Department of Basic Medical Sciences
Division of Molecular Cell Signaling

As the global environment deteriorates alarmingly rapidly by pollution, it is becoming critically vital to learn more about the cellular responses to environmental stresses caused by exposures to, for example, ultraviolet radiation, genotoxins, and oxidants. There is, however, only a rudimentary understanding of the basic mechanisms by which cells respond to these environmental stresses. A conspicuous cellular stress response is activation of the stress-responsive MAP kinases (JNK and p38), which are conserved throughout the eukaryotic world, indicating their fundamental importance in cellular survival and adaptation. Our primary research goal is to elucidate the molecular mechanism of activation of the stress-responsive MAP kinase cascades, using both yeast and human cells as model systems.

1. Role of the GADD45 proteins in activation of the p38 SAPK pathway by Transforming Growth Factor β

Mutsuhiro Takekawa and Haruo Saito

Transforming growth factor β (TGFβ) belongs to a family of multifunctional cytokines that regulate cell adhesion, angiogenesis, cell proliferation and apoptosis. TGFβ expression and responsiveness also regulate tumor development. TGFβ initiates its pleiotropic effects by binding a heteromeric cell surface receptor complex composed of type I and II transmembrane S/T kinase receptors. Upon ligand binding, the type II receptor phosphorylates and activates the type I receptor. Activated type I receptor initiates intracellular signaling through activation of specific Smad proteins that relay signals into the nucleus where they direct transcriptional responses. TGFβ has also been found to activate the stress-responsive p38 MAPK cascade in a variety of cell systems. The mechanism by which TGFβ activates the p38 pathway, however, yet remains to be elucidated.

In order to clarify the molecular mechanisms of TGFβ-induced p38 activation, we first investigated the p38 MAPK activity in TGFβ responsive and unresponsive cell lines. We found that TGFβ-induced p38 activation did not occur in Smad4-deficient cell lines, but that expression of Smad4 in these cell lines restored the p38 activation. Furthermore, expression of the constitutively active TGFβRI activated the p38 pathway, and this activation was further enhanced by co-expression of Smad proteins. Perhaps more important, overexpression of Smad proteins alone was capable of activating the p38 pathway. In addition, the p38 activation induced by the constitutively active TGFβRI was strongly inhibited by expression of the dominant-negative Smad4 mutant. These findings suggest that Smad-dependent gene expression mediates the activation of the p38 pathway in response to TGFβ.

In order to identify the TGFβ-inducible gene whose expression activates the p38 pathway, we investigated expression of the GADD45 family proteins (GADD45α, β, and γ) which are activators of the MTK1 MAPKKK. We found that expression of GADD45β mRNA was specifically and efficiently induced by TGFβ in a Smad-dependent manner, and that the timing of the TGFβ-induced p38 activation was almost parallel to that of GADD45β induction. Overexpression of GADD45β was sufficient, even in Smad4-deficient cell lines, to enhance the p38 activity, presumably through the activation of MTK1.

Moreover, TGFβ-induced p38 activation was strongly inhibited by expression of dominant-negative MTK1 or anti-sense GADD45β. These findings thus suggest that TGFβ activates the p38 pathway, at least in part, through Smad-dependent transcription of GADD45β.

To clarify physiological roles of TGFβ-induced p38 activation, we further screened for genes whose expression were regulated by the cross-talk between TGFβ-mediated signaling and the p38 pathway using a cDNA array system. We identified that expression of thrombospondin 1, a potent inhibitor of tumor cell growth and angiogenesis, is regulated by TGFβ-induced p38 activation, suggesting that the p38 pathway may play an important role in the tumor-inhibitory effect of TGFβ.

2. Analyses of scaffold roles of the S. *cerevisiae* Pbs2 MAPKK in high-osmolarity response pathway

Kazuo Tatebayashi, Mutsuhiro Takekawa and Haruo Saito

Adaptive responses to various environmental stresses is an important mechanism for the survival of living organisms. In budding yeast, one of the MAP kinase cascades, termed the high osmolarity glycerol (HOG) pathway, plays an important role in adaptation to the increased extracellular osmolarity. In response to high osmolarity shock, the phosphorylation signals are transmitted from two independent osmo-sensors, Sho1 or Sln1, to MAP kinase kinase kinase (MAPKKK), Ste11 or Ssk2/22, respectively, and converge on Pbs2 MAP kinase kinase (MAPKK), which finally activates Hog1 MAP kinase (MAPK). Pbs2, which integrates the signals from the upstream branches, has been shown to bind to Sho1, Ste11 and Hog1, serving as a scaffold. The scaffold function of Pbs2 seems important to prevent cross-talk with other MAPK cascade in yeast cells. To investigate the scaffold role of Pbs2 on the molecular basis, we extensively analyzed the N-terminal non-catalytic domain of Pbs2.

Systematic deletion of Pbs2 non-catalytic region revealed that Pbs2 residues 46-56 are important for efficient signal transduction from the Sln1 branch, but not from the Sho1 branch after high osmolarity shock. Furthermore, the amino-acid substitutions in this region, such as V54E, V54G and R53P, specifically blocked the signal from Sln1 branch. Two-hybrid analyses showed that Pbs2 residues 1-67 (Pbs2(1-67)) interacts with the catalytic domain of Ssk2 or Ssk22 MAPKKK, which only function in the Sln1 branch. This interaction is completely abolished by the V54E or V54G mutation in Pbs2. These results indicate that a specific interaction between Pbs2 residues 46-56 and the Ssk2 (or Ssk22) catalytic domain is required for activation of the HOG pathway from the Sln1 branch.

We have also found that a deletion of Pbs2 residues 280-355, which is closely located to catalytic domain, inhibits the activation of Hog1 in response to high osmolarity even if both Ssk2/Ssk22 and Ste11 are intact, implying that the Pbs2(280-355) region is essential for both upstream branches. Interestingly, however, there is a point mutation within this region that specifically inhibits the signal from the Sho1 branch but not from the Sln1 branch. It seems possible therefore that the Pbs2(280-355) region actually contains multiple interaction sites, and deletion of the entire region inhibits both upstream branches.

3. Role of the receptor tyrosine phosphatase Dlar in the organization of actin filaments in *Drosophila* follicular epithelium

Jack Bateman[1], R. Sreekantha Reddy[1], Haruo Saito and David Van Vactor[1]:[1]Harvard Medical School

Regulation of actin structure is instrumental in maintaining proper cytoarchitecture in many tissues. In the follicular epithelium of Drosophila ovaries, a system of actin filaments is coordinated across the basal surface of cells encircling the oocyte. These filaments have been postulated to regulate oocyte elongation; however, the molecular components that control this cytoskeletal array are not yet understood.

We found that the receptor tyrosine phosphatase (RPTP) Dlar and integrin are involved in organizing basal actin filaments in follicle cells. Mutations in *Dlar* and the common β-integrin subunit *mys* cause a failure in oocyte elongation, which is correlated with a loss of proper actin filament organization. Immunolocalization shows that early in oogenesis, Dlar is polarized to membranes where filaments terminate, but becomes generally distributed late in development, at which time β-integrin and the Enabled protein specifically associate with actin filament terminals. Rescue experiments point to the early period of polar Dlar localization as critical for its function. Furthermore, clonal analysis showed that loss of Dlar or mys influences actin filament polarity in wild-type cells that surround mutant tissues, suggesting that communication between neighboring cells regulates cytoskeletal organization. Finally, it was found that tow integrin α subunits encoded by mew and if are required for proper oocyte elongation, implying that multiple components of the extracellular matrix are instructive in coordinating actin fiber polarity.

4. The *dhp1*$^{+}$ gene, encoding a putative nuclear 5'-3' exoribonuclease, is required for proper chromosome segregation in fission yeast

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The *Schizosaccharomyces pombe dhp1*$^+$ gene is an ortholog of the *Saccharomyces cerevisiae RAT1* gene, which encodes a nuclear 5'-3' exoribonuclease, and is essential for cell viability. To clarify the cellular functions of the nuclear 5'-3' exoribonuclease, we isolated and characterized a temperature-sensitive mutant of *dhp1* (*dhp1-1* mutant). The *dhp1-1* mutant showed nuclear accumulation of poly(A)$^+$ RNA at the restrictive temperature, as was already reported for the *rat1* mutant. Interestingly, the *dhp1-1* mutant exhibited aberrant chromosome segregation at the restrictive temperature. The *dhp1-1* cells frequently contained condensed chromosomes, most of whose sister chromatids failed to separate during mitosis despite normal mitotic spindle elongation. Finally, chromosomes were displaced or unequally segregated. As similar mitotic defects were also observed in Dhp1-depleted cells, we concluded that *dhp1*$^+$ is required for proper chromosome segregation as well as for poly(A)$^+$ RNA metabolism in fission yeast. Furthermore, we isolated a multi-copy suppressor of the *dhp1-1* mutant, referred to as *din1*$^+$. We found that the gene product of *dhp1-1* was unstable at high temperatures, but that reduced levels of Dhp1-1p could be suppressed by overexpressing Din1p at the restrictive temperature. Thus, Din1p may physically interact with Dhp1p and stabilize Dhp1p and/or restore its activity.

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Department of Basic Medical Sciences
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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. Seven out of 25 tyrosine residues in the C-terminal cytoplasmic region of GluRε2 were phosphorylated by Fyn *in vitro*. Of these seven residues, Tyr1252, Tyr1336, and Tyr1472 in GluRε2 were phosphorylated in human embryonic kidney fibroblasts when co-expressed with active Fyn, and Tyr1472 was the major phosphorylation site in this system. 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. Importantly, Tyr1472 phosphorylation was greatly reduced in *fyn*-mutant mice. 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.

2. Adhesion molecules and synaptic plasticity

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Adhesion molecules play critical roles in synaptic transmission and plasticity and we have examined functions of several kinds of adhesion molecules and their anchoring proteins. Telencephalin (TLCN) is a cell adhesion molecule selectively expressed in the telencephalon of the mammalian brain. The mutant mice lacking TLCN had no detectable abnormalities

in their neural development and synaptic structures. Ablation of TLCN increased LTP and its saturation level in the CA1 region of the hippocampus. The TLCN mutation selectively enhanced the performance of the radial maze and water-finding tasks, learning tasks with appetitive reinforcers, but not the contextual fear conditioning and Morris water maze tasks with aversive stimuli for conditioning. Furthermore, the TLCN mutant mice showed an increase of prepulse inhibition of the acoustic startle response. These results suggest that TLCN is a determinant of the dynamic range of synaptic plasticity and plays roles in reward-motivated learning and memory and sensorimotor gating.

3. Intracellular calcium regulation and synaptic plasticity

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The concentration of intracellular calcium is controlled by many kinds of molecules including ryanodine receptors (RyRs) and inositol trisphosphate receptors. The precise function of RyRs in synaptic transmission is unknown, while three of their subtypes are expressed in the brain. We examined the role of RyRs in excitatory synaptic transmission in hippocampal slices, using type 3 RyR (RyR3)-deficient mice. The α-amino-3-hydroxy-5-methyl-4-isoxozolepropionic acid (AMPA) receptor-mediated basal synaptic responses in the CA1 region of mutant mice were smaller than those of wild-type mice, while there was no difference in NMDA receptor-mediated responses, suggesting selective postsynaptic modification of AMPA receptors by RyR3. The expression of synaptic AMPA receptors examined by Western blotting or immunohistochemistry was indistinguishable, suggesting that the smaller AMPA synaptic responses in mutant mice were not due to the reduced number of synaptic AMPA receptors. Although the initial potentiation was similar, LTP was smaller in mutant mice. There were no differences in presynaptic electrophysiological properties. We conclude that RyR3 regulates the properties of AMPA receptors postsynaptically.

4. Regulation of synaptic glutamate concentrations and synaptic plasticity

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At the excitatory synapse, the neurotransmitter glutamate released from the presynaptic terminal is subject to the reuptake by glial or neuronal glutamate transporters. Although glutamate transporters maintain low extracellular levels of glutamate in the nervous system, little is known about their roles in synaptic plasticity. Here, using knockout mice lacking GLT-1 that is the most abundant glial subtype of glutamate transporters, we showed that LTP induced by tetanic stimulation in mutant mice was impaired in the hippocampal CA1 region. When tetanic stimulation was applied in the presence of low concentrations of an NMDA receptor antagonist, the impairment was overcome. Consistent with these results, the increased glutamate in the synaptic cleft of mutant mice preferentially activated NMDA receptors. Furthermore, analyses of mutant mice revealed that the magnitude of NMDA receptor-dependent transient synaptic potentiation during low-frequency stimulation depended on the concentration of glutamate in the synaptic cleft. These findings suggest that GLT-1 plays critical roles in LTP induction, as well as in short-term potentiation, through regulation of extracellular levels of glutamate, which enables appropriate NMDA receptor activation.

5. Analysis of muscarinic acetylcholine receptor functions using knockout mice

Minoru Matsui, Fumiko Arima, Toru Shinoe[1], Yuji Kiyama, Ayako M. Watabe, and Toshiya Manabe

We are investigating the biological function of muscarinic acetylcholine receptors (mAChRs) using mutant mice lacking corresponding genes. These mice have been established by Matsui et al. in the Laboratory of Biomedical Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo (Prof. Makoto Mark Taketo's Lab.).

The mAChR belongs to a group of seven transmembrane-spanning receptors and is distributed widely in both central and peripheral nervous systems. Five distinct genes for mAChRs were cloned in rats and humans. Now, it is generally accepted that five classes of affinity (M_1, M_2, M_3, M_4 and M_5) defined by various muscarinic ligands are attributable to these gene products, respectively. Previously, Matsui et al. have cloned the all five genes for mouse mAChRs (*Chrm1*, *Chrm2*, *Chrm3*, *Chrm4*, and *Chrm5*), and determined the chromosomal locations of these genes.

Elucidation of the subtype-specific functions of mAChRs has been a matter of considerable interest because they are suitable targets for various therapeutic drugs. However, due to poor subtype selectivity of the available ligands, pharmacological attempts to elucidate the role of each subtype remain inconclusive. As an alternative and more powerful

strategy, we are studying the receptor function using our knockout mice for M_1, M_2, M_3, M_4, and M_5. In order to minimize the contaminating effects caused by different genetic backgrounds, we are carefully backcrossing these animals to C57BL/6J and DBA/2J strains.

We have previously reported that the M_3 knockout mice are retarded in post-weaning growth and devoid of pilocarpine-induced salivation. The mice also showed partial mydriasis, and male-selective urinary retention. We are now comparing the phenotype of M_3 knockout mice with that of M_2 knockout mice, because M_2 and M_3 are the two major subtypes in smooth muscle organs. Furthermore, we have established a double knockout mouse line lacking both M_2 and M_3 and analyzing their phenotypes.

In addition, mAChRs are supposed to be important in several aspects of 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 functions, employing molecular biology, electrophysiology, and behavioral experiments.

Our mice are now regarded as invaluable resources in the research community and we are organizing many collaborative programs (both domestic and international) as well.

6. Role of MAP kinase signaling in synaptic plasticity

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Several lines of evidence including ours have suggested that the mitogen-activated protein kinase (MAPK) signaling cascade plays a regulatory role in the induction of LTP. We therefore examined the change in synaptic transmission and plasticity in genetically manipulated mice that carry no SynGAP, a GTPase-activating protein known to interact with PSD-95 and negatively regulate MAPK signaling. The mutant mice showed a reduced level of LTP at all examined protocols and showed deficits in a hippocampus-dependent spetial learning test, which can be overcome by excess training. The molecular mechanisms underlying this LTP and learning impairment is still unclear, and the studies to identify the altered MAPK pathways in the mutant mice are now in progress.

7. Modulatory neurotransmitters and synaptic plasticity

Ayako M. Watabe, Hideki Miwa, Thomas J. O'Dell[9], 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.

8. Mechanisms of bidirectional synaptic modification

Ayako M. Watabe, Hideki Miwa, Noriko Kumazawa, Thomas J. O'Dell[9], and Toshiya Manabe

a. Characterization of mGluR-dependent synaptic plasticity

Activity-dependent modification of synaptic strength plays a key role in neural development and some forms of neuronal plasticity. While much focus has been on the LTP mechanisms, not much is known for the molecular mechanisms of LTD, long-lasting suppression of synaptic strength. Recently, it has been reported that activation of the metabotropic glutamate receptor (mGluR) with the group I mGluR agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) induces LTD in the CA1 region of the hippocampus. We investigated potential roles of pre- and postsynaptic processes in the DHPG-induced LTD. DHPG-induced LTD was completely blocked when GDP-βS was delivered into postsynaptic cells, strongly suggesting that DHPG depresses synaptic transmission through a postsynaptic, G protein-mediated signaling pathway. On the other hand, the effect of DHPG was strongly modulated by experimental manipulations that altered presynaptic calcium influx. Also, enhancing calcium influx by

prolonging action potential duration with bath applications of the potassium channel blocker 4-AP strongly reduced the effect of DHPG. Furthermore, while inhibiting both pre- and postsynaptic potassium channels with bath-applied 4-AP blocked the effects of DHPG, inhibition of postsynaptic potassium channels alone with intracellular cesium and TEA had no effect on the ability of DHPG to inhibit synaptic transmission. These results suggest that activation of postsynaptic mGluRs suppresses transmission at excitatory synapses onto CA1 pyramidal cells through presynaptic effects on transmitter release. Further physiological roles of mGluRs in synaptic transmission and activity-dependent modification of synaptic transmission are currently in progress.

b. Molecular mechanisms of metaplasticity

While certain patterns of synaptic stimulation can change synaptic strength, the degree and/or direction of the synaptic modification itself can strongly depend on the previous history of the synaptic stimulation. This effect of the stimulus history onto the plasticity, or plasticity of plasticity (*metaplasticity*), has been implicated from the theoretical point of view in neuronal network development, but its physiological and biochemical mechanisms are still unclear. To elucidate molecular and cellular mechanisms underlying metaplasticity, possible involvement of mGluRs and NMDA receptors and their modulation such as phosphorylation and dephosphorylation are currently investigated.

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Department of Basic Medical Sciences
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Electron microscopy provides a useful means to investigate the structure of biological materials including cells/tissues and macromolecules. If the specimens are properly prepared, It can capture 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 is next to X-ray diffraction, its real unique feature exists in its almost limitless applicability to those whose structure cannot otherwise be pursued.

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

E. Katayama, N. Ichise and T. Shiraishi (In collaboration with Prof. N. Baba's team[1], [1]Kogakuin Univ.)

We investigate the three-dimensional (3-D) architecture of various kinds of macromolecular machinery that might play crucial roles in a number of cell motility and intracellular signal-transduction systems. Some projects are in collaboration with other laboratories, mostly utilizing our expertise technique, the quick-freeze deep-etch replica electron microscopy. With this cryo-EM methodology, all the biological events are instantaneously arrested within one millisecond and the fine structure of individual macromolecules not only in solution but also in cells or tissues can be clearly visualized with high contrast. Since replicas are extremely tolerant to high-dose electron beam irradiation, we can take series of tilted images of each particle to reconstruct its 3-D structure by our new technique, the combination of stereo-photogrammetry and "dynamic shell modeling". Our method, established recently (Patent pending), overcomes the weakness of conventional back-projection method; i.e. impaired resolution along Z-axis due to the "missing-wedge problem", by precise parallax measurement of many reference points on the replica surface to determine their actual heights (Katayama et al., 1996), and finally gives the most reasonable density distribution in each topographic section, so that it satisfies all the density projected onto certain planes, as observed in tilt-images. This method is readily applicable to the structure of macromolecules not only in solution but also in cells and tissues.

One of our continuous projects is to capture transient 3-D configuration of myosin crossbridges supporting actin movement. Combination of quick-freeze deep-etch replica electron microscopy and *in vitro* motility assay systems is suitable for such purpose. We reconstructed 3-D surface features of actin-bound heavy meromyosin (HMM) crossbridges during sliding and obtained the data which we can compare with acto-S1 docking model to examine the structural changes crucial for the movement. As a complementary approach, rotary-shadowed images observed in deep-etch replicas might be simulated by ray-tracing computer software, using atomic coordinates of given molecular model (Patent pending). Since the architecture of acto-S1 rigor-complex has been well characterized by cryo-EM, replica images of rigor acto-HMM complex might be usable as a convenient cross-reference to examine whether our novel approach is applicable to the structural studies of individual molecules. Artificial images of rigor complex simulated form acto-S1 docking model showed good matching with real replica images, suggesting the feasibility and usefulness of our strategy. By an appropriate modification of the original acto-S1 model so that its appearance

best matches respective replica image, we could postulate possible conformational change(s), which might occur in individual particles in the complex captured instantaneously by quick-freezing. When one of two HMM heads bound to actin filament leaving the other head free, the rigor head took the conformation very similar to that in the original acto-S1 model. When both HMM heads were bound, side by side, to the same strand of actin double helix, there must be a considerable degree of distortion in either or both heads to combine the C-terminal ends of the lever-arm continuing to S2 portion. According to our analysis made as above, the structure of the leading head was much less distorted than the following in which the backbone of the heavy chain polypeptide should have been rotated at more than two positions to meet the requirement. Those crossbridges, comprised by a single and double heads might correspond to rear and lead bridges, respectively, as found in insect flight muscle (Taylor et al., 1989). When two heads of HMM crosslinked two actin filaments under rigor condition, the configuration of bound head seemed to be drastically distorted at certain position within the lever-arm. Since the rotation of polypeptide backbone often occurs at glycine, we tentatively rotated Gly-796 in the middle of the lever-arm and compared the simulated image with the replica. The observed replica image was reasonably explained by a passive right-handed rotation of that bond, if neighboring actin filament on the right-side pulled the other head, whereas reverse rotation occurred when the bound head was pulled to the left. Thus, lever-arm might not always behave as a rigid-body. Such structural change could be the origin of spring-like behavior of the lever-arm proposed by Houdusse and Sweeney (2000) to explain the compliance within the crossbridge, which might operate during isometric contraction.

2. "Hot-Spots" along actin filament as induced by energized myosin head

E. Katayama (In collaboration with Dr T.Q.P. Uyeda[2] , Prof. M. Ikebe's team[3] and Prof. T. Yanagida's team[4]; [2]Nat'l Inst. of Adv. Ind. Sci. & Technol.,. [3]Univ. of Massachusetts, [4]Osaka Univ.[***])

The SH-helix of myosin including conservative glycyl residue has been postulated to work as a hinge for the swinging "lever-arm" movement induced by nucleotide binding. *Dictyostelium* G680V myosin II is a natural mutant whose glycine at that position is replaced by a bulky amino acid. Several lines of biochemical and genetic evidence suggest that the mutant is kinetically unique, presenting very much stabilized A.M.ADP.Pi state, which otherwise is short-lived and thus, has hampered direct electron microscopic observation. We expressed the head fragment of that mutant (G680V S1), equivalent to the subfragment-1 of skeletal muscle myosin II, and confirmed that it binds strongly to actin filaments even in the presence of ATP. The resultant crossbridges did not quench the fluorescence of pyrene-actin and were distinct from those in the absence of nucleotides or in the presence of ADP. We examined the structure of these crossbridges by negative staining. Actin filaments decorated with excess amount of G680V S1 showed regular, rigor-like arrowhead appearance even in the presence of ATP. With less G680V S1 molar ratio, it behaved cooperatively, in that some filaments were heavily decorated whereas others left completely bare. Intriguingly, the binding occurred at regular ~36 nm intervals along only one side of actin filaments, up to lengths approaching 1 μm. Similar results were obtained with full length G680V myosin. On the other hand, rigor complex with wild-type S1 or that with myopathy loop-deficient mutant S1 exhibited more or less randomized pattern. We speculate that the binding of an "energized" myosin head might induce certain conformational change within the actin filament and evokes high affinity binding site(s) ~36 nm away from the original site(s).

Unconventional myosin V is known to translocate actin filaments processively with ~36 nm steps. Based on the results of single molecule physiology and electron microscopy, the origin of the processive movement has been attributed to the "striding" of two lever-arms, each of which is comprised of a long neck with six light chains, along one side of actin. According to Tanaka et al. (2002), however, mutant myosin V with drastically shortened neck still preserved the ability to translocate actin filaments with the same ~36 nm steps. Another unconventional myosin VI, naturally possessing short necks, also showed processive movement with ~36 nm steps (Nishikawa et al., 2002), that introduced further controversy to the interpretation by swinging lever-arm hypothesis. Myosin VI in the presence of ATP bound along only one side of actin filaments with ~36 nm intervals, in a similar manner to that of G680V (Nishikawa et al., 2002). We propose that such conformational change induced in actin filament might play a major role in force generation, particularly in the case of 36 nm stepping motion generated by certain classes of myosin.

The other collaborative studies are proceeding mostly on the structural change accompanied with the function of various motility-related protein systems. These projects include the characterization of dynein molecules(cytoplasmic and axonemal) in search for the origin of force to slide microtubules (with Dr. C. Shingyoji's team; Dept of Biology, Univ. of Tokyo and Dr. K. Oiwa's team, Kansai Adv. Res. Ctr,), detailed structures of myosin/metallo-fluoronucleotide complex(with Dr. S. Maruta's team, Soka Univ.), the srtructure of bacterial flagella and

needles(with Dr. C. Sasakawa's team, Div. of Bacterial Infection in this Institute and Dr. S.-I. Aizawa's team, Teikyo Univ.).

3. Molecular mechanism on the regulation of myosin-VI

M. Yoshimura (In collaboration with Prof. M. Ikebe' s team[3])

Myosin is a motor protein which plays a major role in cell contractility, motility, and vesicular trafficking. Myosin-VI is one of the known 18 classes of myosin superfamily and thought to be involved in the vesicular transportation.

Several lines of evidence have suggested that the mechanochemical activities of myosin-VI could be regulated by the phosphorylation of the motor domain and/or the binding of Ca^{2+} to the calmodulin light chain in the neck domain. To clarify the factor(s) responsible for its regulation, we expressed myosin-VI heavy meromyosin (HMM) in Sf9 cells and examined its motor function. As was found in other myosin species, p21-activated kinase 3 phosphorylated myosin-VI, and the site was identified as Thr^{406}. Phosphorylation of myosin-VI significantly facilitated the actin-translocating activity of myosin-VI. On the other hand, Ca^{2+} diminished the actin-translocating activity of myosin-VI, though its actin-activated ATPase activity was not affected. Calmodulin did not dissociate from the heavy chain at high Ca^{2+}, suggesting that a conformational change of calmodulin upon Ca^{2+} binding, but not its physical dissociation, inhibited the motility. These results revealed that myosin-VI is dually regulated, one by phosphorylation of the motor domain and the other by Ca^{2+} binding to the calmodulin light chain.

We also focused on the mechanism that determines the directionality of myosin-VI movement. It was shown recently that myosin-VI moves towards the minus end direction which is opposite from that of the other known myosins. Because there is a large, unique insertion in the myosin-VI head domain between the motor domain and the light-chain-binding domain (the lever arm), it was thought that this insertion alters the angle of the lever-arm switch movement, thereby changing the direction of motility. To test this hypothesis, we determined the direction of motility of chimeric myosins that comprise the motor domain and the lever-arm domain (containing an insert) from myosins that have movement in the opposite direction. The results show that the motor core domain, but neither the large insert nor the converter domain, determines the direction of myosin motility.

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Department of Basic Medical Sciences
Division of Structural Biology (2)

We are running a laboratory open to any researchers of IMSUT who are interested in the morphological analyses of tissue and cells using light and electron microscopes. Besides supportive research works, our own research projects are going on as described below.

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. We are taking three ways of approach to this issue. First, we are searching for the genes which are involved in the development of the nervous system, by screening the P-element insertion mutants whose behavioral phenotype is decreased motor activity in adults. This project is carried out in collaboration with Dr. Chihiro Hama (RIKEN) supported by CREST JST. We have identified three genes so far, *hig, sif and trio*. HIG protein is a member of Ig super family with complement binding domains. It is secreted into the synaptic cleft and required for the functional synapse formation. SIF and TRIO proteins are GDP-GTP exchange factors which activate Rho-family small G-proteins. SIF co-localizes with FAS2 to the pre-synaptic periactive zones. Several lines of evidence indicate that SIF functions cooperatively with FAS2 in synaptic morphogenesis through activating Rac-GTPase. TRIO protein localizes in dendrites and neurite terminals. The function of TRIO in dendrites is under investigation using genetic cell markers.

The second approach is to visualize the dynamic interaction of pre- and postsynaptic cells by single cell labeling methods. This project is collaboration with Dr. Akira Chiba (University of Illinois). We have demonstrated the target specific interaction of cellular processes extending from both pre- and post-synaptic cells, using time lapse recording of the live cells visualized by the genetic labeling of membrane targeted GFP. Furthermore, the ectopic expression experiments of synaptic target recognition molecules revealed that such interaction is regulated by the combination of positive and negative cue molecules. This year we developed a method for observing the synaptic target selection by immuno-scanning electron microscopy. This method makes it possible, to observe three dimentional relation of the pre- and post-synaptic microprocesses and the expression patterns of target recognition molecules on these processes at the same time.

The third approach is to study how the signaling molecules are assembled at the proper subcellular sites of synapses. We have chosen photoreceptor cells as a model system for studying these aspects. This work was done in collaboration with Dr. Charles S. Zuker (UC San Diego). The Drosophila phototransduction is carried out by macromolecular complexes of transduction proteins binding to the PDZ-domain protein, INAD, as is the case for other PDZ-domain proteins in synapses. We have shown that INAD binds TRP (the major membrane ion channel), INAC (eye-protein kinase C that is required for the termination of phototransduction), and NORPA (PLCβ which initiates the PI cascade of phototransduction) by specific PDZ domains. The binding of these proteins to INAD protein was necessary for their proper subcellular localization. These topological couplings of signaling molecules were essential for the phototransduction, indicating that many cellular events require the assembly of the proper molecules at the proper subcellular sites. To

analyze the genetic mechanism of assembly and anchoring of these signaling molecules, we have carried out the expression studies using germ-line transformation. And we found the independent anchoring and assembly mechanisms of INAD complexes.

2. Cell and developmental biology of visual system

Hiroshi Sagara and Emiko Suzuki

a. Drosophila phototransduction

Drosophila photo-transduction is a phosphoinositide (PI)-mediated and calcium-regulated signaling cascade. We have revealed that many of the proteins involved in the PI metabolism, ex. eye-diacylglycerol kinase (eye-DGK), phosphatidylinositol transfer protein (PITP), CDP- diacylglycerol synthase (CDS), are concentrated on the subrhabdomeric cisternae (SRC), the intracellular calcium pool adjacent to photoreceptive rhabdomeres. These findings strongly suggest that PI-metabolism and calcium signalling are topologically coupled on SRC. As for the eye-DGK protein, we studied the involvement of intramolecular domains in the intracellular targeting and expression of DGK activity. The studies on mutated eye-DGK proteins revealed the involvement of the cystein-rich zinc-finger domains in the protein targeting and the enzyme activity.

b. Vitamin A metabolism in vertebrates and invertebrates

In spite of the same photoreceptive function, structures of the photoreceptive organs of vertebrates and invertebrates are quite different. However, use of the same vitamin A derivative, 11-cis retinal, as a chromophore of the visual pigment, rhodopsin, and the induction of the eye under the control of the same homeobox gene Pax/eyeless in both vertebrates and invertebrates indicate that the same basic mechanisms are involved in the formation and maintenance of the photoreceptive organ. In the vertebrate eye, the retinal pigment epithelium (RPE), a single layer of cells adjacent to the photoreceptor cells, has the vital role in visual acuity. Its main functions include the synthesis of 11-cis retinal, which are then transported to the photoreceptor cells to produce rhodopsin. In the course of clarifying the functions of RPE cells, we have prepared several monoclonal antibodies (MAbs) against RPE cells. Among these, three MAbs recognized a 65kDa protein, expressed exclusively in RPE. We molecularly cloned the antigen gene and named it as *RPE65* gene. Phylogenetic analysis of the reactivity of these MAbs to the eye of various vertebrate species indicated that RPE65 proteins, are highly conserved among vertebrates. The genetic knock out of RPE65 carried out by other group showed that the RPE65 deficient retina accumulates all-trans retinyl ester (Redmond et al., 1998). However, no one has demonstrated the precise function of RPE65. To solve this problem, we have decided to clone a homologue of RPE65 in *Drosophila melanogaster* which has great advantage in genetic manipulation. The striking similarity of the amino acid sequence of RPE65 from fish to humans also encouraged us to search for the invertebrate homologues of RPE65. Indeed we have found a gene homologous to vertebrate RPE65 by screening of cDNA library from adult Drosophila heads. The putative Drosophila RPE65 (DRPE65) had 35% -37% homology to vertebrate RPE65s and was specifically expressed in the eyes. The protein sequence alignment revealed several regions highly conserved among Drosophila, putative gene of *C.elegans*, vertebrate RPE65s, β-carotene dioxygenase, and plant 9-cis-epoxylcarotenoid dioxygenase. In parallel to our investigation, von Linting and Vogt (2000) also cloned DRPE65 and found that it has β-carotene dioxygenase activity. From these results, we propose that RPE65-related proteins constitute a carotenoid- and retinoid-metabolizing proteins family throughout the animal and plant kingdom.

c. Development of the zebrafish eye

The zebrafish eye is an excellent model for studying development of vertebrate eyes. We have studied the influences of genetically expressed diphtheria toxin in the lens on the development of neural retina, and found that defective formation of the lens caused by diphtheria toxin severely affects the tissue morphogenesis of the retina. This project was carried out as a collaboration with Dr.Sumiko Watanabe at Department of Molecular and Developmental Biology.

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Department of Basic Medical Sciences
Division of Molecular Neurobiology

Our research interest lies on molecular mechanisms of inositol polyphosphate/calcium signaling and neurobiological activities. We have been studying on the structure and function of genes and their products involved in intracellular calcium dynamics and neuronal development and plasticity.

1. Inositol trisphosphate receptor and Ca^{2+} signaling

Takayuki Michikawa, Mitsuharu Hattori, Takeshi Nakamura[1], Manabu Yoshida[1], Kozo Hamada[2], Kazushi Yamauchi[2], Chihiro Hisatsune[2], Akira Futatsugi[1], Akinori Kuruma[2], Hiroko Bannai[2], Toshifumi Morimura[2], Hirohide Iwasaki[2], Tsuyoshi Uchiyama, Hiroshi Miyauchi, Wei-Hua Cai, Rei Yokoyama, Keiko Uchida, Tomohiro Nakayama, Hideaki Ando, Zhang Songbai, Zhou Hong, Yoko Tateishi, Takayasu Higo, Toru Matsu-ura, Jun-ichi Goto, Ayuko Kurokura, Takafumi Inoue, and Katsuhiko Mikoshiba:[1]Calcium Oscillation Project, ICORP, JST, [2]Laboratory for Developmental Neurobiology, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN)

Inositol 1,4,5 trisphosphate (IP3) is a second messenger produced through the phosphoinositide turnover in response to many extracellular stimuli (hormones, growth factors, neurotransmitters, neurotrophins, odorants, lights, 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 (IP3-induced calcium release; IICR) from intracellular store sites such as endoplasmic reticulum (ER) to cytoplasm. IP3 binds to its specific receptor (IP3R) on the Ca^{2+} store sites. IP3R is an IP3-gated Ca^{2+} release channel, and could be considered as a signal converter that exchanges the IP3 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 relationships of the IP3R, and the physiological roles of IP3R-mediated Ca^{2+} signaling in various cell-types.

We have cloned three distinct types of IP3R and have analyzed the structure, function, and expression of each type of IP3R by means of molecular biological, biochemical, cell biological, physiological and histochemical approaches. We have found that each type has different IP3 binding (e.g., affinity, specificity, Ca^{2+} sensitivity) and modulation (e.g., phosphorylation, calmodulin binding) properties. We analyzed the folding structure of the IP3R-channel by a limited trypsin digestion and have found that the IP3R channel is an assembly of four subunits, each of which is constituted by non-covalent interactions of five major, well folded structural components. The ligand binding site was splited into two tryptic fragments, suggesting that the IP3-binding pocket consists of two non-covalently associated structural domains: the C-terminal domain alone has low affinity for IP3, whereas the N-terminal one alone is incapable of binding but is capable of potentiating binding affinity.

Ca^{2+} signaling via IICR often exhibits dynamic changes in time and space inside a cell (known as Ca^{2+} waves and 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. We found that the positive feedback regulation by cytoplasmic Ca^{2+} is an intrinsic property of the cerebellar IP3R1, whereas

the negative feedback regulation by Ca^{2+} is mediated by calmodulin, a ubiquitous and multifunctional Ca^{2+}-dependent regulator protein. In nonneuronal cells such as *Xenopus* oocytes, IP3 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.

Capacitative Ca^{2+} entry (CCE), the mechanism that replenishes intracellular calcium stores after depletion, is essential to intracellular Ca^{2+} signaling. CCE is mediated by the channels in the plasma membrane generally referred to as "store operated channels (SOCs)". However, the molecular identity of the SOCs has never been determined, and the mechanism of the activation of SOCs remains to be elucidated. Recent studies have demonstrated that 2-aminoethoxydiphenyl borate (2-APB), which has been found to be an antagonist of IP3Rs, inhibits CCE, suggesting that IP3Rs channel activity is essential to the generation of CCE. However, CCE has also been reported to occur normally in IP3R-deficient cells. In order to resolve this discrepancy, we investigated the effect of 2-APB on CCE in IP3Rs-deficient cells. In response to store depletion with thapsigargin or N,N,N9,N9-tetrakis (2-pyridylmethyl) ethylene diamine (TPEN), CCE was generated in IP3Rs-deficient cells the same as in wild-type cells, however, 2-APB abolished CCE in IP3Rs-deficient cells, despite the fact that this cell line does not possess functional IP3Rs. We also examined the effect of 2-APB on several types of TRP Ca^{2+} channels, which exhibit properties similar to those of SOCs. 2-APB had a different inhibitory effect on spontaneous and thapsigargin-induced Ba^{2+} influx in cells that transiently expressed individual TRP subtypes. These results suggest that the channel activity of IP3Rs is not essential to the generation of CCE in this cell line and that 2-APB inhibits CCE independently of the function of IP3Rs.

2. Physiological studies of the Ca^{2+} signaling in CNS

Takafumi Inoue, Takeshi Nakamura[1], Akinori Kuruma[2], 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 IP3R1-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 IP3R1-deficient mice completely lack long-term depression (LTD), a model of synaptic plasticity in the cerebellum. Moreover, a specific antibody against IP3R1, 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 IP3R 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.

3. Studies on the physiological role of IP3R in body patterning

Takeo Saneyoshi and Katsuhiko Mikoshiba

The phosphatidylinositol (PI) cycle has been postulated to function in dorso-ventral (D-V) axis formation of many species, as indicated by the action of lithium. Lithium is assumed to block the recycling of IP3 into inositol by inhibiting the hydrolysis of intermediate inositol phosphates. Application of lithium to cleavage stage embryos of *Xenopus laevis* induced dorsalization by conversion of ventral mesoderm to dorsal mesoderm, with a concomitant reduction in posterior structures. To determine the role of the PI cycle in patterning the body plan, we isolated functional blocking monoclonal antibodies (mAbs) against the *Xenopus* IP3R (XIP3R). Ventral injection of these monoclonal antibodies at 4-cell stage induced the formation of a secondary dorsal axis, whereas dorsal injection of the mAbs or normal mouse IgG showed no obvious effects. This implies that blockage of IICR in the ventral part of *Xenopus* embryos converted ventral mesoderm to dorsal mesoderm, thereby generating an ectopic dorsal axis. These results indicate that an active IP3-Ca^{2+} signal is required for ventral differentiation. To ask which upstream signaling pathways play roles in the ventral differentiation, we introduced constitutively active mutant of Gαq , βARK, a Gβγ inhibitor, or panel of inhibitory antibodies against Gαq/11, Gαs/olf, or Gαi/o/t/z in *Xenopus* embryos. And we obtained a conclusion that activation of the Gαs-coupled receptor relays dorsoventral signal to Gβγ, which then stimulates PLCβ and then the IP3- Ca^{2+} system. This year, we extended our study in attempts to identify possible candidates downstream molecules or upstream molecules of IP3-Ca^{2+} signaling as mediating the ventral signal. We have characterized several

candidates in *Xenopus*.

4. Studies on the ER dynamics

Yuko Aihara, Hiroko Bannai[2], Tomohiro Nakayama, Yoko Tateishi, Mitsuharu Hattori, Takafumi Inoue and Katsuhiko Mikoshiba

The recent view of ER is as a dynamic organelle rather than a classical, stable intracellular structure. We are characterizing dynamic movement and restructuring of ER in variety of cell types including neurons.

ER is the major membranous component present throughout the axon. While other membranous structures such as synaptic vesicles are known to move via fast axonal transport, the dynamics of ER in the axon remained unknown. To study the dynamics of ER in the axon, we directly visualized the movement of two ER-specific membrane proteins, the sarcoplasmic/endoplasmic reticulum calcium-ATPase and the IP3R, both of which were tagged with green fluorescence protein (GFP) and expressed 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, the two ER proteins did not move in a discrete vesicular form. Their movement determined by the fluorescence recovery after photobleaching technique was bi-directional and ten-fold slower (~0.1 μm/sec). The movement of ER was sensitive to temperature, indicating that it is dependent on the active transport system. 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, further suggesting that it is also distinct from the well-documented movement of membranous vesicles in its relation with microtubules.

5. Studies on the molecular mechanisms underlying the development and differentiation of the nervous system

Jun Aruga[2], Mitsuhiro Hashimoto[2], Mitsunori Fukuda[2], Toshio Kojima[2], Toshio Oshima[2], Hiroyuki Kabayama[2], Keiji Ibata[2], Tetsuya Kitaguchi[2], Takashi Inoue[2], Kiyomi Mizugishi[2], Ken-ichiro Kubo, Jun Hoshino[2], Yoshio Koyabu[2], Kazunori Nakajima[3], Kanehiro Hayashi, Miyuki Ogawa, Takahide Toumonda and Katsuhiko Mikoshiba:[3]The Jikei University School of Medicine

The nervous system is composed of neurons and glial cells. Though these cells have distinct characteristics, they arise from a single population of precursor cells, neuroepithelium cells. Only a few factors seem to determine the fate of neuronal and glial cells, since we have observed that a single neuroepithelium cell divides into a neuronal and a glial progenitor cell *in vitro*. To identify such intracellular factors, we have been investigating the mechanisms governing the expression of the genes for proteins specifically produced in neurons and in glial cells. In the series of the developmental process, we focused on the following topics. 1. Generation of neural plate and neural crest cells from ectoderm; 2. molecular mechanism underlying the proper positioning of neurons in the neural tissue; 3. mechanisms of pattern formation and neural circuit formation in the central nervous system. We were able to identify and to characterize some of the key molecules involved in it, such as Zic, CR50-antigen/reelin, and synaptotagmin. In addition, the gene delivery into neuronal and glial cells via adenoviral vectors has been extensively studied.

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Department of Basic Medical Sciences

Division of Molecular Biology(1)

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, but this 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 that translation factors sit in the cockpit in a ribosome 'machine' instead of tRNA and achieve diverse actions. The mechanism of translational control as well as the structural, functional and applied aspects of molecular mimicry are main research interests in this department.

1. Translational Control and Protein-tRNA Molecular Mimicry

Koichi Ito, Makiko Uno, Kuniyasu Yoshimura, Miki Wada, Fumiyoshi Sei, Hanae Sato and Yoshikazu Nakamura

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. Polypeptide release at sense and non-cognate stop codons by localized charge-exchange alterations in translational release factors

The mechanism of stop codon recognition during translation has long been a puzzle. Only recently we have established that a tripeptide in the bacterial release factors, RF1 (UAG/UAA specific) and RF2 (UGA/UAA specific), serves as the 'anticodon' in deciphering stop codons in mRNA. Swaps of each of the conserved domains between RF1 and RF2 led to the identification of a domain which could switch recognition specificity. A genetic selection among clones encoding random variants of this domain showed that the tripeptides Pro-Ala-Thr in RF1 and Ser-Pro-Phe in RF2 determine release factor specificity *in vivo* as well as *in vitro*. However, the molecular basis of the accuracy of stop codon recognition is unknown. Although specific tripeptides in the RFs are primarily responsible for selective reading of cognate stop codons, charge-flip variant RF proteins, altered at conserved Glu residues adjacent to the tripeptide 'anticodon', are shown to be crucial to codon recognition. Changes of these Glu residues are capable of triggering polypeptide release at non-cognate stop codons, and also at sense codons. These changes also reverse the growth inhibition by RFs containing 'harmful' tripeptide-anticodon changes. These findings suggest that electrostatic interactions involving negative charges in domain C of the re-

lease factors mediate their accurate docking in the ribosome. Our results also establish that the charge flipping creates a novel phenotype - translation termination by 'codon bypassing' via relaxed positioning of the RF tripeptide anticodon in the decoding pocket of the ribosome.

b. A tripeptide modulator alters selectivity of stop codon recognition in *Tetrahymena* release factor

In eukaryotes, a single translational release factor, eRF1, deciphers three stop codons. Despite recent achievements in determining the crystal structure of human eRF1, its decoding mechanism remains puzzling. In the ciliate *Tetrahymena thermophila*, UAA and UAG codons are reassigned to glutamine codons, and its eRF1 contains a variant sequence, KATNIKD, for the conservative TASNIKS heptapeptide at the tip of domain 1. A yeast eRF1- domain swap containing *Tetrahymena* domain 1 responded only to UGA *in vitro* and failed to complement a defect in yeast eRF1 *in vivo* at 37˚C. The TASNIKD variant of hybrid eRF1, in contrast, rendered the eRF1-nullified yeast viable. To our surprise, the wild-type eRF1 hybrid fully restored the growth of eRF1-deficient yeast at 30˚C. These results strongly suggest that *Tetrahymena* eRF1 has a potential to decipher three stop codons and that the variant TAS tripeptide can modulate the decoding capacity.

c. Cloning and sequence analysis of translational release factors eRF1 and eRF3 of *Pneumocystis carinii*

To clarify the translation termination apparatus of *Pneumocystis carinii*, we searched for structural genes for eRF1 and eRF3 in *P. carinii* EST database, and found an eRF1 homolog as well as an eRF3 homolog whose C-terminal part is highly homologous to EF-1alpha. Based on these sequences, full-length eRF1 and eRF3 cDNAs were cloned by the RACE method. eRF1 is composed of 432 amino acids and 80% and 78% similar to those of *S. pombe* and *S. cerevisiae*, respectively. eRF3 is 629 amino acid long, and contains unique N-terminal sequence, while the C-terminal sequence is homologous to other eukaryotic eRF3. The activity of these homologs was further investigated by the *in vivo* complementation test using temperature-sensitive eRF1 (*sup45*) and eRF3 (*sup35*) genes of *Saccharomyces cerevisiae* under heterologous conditions. *P. carinii* eRF1 gene restored the growth of ts *sup45* cells while the eRF3 gene failed to restore the *sup35* defect.

The genomic sequences for eRF1 and eRF3 were amplified from *P. carinii* DNA by the PCR method using primer sequences of the cDNA end sequences. The deduced eRF1 and eRF3 genes are composed of 1884 and 2323 nucleotides, respectively. Surprisingly, when compared with their cDNAs, both genes appeared to contain unusually multiple introns, i.e., 13 (Pc-eRF1) and 10 (Pc-eRF3) introns. To our knowledge, this feature is unique among phylogenetically related release factors. Most of yeast and fungus eRF3s do not encode an intron and *S. pombe* eRF3 may be the rare exception as it encodes one intron. All introns of Pc-eRF1 and Pc-eRF3 are similar in the sizes (39-52 bp) and retain the acceptor/donor site consensus sequence for splicing, GT/AG, but do not conserve the apparent branch point consensus as found in other genes. Functional study of *P. carinii* release factors is in progress in this laboratory.

2. Regulation of Ribosome Recycling

Toshinobu Fujiwara, Koichi Ito, Tohru Yamami, Tomohiko Toyoda and Yoshikazu Nakamura

After release of nascent polypeptides, the posttermination complex composed of the ribosome, deacylated tRNA, RF and mRNA needs to be dissociated for the next round of protein synthesis. Ribosome recycling factor (RRF), in concert with elongation factor EF-G, is required for disassembly of the posttermination complex. Three groups including my group have recently solved the crystal structure of RRF. These three molecules are composed of two domains, domain 1 and domain 2, bridged by two loops (a hinge), and superimpose almost perfectly with tRNA except for the amino acid-binding 3' end. It has been proposed that RRF is a near perfect tRNA mimic to explain the mechanistic disassembly of the posttermination ribosomal complex. RRF, however, is architecturally different from tRNA in that the hinge of RRF forms a flexible 'gooseneck' elbow, while the elbow of tRNA is rigid, and that this flexibility of RRF is vital for its function. Moreover, the biochemical findings show that RRF and EF-G split the ribosome into subunits in a reaction that requires GTP hydrolysis. Therefore, the mechanistic significance of tRNA mimicry by RRF remains to be verified. We assume that nature may not have created such protein of a tRNA mimic to simply substitute for tRNA unless protein is required to pursue some function(s) that tRNA cannot do.

a. *In vitro* dissection of ribosomal occupancy of ribosome recycling factor and elongation factor G

We have previously reported a simple *in vitro* spin-down system to assay the ribosome-binding capacity of variant RRF proteins. We have extended this system to dissect, in some detail, the ribosomal occupancy of RRF in relation to other translation factors and antibiotics. The data point out the shared as well as distinct binding sites on the ribosome for RRF and other components such as EF-G.

b. Transmission of elongation factor G motor action to

ribosome disassembly via specific interface between tRNA-mimic domains of EF-G and ribosome recycling factor

Elongation factor G is a G-protein motor that drives two target molecules-tRNAs on the translating ribosome and the ribosome recycling factor (RRF) on the posttermination complex. How the motor action is transmitted to RRF is unknown. *Thermus thermophilus* RRF is nonfunctional in *Escherichia coli* but can be restored by specific surface mutations in the domain of *E. coli* EF-G that mimics tRNA or by swapping that domain for the *T. thermophilus* domain. Functionality was also conferred on *T. thermophilus* RRF by specific surface substitutions on RRF in the region that mimics the anticodon-arm. These 'gain-of-function' phenotypes are allele-specific between two proteins. These results suggest that the EF-G motor action is transmitted to RRF by specific surface contacts between them at the domains that mimic the anticodon arm, thereby providing functional and genetic evidence for the interface between EF-G and RRF for the transmission of the motor movement.

3. Molecular Biology of Yeast Prions

Toru Nakayashiki, Colin G. Crist, Hideyuki Hara 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. Yeast [*PSI*$^+$] prions that are crosstransmissible and susceptible beyond a species barrier through a quasi-prion state

The yeast [*PSI*$^+$] element represents an aggregated form of release factor Sup35p and is inherited by a prion mechanism. A 'species barrier' prevents cross-transmission of the [*PSI*$^+$] state between heterotypic Sup35p 'prions'. *Kluyveromyces lactis* and *Yarrowia lipolytica* Sup35 proteins, however, show interspecies [*PSI*$^+$] transmissibility, susceptibility and a high spontaneous propagation rate. Cross seeding was visualized by coaggregation of differential fluorescence probes fused to heterotypic Sup35 proteins. This coaggregation state, referred to as a 'quasi-prion' state, can be stably maintained as a heritable [*PSI*$^+$] element composed of heterologous Sup35 proteins. *K. lactis* Sup35p was capable of forming [*PSI*$^+$] elements not only in *S. cerevisiae* but in *K. lactis*. These two Sup35 proteins contain unique multiple imperfect oligopeptide repeats responsible for cross-transmission and high spontaneous propagation of novel [*PSI*$^+$] elements, serving good models to unravel the molecular basis of interspecies transmission and susceptibility of prion diseases in mammals.

b. Hsp104-independent [*PSI*$^+$] prion propagation by non-Gln/Asn oligopeptide repeats of yeast Sup35 variants

The N-terminal of Sup35p, necessary for [*PSI*$^+$], contains oligopeptide repeats and multiple Gln/Asn residues, which are characteristic of mammalian prions and polyglutamine disease proteins, respectively. To understand the importance of the unique oligopeptide repeat structure apart from the multiple Gln/Asn residues in [*PSI*$^+$], we replaced the Gln/Asn-rich prion repeats of Sup35p with non-Gln/Asn repeats from heterologous yeast strains. These non-Gln/Asn repeat Sup35ps propagated [*PSI*$^+$] prions that were not eliminated by guanidine treatment or deletion of *HSP104*, unlike known [*PSI*$^+$] prions. Moreover, these [*PSI*$^+$] prions formed strand-like aggregate foci, unlike Gln/Asn-rich prion aggregates, which form dot-like foci. *In vitro*, non-Gln/Asn repeat domains formed amyloid fibers more slowly than did Gln/Asn-rich prion domains. These findings suggest that the non-Gln/Asn oligopeptide repeats interact independent of Hsp104 to stabilize the intermolecular interactions between Sup35ps necessary for [*PSI*$^+$] aggregation, and that multiple Gln/Asn residues accelerate the fiber formation and hence [*PSI*$^+$] propagation with the aid of chaperone Hsp104.

4. Protein Crystallography

Tomohiko Toyoda, Koichi Ito, Masayo Urata, Maria B. Garber[2], Natalia Nevskayaa[2], Stanislav Nikonova[2], and Yoshikazu Nakamura:[2]Protein Research Institute, Pushchino, Russia

a. Structure of ribosomal protein L1 from *Methanococcus thermolithotrophicus*. Functionally important structural invariants on L1 surface

The crystal structure of ribosomal protein L1 from the archaeon *Methanococcus thermolithotrophicus* has

been determined at 2.8 Å resolution. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a=67.4, b=70.0, c=106.2 Å and two molecules per asymmetric unit. The structure was solved by the molecular-replacement method with *AmoRe* and refined with *X-PLOR* to an *R* value of 20.7% and an *R* free of 30.5% in the resolution range 8-2.8 Å. Comparison of this structure with those obtained earlier for two L1 proteins from other sources (the bacterium *Thermus thermophilus* and the archaeon *Methanococcus jannaschii*) as well as detailed analysis of intermolecular contacts in corresponding L1 crystals reveal structural invariants on the molecular surface, which are probably important for binding the 23S ribosomal RNA and the protein function within the ribosome.

5. Pharmaceutical RNA Design and Discovery by SELEX

Takashi Ohtsu, Akihiro Oguro, Eiko Futami, and Yoshikazu Nakamura

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 eIF4G and eIF4A provided by Dr. Nahum Sonenberg (McGill University, Canada). 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.

Several RNA aptamers were successfully raised against eIF4G and eIF4A, and were shown to acquire a strong and specific binding capacity for these proteins. Of two target proteins, eIF4A exhibits RNA-dependent ATPase activity and RNA helicase activity. It was reported that expression of eIF4A1 mRNA is elevated in human melanoma cells. One class of RNA aptamers exhibits a high affinity with eIF4A1 and inhibits the ATPase activity of eIF4A1 efficiently, suggesting that they probably dock the catalytic pocket. Furthermore, these aptamers inhibit cap-dependent protein synthesis in a cell free translation system. This inhibition was reversed by addition of purified eIF4A1 suggesting that RNA aptamers to eIF4A1 inhibits its activity in the cell lysate. Hence, from the therapeutic point of view, it is of great interest whether or not these RNA aptamers can suppress the malignant cells.

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Department of Basic Medical Sciences
Division of Molecular Biology (2)

One genome is a community of genes potentially with different interests. Their collaboration and conflicts underlie various aspects of genome metabolism and genome changes. Our goal is to understand genes, genomes, their interactions, their changes, diseases and evolution from this point of view.

1. Restriction-modification gene complexes as selfish, mobile genetic elements

A gene for a restriction enzyme is often linked to a gene for a methylase, whose role is to protect the recognition site from breakage by the restriction enzyme. Our previous work demonstrated that several restriction-modification gene complexes resist replacement by a competitor genetic element by killing host cells freed of them through chromosome cleavage. Their behavior as selfish genetic elements explains their parasitic life cycle and cellular responses to DNA double-strand breaks. Decoding of bacterial genome has been providing strong evidence for our selfish gene hypothesis.

a. Amplification of a selfish restriction modification gene complex

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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 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. Here we report amplification of a chromosomal RM gene complex. This process, reminiscent of provirus induction to replication, would be advantageous for the maintenance and spreading of the RM complexes, and might help gene evolution through duplication and mutation.

b. Selfish transposition of a restriction modification gene complex

Seishi Ohashi, Yoko Ui, and Ichizo Kobayashi

Loss of several restriction-modification gene complexes from a bacterial cell results in cell death through chromosome cleavage. This finding led to the hypothesis that they behave as selfish genetic elements: they persist by destroying 'non-self' DNA, not properly methylated, whether they be on invaders or host cell's chromosome. Their mobility and horizontal transfer can be inferred from their linkage to mobile genetic elements, and from genome sequence comparisons and evolutionary/informatics analyses. Here 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, thermoresistant survivors carrying *Pae*R7I integrated into the chromosome were recovered. The integration appeared to occur concomitantly with restriction attack on the chromosome and to be *recA*-dependent. 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. Thus restriction-modification gene complexes seem to use the same strategy of restriction attack on the genome for their short-term and long-term persistence.

c. Epigenetics in genome defense: a DNA methyltransferase can protect the genome from post-disturbance attack by a restriction-modification gene complex

Yasuhiro Naito, Noriko Kobayashi-Takahashi, Naofumi Handa, and Ichizo Kobayashi

Dcm in several bacteria methylates DNA to generate 5' C^{m}CWGG. Vsr mismatch repair function prevents C to T mutagenesis enhanced by this methylation but promotes other types of mutation. The reason for the existence of the *dcm-vsr* gene pair has been unclear. We found that failure to replicate *Eco*RII restriction-modification genes, whose products recognize the same sequence as Dcm, leads to chromosome degradation and loss of cell viability. This cell killing was suppressed by *dcm*. Dcm, therefore, can play the role of a "molecular vaccine" by defending the genome against parasitism by a restriction-modification gene complex.

d. Analysis of *in vivo* stability of restriction modification enzyme proteins

To further understand the molecular mechanism underlying the post-segregational host killing by restriction modification gene systems, we are currently examining the *in vivo* stability of the *Eco*RI restriction modification enzyme proteins by pulse-chase analysis and Western blot analysis.

e. Application of the selfish behavior of restriction modification genes to stable maintenance and expression of useful genes

Noriko Kobayashi-Takahashi, Ichizo Kobayashi, Nanae Kotake[4], Hiroko Funaki[4], and Masanori Watahiki[4]:[4]Nippongene

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.

2. Involvement of restriction modification gene complexes in genome evolution as suggested from genome analysis

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

Ikuo Uchiyama[5], Toshio Higuchi[6], and Ichizo Kobayashi:[5]National Institute for Basic Biology, Okazaki National Research Institutes, [6]INTEC Web and Genome Informatics Cooperation

Recently, complete sequences of two or more microbial genomes that are closely related to each other have been determined, and detailed comparison of such genomes became a useful approach for elucidating principles and mechanisms of genome evolution. For such an analysis, 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 comparative genome analysis tool (CGAT) for closely related microorganisms. 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. Genome analysis of meticillin-resistant *Staphylococcus aureus*, a major hospital pathogen

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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 possesses extreme flexibility in adapting to antibiotic pressure, and has acquired resistance to practically all antibiotics so far introduced in clinical practice. Whole genome analysis is a necessary step towards future development of countermeasures for the infection caused by *Staphylococcus aureus*.

Whole genome sequences of two related *Staphylococcus aureus* strains, N315 and Mu50, were determined by shot-gun random sequencing strategy. N315 is an MRSA strain isolated in 1982. Mu50 is an MRSA strain with vancomycin resistance isolated in 1997. The open reading frames (orfs) were identified by using GAMBLER and GLIMMER programs, and annotation of each orf was performed using BLAST homology search, motif analysis, and protein localization prediction (PSORT).

The Staphylococcus genome was composed of complex mixture of genes, many of which seem to have been acquired by lateral gene transfer. Most of the antibiotic resistance genes were carried either by plasmids or by mobile genetic elements including a unique resistance island SCCmec. Three classes of novel pathogenicity islands were identified in the genome: TSST-1 islands (SaPIn1/SaPlm1), exotoxin islands (SaPIn1/SaPlm2), and enterotoxin islands (SaPln3/SAPlm3). In the latter two pathogenicity islands, clusters of enterotoxin and enterotoxin paralogues were found closely linked with other gene clusters encoding putative pathogenic factors. The analysis also identified a total of 70 candidates for novel virulence factors.

Remarkable ability of *Staphylococcus aureus* to acquire useful genes from various organisms was revealed through the observation of genome complexity and evidence of lateral gene transfer. Repeated duplication of genes encoding superantigens explains why Staphylococcus aureus is capable of infecting humans of diverse genetic backgrounds, eliciting severe reaction of human immune system. Investigation on many newly identified gene products including 70 putative virulence factors will revolutionize our understanding of Staphylococcus biology and the processes of infectious diseases caused by *Staphylococcus aureus*.

c. 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 Z2491 (serogroup A), *Neisseria meningitidis strain* MC58 (serogroup B), and a *Neisseria gonorrhoeae* strain. We searched these genomes for DNA methylase 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 an 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 also linked with IS elements. These results lend further support for the hypothesis that RM genes are potentially mobile and involved in genome rearrangements.

3. Recombination machines — 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 conflict 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. Double-strand-break-repair type homologous recombination by bacteriophages

Noriko Kobayashi-Takahashi, and Ichizo Kobayashi

Earlier we demonstrated that a restriction break is repaired by conservative (two-progeny) double-strand break repair in *E. coli* cells with active RecET genes of Rac prophage or with active Red genes of bacteriophage lambda. One *recBC sbcB endA* strain showed high activity of conservative double-strand break repair in apparent contrast to our early demonstration of non-conservative (one-progeny) recombination in a *recBC sbcB* strain. We identified Rac prophage in this strain and demonstrated that mutational inactivation of its *recT* gene eliminates the double-strand break repair activity. An *endA* mutation enhanced the double-strand break repair in a well established *recBC sbcA* strain.

c. Alleviation of restriction by bacteriophage recombination functions

Naofumi Handa, and Ichizo Kobayashi

We now hypothesize that this type of 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. Coevolution of bacterial restriction modification systems and bacteriophages — a theoretical study

Akira Sasaki[18], Ichizo Kobayashi, and Ryota Horie[19]:[18]Department of Biology, Faculty of Science, Kyushu University, [19]Riken

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.

e. Spontaneous accumulation of large linear forms of *Escherichia coli* chromosome

Naofumi Handa, and Ichizo Kobayashi

Large linear forms of *E. coli* chromosome that are produced by spontaneous breakage of its circular forms were detected by pulsed-field gel electrophoresis in various recombination-related mutants. The results were interpreted in terms of the roles of various proteins in the repair and processing of chromosomal breaks.

f. Homology-associated non-homologous recombination

Naofumi Handa, Ayumi Fujita, Yoko Ui, Keiko Sakagami, 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 two 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*. In the other system, the substrate plasmid molecules carried two restriction breaks at the repeats. 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.

g. Random-walk model for interference in meiotic recombination

Youhei Fujitani[17], Shintaro Mori[20], and Ichizo Kobayashi:[20]Department of Physics, School of Science, Kitasato University

A crossing-over between homologous chromosomes apparently suppresses another crossing-over in its neighborhood in meiosis. This crossover interference or chiasma interference has been a subject of various models — some physical/biological and others genetic/mathematical. We here propose a novel model that treats the process as a one dimensional reaction-diffusion process. We suppose that an early contact point searches for global homology between homologous chromosomes to initiate a crossing-over. We treat this contact point as a random-walker that moves along the homology, becomes immobilized and matures into a crossing-over point. The interference is caused by collision between the random-walker with another random-walker or with an immobilized point resulting in its destruction. We numerically show that this simple model with only

two parameters — the initial density of the contact point per physical length, and the efficiency of its processing into a crossing-over point — can describe the interference under a variety of conditions.

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

Youhei Fujitani[17], 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, such as RecG and RuvA. In this work, we take into account this asymmetry in the random-walk model.

4. Basic studies for gene therapy by mutation correction

Asami Ino, and Ichizo Kobayashi

Earlier we demonstrated that virus-mediated gene transfer followed by homologous recombination with the genome provides an accurate means of correcting mutations in mammalian cells. We have been trying to extend this approach to more complex systems. We are also analyzing stability of the viral vector genomes in the cell. Furthermore, we try to use non-viral vector for gene targeting.

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Department of Basic Medical Sciences
Division of Molecular Biology (3)

Our major reserch interest 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. Proteolysis and cell death

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.

a. Caspase-mediated cleavage and downregulation of calpastatin, the endogenous inhibitor for calpain

Masahiko Kato, Takashi Nonaka, Hidehiko Kikuchi and Shinobu Imajoh-Ohmi

Using a novel technique with cleavage-site-directed antibodies we have demonstrated that procalpain μ is activated in apoptotic cells where cell death is induced by cytotoxic anti-Fas antibodies or tumor necrosis factor. Calpain is thought to be suppressive to apoptosis, since calpain inhibitors accelerate cell death induced by tumor necrosis factor or anti-fas antibody. However, it has remained to be elucidated how procalpain is activated during apoptosis.

Recently, we demonstrated that calpastatin, an endogenous inhibitor protein specific for calpain, is cleaved in human T Jurkat cells treated with anti-Fas antibody. Native calpastatin retains four inhibitory units for calpain and shows apparent molecular mass of 120 kDa on SDS-PAGE. Jurkat cells contain a quantitative amount of calpastatin. In 8 h after induction of apoptosis the 120-kDa calpastatin decreased and a 90-kDa polypeptide appeared when analyzed by immunoblotting with an antibody against calboxyl-terminal region of calpastatin, indicating that a cleavage occured around the amino-terminal region. Limited proteolysis of calpastatin preceded activation of procalpain, both of which were suppressed by peptidyl inhibitors for caspases. Activation of procalpain but not degradation of calpastatin was inhibited by synthetic inhibitors for calpain. Recombinant calpastatin expressed in Eschrichia coli was cleaved to a similar molecular size after incubation with extracts from apoptotic cells, which was inhibited by acetyl-Asp-Glu-Val-Asp-CHO, a synthetic inhibitor for caspase-3 and -7. Amino-terminal sequence analysis of the 90-kDa fragment generated by recombinant caspase-7 from recombinant calpastatin revealed that cleavage site was at Asp-233, around which DAID/A was a cleavable sequence for caspases-3 and -7. These findings strongly suggest that calpastatin is cleaved directly by caspases during apoptosis, and that degradation of calpastatin at least in part triggers activation of procalpain. Precise mechanism for activation of procalpain and biological role of calpain in apoptosis are under investigation.

b. Limited proteolysis of actin in neutrophils

Junko Ohmoto, Ryoko Iizuka, Satoshi Toyoshima[2] and Shinobu Imajoh-Ohmi:[2]Hoshi University

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.

2. Phagocytic differentiation and apoptosis

Yuichi Niikura, Ryoko Iizuka, Takashi Nonaka 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 (IFNγ), 1α, 25 dihydroxy vitamin D3 (VD) or retinoic acid (RA). RA- and VD-differentiated U937 cells seem to be more closely related to mature macrophages than IFNγ-differentiated cells, since RA and VD induce cell surface expression of CD11b. Although RA and VD do not change the expression of Fas antigen and tumor necrosis factor receptor, they desensitize these death receptors in U937 cells after differentiation.

To know how the apoptotic machinery is affected or changed during differentiation, we analyzed apoptosis-related proteins in RA-differentiated cells. First, poly(ADP-ribose) polymerase (PARP) was cleaved by apoptotic protease caspase-3 or -7 in undifferentiated and IFNγ-differentiated cells. However, when RA-differentiated U937 cells were treated with cytotoxic anti-Fas antibody, neither activation of procaspase-3 nor proteolysis of PARP was observed. Second, caspase-8 is coupled to Fas and its pro form is autocatalytically converted to an active enzyme upon activation of Fas, which was seen in Fas-mediated apoptosis of U937 cells. In RA-differentiated cells expression of procaspase-8 was slightly enhanced compared with undifferentiated cells. However, activation of procaspase-8 did not occur when the cells were treated with anti-Fas as judged by immunoblotting with active caspase-8-specific antibodies. These results suggest that caspases do not function when Fas is stimulated in RA-differentiated U937 cells. Expression of antiapoptotic members of Bcl-2 family (Bcl-2, Bcl-xL and Mcl-1), some of which were reported to upregulated during monocytic differentiattion, was not changed before and after differentiation. Mobilization of receptor-coupled apoptosis-promoting proteins and apoptosis-suppressing factors in RA-differentiated U937 cells remain to be determined.

When RA-differentiated U937 cells were treated with inhibitors for proteasomes or protein kinases, apoptotic cell death could be induced as well as in undifferentiated and IFNγ-differentiated cells. In such cells proteolytic activity of caspase-3/7 increased and cleavage of PARP was observed. But activation of procaspase-8 remained suppressed in RA-differentiated cells, indicating that activity of this caspase is particularly inhibited in RA-differentiated cells. Interestingly, activation of procaspase-8 was seen in undifferentiated or IFNγ-differentiated U937 cells treated with proteasome inhibitors. Apoptotic signal downstream of caspase-3/7 may come back near the receptor and presumably enhances the death signal. Fas ligand and other death factors are induced by some apoptotic stimuli. On the other hand, procaspase-8 is reported to activated independently of interaction between Fas and Fas ligand. Such positive feedback pathways should function in apoptotic cells.

3. Cell death of monocytic cells and macrophages infected with *Shigella flexneri*

Taku Kuwabara, Takashi Nonaka, Asaomi Kuwae[3], Chihiro Sasakawa[3] and Shinobu Imajoh-Ohmi:[3]Division of Bacterial Infection, Department of Microbiology and Immunology

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.

We employed here a human monoblastic cell line

U937 that is potentially differentiated into cells resembling mature monocytes in the peripheral blood. Cultured with IFNγ or RA, U937 cells become superoxide anion-producible. When S. flexneri was introduced into thus differentiated cells, cell death occurred regardless of differentiation stateas as judged by the dye exclusion viability test. Cell death was also observed in undifferentiated U937 cells, but was strongly promoted in RA-differentiated cells. On the other hand, an avirulent mutant strain deficient in Ipa proteins did not induce cell death, probably because bacteria could not enter the cell. These results clearly indicate that bacterial invasion induces cell death in monocyte-like U937 cells. However, undifferentiated and RA-differentiated U937 cells died exhibiting cytoplasmic swelling but not nuclear condensation and fragmentation, suggesting that these cells underwent necrosis. Surprisingly, IFNγ-differentiated U937 cells showed morphological features typical of apoptosis, though these cells were as sensitive as undifferentiated U937 to virulent Shigella. These observations were confirmed by DNA fragmentation assay on an agarose gel where chromosomal DNA from IFNγ-treated cells was electrophoresed in a ladder-like manner upon *Shigella* infection but that from U937 cells of different differentiation state was not. Furthermore, cleavage of PARP was seen only when IFNγ-differentiated cells were infected with pathgenic *Shigella*. These findings suggest that vilulent *Shigella* induces distinct types of cell death in U937 cells depending on their differentiation state.

4. 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

Yuichi Niikura, Takashi Nonaka, and Shinobu Imajoh-Ohmi

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 and caspase-catalyzed poly(ADP-ribose) polymerase in apoptotic cells.

Similar antibodies are now available for caspase-8 and its endogenous inhibitor (FLIP). These antibodies did not crossreact each other although the two proteins are structurally related. Molecular mechanism for activation and regulation of caspase-8 in apoptosis-induced human monoblastic U937 cells were analyzed by these antibodies during differentiation to macropahge-like cells.

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 struc-

tural 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.

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Department of Basic Medical Sciences
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Malaria kills more than two million people every year in the world. Since most of the victims are children under 5 years of age, it is imperative to develop effective methods to control this disease. To achieve this goal, we focus on the genome research and investigation of chaperones of malaria parasites.

1. Full-length-enriched cDNA library

In a cooperative study with Dr. Sumio Sugano, a full-length-enriched cDNA library was produced from the erythrocytic-stage malaria parasite, *Plasmodium falciparum*. 5' end-one-pass-sequencing of 2490 random clones provided important information which complements the genome sequencing projects that are underway in the U.S.A. and England. The database: FULL-malaria is now available at http://fullmal.ims.u-tokyo.ac.jp/.

2. Chaperone DnaJ homologues of malaria parasites

DnaJ was first described as a gene that regulates phage replication in *E. coli* at our institute. Since then, 20 species of DnaJ homologues, which are characterized by the J domain with 70 conserved amino acids, have been identified in yeast. 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 antigens). Based on the hope that elucidation of the functions of these molecules will lead to elucidation of mechanisms behind parasitism and control of proliferation of this organism, analyses of these homologues are underway.

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Department of Basic Medical Sciences

Department of Molecular and Developmental Biology

Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of metazoan cells with emphasis on intracellular signal transduction, transcription and DNA replication. 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 growth and differentiation, 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.

1. Signal transduction of cells and its application to the stem cell expansion and eye regeneration

We have been analysing signal transduction mechanisms of cytokine receptors using various hematopoietic cells and fibroblasts. On the basis of accumulated knowledge and techniques for cell signaling events, we have recently started to analyse molecular mechanisms of organogenesis. We use the zebrafish system in addition to mouse genetics with particular focus on eye development and hematopoietic cell differentiation.

a. Statb shuttles between cytoplasm and nucleus in cytokine-dependent and -independent manners

Rong Zeng, Yutaka Aoki, Minoru Yoshida, Ken-ichi Arai, and Sumiko Watanabe

Stats are a family of transcription factor which play important roles in cytokine signal transduction. In response to cytokine stimuli, Stats are phosphorylated and translocated to the nucleus to activate target genes. Then, most are dephosphorylated and returned to the cytoplasm. Using Ba/F3 cells, we found that the nuclear export of Stat5B by cytokine depletion was inhibited by leptomycin B (LMB), a specific inhibitor of nuclear export receptor CRM1. Interestingly, LMB treatment in the absence of cytokine led to the accumulation of Stat5B in the nucleus, suggesting that Stat5B shuttles between the nucleus and the cytoplasm as a monomer without cytokine stimulation. This notion is supported by the observation that LMB-induced accumulation of STAT5B in the nucleus was also observed with Stat5B having a mutated tyrosine 699, which is essential for dimer formation. Using a series of mutant Stat5Bs, we identified a part of the coiled coil domain to be a critical region for monomer nuclear import and a more N-terminal region to be critical for the cytokine stimulation dependent import of Stat5B. Taken together, we propose a model in which Stat5B shuttles between the nucleus and cytoplasm by two different mechanisms, one being a factor-independent constitutive shuttling by monomeric form, and the other, a factor stimulation-dependent one regulated by tyrosine phosphorylation and subsequent dimerization.

b. *Ex vivo* expansion of haematopoietic stem cells using human GM-CSF receptor transgenic mouse

Yutaka Aoki, Eishun Mutoh, Koichiro Tsuji[1], Ken-ichi Arai, Sumiko Watanabe:[1]Department of Clinical Oncology

Using Tg mice expressing hGM-CSFR (wild-Tg), we previously showed that hGM-CSF is not a GM-lineage promoting factor but a strong proliferation promoting factor of all the lineages of haematopoietic cells examined. GM-CSF induced not only GM colonies, but also the erythroid colonies were observed within bone marrow cells of wild-Tg mice. To analyse signaling requirement for these activities, we generated Tg mice expressing Fall hGM-CSF mutant receptor, in which all the cytoplasmic tyrosine residues were replaced by phenylalanine. Methyl cellulose assays of bone marrow cells from Fall-Tg, wild-Tg and their litter mate showed that Fall induced lower but still significant numbers of myeloid colonies. Interestingly, immature colonies were major population of Fall induced colonies and differentiated colonies such as erythroid colonies were not observed. Methyl cellulose assay of 5-FU treated mouse bone marrow cells further confirmed this tendency. Taken together, it was suggested that Fall signals preferentially support immature cells. We are now examining whether the Fall signals can expand haematopoietic stem cells by ex vivo expansion system.

c. Identification of genes of which expression is temporally regulated during development of mouse eye

Akihiko Muto, Rika Saitoh, Ken-ichi Arai, Sumiko Watanabe

Development of eye is tightly regulated by intracellular and extracellular factors. A huge number of studies have demonstrated that various transcription factors, which are expressed in spatially and temporally different patterns, are implicated in the regulation of the eye development, suggesting that transcriptionally regulated genes play pivotal roles to maintain the accurate developmental processes. To identify such spatiotemporally regulated genes expressed in the developing mouse eye, we performed differential display using total RNA prepared from various stages of embryonic (E13.5, E15.5 and E18.5) and postnatal (P1, P4, P7, P10, P17 and adult) mouse eyes as templates. Ninety-one bands were differentially displayed and finally 62 independent sequences were identified. Among them, 37 clones had identical sequences to the already known genes, and 25 clones were either similar to genes only reported as EST entries or previously unreported. For the latter 25 unidentified genes, tissue specific expression was examined by RT-PCR using primer sets specific to each gene. All genes examined were expressed at least in retina and some were expressed specifically or predominantly in the central nervous system. At present, we attempt to isolate full-length cDNAs.

d. Functional Analyses on a Novel PH Domain Protein:Its Essential Role in Vertebrate Nervous System Development

Eishun Muto, Ken-ichi Arai, Sumiko Watanabe

In the developmental processes of vertebrate, accurate regulation of the expression of the genes are required for the adequate formation of numerous numbers of organs. To study on the development of nervous system, differential expression cloning based on degenerate RT-PCR was done. One of the cloned genes, Clone22, was identified as a norvel PH domain protein. Expression analyses revealed that its expression is highly restricted in eye and kidney in adult mice, and that, during nervous system development, high-level expression is observed in ventricular zone.

To address the function of Clone22, Loss of function analysis by antisense MO oligo in zebrafish was done. According to its expression pattern, loss of proper expression level of Clone22 leads to abnormal development of central nervous system. To further characterize its function, construction of gene-targeted deletion mouse (KO mouse) have been performed.

e. Expression of diphtheria toxin results in disruption of retinal cell organization in zebrafish

Ryo Kurita, Hiroshi Sagara[2], Yutaka Aoki, Ken-ichi Arai and Sumiko Watanabe:[2]Department of fine morphology

Using a 2.8-kb fragment of the zebrafish αA-crystallin promoter (zαAcry), we expressed the diphtheria toxin A fragment (DTA) in zebrafish embryos in a lens-specific manner. Injection of the zαAcry-DTA plasmid into eggs at the one- or two-cell stage resulted in the formation of small eyes, in which both lens and retina were reduced in size. In the DTA-expressing lenses, their fiber structure was disorganized, indicating that normal lens development had been abrogated. The neural retina also showed abnormal development, although this tissue per se did not express DTA. The laminated structure of the retina did not develop well, and molecular markers for the outer and inner plexiform layers were either abnormally expressed or absent. On the other hand, cell type-specific markers of ganglion and bipolar cells, as well as photoreceptors, were expressed in appropriate positions, indicating that differentiation of these retinal subpopulations occurred in the DTA-expressing embryos. Cell proliferation also normally occurred in these embryos, although apoptosis was enhanced. These results suggest that the lens plays a critical role in the morphogenetic organization of retinal cells, but not in their early differentiation, during eye development in zebrafish embryos.

f. Genetic studies on vertebrate development using zebrafish as a model animal: Development of a transposon-mediated insertional mutagenesis method in zebrafish

Koichi Kawakami, Ken-ichi Arai

In order to understand the genetic basis for developmental processes in vertebrate, we have been using a small tropical fish, the zebrafish, as a model animal. Because it is practically possible to breed and maintain very large numbers of fish in the lab, and because zebrafish embryos develop in water and are transparent, forward genetic approaches (i.e., collecting a large number of mutations affecting developmental processes and analyzing genes responsible for the mutant phenotypes) are feasible in the fish. Actually large-scale chemical mutagenesis screens in zebrafish had successfully collected hundreds of mutants defective in early developmental processes. It has, however, not been easy to identify the mutated genes since most of the chemically induced mutations were point mutations and positional cloning approaches are required to clone the genes. Therefore it is important to develop insertional mutagenesis methods in zebrafish, such as the P transposon system in Drosophila.

Previously we have successfully developed an insertional mutagenesis method in zebrafish using a pseudotyped retrovirus. By this approach, we isolated a dominant mutation *hagoromo* (*hag*), which caused disorganized stripe patterns on the skin of adult fish. In the *hag* mutant, the provirus was located within the fifth intron of a novel gene encoding a novel F-box/WD40-repeat protein. Interestingly, the mouse ortholog of the *hag* gene was mutated in the *Dactylaplasia* (*Dac*) mutant mouse. The *Dac* mutation is also dominant and causes defects in digit formation in fore- and hindlimbs. Thus, the *hag/Dac* locus is important for pattern formation in vertebrates but is involved in distinct morphogenetic events in different animals. We have characterized subcellular localization of the mouse *Dac* protein and have shown by biochemical analyses that it is a component of a SCF complex of a ubiquitin ligase.

The *Tol2* element is a transposable element identified in the genome of the medaka fish. We have been developing a novel insertional mutagenesis using *Tol2*. We cloned cDNA of *Tol2* and disclosed it encoded a fully functional transposase, which catalyzes its transposition in the zebrafish germ lineage. Using this *Tol2* transposon system, we have established a very efficient method to make transgenic zebrafish. Our next goal is to establish a gene trap strategy in zebrafish using the *Tol2* transposon system. Efforts are in progress toward this goal. Also activities of this transposon in other vertebrates are currently under investigation.

g. Nuclear and chromatin structure in embryonic stem (ES) cell

Noriko Sato and Ken-ichi Arai

Embryonic stem (ES) cell is capable to proliferate with maintaining totipotency . It is thought that specific sets of signal transduction pathways such as LIF-receptor - Stat 3, and/or transcripition factors such as Oct3/4 play a major role in stem cell renewal. It is indeed important to clarify transcriptional control which is responsible for either maintenance of undifferentiated state or determinant of differentiation. However, even if we could identify local regulation of respective gene expression, it will not be easy to answer how pluripotency is maintained only in undifferentiated ES cell. Then we hypothesized that there should be a certain functional architecture specific to ES (and possibly immature embryo cell) nuclei. In order to analyze dynamics of functional chromosomal regions, we have investigated the chromosomal positioning process after mitosis and the nuclear higher order compartmentalization of chromatin according to its replication timing. Our goal is to elucidate the regulation of nuclear structure in the course of cell differentiation in *in vitro* ES cell differentiation system.

Undifferentiated ES cells complete to form interphase nuclear structure at about 1.5 hours after metaphase. At the same time, cells start to replicate chromosomal DNA. Several clamps of heterochomatin regions are detected throughout the S phase. On the contrary, initiation of DNA replication is delayed in differentiated cells because of acquisition of long G1 phase. Heterochromatin regions are more dispersed in the differentiated nuclei. A clamp of heterochromatin in undifferentiated nuclei are formed by a clusters of centromeric regions and HP1 proteins are co-localized. Even in the undifferetiated nuclei, clear compartmentalization according to the order of replication timing is observed. We are currently examining whether replication timing and accessibility to heterochromatin is altered during differentiation in the case of chromosomal region of Oct3/4 gene.

2. Regulation of lymphocyte differentiation and activation by transcription factors

T helper cells can be divided into two subsets called Th1 and Th2. Th1 and Th2 cells are characterized by the spectrum of cytokines they produced. Th1 cells produce mainly interferon gamma and lymphotoxin, while Th2 cells produce interleukin (IL)- 4, 5, 6, 10 and 13. These differentially expressed cytokines determine effector function of T helper cells. Th1 cytokines regulate cellular immune responses and macrophage activation. On the other hand, Th2 cytokines help B cells to produce antibod-

ies and activate eosinophils in order to eliminate extracellular pathogens. Interestingly, inappropriate activation of these subsets induces various immunological disorders. Several autoimmune diseases, such as multiple sclerosis are caused by activation of Th1 cells. Allergic diseases such as asthma and atopic dermatitis are triggered by Th2 activation. Therefore, regulation of differentiation towards each subset becomes key issue for controlling these diseases. There are many factors that regulate differentiation of T helper cells. Among them, cytokine IL-4 and IL-12 are well known and studied as regulators for Th2 and Th1 differentiation, respectively. Addition of IL-4 together with TCR stimulation induces Th2 differentiation via activation of transcription factor GATA3 and STAT6.

IL-4 and IL-13 produced by activated Th2 cells sometime affect directly to the organs that have their receptors, and induce allergic diseases. Hence, STAT6 that is down stream signaling molecule for both IL-4 and IL-13 turn out be important molecule for generation of allergic diseases. Our goal of study is to perceive the role of transcription factors on Th2 differentiation and effector function *in vitro* and *in vivo*.

a. The role of STAT6 in the Th2 diffrentiation and the pathology of airway hyperresposiveness

Yumiko Kamogawa, Sahori Namiki, Kazuho Shigemoto, Miho Nagoya, Shoichiro Miyatake[3], and Ken-ichi Arai:[3]Tokyo Metropolitan Institute of Medical Science

IL-4 is a crucial cytokine for the differentiation of Th2 cells which induce allergic diseases. It has been shown that IL-4 and 13 may play important roles in the generation of allergic asthma not only through induction of Th2 cells but also directly affecting target airway tissues. STAT6 is a signaling molecule which functions down stream of both IL-4 and IL-13 receptors. Disruption of STAT6 failed to cause allergic asthma upon proper immunological stimulation. Thus, STAT6 became an important drug target for allergic diseases.

We have been utilizing STAT6-hormone-binding domain of estrogen receptor fusion protein(STAT6ER) to elucidate role of STAT6. Estrogen analog 4-hydroxitamoxifen (4-HT) induces dimerization of the estrogen receptor, thereby resulting in dimerizatrion and activation of STAT6ER without activation of another down stream molecules of IL-4 or IL-13 receptors. We could showed that activation of STAT6 upon TCR stimulation induce Th2 response *in vitro*. The introduction of STAt6ER into airway epitherial cells by adenovirus infection induced chemokine upregulation *in vitro*. To study the role of STAT6 in actual allergic asthma model, we generated transgenic mice that expressed either STAT6ER or dominant negative STAT6ER in airway cells. Now those animals are in progress to study the role of STAT6 in allergic asthma.

b. The role of NFATx in cytokine production

Chen Jingtao, Yoshiharu Amasaki, Yumiko Kamogawa, Shoichiro Miyatake[3], Ken-ichi Arai

NFAT family transcription factors have been imprecated in cytokine production in various cell types. There are four NFAT family members, NFAT1, NFATc, NFAT3 and NFATx. These genes are expressed in various tissues and gene disruption analysis revealed that some of the family members show functional redundancy. Cytokine production of T cells isolated from NFATx deficient mice does not show any modulation. In many promoters of cytokine genes, NFAT forms a complex with AP-1 and binds to the NFAT/AP-1 composite sites. NFAT activation can be induced by Ca ionophore treatment and AP-1 activation is induced by phorbol ester (PMA). By deleting several critical regions for Ca regulation in NFATx, active NFATx mutant that localizes in nucleus without Ca signal and can fully activate cytokine promoters in the presence of PMA alone was created. Naive CD4+ T cells isolated from this NFATx mutant transgenic mice can produce IL-2 and proliferation is induced upon PMA treatment alone. When the naive T cells were differentiated into Th1 subset, Th1 cytokines, IFNγ and TNFα are both up-regulated by the active NFATx. Interestingly when the naive cells were differentiated into Th2 subset, the production of Th2 cytokines, IL-4 and IL-5 were severely impaired, indicating that NFATx is a negative regulator of Th2 cytokine genes. T cells isolated from NFAT1 deficient mice show elevated level of IL-4 production, suggesting that NFAT1 is also a negative regulator of IL-4 gene. However both NFAT1 and NFATc are required for the transcription of IL-4. These data suggest that the expression of the Th2 cytokine genes is regulated by the complicated network of NFAT family genes.

c. Studies on regulation of DNA replication

DNA replication is an essential process for cell proliferation, differentiation and development. We are interested in three levels of regulation which are imposed on DNA replication to permit stable and accurate inheritance of genetic materials.

1) genetic program which determines the spatial and temporal pattern of origin activation, 2) cell cycle regulation of DNA replication in response to various growth and differentiation signals, and 3) cellular responses to replication fork arrest to ensure stable maintenance of genetic integrity.

We hope to apply the knowledge obtained through these studies to development of novel tech-

nologies to manipulate self renewal and development of various stem cells and to curve the growth of cancerous cells.

c. Functional analyses of ASK, the regulatory subunit of human Cdc7 kinase

Noriko Sato, Masahito Nakayama, Hisao Masai[3] and Ken-ichi Arai

Activation of Cdc7 kinase, which is required for activation of prereplicative complex (preRC), is regulated locally at individual origins. Fluctuation of the protein level of ASK, the regulatory subunit, during the cell cycle, plays crucial roles in cell cycle regulation of huCdc7 kinase activity. In order to investigate spatial regulation of huCdc7-ASK kinase complex, we fused these proteins to GFP or its derivatives. We ectopically expressed GFP-ASK variants (delta M [Δ193-270], lacking the entire motif M region and incapable to activate Cdc7, minimum ASK [174-350] bearing both motif-M and motif-C, capable of activating Cdc7, and the full-length) on a tet-inducible adenovirus expression vector. Overexpression of these ASK variants did not cause any significant influence on cell cycle progression. This may be because ASK protein may not be a rate-limiting factor for DNA replication. Alternatively, only a small portion of Cdc7-ASK complex may be necessary for origin activation and the level of the exogeneous proteins expressed may not be sufficient to cause a dominant effect. In fact, we have observed only a small fraction of the endogenousCdc7-ASK binds to chromatin. In cycling cells, the replication complexes on origins are reset at each mitosis. During progression of cell division, cytoplasmic ASK and Cdc7 are relocated into daughter nuclei. We found that the timing of nuclear accumulation of ASK and Cdc7 during cell cycle was different. ASK as well as MCM2 starts to accumulate in the nuclei at telophase, while Cdc7 accumulation occurs after decondesation of chromatin in the G1 phase. Our results suggest a possibility that Cdc7 and ASK subunits dissociate in the mitotic phase and are sorted into daughter nuclei by different mechanisms.

d. Phosphorylation of MCM2 protein by Cdk and huCdc7 kinases

Minkwon Cho[3], Etsuko Matsui, Chika Taniyama, Yukio Ishimi[4], Katsuyuki Tamai[5], Noriko Sato, Jung Min Kim, Hisao Masai[3] and Ken-ichi Arai: [4]Mitsubushi Kagaku Institute of Life Sciences, [5]MBL

Cdc7 kinase regulates initiation of DNA replication at origins by phosphorylating components of replication complexes. Genetic and biochemical evidence indicates that MCM2, among them, is a critical substrate for firing of origins. Cdc7 can efficiently phosphorylate MCM2 in the MCM2-4-6-7 complex *in vitro*. Tryptic peptide mapping and mobility-shift on SDS-PAGE indicate phosphorylation of distinct residues on MCM2 in the complex. Analyses of MCM2 within the cells indicate that MCM2 in a complex is the major target of Cdc7 *in vivo*. The objective of our research is 1) identify Cdc7-mediated phosphorylation sites on MCM2 (both *in vitro* and *in vivo*), 2) elucidate the physiological significance of this phosphorylation on regulation of functions of MCM complex, and 3) ultimately understand how DNA replication is triggered by Cdc7 at each origin. Toward this goal, we analyzed biochemical characteristics of mutant MCM complexes and examined their phosphorylation by huCdc7-ASK kinase complex *in vitro*. We have identified several *in vitro* phosphorylation sites on MCM2. Various *in vivo* and *in vitro* assays using phosphorylation site mutants and phosphopeptide antibodies indicate that chromatin association, interaction with other proteins, and cellular localization may be regulated by the phosphorylation.

e. Inactivation of Cdc7 kinase in mouse embryonic stem cells results in S phase arrest and p53-dependent cell death

Jung Min Kim, Nobuyuki Yamashita, Hisao Masai[3] and Ken-ichi Arai

We have established a mutant ES cell line lacking both alleles of muCdc7 in the presence of a loxP-flanked transgene expressing a muCdc7 cDNA. Upon removal of the transgene by of the Cre-recombinase, the mutant ES cells cease DNA synthesis, arresting growth with S phase DNA content. Rad51 foci are generated in nuclei and M phase is blocked by increased Tyr15 phosphorylation of Cdc2 kinase, followed by cell death with concomitant increase and activation of p53 protein. muCdc7$^{-/-}$p53$^{-/-}$ embryos survive up to E8.5 and their blastocysts generate inner cell mass of a significant size by E8.5 *in vitro*, whereas muCdc7$^{-/-}$p53$^{+/-}$ counterpart undergoes complete degeneration of inner cell mass under the same condition. Furthermore, inhibition of p53 partially rescued survival of the mutant ES cells lacking muCdc7. These results show that loss of Cdc7 functions in ES cells results in rapid cessation of DNA synthesis within S phase, which triggers checkpoint responses leading to recombinational repair, G2/M arrest and eventually to p53-dependent cell death. Since p53 is not activated by replication block in normal differentiated cells, our results suggest a possibility that cellular responses to replication fork arrest may vary depending on the cell types. Efficient removal of damaged cells by cell death may help tautipotent stem cells to maintain its genomic integrity as a whole. We are also improving

the methods for generation of conditional knock-out ES cells by utilizing Flp/FRT-directed integration of a trans-gene.

f. *E. coli* PriA protein in recognition of arrested replication forks and reassembly of replication complexes

Taku Tanaka, Toshimi Mizukoshi[6], Chika Taniyama, Daisuke Kohda[6], Hisao Masai[3] and Ken-ichi Arai:[6]Biomolecular Engineering Research Institute

In response to replication fork arrest, cells need to temporally suspend the cell cycle progression, repair any lesions, and restart DNA replication. In *E. coli*, PriA protein, a DEXH-type DNA helicase, plays a central role in this process presumably by recognizing the sites of replication fork arrest and inducing recombination-dependent DNA replication. *In vitro*, PriA binds specifically to a synthetic D-loop like structure, and triggers assembly of a primosome capable of unwinding duplex DNA and initiating DNA chain elongation. We have developed an antibody specific to PriA protein and showed that its amount, 30-50 copies per cell, is constant after replication fork arrest. Partial tryptic digestion of PriA protein revealed the existence of D-loop binding activity in the N-terminal segment. In fact, the isolated N-terminal 181 amino acid segment, carrying a novel "WYY" motif, can bind to D-loop. However, D-loop binding activity significantly decreased by mutations in C-terminal helicase domains as well. All the D-loop binding mutants are incapable of supporting RecA-dependent inducible stable DNA replication. These results suggest that binding of PriA to arrested forks involves precise configuration of the N-terminal "D-loop recognition" domain and C-terminal helicase domain and demonstrate essential roles of PriA's ability to recognize fork-like structures in recombination-dependent reassembly of replication forks.

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