specific transcription factors.

A number of distinct MAPKs have been described in eukaryotic organisms: 5 in *Saccharomyces cerevisiae*, and more than 10 in mammals. Different external stimuli, such as mitogenic growth factors, pro-inflammatory cytokines, and osmotic and oxidative stresses, activate distinct subsets of MAPKs. There are also similarly large numbers of MAPKKs and MAPKKKs in each organism, potentially permitting an enormous number of MAPKKK-MAPKK-MAPK combinations. However, because proper responses to external stimuli are critical to cells and organisms, several mechanisms exist to ensure highly selective recognition among homologous kinases. Specific substrate-enzyme interaction, *i.e*. recognition of the substrate phosphorylation site(s) by the kinase catalytic site, must obviously be important, but is not selective enough. Therefore, other mechanisms exist that contribute to the specificity of the interaction. For example, in many cases, a scaffold protein tethers three kinases together thus limiting the flow of signal. Furthermore, it recently became evident that MAPKs use a docking domain to facilitate specific interactions with their activators (MAPKKs), inhibitors (protein phosphatases), and substrates (transcription factors). Both a docking domain and a scaffold protein contribute in varying degree to the final specificity between the two components.

There are greater structural diversities among MAPKKKs than among MAPKKs and MAPKs, perhaps reflecting similarly diverse upstream stimuli. Thus, specific MAPKKK-MAPKK inter-

action is a potential fate-determining event in cellular responses to external stimuli. Nonetheless, not much is yet known about how MAPKKK-MAPKK specificity is determined.

In the yeast MAPK cascades, the Ste11 MAPKKK can be activated by at least three distinct stimuli: high osmotic stress, mating factor, and nitrogen limitation. When activated, Ste11 can activate (*i.e.*, phosphorylate) two different downstream MAPKKs, Ste7 and Pbs2. Surprisingly, however, when Ste11 is activated by mating factors or by nitrogen limitation, it only transduces signals to Ste7, whereas when Ste11 is stimulated by osmotic stress, it only activates Pbs2. Specificity of Ste11 signaling appears to be regulated by the scaffold proteins Ste5 and Pbs2, which specifically tether multiple components of only one or the other pathway, although a role of the Hog1 MAPK in cross-talk suppression has also been reported. The Pbs2 MAPKK can also be activated by Ssk2 and Ssk22 MAPKKKs, which are activated only by osmotic stress. Unlike Ste11, the Ssk2/Ssk22 MAPKKKs only activate Pbs2, and never activate Ste7.

We found that this was due to an Ssk2/Ssk22-specific docking site in the Pbs2 N-terminal region. The Pbs2 docking site constitutively bound the Ssk2/Ssk22 kinase domain. Docking site mutations drastically reduced the Pbs2-Ssk2/Ssk22 interaction and hampered Hog1 activation by the SLN1 branch. Fusion of the Pbs2 docking site to a different MAPKK, Ste7, allowed phosphorylation of Ste7 by Ssk2/Ssk22. Thus, the docking site contributes to both efficiency and specificity of signaling. During these analyses, we also found a nuclear export signal and a possible nuclear localization signal in Pbs2.

3. Functions of the ectodomain and cytoplasmic tyrosine phosphatase domains of receptor protein tyrosine phosphatase Dlar in vivo

Neil X. Krueger[1], R. Sreekantha Reddy[1], Karl Jonson[1], Jack Bateman[1], Nancy Kaufmann[1], Daniella Scalice[1], David Van Vactor[1], and Haruo Saito: [1]Harvard Medical School

Protein tyrosine phosphorylation is a critically important posttranslational modification in the eukaryotic signal transduction pathways that regulate cell growth, differentiation and development. The phosphorylation status of a given tyrosine residue is regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). The essential role of PTKs in these pathways is now well established: misregulation of PTKs can have dire consequences, including cell death, cell transformation and tumorigenesis. Far less is understood about the functions of PTPases in signal transduction, especially for the receptor-like PTPases (RPTPs).

The PTPase gene family is large and varied. Mammalian LAR, PTPδ, PTPσ, and *Drosophila* Dlar comprise a subfamily of RPTPs that have ectodomains consisting of N-terminal Immunoglobulin-like (Ig-like) domains and membrane-proximal Fibronectin-type III (FnIII) repeat motifs, and cytoplasmic regions consisting of two tandemly-repeated PTPase-like domains, PTP-D 1 and PTP-D2. One of the FnIII repeat units of human LAR has been implicated in binding to the extracellular matrix (ECM) complex of laminin and nidogen, and Dlar cooperates with integrins to regulate actin polymerization during oogenesis by integrating signals from the ECM. So far, no physiologically-relevant ligand has been found that binds to the Ig-like domains of mammalian LAR family members, despite the fact that Ig-like domains are typically ligand binding domains. It is likely that other physiologically-relevant ligands for all of these RPTPs still remain to be discovered.

The PTPase domains of the LAR subfamily are extremely well conserved; the PTPase domains of *Drosophila* Dlar and human LAR share 74% amino acid sequence identity. We have previously reported that only the membrane-proximal PTPase domain, PTP-D1, of mammalian RPTPs has a physiologically-significant amount of catalytic activity. We found that this is also the case with Dlar. Despite the lack of detectable PTPase catalytic activity from the C-terminal PTPase-like domain, PTP-D2, it is apparent that PTP-D2 has important functions. PTP-D2 of LAR family members interacts with and tightly binds to several cytosolic proteins, including the liprin family of coiled-coil cytoskeletal proteins, the multi-functional protein kinase/guanine nucleotide exchange factor Trio, the Abl tyrosine kinase, and its substrate Enabled.

In *Drosophila*, Dlar has been implicated in regulating the development of the embryonic central nervous system (CNS), axon guidance, and development of the optic lobe. In developing fly embryos, Dlar mRNA is supplied to the syncytial blastoderm by maternal expression that disappears around the cellularization stage. Dlar then reappears late in embryogenesis specifically in the central nervous system (CNS) 12-16 hrs post-fertilization. Dlar transcripts are also expressed in adult flies during oogenesis. Loss-of-function mutations of the *Dlar* gene are lethal when homozygous, and cause multiple neuronal defects, including breaking and thinning of the CNS, aberrant, failed or ectopic muscle/motoneuron synapse formation, and defective visual

cognition. In *Dlar* loss-of-function mutants, Intersegmental Nerves b and d (ISNb and ISNd) fail to properly innervate their target muscles, a phenotype that is exacerbated if other RPTP genes, including *RPTP99A* and *RPTP10D* are simultaneously disrupted. Non-neuronal phenotypes are also seen in *Dlar* loss-of-function genotypes: defects in oogenesis are observed in germ -line $Dlar^{-/-}$ clones; mutant embryos exhibit loss of cell polarity, resulting in a round egg that is not oviposited. The implication of the phenotype is that Dlar present in the follicular epithelium surrounding the oocyte collaborates with integrin receptors to organize actin polymer arrays and cell polarity in the developing egg.

In order to better understand the function of RPTPs *in vivo*, we have constructed a series of point mutant and deletion mutant *Dlar* transgenes and introduced them into the *Drosophila* genome via P-element transformation. These mutant transgenes were expressed using the bipartite GAL4-UAS transcription system, which allows tissue-specific expression of transgenes. We designed the transgenes to probe several unanswered questions about RPTP structure, including which portions of the complex Dlar ectodomain are required for PTPase function, and which of the cytoplasmic PTPase-like domains are required for *in vivo* function. We found that both the Ig-like domains and FnIII domains of the Dlar ectodomain serve important, non-redundant functions. The Ig-like domains, but not the FnIII domains, are essential for survival. Conversely, the FnIII domains, but not the Ig-like domains are required during oogenesis, suggesting that different domains of the Dlar ectodomain are involved in distinct functions during *Drosophila* development. Surprisingly, we found that the catalytically-inactive mutants of Dlar were able to rescue $Dlar^{-/-}$ lethality nearly as efficiently as wild type *Dlar* transgenes, while this ability was impaired in the PTP-D2 deletion mutants *DlarΔPTP-D2* and $Dlar^{bypass}$. *Dlar-C1929S*, in which PTP-D2 has been inactivated, increases the frequency of bypass phenotype observed in $Dlar^{-/-}$ genotypes, but only if PTP-D1 is catalytically active in the transgene. These results indicate multiple roles for PTP-D2, perhaps by acting as a docking domain for downstream elements and as a regulator of PTP-D1.

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Department of Basic Medical Sciences

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

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

1. NMDA receptor phosphorylation and synaptic plasticity

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In the hippocampus, excitatory synaptic transmission is regulated dynamically depending on the pattern of synaptic activation: high-frequency activation induces long-lasting enhancement of synaptic efficacy referred to as long-term potentiation (LTP), and prolonged lower-frequency activation causes long-term depression (LTD) of synaptic transmission. Excitatory synaptic transmission is mediated by glutamate receptors and the N-methyl-D-aspartate (NMDA) receptor, one of the glutamate receptor subtypes, plays crucial roles in LTP and LTD induction.

Tyrosine phosphorylation of NMDA receptors by Src-family tyrosine kinases such as Fyn is implicated in synaptic plasticity. We identified Fyn-mediated phosphorylation sites on the GluRε2 (NR2B) subunit of NMDA receptors and Tyr 1472 was the major phosphorylation site. We then generated rabbit polyclonal antibodies specific to Tyr1472-phosphorylated GluRε2, and showed that Tyr1472 of GluRε2 was indeed phosphorylated in murine brain using the antibodies. Moreover, Tyr1472 phosphorylation grew evident when mice reached the age when hippocampal LTP started to be observed and its magnitude became larger. Finally, Tyr1472 phosphorylation was significantly enhanced after the induction of LTP in the hippocampal CA1 region. These data suggest that Tyr1472 phosphorylation of GluRε2 is important for synaptic plasticity. We are currently examining mutant mice that have a point mutation in this residue (tyrosine→phenylalanine) electrophysiologically and behaviorally.

2. Analysis of muscarinic acetylcholine receptor functions using knockout mice

Minoru Matsui, Shinji Kusakawa, Yuji Kiyama, Hideki Miwa, Toru Shinoe, Sayuri Inagaki, and Toshiya Manabe

We are investigating the biological function of muscarinic acetylcholine receptors (mAChRs) using mutant mice lacking corresponding genes (mAChR KO mice). These mice have been established by Matsui *et al*. at Laboratory of Biomedical Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo (Prof. Makoto M. Taketo Lab). The mAChRs (M_1, M_2, M_3, M_4 and M_5) belong to a group of seven transmembrane-spanning receptors and are distributed widely in both the central and peripheral nervous systems. Elucidation of the subtype-specific functions of mAChRs has been a matter of considerable interest, especially because they are suitable targets for pharmacological therapeutics. However, because of poor subtype-selectivity of the available ligands, pharmacological approaches to discriminate their roles remain inconclusive.

The use of mAChR KO mice is an alternative strategy to achieve complete subtype specificity. In order to minimize the concomitant effects reflecting the possible difference in the genetic background, we have backcrossed most of these mutant lines to two representative inbred strains, C57BL/6J and DBA/2J, for more than 10 generations. We have established an efficient system of mouse breeding and a total of 4,900 pups have been born within this year. Various compound mutant mice (M_1/M_2, M_1/M_3, M_1/M_5, M_2/M_3 and M_2/M_4) are also available.

Our original research articles of this year are as follows. Using a DBA/2J congenic strain, we have revealed that the M_4 receptor is a target of anticholinergic therapy of catalepsy induced by haloperidol (Karasawa et al., 2003). We have collaborated with Dr. Ehlert, UC Irvine, about the roles of M_2 in smooth muscle contractility (Matsui et al., 2003; Griffin et al., 2004). We have collaborated with Dr. Kano, Kanazawa Univ., and discovered that both M_1 and M_3 are responsible for the muscarinic enhancement of retrograde endocannabinoid signaling in the hippocampus (Ohno-Shosaku et al., 2004). We have also collaborated with Dr. Okabe, Kyoto Pharmaceutical Univ., and found that M_3 is essential for normal gastric secretion (Aihara et al., 2003).

Muscarinic receptors are supposed to be important in various brain functions. These include learning and memory, drug addiction, sleep and respiratory control, and striatal function. We are investigating the role of each subtype in these aspects, employing molecular biology, electrophysiology, and behavioral experiments.

3. Identification and characterization of a novel GTPase activating protein p250GAP

Ayako M. Watabe, Takanobu Nakazawa[2], Tohru Tezuka[2], Tadashi Yamamoto[2], and Toshiya Manabe

N-methyl-D-aspartate (NMDA) receptors regulate structural plasticity by modulating actin organization within dendritic spines. We have reported identification and characterization of p250GAP, a novel GTPase-activating protein for Rho family proteins that interacts with the GluRε2 (NR2B) subunit of NMDA receptors in vivo. The p250GAP mRNA was enriched in brain, with high expression in cortex, corpus striatum, hippocampus, and thalamus. Within neurons, p250GAP was highly concentrated in the postsynaptic density and colocalized with the GluRε2 (NR2B) subunit of NMDA receptors and with postsynaptic density-95. p250GAP promoted GTP hydrolysis of Cdc42 and RhoA in vitro and in vivo. When overexpressed in neuroblastoma cells, p250GAP suppressed the activities of Rho family proteins, which resulted in alteration of neurite outgrowth. Finally, NMDA receptor stimulation led to dephosphorylation and redistribution of p250GAP in hippocampal slices. Together, p250GAP is likely to be involved in NMDA receptor activity-dependent actin reorganization in dendritic spines.

4. Role of Ras signaling in synaptic plasticity

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The small GTPase Ras as well as Ras regulators and effectors are associated with NMDA-type glutamate receptors in the postsynaptic density of excitatory synapses. Although the role of Ras in NMDA receptor-mediated signaling has not been well characterized, several findings indicate that Ras signaling pathways have an important role in NMDA receptor-dependent forms of synaptic plasticity, such as long-term potentiation (LTP). For instance, mice with mutations affecting H-Ras or SynGAP (a synaptic Ras-GTPase activating protein) have alterations in hippocampal LTP. Moreover, pharmacological inhibition of the Ras effectors phosphatidylinositol 3-kinase (PI3-kinase) and the p44/42 MAPK (mitogen-activated protein kinase) pathway disrupts LTP. Although Ras-activated signaling pathways are clearly involved in LTP, the molecular details of how these pathways contribute to an enhancement of synaptic strength remain unclear. We therefore examined the role of PI3-kinase and ERK in LTP at excitatory synapses in the CA1 region of the mouse hippocampus. Consistent with the notion

that PI3-kinase links NMDA receptors to the ERK pathway, PI3-kinase inhibitors significantly reduced both NMDA and high-frequency stimulation-induced increases in ERK2 phosphorylation. We found, however, that PI3-kinase inhibitors suppress LTP under conditions in which blocking ERK activation with MEK (MAP kinase kinase) inhibitors has no effect. Thus, although PI3-kinase contributes to NMDA receptor-mediated ERK activation, our results demonstrate that the induction of LTP is also dependent on PI3-kinase signaling through ERK-independent pathways.

5. Modulatory neurotransmitters and synaptic plasticity

Ayako M. Watabe, Fumiko Arima, Shizuka Kobayashi, Thomas J. O'Dell[3], and Toshiya Manabe

Several signaling mechanisms that are crucial for the induction of LTP by theta frequency (5 Hz) trains of synaptic stimulation are altered in aged animals. Thus, to determine whether the induction of LTP by theta frequency stimulation is particularly sensitive to changes in synaptic function that occur in aged animals, we compared the effects of three different trains of synaptic stimulation pulses delivered at 5 Hz (theta pulse stimulation, TPS) on synaptic strength in the hippocampal CA1 region of aged and young mice. In addition, we investigated whether the modulation of TPS-induced LTP by β-adrenergic and cholinergic receptor activation showed deficits with aging. Our results indicated that TPS-induced LTP was not diminished in the aged hippocampus but showed pronounced dependence on L-type calcium channels that was not seen in slices from young animals. In addition, we observed that the enhancement of TPS-induced LTP by co-activation of β-adrenergic and cholinergic receptors was significantly reduced in slices obtained from aged animals. Since TPS-induced LTP was not altered in aged mice, our results suggest that deficits in modulatory pathways that regulate activity-dependent forms of synaptic plasticity may contribute to memory impairments in older animals. The molecular and biochemical mechanisms underlying this alteration in aged animals are currently under investigation.

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Department of Basic Medical Sciences

Division of Structural Biology (1)
分子構造解析分野(1)

Professor Eisaku Katayama, M.D., Ph.D.

教 授 医学博士 片 山 榮 作

Electron microscopy provides a useful and unique means to investigate the structure of biological materials including cells/tissues and purified macromolecules. If the specimens are properly prepared, we can preserve the instantaneous structure of functioning molecules not only in solution but also in live cells, and visualize their details with high contrast. Though the spatial resolution may not compete with X-ray crystallography, the real superior feature of the method is its almost unlimited applicability to those whose structure cannot otherwise be pursued

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

E. Katayama, T. Tojo, Y. Kimori and T. Shiraishi: in collaboration with Prof. N. Baba's team, Kogakuin University

We have been studying 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. In the field of molecular motor research, a unique experimental approach, "single molecule physiology" started 20 years ago and completely innovated the conventional knowledge on the intrinsic properties of various motor proteins and their interactions. The most important message learned from the new concept is that the behavior of individual molecules might be different from that of the others and that important information could be revealed by observation and separately measuring unaveraged properties of each single molecule. Thus, various experimental techniques to deal single molecules have already been established 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, and apparently is not compatible to "single molecule" matter. Electron microscopy is unique in terms that it has a potential to visualize the structure of individual molecules. In order to obtain the structural information of functioning actomyosin motor comparable to the results of single molecule physiology, we have been utilizing quick-freeze deep-etch replica electron microscopy with mica-flake technique to capture transient 3-D configuration of myosin crossbridges supporting actin movement *in vitro*. In this way, various molecular events extensively studied under fluorescence microscope can be instantaneously arrested within one millisecond and the structure of individual protein molecules under well-characterized experimental conditions might be clearly visualized with a resolution that enables to recognize subdomain arrangement of "individual" protein molecules, by high contrast metal-shadowing. Since replica specimens are extremely tolerant to high-dose electron beam irradiation, it should be possible to take many micrographs of the same field, and to reconstruct the 3-D constitution of individual protein parti-

cles by a tomography technique, if "missing data-range problem" can be adequately avoided. As a matter of fact, this problem has been the biggest obstacle that hampers high-resolution 3-D electron microscopy of biological material even now. We devised new method along this line to overcome the problem (patent pending) and applied it to obtain 3-D image of a single molecule from tilt-series micrographs. As a complementary approach to characterize the 3-D structure of the target particles, we have been simulating the replica images of protein molecules from their atomic coordinates. Since the image contrast of replica specimen arises by heavy metal-shadowing, virtual model in the shape of protein particle was placed in cyberspace, and its image was rendered by a ray-tracing computer program, as if it was illuminated by a number of surrounding light-sources from the appropriate elevation angles (patent pending). We were encouraged by initial trials with the models of actin filament and actomyosin rigor complex, which not only showed generally good matching to real replica images, but also revealed some subtle differences in the configurations between real and artificial images, suggestive for the feasibility and usefulness of this unconventional approach. Hence, we applied such strategies to examine 3-D structural features of myosin heads under a variety of conditions; free in solution with various bound nucleotides, and/or associated with actin filament. In this year, our research activities were directed to two ways; one, development of new experimental tools, computer programs for the image analyses and several microscopic probes dedicated for our purpose to specifically label intracellular target proteins of interest, both to realize "Structural Biology of Single Molecules", and the other, their application to actual biological materials including the functioning molecules *in situ* in live cells. Shown below is one good example to exhibit the utility of our methods. We had previously found that the body of crossbridges supporting actin during sliding was often bent to hold actin filament inside its curvature. According to the most popular "swinging lever-arm hypothesis" as the mechanism of actomyosin motor, the motor-domain is presumed to stay attached to actin in the same configuration throughout its power-stroke and the lever-arm moiety bends along the direction almost parallel to actin filament axis. Then, putative actin-binding site should come outside the curvature of bent S1 body. Now, the configuration we observed very often in actual replica images cannot reconcile with any of the transient states of lever-arm swinging. The hypothesis also postulates that each chemical state of myosin in its ATP-hydrolysis cycle corresponds to certain structural state of the cross-bridge configuration. Since the above configuration we observed during sliding does not seem to match with any one of the crystal structures so far reported, we sought for the possibility if myosin head could take such unusual configuration only transiently during its ATP-hydrolysis. Vertebrate skeletal myosin has two highly reactive thiol groups called SH1 and SH2 whose chemical modification greatly affects myosin's intrinsic enzymatic activity. X-ray structure of S1 revealed that they are actually located at both ends of a single α-helix (named as SH-helix) in the heart of myosin. From this structural feature, it is apparent that they cannot come close as long as that helix is kept stable. It has been reported that these two thiols react each other to be cross-linked by certain bifunctional reagents under limited conditions in the presence of ADP. p-Phenylenedimaleimide (pPDM) is one of such reagents whose span between reactive groups is approximately 12Å. In our deep-etch replica images, each head of pPDM cross-linked HMM/ADP was bent and appeared similar to that of well characterized kinked head, at first sight. We noticed, however, that the polarity of the head curvature was opposite to that we have observed previously for ATP-bound or ADP/Vi-bound HMM (Vi-form). This cannot be a simple mirror image of Vi-form configuration, because the proximal segment of S1 head continuing to S2 appeared strangely crooked or twisted, whereas smoothly curved Vi-form S1 continued quite naturally to S2 moiety. To characterize possible new configuration more in detail, we examined the structure of those particles by 3-D reconstruction and computer-simulation as above. Because our purpose here was to determine which side of S1 molecule is facing toward outside of the curvature, we laid our stress on the simulation of surface profiles of S1 and compared them with replica images actually observed under electron microscope. We modified S1 backbone structure of its X-ray atomic model and searched for the candidates in which the lever-arm comes closer to the opposite side of scallop Vi-form. In the structure we chose, the lever-arm orientation was about 150 degrees deviated from the original position. Simulated images of the modified structure placed in appropriate orientation showed nice matching with the delicate surface features of real replica images of pPDM-HMM. Though the initial trials for the matching were done manually by comparing simulated images and micrographs by human eyes, we have now developed semi-automated system to search for the best-matching face of the model structure. Thus,

we might be able to say more safely that the actin-binding site of pPDM-HMM head faces toward the inner side of the curvature. Since actin -bound HMM represents essentially similar surface features to that of pPDM-HMM, we concluded that one of the most abundant actin-bound configuration during sliding would be a short-lived transient state, where SH-helix might be disrupted. This unusual configuration might possibly represent putative energy-preserving structure as claimed by Prof. Yanagida's team. Further, we found that each of two heads of single HMM during sliding shows different configuration from each other; pPDM-form if it was associated with actin, but Vi-form, if not. It is apparent that such kind of information might be available only through structural analyses of single molecules. In collaboration with Dr. Maruta's team (Dept of Bio-engineering, Soka Univ.), we also obtained several spectroscopic evidences indicating that pPDM-crosslinked myosin and actin-activated myosin heads commonly take a unique configuration (probably pPDM-form) distinct from that of ATP-bound myosin structure (*i.e.* Vi-form). We expect that our approach "Structural biology of Single Molecules" would find fruitful future applications along this line.

In conclusion, we confirmed the presence of prominent structural change, the bending of the crossbridges during its sliding along actin filament. However, the direction of the bending is almost opposite to that predicted by swinging lever-arm hypothesis. Thus, we cannot heip considering the other possibilities for the molecular event that evokes muscle-contraction and the other cell motility phenomena.

The other collaborative studies are proceeding mostly on the structural change accompanied with the function of various motility-related protein systems and receptor molecules. These projects include the conformational change of myosin in myofiber *in situ* (with Drs T.Q.P. Uyeda, AIST, and H. Iwamoto, SPring-8), the stability of microtubule structure (with Dr. Y. Atomi's team, Dept of Life Sci., Grad. Sch. of Arts and Sci.) and the structure and function of bacterial exporting apparatus (with Dr. C. Sasakawa's team, Div. of Bacterial Infection in this Institute and Dr. S.-I. Aizawa's team, CREST.). Structural studies of ryanodine receptor molecule are in progress also as a collaboration with Drs T. Murayama (Juntendo Univ.) and H. Oyamada (Showa Univ.).

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Department of Basic Medical Sciences

Division of Structural Biology (2) Laboratory of Fine Morpholology

分子構造解析分野(2) 微細形態室

Research Associate Emiko Suzuki, Ph.D.
Research Associate Hiroshi Sagara, Ph.D.

助 手 医学博士 鈴 木 えみ子
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This laboratory has two major activities. One is to offer various supports for the research projects using electron microscopes. This includes the development of new techniques for electron microscopy. The other activity is our own and collaborative research works on various tissues and cells, mainly of nervous systems, by the combination of fine morphology and molecular biology.

1. Genetic and molecular anatomy of the Drosophila nervous system

Emiko Suzuki

Molecular and genetic studies on the Drosophila nervous system have demonstrated the elaborate genetic programs that control the development of neuronal networks. We are interested in how such programs are carried out at the subcellular level.

a. Synapse formation

The neuromuscular junction of Drosophila embryonic body wall musculature is one of the ideal models for studying development and function of synapses. Each hemisegment of an embryo/larva has 30 muscle cells, innervated by about 40 motoneurons. The neuromuscular projection forms in the late embryos. During these stages, one can fillet dissect the embryos and observe or manipulate the development of neuromuscular networks under a light microscope. We are focusing on the mechanism of the initial step of synapse formation, the synaptic target recognition. Recently, we found that the postsynaptic muscle cells project filopodia (myopodia) from their cell surfaces prior and during this process (Ritzenthaler et al., Nature neurosc., 2000). We also found that the myopodia interact with the filopodia of axonal growth cones of their synaptic partners. The ectopic expression of target recognition molecules suggested that it is controlled by these molecules (Suzuki et al., J Neurobiol., 2000). We were interested to learn how such molecules are expressed on the filopodia and how they play their roles at the ultrastructural levels. To study this issue, we have developed a new method to observe such filopodial interactions three dimensionally at the resolution of several nm using immuno-scanning electron microscopy. Each filopodium could be identified by the specific labeling with the antibodies conjugated with 5 or 10 nm colloidal gold particles. By this method, we could observe all the aspects of growing neuronal processes and muscle cells with reference to the cell surface molecules, and found that the filopodial interaction ocurrs not only between neural growth

cones and target muscle cells, but also between the axonal shafts an the muscle cells under their ways.

b. Phototransduction in the visual system

The phototransduction in Drosophila photoreceptor cells is a G-protein coupled phosphoinositide (PI)-signaling cascade. Its response is very rapid (receptor potential is generated within ~20 m seconds after light stimulation) and highly regulated. We are interested in the regulatory mechanism of this system. Our recent studies have shown that the topological coupling of the molecules of the major signaling cascades and the related metabolic pathway is essential (Suzuki, in "Atlas of Arthropod sensory receptors" Springer-Verlag, 1999). Previously we focused on the genetic mechanism of the intracellular targeting of eye-diacylglycerol kinase (eye- DGK/RdgA protein), that is a key enzyme for the regulation of intracellular level of DG and phosphatidic acid. In Drosophila phototransduction, DG is thought to regulate the gating of the light-sensitive membrane channels, and phosphatidic acid is a key precursor for the regeneration of membrane phophatidyl inositol bisphosphate that is essential for the initiation of phototransduction. We have demonstarted that eye-DGK localizes to the specialized cellular compartment called subrhabdomeric region, which is close to the photoreceptive organellae (rhabdomere) that contain most of the molecules of phototransduction (rhodopsin, G-protein, phospholipase C, light-sensitive channels etc). We were interested in how this enzyme is localized to this region. By the expression of the eye-DGK in which certain domains are deleated in photoreceptor cells, we found that the cystein-rich domains (CRDs) of eye-DGK is required for the function of eye-DGK. This is the first demonstration of the function of CRDs in DGKs *in vivo*. As for the intracellular localization of this molecule, we made double mutants of *rdgA* and *norpA* gene to block the degeneration phenotype of *rdgA* mutants. In such mutant photoreceptors, the eye-DGK protein with the deletion of CRDs mislocalized in the cell bodies and the axon terminals.

2. Cell and developmental biology of the visual system

a. Vitamin A metabolism in vertebrates and invertebrates

Hiroshi Sagara and Emiko Suzuki

Many of the proteins involved in vitamin A metabolism are phylogenetically conserved. Among these, RPE65 protein, which we previously identified in chick retinal pigment epithelial (RPE) cells, shows striking homology throughout vertebrates, more than 90% amino acid identities from fish to human, suggesting that this protein has essential roles in vitamin A metabolism. Although many researchers have studied this protein, its precise function in vitamin A metabolism is still obscure. For the purpose of clarifying this issue, we studied the homologue of RPE65 in Drosophila in which various molecular tools can be applicable. We found that Drosophila has one orthologue of RPE65, DRPE65. Sequence analysis indicated that DRPE65 protein is encoded by *ninaB*, one of the phototransduction genes. Recently, von Linting and Vogt showed that this protein has beta-carotene dioxygenase activity. This year, we analyzed the cellular localization of DRPE65 by immunohistochemistry. We found that it exists in eyes and digestive tracts. In eyes, it localized to the cone cells that are thought to contain vitamin A metabolizing enzymes for visual cycle. In the digestive tract, the basal region of the epithelial cells in the oesophagus was immunopositive. These results indicate that DRPE 65 has functions not only in visual cycle but also in vitamin A metabolism in the digestive system.

b. Development of the photoreceptor outer segments in vertebrates

Hiroshi Sagara, Yoko Tabata[1], Ken-ichi Arai[1] and Sumiko Watanabe[1]: [1]Division of Molecular and Developmental Biology, Department of Basic Medical Sciences

Photoreceptor outer segment consists of a stack of photo-receptive membrane discs, and is connected to the inner segment by a connecting cilium. Light energy is converted to electrical signals via the phototransduction cascade in the outer segment. During the morphogenetic process of the outer segment, the membrane discs are formed by the invagination of the plasma membrane, the constituents of which are synthesized in the inner segment. When the developing mouse retina was dissected out and cultured on the porous membrane, it can develop into the normally layered retina. When the culture was performed without RPE cells, even the cells in the outer nuclear layer formed well developed inner segments, the outer segments were never formed in these cells. When the retinal explants were cultured in the presence of RPE cells, some photoreceptor cells formed the outer segments. This indicates the presesnce of the

outer segment promoting factor(s) in the RPE cells. Molecular nature of the factor(s) inducing the formation of outer segments are now under investigation.

3. Other collaborative research works

a. Molecular and structural analysis of the influenza virus and Ebola virus.

Takeshi Noda[2], Hiroshi Sagara, Emiko Suzuki and Yoshihiro Kawaoka[2]; [2]Division of Virology, Department of Microbiology and Immunology

b. Electron microscopic analysis of the migrating edge cells of the regenerating capillaries.

Ikuo Yana[3], Hiroshi Sagara, Emiko Suzuki and Motoharu Seiki[3]: [3]Division of Cancer Cell Research, Department of Cancer Biology

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Department of Basic Medical Sciences

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We study the molecular mechanisms of expressing diversity and specificity unique to the nervous system and compare them with those of other tissues, and we undertake to understand the scenario of the development and differentiation of the nervous system. We are studying the following subjects by introducing molecular imaging, electrophysiology, molecular biology, cellular biology and biochemistry.
1) Role of IP_3 receptor/Ca^{2+} signaling in cell fuction:
We have already identified the importance of Ca^{2+} signaling in fertilization, cell division and dorsoventral axis formation. We further analyze how Ca^{2+} oscillation is involved in the brain development and differentiation as well as in the higher brain function.
2) Role of Zic (zinc finger protein enriched in the celebellum) in development:
We are studying the role of a gene (zic), in neural induction, morphogenesis of the brain, and right and left axis formation.
3) Molecular mechanism of neuronal positioning in the brain:
We are studying the role of genes (reelin, cdk5, disabled 1) in neuronal positioning and neurite extension.

1. Role of IP_3 receptor /Ca^{2+} signaling in cell function

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Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN)

We have been long working on the protein (P 400) which increases during development but is greatly decreased in the cerebellar mutant mice where Purkinje cells are deficient or spins of Purkinje cells are absent. We discovered that this developmentally regulated P400 protein is IP_3 receptor (IP_3R).

It is now known that inositol 1,4,5-trisphosphate (IP_3) is a second messenger produced through the phosphoinositide turnover in response to many extracellular stimuli (hormones, growth factors, neurotransmitters, neutrophines, odorants, light, etc.), and controls a variety of Ca^{2+}-dependent cell functions (cell proliferation, differentiation, fertilization, embryonic development, secretion, muscular contraction, immune responses, brain functions, chemical sense, light transduction, etc.) by inducing Ca^{2+} release (IP_3-induced calcium release; IICR) from intracellular Ca^{2+} store sites such as endoplasmic reticulum (ER) to cytoplasm. IP_3 binds to its specific receptor (IP_3R) on the Ca^{2+} store sites. IP_3R is an IP_3-gated Ca^{2+} release channel, and could be considered as a signal converter that exchanges the IP_3 signal to the Ca^{2+} signal that physiologically acts on a wide variety of targets. Our goal in this research is to elucidate the structure-function relationship of the IP_3R and the physiological roles of IP_3R-mediated Ca^{2+} signaling in various cell-types.

a. Structure -functuion analysis of IP_3 receptor type 1

We have cloned three types of IP_3R (IP_3R1, IP_3 R2, and IP_3R3) and have analyzed the structure, its function of each type of IP_3R by means of molecular biological, biochemical, cell biological, physiological and histochemical approaches. We have found that each type has different IP_3 binding (e.g., affinity, specificity, Ca^{2+} sensitivity) and modulation (e.g., phosphorylation, calmodulin binding) properties. The IP_3R is a polypeptide (~2,700 amino acids) with five major functionally distinct domains: the amino-terminal coupling domain, the IP_3 binding domain, the internal coupling domain, the transmembrane domain, and the carboxy-terminal gatekeeper domain. Four IP_3R subunits assemble to form a functional IP_3-gated Ca^{2+} release channel, and both homo- and heterotetrameric channels are detected. We analyzed the folding structure of the IP_3R channel by limited trypsin digestion and have found that the IP_3R channel is an assembly of four subunits, each of which is constituted by non-covalent interactions of five major, well folded structural components. The IP_3 binding core, a minimum essential region for specific IP_3-binding, resides among residues 226-578 of the mouse IP_3R1.

Ca^{2+} signaling via IICR often exhibits dynamic changes in time and space inside a cell (known as Ca^{2+} waves and Ca^{2+} oscillations). These complex spatiotemporal patterns are not produced by simple diffusion of cytoplasmic Ca^{2+}. The essential ingredients to generate repetitive Ca^{2+} spikes are positive feedback, cooperativity, deactivation (including negative feedback) and reactivation. The IP_3R1 is regulated by cytoplasmic Ca^{2+} in a biphasic manner. We found that the positive feedback regulation by cytoplasmic Ca^{2+} is an intrinsic property of the IP_3R1, whereas the negative feedback regulation by Ca^{2+} is mediated by calmodulin, a ubiquitous and multifunctional Ca^{2+}-dependent regulator protein. This finding suggests that the Ca^{2+}-dependent activation of the IP_3R is a fast process, while Ca^{2+}-dependent inactivation is a relatively slow process.

b. Structural property of IP_3 receptor type 1

a) IP_3 binding core

Bosanac, I., Alattia, J. R., Mal, T. K., Chan, J., Talarico, S., Tong, F. K., Tong, K. I., Yoshikawa, F., Furuichi, T., Iwai, M., Michikawa, T., Mikoshiba, K. & Ikura, M.

We unveiled three-dimensional structure of the IP_3-binding core complexed with IP_3 at a resolution of 2.2-Å. The asymmetric, boomerang -like structure consists of an N-terminal β-trefoil domain and a C-terminal α-helical domain containing an 'armadillo repeat'-like fold. The cleft formed by the two domains exposes a cluster of arginine and lysine residues that coordinate the three phosphoryl groups of IP_3. Eleven amino acid residues within the IP_3-binding core are involved as IP_3-coordinating residues.

b) Three-dimensional rearrangements within IP_3 receptor by calcium.

Hamada, K., Terauchi, A. & Mikoshiba, K.

We found that Ca^{2+} induces structural changes in the tetrameric IP_3R purified from mouse cerebella. Electron microscopy of the IP_3R particles revealed two distinct structures with 4-fold symmetry: a windmill structure and a square structure. Ca^{2+} reversibly promoted a transition from the square to the windmill with relocations of four peripheral IP_3-binding domains. Ca^{2+} appeared to regulate IP_3 gating activity through the rearrangement of functional domains.

Allosteric binding of calcium ion (Ca^{2+}) to IP_3 R controls channel gating of IP_3R. Here, we present biochemical and electron microscopic evidence of Ca^{2+}-sensitive structural changes in the three-dimensional (3D) structure of IP_3R1. Low concentrations of Ca^{2+} and high concentrations of Sr^{2+} and Ba^{2+} were shown to be effective for the limited proteolysis, but Mg^{2+} had no effect on the proteolysis. The electron microscopy and the limited proteolysis consistently demonstrated that the effective concentration of Ca^{2+} for conformational changes was less than 10^{-7} M, that this low concentration of Ca^{2+} only acted as an activator, and that the IP_3 scarcely affected the conformational states. The structure without Ca^{2+}, as reconstructed by 3D electron microscopy, had a "mushroom-like" appearance consisting of a large square-shaped head and a small channel domain linked by four thin bridges. The projection image of the "head-to-head" assembly comprising two particles confirmed the mushroom-like side view. The "windmill-like" form with Ca^{2+} also contains the four bridges connecting from the IP_3-binding domain towards the channel domain. These data suggest that the Ca^{2+}-specific conformational change structurally regulates the IP_3-triggered channel opening.

c. 4.1N, a molecule that translocates IP_3 receptor type 1 to plasma membrane

Zhang, S., Mizutani, A., Hisatsune, C., Higo, T., Bannai, H., Nakayama, T., Hattori, M. & Mikoshiba, K

Recently, we identified protein 4.1N as a binding molecule for the C-terminal cytoplasmic tail of the IP_3R1 using a yeast two-hybrid system. 4.1N and IP_3R1 associate in both subconfluent and confluent Madin-Darby canine kidney (MDCK) cells, a well studied tight polarized epithelial cell line. In subconfluent MDCK cells, 4.1 N is distributed in the cytoplasm and the nucleus; IP_3R1 is localized in the cytoplasm. In confluent MDCK cells, both 4.1N and IP_3R1 are predominantly translocated to the basolateral membrane domain; whereas 4.1R, the prototypical homologue of 4.1N, is localized at the tight junctions and other ER marker proteins are still present in the cytoplasm. Moreover, the 4.1N-binding region of IP_3R1 is necessary and sufficient for the localization of IP_3R1 at the basolateral membrane domain. A fragment of the IP_3 R1-binding region of 4.1N blocks the localization of co-expressed IP_3R1 at the basolateral membrane domain. These data indicate that 4.1N is required for IP_3R1 translocation to the basolateral membrane domain in polarized MDCK cells.

d. IRBIT, a novel IP_3 receptor binding protein, is released from the IP_3 receptor upon IP_3 binding to the receptor

Ando, H., Mizutani, A., Matsu-ura, T. & Mikoshiba, K.

IP_3Rs are IP_3-gated Ca^{2+} channels on intracellular Ca^{2+} stores. Here, we report a novel protein, termed IRBIT (IP_3R binding protein detected in inositol 1,4,5-trisphosphate-eluate), which interacts with IP_3R (IP_3R1 and was released on IP_3 binding to IP_3R1. IRBIT was purified from high salt extract of crude microsome of rat brain with IP_3 elution of affinity column immobilized with huge N-terminal cytoplasmic region of IP_3R1 (residues 1-2217). IRBIT, consisting of 530 amino acids, had a domain homologous to S-adenosylhomocysteine hydrolase in C-terminal and, in N-terminal, appendant 104 amino acids stretch containing multiple potential phosphorylation sites. *In vitro* binding experiments showed that the N-terminal region of IRBIT was essential for the interaction and that the IRBIT binding region of IP_3R1 was mapped on the IP_3-binding core. IP_3 dissociated IRBIT from IP_3R1 with EC50 ~0.5 mM, which was 50 times as potent as other inositol polyphosphates. Moreover, alkaline phosphatase treatment abolished the interaction, suggesting that the interaction was dualistically regulated by IP_3 and phosphorylation. Immunohistochemical studies and coimmunoprecipitation assay showed the relevance of the interaction in a physiological context. These results suggest that IRBIT is released from activated IP_3R, raising the possibility that IRBIT acts as a signaling molecule downstream of IP_3 R.

e. Critical regions for activation gating of the IP_3 receptor type 1

Uchida, K., Miyauchi, H., Furuichi, T., Michikawa, T. & Mikoshiba, K.

To understand the molecular mechanism of ligand-induced gating of the IP_3R/Ca^{2+} release channel, we analyzed the channel properties of delection mutants retaining both the IP_3-binding and channel-forming domains of IP_3R1. Using intrisically IP_3R-deficient cells as the host cells for receptor expression, we determined that six of the mutants, those lacking residues 1-223, 651 -1130, 1267-2110, 1845-2042, 1845-2216, and 2610 -2748, did not exhibit any measurable Ca^{2+} release activity, whereas the mutants lacking residues 1131-1379 and 2736-2749 retained the activ-

ity. Limited trypsin digestion showed that not only the IP_3-gated Ca^{2+}-permeable mutants lacking residues 1131-1379 and 2736-2749, but also two nonfunctional mutasntd lacking residues 1-223 and 651-1130, retained the normal folding structure of at least the C-terminal channel-forming domain. These results indicate that two regions of IP_3R1, *viz*. residues 1-223 and 651-1130, are critical for IP_3-induced gating. We also identified a highly conserved cysteine residue at position 2613, which is located within the C-terminal tail, as being essential for channel opening. Based on these results, we propose a novel fiver-domain structure model in which both N-terminal and internal couplong domains transduce ligand-binding signals to the C-terminal tail, which acts as a gatekeeper that triggers opening of the activation gate of IP_3R1 following IP_3 binding.

f. Physiological studies of the Ca^{2+} signaling in central nervous system

a) Long-term depression in the cerebellum of lacking IP_3 type 1 receptor

Takafumi Inoue, Akinori Kuruma[2], Takeshi Nakamura[1], Akira Futatsugi[1], Jun-ichi Goto, Ayuko Kurokura and Katsuhiko Mikoshiba

Analysis of the functional roles of the Ca^{2+} signaling in mammalian brain is one of the most focused topics in our research. We have shown that a neuronal IP_3R1-deficient mouse strain generated by gene-targeting technique exhibits significant reduction of birthrate and abnormal behavior (ataxia and seizure). We found that cerebellar slices prepared from IP_3R1-deficient mice completely lack long-term depression (LTD), a model of synaptic plasticity in the cerebellum. Moreover, a specific antibody against IP_3 R1, when introduced into wild-type Purkinje cells through patch pipettes, blocked the induction of LTD. These data indicate that, in addition to Ca^{2+} influx through Ca^{2+} channels on the plasma membrane, Ca^{2+} release through IP_3R plays an essential role in the induction of LTD in Purkinje cells. This year, we have been focusing on detailed Ca^{2+} dynamics evoked by synaptic activation in Purkinje cell dendrite, and have been revealing Ca^{2+} concentration characteristics in time and space, which will be a basis for understanding consequence of Ca^{2+} dynamics and Ca^{2+} signaling in neural dendrite.

In nonneuronal cells such as *Xenopus* oocytes, IP_3 is a global messenger that liberates Ca^{2+} throughout cytoplasm, but in cerebellar Purkinje cells, repetitive activation of parallel fiber-Purkinje cell synapses causes Ca^{2+} release that is restricted to individual postsynaptic local domains. The spatially restricted IICR might contribute the input specificity of the synaptic plasticity observed in the parallel fiber-Purkinje cell synapses. Our data suggests that the negative feedback regulations of Ca^{2+} mediated by calmodulin determine the spatial and temporal patterns of Ca^{2+} signaling in cerebellar Purkinje cells by limiting the amount of Ca^{2+} being released.

b) Long-term potentiation and long-term depression in hippocampal CA1 neurons of mice lacking the IP_3 type 1 receptor

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To investigate the role in synaptic plasticity of Ca^{2+} released from intracellular Ca^{2+} stores, mice lacking the IP_3R1 were developed and the physiological properties, long-term potentiation, and long-term depression of their hippocampal CA1 neurons were examined. There were so significant differences in basic synaptic functions, such as membrane properties and the input/output relationship, between homozygote mutant and wild-type mice. Enhanced paired-pulse facilitation at interpulse intervals of less that 60 ms and enhanced post-tetanic potentiation were observed in the mutant mice, suggesting that the presynaptic mechanism was altered by the absence of the IP_3R1. Long-term potentiation in the field-excitatory postsynaptic potentials induced by tetanus (100Hz, 1s) and the excitatory postsynaptic currents induced by paired stimulation in hippocampal CA1 pyramidal neurons under whole-cell clamp conditions were significantlly greater in mutant mice than in wild-type mice. Homosynaptic long-term depression of CA1 synaptic responses induced by low-frequency stimulation (1Hz, 500pulses) was not significantly different, but heterosynaptic depression of the non-associated pathway induced by tetanus was blocked in the mutant mice. Both long-term potentiation and long-term depression in mutant mice were completely dependent on N-methyl-d-aspartate receptor activity. To rule out the possibility of an effect compensating for the lack of the IP_3R1 occurring during development, an anti- IP_3R1 monoclonal antibody that blocks receptor function was diffused into the wild-type cell through a patch pipette, and the effect of acute block of IP_3R1 on long-term potentiation was observed compared with after control IgG, suggesting that developmental redundancy was not responsible for the increase in long-term potentiation amplitude observed in the

mutant mouse.

g. Studies on the ER dynamics

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Recently, ER is regarded as a dynamic organelle rather than a static and stable membranous structure as was classically considered. We are characterizing dynamic movement and restructuring of ER in variety of cell types including neurons.

In the neuron, ER is the major membranous component present throughout the axon. While other membranous structures such as synaptic vesicles are known to be transported via fast axonal transport, the dynamics of ER in the axon remained unknown. To elucidate them, we directly visualized the movement of two ER-specific membrane proteins, the sarcoplasmic/endoplasmic reticulum calcium-ATPase and the IP_3R, both of which were tagged with green fluorescent protein (GFP) in cultured chick dorsal root ganglion neurons. In contrast to GFP-tagged synaptophysin which moved as vesicles at 1 μm/sec predominantly in the anterograde direction in the typical style of fast axonal transport, GFP-tagged ER proteins did not move in a discrete vesicular form. Their movement detected by the fluorescence recovery after photobleaching technique was bi-directional, and the rate of the movement was ten-fold slower (~0.1 μm/sec) than fast axonal transport and temperature sensitive. The rate of movement of ER was also sensitive to low doses of vinblastine and nocodazole which did not affect the rate of synaptophysin-GFP. These results suggest that ER dynamics in the axon is dependent on the active transport system in which microtuble and motor proteins are probably involved, but is distinct from the well-documented movement of membranous vesicles.

2. Role of Zic (Zinc finger protein enriched in the cerebellum) in development.

a. Studies on the molecular mechanisms of neural and neural crest formation

J. Aruga[2], T.Kitaguchi, Y. Koyabu[2], T. Inoue, J. Hoshino, T. Tohmonda, K. Mikoshiba

Zic family was found as being expressed abundantly in the cerebellar granule cell lineage. The Zic genes encode transcription factor with zinc finger motifs. We previously showed that the genes are vertebrate homologues of Drosophila pair-rule gene, odd-paired and that the zinc finger motifs of Zic are highly similar to those of Gli family, which has been characterized as a factor involved in the Sonic hedgehog mediated signaling cascade. Furthermore, Zic proteins can bind to the target sequence of Gli proteins. Recently, we characterized the Xenopus Zic3 as determining the ectodermal cell fate and promote the earliest step of neural and neural crest development. In 2003, we generated Zic5-deficient mice by homologous recombination, and investigated the role of Zic5 in neural crest development. In the Zic5-defienct mice, we found a hypoplasia of the cephalic neural crest derivatives, such as mandibular bone and cranial ganglia. The result suggests that Zic5 has a role in the promotion of neural crest generation. Zic5 function is considered to be uniquely enhanced in the control of neural crest development among mouse Zic genes. This was the first indication of the involvement of mammalian Zic protein in the control of cephalic neural crest development.

b. Studies on the regional specification of the nervous system

J. Aruga[2], T.Kitaguchi , Y. Koyabu[2], T. Inoue, J. Hoshino, T. Tohmonda, K. Mikoshiba

We also found that the same gene family are involved in the regional specification of neural tissues besides the role of Zic family in the early stage of neural development. Gene targeting study of Zic families which are in progress in our laboratory showed that some of the Zic genes have essential roles in the development of dorsal neural tissue including cerebella. Mutations in human ZIC2 or ZIC3 causes congenital anomalies.

c. Zic2 patterns biocular vision by specifying the uncrossed retinal projection.

Herrera, E., Brown, L., Aruga, J., Rachel, R. A., Dolen, G., Mikoshiba, K., Brown, S. & Mason, C.A

We investigated the role of Zic2 proteins in retinal axon development using Zic2 mutant mice. Zic2 proteins were detected in the retinal ganglion cells in ventro-temporal region at certain developmental stages when these cells projects to ipsilateral optic tectum. In the Zic2 mutant mice, we revealed the proportion of ipsilaterally projecting fibers were decreased. It is considered that Zic2 controls the gene expression of proteins controlling the retinal axon guidance. We also found there is a correlation between the

location of Zic2 protein in the ventro-temporal retinal ganglion cells and the presence of ipsilateral projections through the analysis of several animal models. It had been known that the ipsilateral projection is essential for animals to recognize depth and distance of visual objects. (This work was done in collaboration with Dr. Carol Mason at Columbia University.)

3. Molecular mechanism of neuronal positioning in the brain.

T. Ohshima[2], K. Saruta[2], H. Suzuki[2], K. Hayashi, K. Mikoshiba

We have been analyzing several molecules that are involved in the control of neuronal alignment in the brain. We have shown the critical roles of Reelin, disabled-1 (Dab1) and Cdk5/p35 kinase in the positioning of cortical neurons through the analyses of the mutant mice for these genes. We had demonstrated the genetic interaction of *Cdk5/p35* and *Reelin/Dab1* in the positioning of the cortical neurons using double mutant mice. We have studied biochemical relation between Cdk5/p35 and Dab1 and found Cdk5/p35 modulates Reelin signaling via the phosphorylation of Dab1 *in vivo*. We recently identified a novel Cdk5 substrate in embryonic brain and we are now studying the significance of its phosphorylation in the brain development.

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Department of Basic Medical Sciences

Division of Molecular Neurobiology (2)
脳神経発生・分化分野(2)

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Malaria kills more than two million people worldwide every year, most of whom are children under 5 years of age. This death toll makes it imperative to develop effective methods to control this disease. To achieve this goal, we are focusing on genome research, DNA vaccine development and investigation of chaperones of malaria parasites. We have also started to approach this parasite in the context of comparative biology by studying other apicomplexan parasites, including murine malaria parasites, Toxoplasma gondii and Theileria species.

1. Full-length-enriched cDNA library

As of October 2002, the entire genomic nucleic acid sequence of *Plasmodium falciparum* (23 Mb on 14 chromosomes) had been deciphered, and 5286 genes had been predicted. However, analyses of mRNA are mandatory for accurate characterization of the expressed genes. In a cooperative study with Dr. Sumio Sugano, three full-length-enriched cDNA libraries were produced from the erythrocyte-stage malaria parasites. 5′ end-one-pass-sequencing of random clones provided important information which complements the genome sequencing projects. We have developed a mapping viewer that visualizes the sequences of cDNA clones on the determined genome. Furthermore, as a comparative biological approach, a full-length cDNA library of a murine malaria parasite, *Plasmodium yoelii*, was produced and sequenced. 5x draft genome sequences of *P. yoelii* and 5′ end sequences were aligned with the genome sequences of *P. falciparum*.

The database "FULL-malaria" is now available at http://fullmal.ims.u-tokyo.ac.jp.

2. Development of a novel DNA vaccine against the malaria parasite

Using a full-length cDNA library that was constructed using the RNA from erythrocyte-stage parasites of a lethal murine malaria, *P. berghei*, and an expression vector, we have started the screening of potential DNA vaccines in a murine malaria model. The data strongly suggest that vaccine immunization prolongs the survival of infected mice.

3. Chaperone DnaJ homologues of malaria parasites

DnaJ was first described by researchers at our institute as a gene that regulates phage replication in *E. coli*. In the genomic sequence of *Plasmodium falciparum*, 53 species of DnaJ homologues, which are characterized by a so-called J domain consisting of 70 conserved amino acids, have been identified. Though their ubiquitous existence in all organisms indicates the importance of these molecules, little is known about their functions. Malaria parasites contain quite unique DnaJ homologues (RESA; ring-infected erythrocyte surface antigens). We have focused on Pfj2 (Pbj2 in murine malaria), which is localized in the ER and unique to Apicomplexa species. Gene knockout experiments suggest that Pfj 2 is an essential gene. Systematic analyses will reveal the exact functions of these DnaJ homo-

logues in parasitism.

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Department of Basic Medical Sciences

Division of Molecular Biology (1)
遺伝子動態分野(1)

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Mimicry is a sophisticate program developed in animal, fish or plant to cheat objects by imitating a shape or a color for diverse purposes such as to prey, evade, lure, pollinate or threaten. Mimicry is not restricted to a 'macro-world' but can be extended to a 'micro-world' as 'molecular mimicry'. Recent advances in the structural and molecular biology uncovered that a set of translation factors resembles a tRNA shape and, in one case, even mimics a tRNA function for deciphering the genetic code. Nature must have evolved this 'art' of molecular mimicry between protein and ribonucleic acid using different protein architectures to fulfill the requirement of the ribosome. The mechanism of translational control as well as the structural, functional and applied aspects of molecular mimicry is main research interests in this department.

1. Translational Control and Protein-tRNA Molecular Mimicry

Koichi Ito, Miki Wada, Hanae Sato, Tomohiko Toyoda, Yuya Watanabe, Norbert Polacek[1], Maria J. Gomez[1], Liqun Xiong[1], Alexander Mankin[1], Zemfira N. Karamysheva[2], Andrey L. Karamyshev[2], Senya Matsufuji[3] and Yoshikazu Nakamura: [1]Center for Pharmaceutical Biotechnology, University of Illinois, [2]Department of Biochemistry and Biophysics, Texas A&M University, [3]Department of Biochemistry II, Jikei University, School of Medicine.

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

a. Making sense of mimic in translation termination

Recent crystallographic evidence suggests that the eukaryotic release factor (eRF1), the bacterial release factor (RF2) and the ribosome recycling factor (RRF) all mimic a tRNA shape, while biochemical and genetic evidence supports the idea of a tripeptide 'anticodon' in bacterial release factors RF1 and RF2. However, the suggested structural mimicry of RF2 is not in agreement with the tripeptide 'anticodon' hypothesis and furthermore very recent structures determined by cryo-electron microscopy show that RF2 has a conformation on the ribosome that is distinct

from the RF2 crystal structure. Also, hydroxyl radical probings of RRF on the ribosome is not in agreement with the simply idea of RRF mimicking a tRNA in the ribosome A site. All of this evidence seriously question a simple concept of mimicry in shape between proteins and RNA, and thus leaves only mimicry in function of protein factors of translation to be studied.

b. The critical role of the universally conserved A2602 of 23S ribosomal RNA in the release of the nascent peptide during translation termination

The ribosomal peptidyl transferase center is responsible for two fundamental reactions, peptide bond formation and nascent peptide release, during the elongation and termination phases of protein synthesis, respectively. We used *in vitro* genetics to investigate the functional importance of conserved 23S rRNA nucleotides located in the peptidyl transferase active site for transpeptidation and peptidyl-tRNA hydrolysis. While mutations at A2451, U2585, and C2063 (*E. coli* numbering) did not significantly affect either of the reactions, substitution of A2602 with C or its deletion abolished the ribosome ability to promote peptide release but had little effect on transpeptidation. This indicates that the mechanism of peptide release is distinct from that of peptide bond formation, with A2602 playing a critical role in peptide release during translation termination.

c. Antizyme frameshifting as a functional probe of eukaryotic translational termination

Translation termination in eukaryotes is mediated by the release factors eRF1 and eRF3, but mechanisms of the interplay between these factors are not fully understood, due partly to the difficulty of measuring termination on eukaryotic mRNAs. Here, we describe an *in vitro* system for the assay of termination using competition with programmed frameshifting at the recoding signal of mammalian antizyme. The efficiency of antizyme frameshifting in rabbit reticulocyte lysates was reduced by addition of recombinant rabbit eRF1 and eRF3 in a synergistic manner. Addition of suppressor tRNA to this assay system revealed competition with a third event, stop codon readthrough. Using these assays, we demonstrated that an eRF3 mutation at the GTPase domain repressed termination in a dominant negative fashion probably by binding to eRF1. The effect of the release factors and the suppressor tRNA showed that the stop codon at the antizyme frameshift site is relatively inefficient compared to either the natural termination signals at the end of protein coding sequences or the readthrough signal from a plant virus. The system affords a convenient assay for release factor activity and has provided some novel views of the mechanism of antizyme frameshifting.

2. Regulation of Ribosome Recycling

Toshinobu Fujiwara[1], Tohru Yamami, Koichi Ito, and Yoshikazu Nakamura: [1]Department of Biology, Graduate School of Science and Technology, Kobe University.

a. Ribosome recycling factor disassembles the posttermination ribosomal complex independent of the ribosomal translocase activity of elongation factor G

Ribosome recycling factor (RRF) disassembles posttermination ribosomal complexes in conjunction with elongation factor EF-G freeing the ribosome for a new round of polypeptide synthesis. RRF is structurally similar to tRNA and is therefore thought to functionally mimic tRNA and be translocated by EF-G during ribosome disassembly. However, EF-G variants that remain active in GTP hydrolysis but are defective in tRNA translocation fully activate RRF function *in vivo* and *in vitro*. Furthermore, RRF and the GTP form of EF-G do not co-occupy the terminating ribosome *in vitro*; RRF is ejected by EF -G from the preformed complex. These findings suggest that RRF action does not mimic tRNA and is independent of the translocation activity of EF-G.

b. Functional domain in RRF necessary for coordination with the catalytic action of EF -G-driven posttermination ribosome disassembly

Aquifex aeolicus RRF binds *Escherichia coli* ribosomes but is nonfunctional and toxic in *E. coli*. While other nonfunctional RRFs from different species can be activated in *E. coli* upon coexpression of homologous EF-Gs, *A. aeolicus* RRF remains inactive in the presence of *A. aeolicus* EF-G. It became functional by replacing the *A. aeolicus* EF-G domains I-III by the *E. coli* domains, showing that the concerted action of G domain of EF-G is crucial for RRF function. *A. aeolicus* RRF could also be activated in the presence of *E. coli* EF-G by introducing surface substitutions in the L-shape armpit region of RRF, which are distinct from any mutations previously isolated. These findings suggest that RRF action can be divided into, at least, three func-

tional steps, i.e., posttermination ribosome binding, EF-G interaction, and functional (or dynamic) coordination with the ribosome and EF-G, and that identified amino acids in the armpit surface region are involved in the coordination with the catalytic motion of the ribosome triggered by EF-G on the posttermination ribosome.

3. Molecular Biology of Yeast Prions

Toru Nakayashiki, Colin G. Crist, Hideyuki Hara, Hiroshi Kurahashi, Hiroyuki Kodama 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. $[PHI^+]$, a novel Sup35-prion variant propagated with non-Gln/Asn oligopeptide repeats in the absence of the chaperone protein Hsp104

The $[PSI^+]$ element of the budding yeast is an aggregated form of the translation release factor Sup35 that is propagated and transmitted cytoplasmically in a manner analogous to that of mammalian prions. The N-terminal of Sup35, necessary for $[PSI^+]$, contains oligopeptide repeats and multiple Gln/Asn residues. We replaced the Gln/Asn-rich prion repeats of Sup35 with non-Gln/Asn repeats from heterologous yeast strains. These non-Gln/Asn repeat Sup35s propagated a novel $[PSI^+]$ variant, $[PHI^+]$, that appeared *de novo* 10^3 times more frequent than $[PSI^+]$. $[PHI^+]$ was stably inherited in a non-Mendelian fashion, but not eliminated upon the inactivation of Hsp104, unlike known $[PSI^+]$ elements. *In vitro*, non-Gln/Asn repeat domains formed amyloid fibers that were shorter and grew more slowly than did Gln/Asn-rich prion domains, while $[PHI^+]$ aggregates were smaller than $[PSI^+]$ aggregates *in vivo*. These findings suggest the existence of an alternative, Hsp104-independent pathway to replicate non-Gln/Asn variant Sup35 prion seeds.

b. Prion domain interaction responsible for species discrimination in yeast $[PSI^+]$ transmission

The yeast $[PSI^+]$ factor is transmitted by a prion mechanism involving self-propagating Sup35 aggregates. As with mammalian prions, a species barrier prevents prion transmission between yeast species. The N-terminal of Sup35 of *Saccharomyces cerevisiae*, necessary for $[PSI^+]$, contains two species-signature elements; a Gln/Asn-rich region (residues 1-41; designated NQ) that is followed by oligopeptide repeats (designated NR). We found that *S. cerevisiae* $[PSI^+]$ is transmissible through plasmid shuffling and cytoplasmic transfer to heterotypic Sup35s whose NQ is replaced with the *S. cerevisiae* NQ. In addition to homology, the N-terminal location is essential for NQ mediated susceptibility to $[PSI^+]$ transmission amongst heterotypic Sup35 s. *In vitro*, a swap of NQ of *S. cerevisiae* Sup35 led to cross seeding of amyloid formation. These findings suggest that NQ discriminates self from non-self, and is sufficient to initiate $[PSI^+]$ transmission irrespective of whether NR is heterotypic. NR as well as NQ alone coalesces into existing $[PSI^+]$ aggregates, showing their independent potentials to interact with the identical sequence in the $[PSI^+]$ conformer. Therefore, two sequential events must occur for $[PSI^+]$ transmission between heterologous Sup35s. First, in a homology dependent manner, the N-terminal discriminator allows for the seeding of $[PSI^+]$. Second, in a homology-independent manner, oligopeptide repeats may stabilize the growing amyloid via intramolecular interactions. Our observations, that $[PSI^+]$ could be transmitted amongst Sup35s with a homologous NQ, while homologous NR only propagated a $[PSI^+]$ -like phenotype in a 'quasi-prion' state, supports this model. As long as the NQ region was homologous, the $[PSI^+]$ conformer could be transmitted to a heterologous NR containing Sup35 that subsequently propagated a distinct, stable $[PSI^+]$ independently. As shown by our 'quasi-prion' phenotype, homologous NR, important for stability of $[PSI^+]$, was sufficient to demonstrate co-aggregation and recruitment of a Sup35 chimera to existing $[PSI^+]$ aggregates. However, since the NQ region is heterologous, the new Sup35 did not adopt and propagate an independent $[PSI^+]$ of its own, hence loss of $[PSI^+]$ upon removal of the inducer Sup35. The

present finding contributes insight into the molecular basis of functional interactions for these two prion 'species signatures'.

4. Protein Crystallography

Koichi Ito, Miki Wada, Tomohiko Toyoda, Masayo Urata, Chunguang Kong[1], Martin A. Walsh1, Yuying Liu[1], Sundramurthy Kumar[1], Haiwei Song[1], and Yoshikazu Nakamura: [1] Laboratory of Macromolecular Structure, Institute of Molecular and Cell Biology, Singapore.

This laboratory is fully equiped with a facility and researcher for the X ray crystallography and contributes not only to this laboroatory study but also to the institutional need as a core service unit.

a. Crystal Structure and Functional Analysis of the Eukaryotic Cass II Release Factor eRF3 from *S. pombe*

Translation termination in eukaryotes is governed by two interacting release factors, eRF1 and eRF3. The crystal structure of the eEF1α-like region of eRF3 from *S. pombe* determined in three states (free protein, GDP- and GTP-bound forms) reveals an overall structure that is similar to EF-Tu, although with quite different domain arrangements. In contrast to EF-Tu, GDP/GTP binding to eRF3c does not induce dramatic conformational changes, and Mg^{2+} is not required for GDP binding to eRF3c. Mg^{2+} at higher concentration accelerates GDP release, suggesting a novel mechanism for nucleotide exchange on eRF3 from that of other GTPases. Mapping sequence conservation onto the molecular surface, combined with mutagenesis analysis, identified the eRF1-binding region, and revealed an essential function for the C terminus of eRF3. The N-terminal extension, rich in acidic amino acids, blocks the proposed eRF1-binding site, potentially regulating eRF1 binding to eRF3 in a competitive manner.

5. Pharmaceutical RNA Design

Takashi Ohtsu, Akihiro Oguro, Taichi Sakamoto, Shin Miyakawa, Shoji Ohuchi, Gota Kawai[1], Kiyotaka Mochizuki, Eiko Futami-Takada, Yukiko Iwata, Katsushi Koda, Nahum Sonenberg[2] and Yoshikazu Nakamura: [1]Department of Industrial Chemistry, Faculty of Engineering, Chiba Institute of Technology, [2] Department of Biochemistry and McGill Cancer Center, McGill University.

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. We have initiated SELEX experiments using mammalian translation initiation factors including eIF4E, eIF4G, eIF1A and eIF4A provided by Dr. Nahum Sonenberg (McGill University, Canada) and Dr. John Hershey (UC-Davis, USA). eIF4G and eIF4A proteins are known to be crucial for catalyzing the initiation of protein synthesis by playing as a multipurpose ribosome adapter bridging eIF4E (cap-binding protein), eIF3 (40S subunit binding protein), eIF4A and Pab1p (poly-A binding protein), and an RNA unwinding helicase, respectively. Importantly, the abnormality in the protein level or the activity of either initiation factor is known to cause cell proliferation. We aim to test the possibility of developing anti-eIF RNA aptamers for novel diagnostic and therapeutic tools.

a. RNA aptamers to initiation factor 4A helicase hinder cap-dependent translation by blocking ATP hydrolysis

The mammalian translation initiation factor 4 A (eIF4A) is a prototype member of the DEAD-box RNA helicase family that couples ATPase activity to RNA binding and unwinding. In the crystal form, eIF4A has a distended "dumbbell" structure consisting of two domains, which probably undergo a conformational change, upon binding ATP, to form a compact, functional structure via the juxtaposition of the two domains. Moreover, additional conformational changes between two domains may be involved in the ATPase and helicase activity of eIF4A. However, the molecular basis of these conformational changes is not understood. Here, we generated RNA aptamers with high affinity for eIF4A by *in vitro* RNA selection-amplification. Upon binding, the RNAs inhibit ATP hydrolysis. One class of RNAs contains members that exhibit dissociation constant of 27 nM for eIF4A and severely inhibit cap-dependent *in vitro* translation. The binding affinity was increased upon Arg substitution in the conserved motif Ia of eIF4A, which probably improves a predicted arginine network to bind RNA substrates. Selected RNAs, however, failed to bind either domain of eIF4A that had been split at the linker site. These findings suggest that the selected RNAs interact cooperatively with both domains

of eIF4A, either in the dumbbell or the compact form, and entrap it into a dead-end conformation probably by blocking the conformational change of eIF4A. The selected RNAs, therefore, represent a new class of specific inhibitors which are suitable for the analysis of eukaryotic initiation, and which pose a potential therapeutic against malignancies that are caused by aberrant translational control.

b. Solution structures of two hairpins found in an RNA aptamer to Initiation Factor 4A

Structural information of RNA aptamers is able to enhance our understanding of protein-RNA interactions at the atomic level, and facilitate extensive manipulation and design of RNA aptamers for therapeutic purpose. We have determined the structures of the AUCGCA loop and the ACAUAGA loop found in the above RNA aptamer to human eIF4A using nuclear magnetic resonance (NMR) spectroscopy. The AUCGCA loop is stabilized by a U-turn motif and contains a non-canonical A:A base pair (the single hydrogen bond mismatch: Hoogsteen/Sugar-edge). The ACAUAGA loop is stabilized by an AUA tri-loop motif and contains the other type of A:A base pair (the single hydrogen bond mismatch: Watson-Crick/Watson-Crick). Considering structural and biochemical analysis of the aptamer and its variants, it is likely that the AUCGCA loop are involved in direct interaction with eIF4A, and the flexibility of the ACAUAGA loop is important for the interaction. The Watson-Crick edges of C7 and C9 in the AUCGCA loop are supposed to interact with eIF4A.

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Department of Basic Medical Sciences

Division of Molecular Biology (2)
遺伝子動態分野(2)

Associate Professor Ichizo Kobayashi, Ph.D.

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One genome is a community of genes potentially with different interests. Their collaboration and conflicts underlie various life and death processes. Our goal is to understand genome dynamics, deaths, diseases, genome changes, and genome evolution from this point of view. Genes coding for a restriction-modification gene complex will provide a clue to these problems. This understanding will open a door to a new type of medicine and biotechnology.

1. Restriction-modification gene complexes as selfish, mobile elements programming cell death and genome rearrangement.

A gene for a restriction (R) enzyme, which cuts DNA at a specific sequence, is often linked to a gene for a modification (M) enzyme, which methylates the same sequence to protect it from cleavage. These systems have been regarded as bacterial tools of defense that attack invading unmethylated DNA but protect the bacterium's own methylated DNA. However, some RM gene complexes kill host bacteria that have threatened their presence or lost them, using restriction cleavage of the chromosome. This and other observations led to the hypothesis that some RM gene complexes behave as selfish mobile genetic elements, similar to viruses and transposons. The increasing evidence in support of this hypothesis includes restriction site avoidance in bacteria, the life cycle and mutual competition of RM complexes, their potential mobility and horizontal transfer, and their association with genome rearrangements.

a. Multiplication of a restriction-modification gene complex.

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Previous works have suggested that some gene complexes encoding a restriction (R) enzyme and a cognate modification (M) enzyme may behave as selfish mobile genetic elements. RM gene complexes, which destroy 'non-self' elements marked by the absence of proper methylation, are often associated with mobile genetic elements and are involved in various genome rearrangements (Kobayshi, 2001). Here we found amplification of a restriction-modification gene complex. BamHI gene complex inserted into *Bacillus* chromosome showed resistance to replacement by a homologous stretch of DNA. Some cells became transformed with the donor without losing BamHI. In most of these transformants, multiple copies of BamHI and the donor allele were arranged as tandem repeats. When a clone carrying one copy of each allele was propagated, extensive amplification of BamHI and the donor unit was observed in a manner dependent on restriction enzyme gene. This suggests restriction cutting of

the genome participates in the amplification. Visualization by fluorescent *in situ* hybridization revealed that the amplification occurred in single cells in a burst-like fashion that is reminiscent of induction of provirus replication. The multiplication takes place in a bacterium with natural capacity for DNA release, uptake, and transformation and might contribute to spreading of RM gene complexes in a virus like life cycle.

b. Intragenomic movement of a restriction-modification gene complex.

Seishi Ohashi, Noriko Takahashi, Yoko Mizutani-Ui, and Ichizo Kobayashi

Potential mobility and horizontal transfer of restriction-modification gene complexes can be inferred from their linkage to mobile genetic elements, and from genome sequence comparisons and evolutionary/informatics analyses. We detected movement of a restriction-modification gene complex within a cell when attempts were made to eliminate it from the cell. After blocking replication of a thermosensitive plasmid carrying *Pae*R7I restriction-modification gene complex in *Escherichia coli* by a temperature shift, thermo-resistant survivors carrying *Pae*R7I integrated into the chromosome were recovered. The products could be classified at the sequence level into: (i) those that had the plasmid and the chromosome apparently co-integrated at chromosomal transposons (IS1 or IS5); (ii) those that were due to *de novo* insertion of the IS1 together with the entire plasmid except for a 1-3 bp long deletion; (iii) those that resulted from reciprocal crossing-over between the plasmid and the chromosome at a 1-3 bp region of homology. We discuss mechanisms of the underlying cooperation between restriction-modification gene complexes, IS and host homologous recombination functions. The restriction-modification gene complexes may use the same strategy of restriction attack on the genome for their short-term and long-term persistence.

c. Molecular mechanism underlying post-segregational host cell killing by restriction modification enzyme systems.

Asao Ichige and Ichizo Kobayashi

Previous works in this laboratory have demonstrated that certain restriction modification gene systems can kill host cells when the gene systems are eliminated from the host cells. The molecular mechanism underlying these post segregational host killing by restriction modification gene system is not completely understood, although several lines of evidence strongly suggest that cleavage of the host chromosome by restriction enzyme is responsible for the host cell killing. In particular, little is known about the molecular mechanism of how the restriction sites on the host chromosome, which are initially protected by modification enzyme, become sensitive to cleavage by restriction enzyme in the process of post segregational host cell killing. To elucidate the underlying mechanism, we are currently examining how cellular level of restriction enzyme and that of modification enzyme change when post segregational host killing is induced. We are especially interested in examining whether difference in protein stability between restriction enzyme and modification enzyme proteins play a role in the host cell killing, as shown for other proteic toxin-antitoxin systems that cause post segregational host cell killing.

e. Death as a principle of symbiosis of genetic elements in a genome—a hypothesis for involvement of mitochondria in programmed cell death.

Ichizo Kobayashi

Once some gene sets are established in a genome, their products kill the host organism when the persistence of these genes is threatened. A simple example is provided by a gene complex, such as *Eco*RI RM, encoding a restriction enzyme and a cognate modification methyltransferase. The descendants of cells that lose the RM gene complex are unable to modify a sufficient number of recognition sites in their chromosomes to protect them from lethal attack by the remaining molecules of restriction enzyme. When loss of a gene leads to death, the gene is called essential. However, since its first appearance in the genome as a 'dispensable' gene, from outside or from inside, the gene may have co-evolved with the host so that it can now program death upon their curing. This form of programmed cell death, post-disturbance killing or addiction, might be a general principle in symbiosis of genetic elements within a genome. Eukaryotic programmed cell death often proceed through release of toxic molecules from mitochondria. The capacity of mitochondria to kill their host eukaryotic cell, upon disturbance, may have stabilized their symbiosis at the initial stage. This scenario of symbiosis-through-death may provide a paradoxical answer to the question of how symbiosis can ever evolve from interaction between genetic elements with potentially different interests.

f. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes.

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Since 1973, restriction endonucleases (REases) and DNA methyltransferases (MTases) have been named based on an original suggestion of Smith & Nathans. They proposed that the enzyme names should begin with a three-letter acronym in which the first letter was the first letter of the genus from which the enzyme was isolated and the next two letters were the first two letters of the species name. Extra letters or numbers could be added to indicate individual strains or serotypes. Thus, the enzyme *HindII* was one of four enzymes isolated from *Haemophilus influenzae* serotype d. The first three letters of the name were italicized. As more enzymes have been found, often from different genera and species with names whose three-letter acronyms would be identical, considerable laxity in naming conventions has appeared. In addition, we now know that each major type of enzyme can contain sub-types. This especially applies to the Type II enzymes, of which more than 3,500 have been characterized . In this work, we revisit the naming conventions and outline an updated scheme that incorporates current knowledge about the complexities of these enzymes. A nomenclature will be presented for restriction endonucleases, DNA methyltransferases, homing endonucleases and related genes and gene products. It provides explicit categories for the many different Type II enzymes now identified and provides a system for naming the putative genes found by sequence analysis of microbial genomes.

2. Involvement of restriction-modification gene complexes and other elements in genome rearrangements and genome evolution as suggested from genome comparison.

Recently complete sequences of two or more microbial genomes that are closely related to each other have been determined. Detailed comparison of such genomes has become a useful

approach for elucidating principles and mechanisms of genome evolution.

a. CGAT: Comparative genome analysis tool for closely related microbial genomes.

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For comparison of closely related bacterial genomes, it is required not only to compare the sequences themselves but also to compare various aspects of sequence features, combining with the alignment of the genomic sequences. To this goal, we are developing a genome analysis tool (CGAT). In this tool, an user can compare several feature segments identified in each genome by various sequence analysis programs, by overlaying them onto the alignment of homologous or orthologous segments identified by all-against-all homology search.

b. Relation between restriction modification genes and genome rearrangements suggested from genome sequence comparison within genus *Neisseria*

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The complete genome sequences of three different lines in the genus *Neisseria*, became available-those of *Neisseria meningitidis* strain Z 2491 (serogroup A), *Neisseria meningitidis strain* MC58 (serogroup B), and a *Neisseria gonorrhoeae* strain. We searched these genomes for DNA methyltransferase homologues and compared two genome sequences in their neighborhood by CGAT. Each genome was characterized by abundance of highly repetitive elements. In intraspecific comparison, we identified insertion of an RM gene complex into a putative operon. We also identified insertion of a long DNA segment with an RM gene complex. In interspecific comparison, we identified transposition of RM gene complex and more examples of operon insertion. In some cases, the polymorphism is linked with IS elements. These results lend further support for the hypothesis that RM genes are potentially mobile and involved in genome rearrangements.

c. Mechanism of genome rearrangements and genome evolution suggested from comparison between two complete genome sequences of *Staphylococcus aureus*

Takeshi Tsuru, Yoko Mizutani-Ui, Ikuo Uchiyama[44], Mikihiko Kawai, Ichizo Kobayashi

Staphylococcus aureus is one of the major pathogens causing both community-acquired and hospital-acquired infections. It produces a variety of toxins that elicit both regional and systemic inflammation in human body. Many of the toxins are known as super-antigens that cause unique disease entities such as toxic shock syndrome and staphylococcal scarlet fever. *S. aureus* has acquired resistance to practically all antibiotics so far introduced in clinical practice. Whole genome sequences of two related *Staphylococcus aureus* strains, N315 and Mu50, were determined. The two genome sequences were compared by CGAT to detect large-scale genome polymorphisms. How they were generated was considered. The restriction modification homologues found inserted into their drug-resistance island and into their genomic islands were analyzed. A hypothesis for their evolution in relation to these mobile elements and for their role in toxin function and evolution was proposed.

d. Identification in a methicillin-susceptible *Staphylococcus hominis* of an active primordial mobile genetic element for the Staphylococcal Cassette Chromosome *mec*, (SCC*mec*) which—in Methicillin-Resistant, *Staphylococcus aureus*—is the carrier of the resistance gene *mecA*

Yuki Katayama[47], Fumihiko Takeuchi[48], Teruyo Ito, Xiao Xue Ma[47], Yoko Ui-Mizutani, Ichizo Kobayashi, and Keiichi Hiramatsu: [47]Department of Bacteriology, Juntendo University, [48]Research Institute, International Medical Center of Japan and The Organization for Pharmaceutical Safety and Research

We previously reported that methicillin resistance gene *mec*A is carried by a novel type of mobile genetic element, SCC*mec* (Staphylococcal cassette chromosome *mec*) in the chromosome of methicillin-resistant *S. aureus* (MRSA). These elements are precisely excised from the chromosome and integrated into a specific site on the recipient chromosome by a pair of recombinases encoded by the *ccr* (cassette chromosome recombinase) *A* and *B* genes. In the present work, we detected homologues of the *ccr* genes in *S. hominis* type strain GIFU12263 (equivalent to ATCC 27844), which is susceptible to methicillin. Se-

quence determination revealed that the *ccr* homologues in *S. hominis* were classified into type-1 *ccr* genes (*ccrA1, ccrB1*) and were localized on a genetic element structurally very similar to SCC*mec* except for the absence of methicillin-resistance gene, *mecA*. The element had mosaic-like patterns of homology with extant SCC*mec* elements, which we designated SCC_{12263} as a representative of type-1 SCC. The *ccrB1* gene identified in the *S. hominis* strain was the first type-1 *ccrB* that retained its function as judged from two criteria: 1) SCC_{12263} was spontaneously excised during cultivation of the strain, and 2) introduction of the *S. hominis ccrB1* into an MRSA strain carrying type-I SCC*mec* whose *ccrB1* gene is inactive generated SCC*mec* excisants at high frequency. Existence of a SCC without *mec* determinant indicates a staphylococcal site-specific mobile genetic element that serves as a vehicle of transfer for various genetic markers across staphylococcal species.

3. Novel biotechnology based on behavior of restriction-modification systems as selfish mobile elements.

a. Application to maintenance and expression of useful genes.

Noriko Takahashi-Kobayashi, Nanae Kotake[58], Hiroko Funaki[49], and Masanori Watahiki[58], Ichizo Kobayashi: Nippongene[49]

The restriction modification (RM) gene pair has a function to force their stable maintenance to their host. This provides the opportunity for stable maintenance and expression of useful genes. Plasmids that carry lactose operon, a model useful gene cluster, connected to *Eco*RI RM genes (R+/R− and M+), were introduced into a *lac- E. coli* strain. The plasmid stability as well as LacZ activity were greatly increased by the presence of the R gene in the absence of antibiotic selection. A similar stabilization in the maintenance and expression was observed with chloramphenicol acetyltransferase (CAT) gene at a larger industrial scale.

b. Experimental genome evolution.

Youko Asakura[50], Ichizo Kobayashi: [50]Ajinomoto

Works from our laboratory demonstrated that an attempt to replace chromosomally-located restriction-modification gene complex by a homologous stretch of DNA leads to a variety of large-scale genome rearrangements. This may provide a novel procedure of breeding of microorganisms.

c. Novel restriction enzymes from sequenced genomes.

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Comparison of closely related bacterial genome sequences and other bioinformatic/evolutionary analyses allowed us to predict putative restriction enzyme genes in sequenced bacterial genomes. We are expressing them in order to find out novel restriction enzymes and to determine their three-dimensional structure.

4. Recombination machinery in the genome community——homologous, site-specific and illegitimate—in interaction with restriction modification systems and other elements.

Action of various machines of DNA recombination in the cells is understood well in terms of conflicts with genetic elements within a genome such as restriction modification systems.

a. Chromosomal site-specific recombination defends genome from post-segregational killing by a restriction-modification system.

Yoji Nakamura, Asao Ichige, Naofumi Handa, and Ichizo Kobayashi

XerCD forms a site-specific recombinase acting at a specific site (*dif*) of *E. coli* chromosome. We found that *xerC* and *dif* mutations enhance cell death after loss of a restriction-modification gene complex. Our analysis of cell shape and chromosomes is in accord with the hypothesis that recombination repair of chromosomal restriction breaks leads to a chromosomal multimer, which is resolved by the site-specific recombination into monomer chromosomes.

b. An *Escherichia coli* strain, BJ5183, that shows highly efficient conservative (two-progeny) DNA double-strand break repair of restriction breaks.

Noriko Takahashi, Hiroshi Yoshikura, and Ichizo Kobayashi

We examined the mode of recombination in an *Escherichia coli* strain, BJ5183, which has been frequently used in recovery and cloning of eu-

karyotic DNA. One of the important criteria in characterizing a homologous recombination mechanism is whether it produces two recombinant DNA molecules or only one recombinant DNA molecule out of two parental DNA molecules. Our previous work transferring plasmid molecules with a restriction break into *Escherichia coli* cells distinguished two modes in recombination stimulated by a double-strand break. In a recBC sbcA mutant strain, where recET genes on the Rac prophage are responsible for recombination (RecE pathway), recombination is often conservative, in the sense that it generates two recombinants out of two parental DNAs. In a recBC sbcBC mutant strain, in which recA and recF genes are responsible (RecF pathway), recombination is non-conservative, in the sense that it generates only one recombinant out of two parental DNAs. Unexpectedly, BJ5183, described as recBC sbcBC, showed very efficient conservative (two-progeny) double-strand break repair. Moreover, this recombination was not eliminated by disruption of its recA gene, which is essential to the RecF pathway. Our polymerase chain reaction analysis detected a recET gene homologue in this strain. This region was easily replaced by a recT::Tn10 through general transduction and the resulting recT-negative derivative was defective in the conservative double-strand break repair. These results led us to conclude that, in strain BJ5183, the action of recET homologue is responsible for the conservative double-strand break repair as in the RecE pathway. BJ5183 carries a mutation in the endA gene, which codes for Endonuclease I. An endA mutation conferred a higher double-strand break-repair activity to a recBC sbcA mutant strain.

c. Alleviation of restriction by bacteriophage recombination functions.

Naofumi Handa, and Ichizo Kobayashi

We now hypothesize that the conservative double-strand-break-repair recombination plays the role to repair bacteriophage genomes after restriction attack. In support of the above hypothesis, *recET* genes of Rac prophage and *red* genes of bacteriophage lambda were shown to alleviate restriction.

d. Accumulation of large linear forms of bacterial chromosomes.

Naofumi Handa, and Ichizo Kobayashi

Double-strand breakage of chromosomal DNA is obviously a serious threat to cells because various activities of the chromosome depend on its integrity. However, recent experiments suggest that such breakage may occur frequently during "normal" growth in various organisms —from bacteria through vertebrates, possibly through arrest of a replication fork at some endogenous DNA damage. In order to learn how the recombination processes contribute to generation and processing of the breakage, large (>2000 kb) linear forms of *Escherichia coli* chromosome were detected by pulsed-field gel electrophoresis in various recombination-defective mutants. The mutants were analyzed in a rich medium, in which the wild-type strain showed fewer of these huge broken chromosomes than in a synthetic medium, and the following results were obtained: (i) Several *recB* and *recC* null mutants (in an otherwise rec^+ background) accumulated these huge linear forms, but several non-null *recBCD* mutants (*recD, recC1001, recC 1002, recC1003, recC1004, recB2145, recB2154*, and *recB2155*) did not. (ii) In a *recBC sbcA* background, in which RecE-mediated recombination is active, *recA, recJ, recQ, recE, recT, recF, recO,* and *recR* mutations led to their accumulation. The *recJ* mutant accumulated many linear forms, but this effect was suppressed by a *recQ* mutation. (iii) The *recA, recJ, recQ, recF* and *recR* mutations led to their accumulation in a *recBC sbcBC* background. The *recJ* mutation showed the largest amount of these forms. (iv) No accumulation was detected in mutants affecting resolution of Holliday intermediates, *recG, ruvAB* and *ruvC*, in any of these backgrounds. These results are discussed in terms of stepwise processing of chromosomal double-strand breaks.

e. Non-homologous end-joining promoted by homologous recombination function.

Naofumi Handa, Ayumi Fujita-Kusano, Yoko Ui, Keiko Sakagami, and Ichizo Kobayashi

The above work and other works have revealed that a double-strand break is frequently generated and processed on the chromosomes from bacteria to vertebrates. The contrasting routes of its processing include precise end-joining, non-homologous end-joining, and repair through homologous recombination. We developed a sensitive assay for non-homologous end-joining in bacterium, *Escherichia coli*, and demonstrated presence of non-homologous end-joining promoted by homologous recombination functions.

f. Homology-associated non-homologous recombination.

Kohji Kusano and Ichizo Kobayashi

We earlier identified non-homologous recombination products that may have been generated by long-range homologous interaction between two DNAs in bacterial and mouse cells. We developed assay systems in order to characterize this type of recombination in *E. coli*. In one system, a plasmid carrying inverted repeats, one with a type II restriction break and the other intact, was subjected to type I restriction in vivo. Dependence on the *rec* genes was demonstrated, and the product structures were determined at the sequence level. The results provided support for the hypothesis of the illegitimate recombination dependent on homologous interaction.

5. Towards system biology of intra-genomic conflicts, genome dynamics and genome evolution.

We employ mathematical approach, both analytical methods and simulation methods, to understand essence of the above processes revealed by experimental work.

a. Asymmetric random-walk model in a reaction intermediate of homologous recombination

Youhei Fujitani[46] and Ichizo Kobayashi

Homologous recombination can take place between a pair of homologous regions of DNA duplexes. Its typical pathway begins with connection of two strands coming from two recombining partners. A resultant connecting point (Holliday structure) migrates along the homologous region. We have formulated this migration in terms of a one-dimensional random-walk to succeed in explaining various phenomena: the dependence of recombination frequency on the homology length, the map expansion, and the very rapid drop-off of recombination frequency associated with sequence divergence. Our model has supposed symmetric random-walk; its forward transition rate equals to its backward one. However, they can differ because of possible polarity of enzymatic machinery driving the migration. In this work, we took into account this asymmetry in the random-walk model. Our analytical results fitted well with the experimental observations with synthetic recombination intermediates.

c. Co-evolution of bacterial restriction modification systems and restriction sites on bacteriophage genomes.

Akira Sasaki[53], Ichizo Kobayashi, Ryota Horie[54]: [53]Department of Biology, Faculty of Science, Kyushu University, [54]Riken

The genome decoding projects revealed that the genomes are full of cis-elements such as transcription signals. Understanding their evolution is a challenging subject in genome biology and system biology. The restriction sites along the genome may provide a simple system to study evolution of such cis-elements in the genome sequence by selection. We constructed a mathematical model for population dynamics of bacteria carrying various restriction modification systems and bacteriophages carrying various restriction sites. We looked for conditions for maintenance of many restriction modification systems in a bacterial genome and for conditions for evolution of recognition sequences.

6. Basic studies for gene therapy by mutation correction.

Asami Ino, and Ichizo Kobayashi

Earlier we demonstrated that adenovirus-mediated gene transfer followed by homologous recombination with the genome can provide efficient and accurate means of correcting mutations in mammalian cells. We have been trying to extend this approach to *in vivo* gene correction. We are also trying chimeric oligonucleotides for gene correction *in vivo*. We are also analyzing rearrangements of viral vector genomes in mammalian cells.

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小林一三，石川健：制限酵素修飾酵素遺伝子の自己増殖の発見．海洋，号外33号，128-136.（2003）

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Our major concept is to understand various cellular phenomena on the basis of structure and function of proteins. We have developed novel antibodies that discriminate post-translational modification of proteins such as phosphorylation and limited proteolysis. The powerful immunocytochemical probes visualize emzymatic reactions in situ and enable us to perform biochemical analysis of growing, differentiating and dying cells without any cell sorting.

1. Establishment of novel antibodies as tools available for in situ analyses of post-translational modification of proteins

After biosynthesis proteins undergo various post-translational modifications, and their functions are modulated. In order to understand such biochemical reactions in a single cell, we have been making modification-specific antibodies as probes for such in situ analyses; cleavage-site-directed antibodies for proteolysis, phosphorylation-site-specfic antibodies, myristoilated peptide-specific antibodies, ubiquitination-specific antibodies, inhibitor-bound enzyme-specific antibodies etc. These antibodies should be useful tools for research in cellular biochemistry.

a. Cleavage-site-directed antibodies

Ryoko Iizuka[1], Katsuyoshi Chiba[1] and Shinobu Imajoh-Ohmi

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We have previously demonstrated that technics of peptide synthesis and anti-peptide antibody production enable us to obtain antibodies to neoantigens generated by proteolysis. Such cleavage-site-directed antibodies specifically bind to terminal regions of proteolyzed fragments including either amino or carboxyl group newly ionized by hydrolysis of the peptide bond. The most remarkable characteristic of cleavage-site-directed antibodies is that they do not cross-react with unproteolyzed native polypeptides although the same sequence exists internally in the polypeptide. The strict specificity of antibodies garantee in situ analysis of proteolysis without fractionation of proteins by biochemical methods. To obtain a cleavage-site-directed antibody a synthetic peptide mimicking the terminal region of a proteolyzed protein is used as a hapten, where molecular design of the hapten is critical. We have so far established cleavage-site-directed antibodies for various proteins: active forms of calpains, calcium-dependent proteases with high- and low-calcium sensitivities; calpain -catalyzed fragments of protein kinase C species; compliment component C1s; caspase-catalyzed poly (ADP-ribose) polymerase in apoptotic cells; and an ovalbumin peptide 329-336 generated during antigen presentation in B lymphoma cells.

It has been suggested that transglutaminase 1 is proteolytically activated upon the terminal differentiation of the keratinocyte, but the mechanisms are not well understood. We have

established two mouse hybridoma cell lines producing monoclonal antibodies that specifically detect proteolytically cleaved transglutaminase 1. One detects the amino-terminus of the fragment produced by cleavage between Arginine 93 and Glycine 94, and the other detects the amino-terminus of the fragment produced by cleavage between Arginine 573 and Glycine 574. Using these two antibodies, immunohistochemical analyses of the epidermis revealed that the cleavages of the transglutaminase 1 protein occur early in the terminal differentiation of keratinocytes in the basal layer of the epidermis, that the cleavage between Arginine 573 and Glycine 574 (producing the 574G fragment) precedes the cleavage between Arginine 93 and Glycine 94 (producing the 94G fragment), that the 94G fragment is localized to the plasma membrane of keratinocytes and has cross-linking activity, whereas the 574G fragment is dispersed in the cytosol and does not have detectable levels of activity on in situ transglutaminase assay, and that 1-alpha-25-dihydroxycholecalciferol or all-trans retinoic acid treatment and ultraviolet B exposure disturb the localization of the transglutaminase 1 fragments with changes in the morphology of differentiating keratinocytes. All these results demonstrate that the antibodies generated in this work are useful to dissect the mechanism by which transglutaminase 1 is activated, and would provide us with novel insights into the biogenesis of the epidermis.

b. A novel method for hunting substrates of limited proteolysis

Masahiko Kato, Hiroyuki Fukuda, Takashi Nonaka and Shinobu Imajoh-Ohmi

During the course of study on calpain/calpastatin system in apoptosis we have found that a cleavage-site-directed antibody recognizes a novel molecule unrelated to the expected target protein. To analyze intracellular mobilization of calpastatin antibodies were raised against peptidyl haptens mimicing terminal regions of calpastatin polypeptides generated by caspases. A cleavage-site-directed antibody stained the amino-terminal 30-kDa fragment of recombinant human calpastatin cleaved in vitro by caspase-7. However, calpastatin was not detected by the same antibody in apoptotic cells, suggesting that the calpastatin fragment underwent further degradation. Instead, a 95-kDa polypepted was recognized by immunoblotting with this antibody during apoptosis. The 95-kDa band was seen specifically in apoptotic cells, and diminished in the presence of caspase inhibitors. Under less stringent conditions a 110-kDa polypeptide was also observed in non-apoptotic cells, but decreased in apoptotic cells in parallel with appearance of the 95-kDa band, suggesting that the 110 kDa protein was cleaved to 95K by caspases during apoptosis. By further structural analysis of the two antibody-stained polypeptides by Edman degradation and mass spectrometry, however, the 110 kDa and 95-kDa polypeptides were identified as APG-2, a member of heat shock protein, and a caspase-cleaved heavy chain of myosin II-A, a non-muscle type myosin, respectively. Furthermore, we found several targets for caspases, some of which remain to be identified, by another type of cleavage-site-directed antibodies.

c. Proteomic approach for identification of cysteine proteases in *Caenorhabditis elegans*

Jin Ling, Hiroyuki Fukuda and Shinobu Imajoh-Ohmi

E64c, [L-3-trans-carbonyloxirane-2-carbonyl]-L-leucine (3-methylbutyl) amide, is a synthetic inhibitor for cysteine proteases such as cathepsins B, H, L and calpain. To inhibit intracellular cysteine proteases E64d, [L-3-trans-ethoxycarbonyloxirane-2-carbonyl]-L-leucine (3-methylbutyl) amide, a membrane-permeable derivative of E64c is used instead of E64c. E64d penetrates into the cell where cellular esterases convert it to E64c that covalently binds to the SH group of active center in enzymes. Thus, anti-E64c antibody is a useful probe for in vivo analysis of cysteine proteases.

We have succeeded in making an antibody to E64c. First, we tried to establish an antibody against E64c-bound calpain. A peptide corresponding to the active center of calpain was synthesized by using the multiple-antigen peptide system. E64c was chemically introduced into the SH group of active center cysteine under reducing conditions. Rabbits were immunized with the E64c-conjugated calpain-derived peptide without further conjugation with a carrier protein. Unexpectedly, an antibody thus prepared reacted not only with E64c-inactivated calpain but also with E64c-bound other cysteine proteases such as papain and cathepsins. Low antigenicity of peptide region in the immunogen may result in such broad specificity of the antibody. Our antibody is expected to be used for identification of E64c-targeted novel proteases. When cells were treated with E64d, cell growth was suppressed and several proteins were labeled by E64c that is visualized with this antibody on immunoblotting. Structural analysis of

these proteins may lead identification of novel cysteine proteases.

Homogenates of *C. elegans* were treated with E64c in the presence or absence of calcium ion, and subjected to electrophoresis/immunoblotting using an anti-E64c antibody. A 55-kDa polypeptide (p55) was labelled with E64c in a calcium ion-dependent manner. In C. elegans several calpain-related gene products were identified at the mRNA level, but their physiological function remains to be elucidated. p55 is to be analyzed by mass spectrometry.

2. Proteolysis and cell death

Junko Ohmoto and Shinobu Imajoh-Ohmi

Cell death involves various intracellular proteolytic enzymes such as caspase, a series of cysteine proteases cleaving substrates after aspartate residue; proteasomes, a protein hydrolysis system regulated by ATP and ubiquitin; and calpain, calcium-dependent protease existing in the cytosol as an inactive precursor form. Among them caspases are now established as pivotal apoptosis-executing enzymes that cleave various substrates. Endogenous or viral proteins and synthetic substances inhibitory for caspases suppress the apoptotic cascade and rescue cells from cell death. On the other hand, proteasomes drive the cell cycle by degrading cyclins etc., and also play important parts in apoptosis, since proteasome inhibitors induce apoptotic cell death in growing cells but suppress apoptosis of some cells that is in quiescent state. Furthermore, in some specific cells such as polymorphonuclear leukocytes, other proteases might be involved in cell death.

Polymorphonuclear neutrophils (PMNs) undergo spontaneous apoptosis during cultivation *in vitro*. Various proteases are also activated and many target proteins have been reported in apoptotic PMNs. Actin is proteolyzed to a 40-kDa fragment that lacks amino-terminal region involved in polymerization. To investigate the role of actin proteolysis we made a cleavage-site -directed antibody for the 40-kDa form of actin using synthetic peptide as a hapten. The antibody stained the 40-kDa polypeptide but did not recognize native actin abundant in cell lysates. First, we found that the 40-kDa fragment is generated during isolation of PMNs from peripheral bood. By using diisopropyl fluorophosphate, an inhibitor for serine proteases, PMNs with native actin could be prepared. Furthermore, elastase was identifited as the enzyme responsible for the limited proteolysis of actin. In fact, when isolated PMNs were incubated with elastase, the 40-kDa fragment was observed, providing us with a question how extracellular elastase attacks actin.

3. Phagocytic differentiation and apoptosis

Taku Kuwabara and Shinobu Imajoh-Ohmi

Among phagocytes macrophages are long-lived to play important roles in the defense system of the host after settlement in various tissues. On the other hand, polymorphonuclear leukocytes spontaneously undergo apoptosis. Using a human monoblast U937 cell line we have investigated relationship between differentiation and cell death. The U937 cells differentiate to superoxide anion-producible cells after cultured with interferon γ (IFN), 1α, 25 dihydroxy vitamin D3 (VD3) or retinoic acid (RA). RA- and VD3-differentiated U937 cells seem to be more closely related to mature macrophages than IFN-treated cells, since RA and VD3 induce cell surface expression of CD11b. IFN-treated U 937 cells become highly sensitive to Fas-mediated apoptosis. On the other hand, RA- and VD3-differentiated cells showed resistance against cytotoxic anti-Fas antibody.

Lipopolysaccharide (LPS), a membrane constituent of gram-negative bacteria, induces apoptosis in U937IFN cells. LPS treatment led to the appearance of characteristic markers of apoptosis such as nuclear fragmentation and activation of caspases. While the caspase inhibitor Z-VAD-fmk prevented LPS-induced apoptosis as judged by its inhibition of nuclear fragmentation, it failed to inhibit cytochrome c release and loss of mitochondrial membrane potential. Transfection of peptides containing the BH4 (Bcl-2 homology 4) domain derived from the anti-apoptotic protein Bcl-XL blocked LPS-induced nuclear fragmentation and the limited digestion of PARP. These results suggest that LPS does not require caspase activation to induce mitochondrial dysfunction and that mitochondria play a crucial role in the regulation of LPS-mediated apoptosis in U937IFN cells.

4. Cell death of monocytic cells and macrophages infected with pathogenic bacteria

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Upon invasion into tissues bacterial phathogens are phagocytosed by resident macrophages to be killed and digested. Some bacteria can escape into the cytosol and induce cell death of the host cell. *Shigella flexneri* is also reported to

induce apoptosis in murine macrophages where a bacterial invasion plasmid antigen B (IpaB) activates a cellular protease triggering apoptotic cell death. However, other investigators observed necrotic cell death in *Shigella*-infected human macrophages derived from peripheral monocytes. Cell death of macrophages caused by bacterial invasion remains to be characterized on the basis of molecular interaction.

It is currently unclear whether *Shigella* kills its host cells by apoptosis or necrosis. Here we show that rapid necrosis ensues in macrophage-like cell lines (U937 cells differentiated by *all trans*-retinoic acid and J774 cells) infected with the *S. flexneri* strain YSH6000. The infected cells rapidly lose membrane integrity, a typical feature of necrosis, as indicated by the release of the cytoplasmic lactate dehydrogenase and the exposure of phosphatidylserine (PS) associated with the rapid uptake of propidium iodide (PI). The infected cells exhibit DNA fragmentation without nuclear condensation and substantial involvement of either caspase-3/-7 or caspase-1 was not detected, which is also contrary what is normally observed in apoptosis. Cytochalasin D potently inhibited *Shigella*-induced cell death, indicating that only internalized *Shigella* can cause necrosis. Osmoprotectants such as polyethylene glycols could suppress cell death, suggesting that insertion of a pore by *Shigella* into the host cell membrane induces the necrosis. The pore was estimated to be 2.87 ± 0.4 nm in diameter. *Shigella* was also found to be able to induce apoptosis but only in one of the lines tested and under specific conditions, namely, U937 cells differentiated with interferon-g (U937IFN). Caspase-3/-7 but not caspase-1 activation was observed in these infected cells and the exposure of PS occurred without the uptake of PI. An avirulent *Shigella* strain, wild-type *Shigella* killed with gentamicin, and even *E. coli* strain JM109 could also induce apoptosis U937IFN cells, and cytochalasin D could not prevent apoptosis. It appears therefore that *Shigella*-induced U937IFN cell apoptosis is unrelated to *Shigella* pathogenicity and does not require bacterial internalization. Thus, *Shigella* can induce rapid necrosis macrophage-like cells in a virulence-related manner by forming pores into host cell membrane while some cells can be killed through apoptosis in a virulence-independent fashion.

5. Topoisomerase IIα inhibitor ITIIα regulates the protein level of topoisomerase IIα

Akira Nakanishi[4], Ryoko Iiduka1, Yoshio Miki[4], and Shinobu Imajoh-Ohmi [4]Dept. Mol. Diagnosis, The Cancer Inst. JFCR.

We have reported that a novel inhibitor of topoisomerase IIα (IT IIα) may provide a molecular basis for key activities of topoisomerase IIα. To investigate the physiological significance of ITIIα levels in the regulation of topoisomerase IIα in vivo, we manipulated the expression of ITIIα using antisense technology. Transfection of NB1-RGB cells with ITIIα antisense phosphorothioate oligonucleotides at concentrations of 83 nM, 125 nM, 167 nM and 250 nM in the presence of cationic lipids reduced ITIIα protein levels after 48 h by 5.0%, 27.5%, 63.0% and 71.0%, respectively. In contrast, sense control, random control and sense reverse control oligonucleotides at 250 nM led to an inhibition of ITIIα expression by at most 23%. We observed no change in the levels of actin, and cationic lipids alone did not affect protein levels at the concentrations used. To determine whether there is a strict correlation between the levels of ITIIα and the presence of ITIIα antisense oligonucleotides, we conducted a time-course analysis. ITIIα protein levels were reduced 12 h after the addition of 250 nM ITIIα antisense oligonucleotide. Topoisomerase IIα protein levels increased up to ten-fold following treatment of cells with ITIIα antisense oligonucleotides for 12 h, and remained high for 48 h. Sense control and random control oligonucleotides had no effect on the levels of topoisomerase IIα. We next carried out immunohistochemical analysis to determine the subcellular localizations of ITIIα and topoisomerase IIα in NB1-RGB cells transfected with antisense oligonucleotides. Forty-eight hours after lipofectin-mediated transfection, the cells were fixed and stained with specific antibody to ITIIα or topoisomerase IIα and then with FITC- or Texas red-conjugated secondary antibody, respectively. Transfection of cells with ITIIα-antisense oligonucleotides, but not with sense oligonucleotides, inhibited the induction of ITIIα expression. Topoisomerase IIα? is normally found in the interphase nuclei of proliferating cells. However, we found that it was present in both the nucleus and cytoplasm of cells treated with ITIIα antisense oligonucleotides. The specific reduction of ITIIα levels by antisense oligonucleotides could be observed not only in normal human fibroblasts but also in other human cell lines, such as A549, HeLa, MCF7.

One major difference between normal mammalian cells and cancer cells is their proliferative potential. Normal mammalian cells can undergo a limited number of doublings, whereas cancer cells are referred to as immortalized. SV40-transformed human keratinocytes are well-established immortalized cells that exhibit features of normal human keratinocytes. Telom-

erase activity is present in 80-90% of human cancers and is a potential molecular marker of immortalization. Our results demonstrate that the SV40 large T-antigen is sufficient for the immortalization of human keratinocytes and that the immortalized cells exhibit a decrease in the expression of ITIIα and a concomitant ncrease in the levels of topoisomerase IIα. These results suggest that ITIIα directly and/or indirectly affects the expression of topoisomerase IIα. Decreased expression of ITIIα protein correlates with cancer development and may thus be an important new marker of cancer progression. In conclusion, our studies suggest that topoisomerase IIα is regulated by ITIIα, and that ITIIα may be a useful target for therapeutic intervention in tumors.

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染色体制御分野

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Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells as well as differentiated cells with emphasis on intracellular signal transduction. For this purpose, we are using systems ranging from zebrafish, chick, mouse and culture cells. The major research areas of interest are on: 1) development and regeneration of eye, 2) roles of cytokines and their receptors in hematopoietic stem cells, 3) Th1 and Th2-specific cytokine genes in activated T cells, 4) regulation of DNA replication and cell cycle. On the basis of these efforts, we intend to develop technologies to manipulate growth and differentiation of various stem cells with high fidelity, which is important for cell and gene therapy.

Ex vivo expansion of haematopoietic stem cells using mutant hGM-CSF receptor signals

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We established transgenic mice expressing wild type human granulocyte-macrophage colony stimulating factor (hGM-CSF) receptor (wild -Tg) and Fall mutant (Fall-Tg), whoes all the cytoplasmic tyrosine residues were mutated to phenylalanine. We used MHC classI promoter to express both α and β subunits of these receptors ubiquitously. In this work, we examined whether the hGM-CSF signals can be used for expansion of haematopoietic stem cells (HSC) proliferation *in vitro* using bone marrow (BM) cells of these Tg mice.

When we cultured lin^- cells from BM of Fall-Tg mice *in vitro* in the presence of hGM-CSF and SCF, the number of $Sca1^+/c\text{-}kit^+$ immature cells was increased but the total number of these cells was equivalent with that of wild-Tg mice, suggested that the Fall signals were sufficient to stimulate proliferation of immature cells, and tyrosine residues dependent signals promoted differentiation signals. In accordance with this, colony formation analysis of $CD34^{-/low}$ $c\text{-}Kit^+$ $Sca\text{-}1^+$ Lin^- cells resulted in the expansion of immature cells from Fall-Tg in the presence of hGM-CSF and SCF. We then analysed long term reconstitution (LTR) activities of *ex vivo* expanded progenitor cells from Tg mice. Immature cells from either wild-Tg or Fall-Tg were cultured in the presence of hGM-CSF and SCF for 1 week and transplanted into irradiated mice. LTR activity was observed with both wild-Tg and Fall-Tg derived cells indicated that the Fall signals were sufficient for maintenance and expansion of

HSC.

Retinal fate specification of mouse embryonic stem cells by ectopic expression of Rx/rax, a homeobox gene

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Neural retina is a part of central nervous system (CNS) and regeneration of retinal cells from retinal stem cells is critical issue. Embryonic stem (ES) cells have offered much hope as an alternative source for regeneration of neural tissues. We aimed to generate retinal cells from mouse ES cells by exogenous gene transfer. We introduced RX/rax transcription factor, which is expressed in immature retinal cells, into feeder free mouse ES cells, CCE. CCE cells expressing RX/rax as well as EGFP (CCE-RX/E cells) proliferated and kept un-differentiated state in the presence of LIF as parental ES cells. We made use of mouse embryo retinal explant cultures to address the differentiation ability of grafted ES cells. Dissociated EBs which were treated with retinoic acid were prepared as donor cells and co-cultured with retina explant for two weeks. In contrast to that the parental CCE cells could not migrate into host retinal cultures, CCE-RX/E cells migrated into the host retina and extended their process-like structures between the host retinal cells. Most of grafted CCE-RX/E cells located in ganglion cell and inner plexiform layers and expressed ganglion and horizontal cell markers. Furthermore, these grafted cells had electorphysiological properties expected of ganglion cell. It thus suggested that subpopulations of retinal neurons can be generated in retinal explant cultures from grafted mouse ES cell expressing ectopically transcription factor Rx/rax.

Analysis of DD76 in developing mouse eye

Akihiko Muto, Asako Usui, Ken-ichi Arai and Sumiko Watanabe

DD76 has originally been identified by differential display as a gene of which expression level varies during mouse eye development. The mouse DD76 (mDD76) gene is specifically expressed in neural tissues including retina and encodes a protein of 635 amino acids containing a single EF-hand and two leucine-zipper motifs at regions proximal to N- and C-termini, respectively. Isolation of a zebrafish orthologue of DD 76 (zDD76) revealed that these characteristic features in structure and the expression pattern were highly conserved among species. In last year, we performed the knockdown experiment of zDD76 by injecting morpholino antisense oligo into zebrafish embryos and showed that zDD76 is involved in development of zebrafish retina.

To further clarify the functions of DD76, we examined the effects of DD76 misexpression in developing mouse retina. We used retrovirus vectors designed to express either EGFP alone (control) or both DD76 and EGFP connected by IRES (DD76 virus) and retinal explants prepared from mouse embryos at E17.5 were infected with these viruses. After two weeks of culture, more than 80% of virus-infected cells, which were detected by the expression of EGFP, were located in the outer nuclear layer (ONL) and remaining (about 20%) cells were in the inner nuclear layer (INL) in a control virus-infected retina. On the other hand, when the DD76 virus was infected, about 50% of the EGFP+ cells were found in the INL. Analysis of cell differentiation revealed that the ratio of Muller glia was increased at a slightly higher level (4-fold) than those of other neurons in the INL (2.5-fold). Localization shift of virus-infected cells in retinal explant was first observed at a day corresponding to postnatal day 3 of the normal development *in vivo*, at which time expression of endogenous DD76 starts to be decreased in the ONL. The proportion of BrdU-incorporated cells at the same day was significantly lower in DD76 virus-infected cells than in control virus-infected cells. In contrast, cell death was not affected by expression of DD76. Therefore, misexpression of DD76 affected on proliferation and/or differentiation of retinal cells in a stage-specific manner. To determine functional domains of DD76 involved in these effects on retinal cells, mutants of mDD76, mDD76ΔN and mDD76ΔC, were constructed and misexpressed in retinal explants. As described above, several conserved motifs have been found in the DD76 protein and, in addition, it was recently reported that a domain involved in interaction with a member of Rab-family of small G-protein (Rab binding domain; RBD) was identified in most C-terminal region of a human orthologue of DD76. mDD76 ΔN and mDD76ΔC lack N- and C-terminal regions containing the conserved EF-hand motif and RBD, respectively. We demonstrated that although the RBD, which is perfectly conserved in mouse and zebrafish DD76, was essential for binding to the Rab protein, mDD76ΔC lacking RBD exhibited comparable effects to the full-length mDD76 on the localization of retinal cells as it was misexpressed, suggesting that the interaction to the Rab protein is dispensable for

the function of DD76 in the development of retina. Interestingly, deletion of N-terminal region resulted in complete loss of DD76 function on retinal cells. These all results indicated that DD 76 plays roles in development of mouse retina and the function is mediated by its N-terminal region rather than the C-terminal region required for interaction to the Rab protein.

Melk-like kinase plays a role in haematopoiesis in the zebrafish

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We cloned a serine/threonine kinase, DD29, that was expressed in mouse embryonic neural tissues, by differential display using mouse brain and eye mRNA from various developmental stages. Full-length DD29 was identical with the previously reported serine/threonine kinase Melk, which was initially cloned in oocytes as a maternal gene, but its function had not been known. *Melk* transcripts were expressed in the ventricular zone of the brain and in the eyes at E13 in mouse embryos, and the expression level sharply decreased after birth. In adult mice, *Melk* was strongly expressed in thymus and bone marrow, suggesting a role for *Melk* in haematopoiesis. We then cloned a Melk-like gene from zebrafish (z). *zMelk-like gene* was expressed in brain and lateral mesoderm at 12 hpf and in several tissues of adult fish including kidney and spleen, both of which are known to be haematopoietic tissues in zebrafish. Abrogation of *zMelk-like gene* function by *zMelk-like gene*-specific MO resulted in abnormal swelling around the tectum region. The start of blood circulation was severely delayed; but, in contrast, the vessel formation seemed normal. Expression of *scl* and *gata1* were down regulated at 12-14 hpf in the zMelk-like gene MO injected embryos. Taken together, our data suggest that the *zMelk-like gene* may play a role in primitive haematopoiesis by affecting the expression of *scl* and *gata1*.

Analysis of the role of p73 in zebrafish development

Shinya Satoh, Ken-ichi Arai, Sumiko Watanabe

p53-related protein p73 has many functions similar to that of p53, including the ability to induce cell-cycle arrest and apoptosis. However, despite the similarities between p53 and p73 activity, it has become evident that they are not equivalent functionally. In order to study the role of this protein in development, we cloned p 73 cDNA from zebrafish (*Danio rerio*) and analyzed its expression pattern as well as the phenotypic consequences of its overexpression and knock-down using zebrafish embryos. We found that two types of splicing variant mRNAs for p 73 were expressed in zebrafish. One is corresponding to human α isoform and the other is produced by using alternative splicing acceptor site, which we named zp73α and zp73θ, respectively. Both proteins activated promoter with the p53-binding consensus sequences in mammalian cells, but to different degrees. Overexpression of zp73 in zebrafish embryos caused the developmental perturbation, such as tail truncation or loss of a part of the head, which was also observed when human p53 or p73 was overexpressed. These results indicate that zp73 proteins appear to have the same features of human p53-family proteins. Whole mount in situ hybridization revealed that zp73 expression was detected in the olfactory placodes, olfactory bulb, and pharyngeal arches of the zebrafish embryos. Knock-down of zp73 function resulted in malformation of jaw and pharyngeal arches, suggesting that zp73 plays important role in its development.

Structure and dynamics of chromatin in the regulation of gene expression and stem cell differentiation

Noriko Sato and Ken-ichi Arai

a. Fluctuation of chromatin unfolding associated with variation in the level of gene expression

We examined whether spontaneous alteration of chromatin structure, if any, correlates with variation in gene expression. Gene activation is associated with changes in chromatin structure at different levels. Large-scale chromatin unfolding is one of such changes detectable under the light microscope. We established cell clones carrying tandem repeats (more than fifty copies spanning several hundred kb) of the GFP-ASK reporter genes driven by a tetracycline responsive promoter. These clones constitutively express the transcriptional transactivator. Flow cytometry and live-recording fluorescence microscopy revealed that, although fully activated by a saturating amount of doxycycline, GFP-ASK expression fluctuated in individual cell clones, regardless of the cell cycle stage. The GFP-ASK expression changed from lower to higher levels and vice versa within a few cell cycles. Furthermore, the levels of GFP-ASK expression were correlated with the degrees of chromatin unfolding of the integrated array as detected by FISH. The chromatin unfolding was not coupled to a mitotic event; around one-third of the daughter-

pairs exhibited dissimilar degrees of chromatin unfolding. We concluded that fluctuation of chromatin unfolding was likely to result in variation in gene expression, although the source of the fluctuation of chromatin unfolding remains to be studied.

b. Chromatin structure and the control of gene expression in pluripotent ES cells

We are interested in identifying the molecular features of chromatin structure and nuclear organization that distinguish pluripotent stem cells from differentiated cells. OCT-3/4 gene is the only gene known to play an essential role in maintaining pluripotency of embryonic cells. We have and are continuing to study changes in chromatin structure of OCT-3/4 gene locus during differentiation of mouse ES cells in culture. Alteration of expression levels of OCT-3/4 and its repressor GCNF (germ cell nuclear factor) were examined by RT-PCR. OCT-3/4 transcription was drastically repressed whereas GCNF expression, inversely, was transiently elevated at eight days after LIF removal. We analyzed the promoter and the intron 1 regions for methylation at CpG residues in undifferentiated ES cells and in day 8 differentiated cells using the bisulfite technique. While these regions were totally unmethylated in undifferentiated ES cells, some cytosines within these regions were methylated in differentiated cells. In order to investigate whether higher-level chromatin organization is modulated upon gene repression, we analyzed FISH signals to nuclear halos of undifferentiated ES cells and of day 10 differentiated cells. Signals of fluorescence hybridization to OCT-3/4 locus were detected within the residual nuclei of both types of cells, regardless of transcriptional activities. Moreover, replication timing of OCT-3/4 locus remained early in differentiated cells. The results suggest that differentiation-induced GCNF expression turns off OCT-3/4 and this repression is maintained by DNA methylation, without alteration of the replication timing and nuclear matrix binding property of OCT-3/4 loci.

Role of STAT6 in allergic asthma

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Th2 cytokine IL-4 plays a crucial role in allergic asthma and that transcription factor STAT6 (Signal Transducer and Activator of Transcription 6) is one of the important signaling molecules downstream of IL-4 receptor. To study molecular mechanisms causing asthma in mice, we generated transgenic mice expressing STAT6 -estrogen receptor hormone binding domain fusion protein (STAT6ER) in lung, heart, brain, and lymphocytes. The proteins can be activated upon administration of 4-hydroxy-tamoxifen(4 HT). The treatment of STAT6ER transgenic mice with 4-HT *in vivo* resulted in airway hyperresposiveness in both RAG knockout and STAT6 knockout background mice. These results indicated that STAT6 not only induces of Th2 differentiation of lymphocytes but also generate asthma phenotype in lung epithelial cells and lung smooth muscle cells. The etiology of bronchial asthma was examined at molecular level by analyzing 4HT inducible genes in the lung from Stat6 transgenic mice by RDA(Representative Difference Analysis) and microarray analyses.

Molecular cloning of Interferon producing cell-specific cell surface antigen Ly49Q

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Dendritic cells are highly motile cells that operate at the interface of innate and acquired immunity. They are specialized for uptake, transport, processing and presentation of antigen to T cells. Among DC subsets, type I interferon producing cells (IPC)/plasmacytoid dendritic cells (PDC) had been reported as immature DCs and high producer of type I IFN upon viral infection and the other stimuli such as bacterial CpG in both human and mouse. Through release of cytokines, IPCs control myeloid DC and activate macrophages and NK cells. In addition, upon viral infection, IPCs have the ability to differentiate into mature dendritic cells, which are capable of inducing naive T cell proliferation and expansion of regulatory T cells. In both human and mouse, the population of IPCs in blood and secondary lymphoid organs is very small. Therefore, it is difficult to study the nature of this subset of cells. In addition, there are no available cell surface markers specific for murine IPCs. In order to clarify the role of IPCs in immune system, we generated monoclonal antibodies recognizing the cell surface molecules of murine IPCs, and cloned the gene encoded the antigen recognized by this antibody.

The cloned gene is the Ly49Q encoding a type II C-type lectin membrane-associated polypeptide of 247 amino acids containing a single CRD motif at the COOH-terminal end and ITIM motif in its cytoplasmic region. The other Ly49 family proteins are expressed on the surface of

NK cells as disulfidelinked homodimer, bind to MHC class I molecules and regulate NK cell functions. However, within Ly49 family, Ly49Q is expressed by only IPC and granulocytes but not by NK cells or other subsets of DCs. Since newly cloned Ly49Q has an inhibitory domain in the cytoplasm and its expression is upregulated upon viral infection, this molecule may act as a negative regulator for IPC activation or differentiation upon viral infection.

Cdc7 kinase knockout and knockdown: Effects on cell growth and survival in ES cells and MEFs

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Cdc7 kinase is essential for initiation of DNA replication. *Cdc7*$^{-/-}$ mouse embryonic stem (ES) cells are non-viable but their growth can be rescued by an ectopically expressed transgene (*Cdc 7*$^{-/-}$tg). We previously reported that removal of the transgene from *Cdc7*$^{-/-}$tg ES cells lead to S phase arrest and eventual p53-dependent cell death. This indicates continuous requirement of Cdc7 kinase for progression of S phase, and the presence of p53-dependent tight monitoring of DNA replication in undifferentiated ES cells.

We have shown that, despite the normal growth capability of *Cdc7*$^{-/-}$tg ES cells, the mice with the identical genetic background exhibit growth retardation. Concomitantly, *Cdc7*$^{-/-}$tg embryonic fibroblasts grow poorly with reduced ability to enter S phase. Furthermore, *Cdc7*$^{-/-}$tg mice display testicular atrophy and disrupted spermatogenesis in stages preceding or at the beginning of meiotic prophase I. The impairment in spermatogenesis correlates with the extremely low level of Cdc7 protein in testes, and is rescued by introducing an additional allele of transgene, which results in increase of Cdc7 expression. The increased level of Cdc7 also recovers the growth of *Cdc7*$^{-/-}$tg mice, indicating that the developmental abnormalities of *Cdc7*$^{-/-}$tg mice are due to insufficiency of Cdc7 protein. Our results indicate the requirement of a critical level of a cell-cycle regulator for mouse development and provide genetic evidence that Cdc7 kinase plays essential roles in meiotic processes in mammals.

Furthermore, we found that expression of S phase factors including Cdc6, CyclinA and ASK (an activation subunit for Cdc7), is significantly decreased in *Cdc7*$^{-/-}$tg MEFs compared to the wild-type MEF, suggesting the presence of a mechanism which coordinates the growth rate with the expression levels of these factors.

A critical role of the 3′ terminus of nascent DNA chains in recognition of stalled replication forks

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Arrest of replication forks by various internal and external threats evokes a myriad of cellular reactions, collectively known as DNA replication checkpoint responses. The initial phase of this response is the recognition of the arrested forks by a sensor protein, which may be recruited to the site of the fork arrest and transduce various signals for further downstream cellular responses. However, the mechanisms of how arrested forks are recognized by a sensor protein are not known. *Escherichia coli* PriA protein is a DEXH-type DNA helicase and is highly conserved in eubacteria. A null mutant of PriA is sensitive to DNA damaging agents and is defective in RecA-dependent DNA replication induced by replication fork arrest. Since PriA binds to D-loop, recombination intermediate generated by recombinational proteins, and assembles replication machinery at this site, it has been proposed that PriA is essential for restoration of stalled replication forks and recombinational repair of double-stranded DNA breaks. We hypothesized that this protein is a strong candidate for component that first directly recognizes arrested forks to restart DNA replication. We recently reported that PriA binds to arrested fork-like structures through cooperation of the N-terminal 181 amino acid DNA binding domain and C-terminal DNA helicase domain. Furthermore, we show that PriA protein specifically recognizes the 3′ termini of arrested nascent DNA chains at model stalled replication forks *in vitro*. Mutations in the putative "3′ terminus binding pocket" present in the N-terminal segment of PriA result in reduced binding to stalled replication fork structures and loss of its biological functions. The results suggest a mechanism by which stalled replication forks are recognized by a sensor protein for checkpoint responses. Furthermore, these findings provide novel strategies in search for eukaryotic proteins with similar structures and

functions.

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