Department of Basic Medical Sciences

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

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We study various aspects of cellular signal transduction with a particular emphasis on the role of protein phosphorylation and dephosphorylation. Protein phosphorylation and dephosphorylation are among the most important intracellular signaling mechanisms, and are mediated respectively by protein kinases and protein phosphatases.

1. Structural basis for the function and regulation of the receptor protein tyrosine phosphatase CD45.

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Protein tyrosine phosphorylation is a key mechanism for nearly every aspect of cell regulation, ranging from cell survival and proliferation to apoptotic cell death, in multi-cellular eukaryotes. Tyrosine phosphorylation itself is regulated by a concerted action of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). The human genome encodes as many as 38 classical PTPs, which share highly homologous PTP domains. These PTPs are divided into two major subfamilies: 17 non-receptor (or cytoplasmic) PTPs and 21 receptor-like (transmembrane) PTPs. CD45, also known as the leukocyte common antigen (LCA), is the prototype of the receptor-like PTP (RPTP) subfamily, and is found in all nucleated hematopoietic cells. CD45 is essential for development and antigen-induced activation of T- and B-cells. Mutations in the human CD45 gene have been associated with Severe Combined Immunodeficiency (SCID) and multiple sclerosis. CD45 controls immune response, both positively and negatively, by dephosphorylating a number of signaling molecules including Src-family kinases (Lck, Fyn), the CD3ζ chain of TCR, and ZAP-70 kinase.

Like many other RPTPs, CD45 consists of an extracellular receptor-like region, a short transmembrane segment, and a cytoplasmic region comprising tandem PTP domains. The length of the extracellular segment varies among the CD 45 isoforms generated by alternative splicing. Mutational analyses have shown that the membrane proximal PTP domain 1 (D1), but not the membrane distal domain 2 (D2), is catalytically active. The entire cytoplasmic region of CD45 is conserved among vertebrates, from shark to mammalian. The strong conservation of the CD 45 D2 primary structure implies a functional role, but this role is not yet clearly defined. Nonetheless, there are observations that suggest a regulatory role of the catalytically inert D2 domain. D2 perhaps influences the activity of D1 by direct inter- and intra-molecular interaction, as shown by *in vitro* binding studies. It was also

reported that CD45 D2 domain could, by itself, bind one of the important substrates, Lck, facilitating its dephosphorylation by D1 domain. In the case of another RPTP, leukocyte common antigen related (LAR) protein, we observed high degree of similarity between the crystal structures of the D1 and D2 domains. Perhaps consistent with the structural similarity of D2 and D1, only two amino acid substitutions were required to convert the otherwise inert D2 domain of LAR into an active enzyme.

A generalized model, known as the dimeric inhibition model or the wedge hypothesis, for the regulation of RPTP activity has been proposed based on the crystal structure of the RPTPα D1 domain. The RPTPα D1 domain formed a homodimeric structure in the crystal, in which the active site of one monomer was blocked by an N-terminal helix-turn-helix wedge motif of the other monomer, suggesting that dimer formation on the membrane negatively regulates the PTP activity.

This year, we reported the first crystal structures of the CD45 cytoplasmic region containing both the D1 and D2 domains. We also determined crystal structures of the CD45 cytoplasmic region bound to phospho-peptide substrates; one bound to a nonspecific short phospho-peptide, and the other bound to the membrane-proximal immunoreceptor tyrosine-based activation motif (ITAM) of the CD3ζ chain. We found several key features of CD45 and its substrate interactions, and analyzed the implications of these structures as they impact on our understanding of the mechanism of D1 and the potential function of D2. In these structures, CD 45 exists as a monomer with the D1 active site cleft, as well as the D2 site, clearly unobstructed by the rest of the protein chain. The observed intra-molecular orientation of the D1 and D2 domains precludes the formation of a dimer of the type predicted by the dimeric inhibition model. Such a D1 dimer interaction is impossible because of significant steric hindrance generated from the overlap of the attached D2 domains.

2. GADD45β/GADD45γ and MTK1/MEKK4 comprise agenetic pathway mediating STAT4-independent IFNγ production in T cells

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Stress-activated MAP kinase (MAPK), including p38 and JNK, regulate a variety of intracellular processes in response to environmental stresses, growth factors, cytokines, and other stimuli. p38 and JNK are activated via signaling cascades involving a MAPK kinase (MAPKK) that is responsible for phosphorylation of the appropriate MAPK, and a MAPK kinase kinase (MAPKKK or MAP3K) that phosphorylates and activates MAPKK. Recent studies have suggested a new mode of p38/JNK activation, which involves an interaction between GADD45 proteins and a MAP3K, MTK1 (MEKK4 in mouse). GADD45 proteins include three closely related members (α, β, and γ) that are transcriptionally induced by environmental stress. These proteins bind MTK1 and augment its activity. Overexpression of each individual GADD45 protein by transient transfection activates p38/JNK and causes apoptosis, which can be partially suppressed by coexpression of a dominant inhibitory MTK1 protein. These studies suggest that GADD45 may mediate activation of the p38 /JNK pathway, through MTK1, in response to environmental stress.

The function of GADD45 proteins in regulating stress-activated MAPK is starting to be explored in the immune system, with the use of T helper type 1 (Th1) cells as a model system. Th1 cells produce a signature cytokine, IFNγ, in response to antigen receptor challenge or combined stimulation by IL12 and IL18. IL12/IL18 stimulation induces high-level expression of GADD45β and GADD45γ. Retroviral overexpression of GADD45β leads to sustained p38 activation and enhanced IFNγ production in IL12/IL 18-stimulated Th1 cells. A study from our group has found that GADD45γ is strongly induced during Th1 cell differentiation. Th1 effector cells deficient in GADD45γ show reduced IFNγ production and p38/JNK activation in response to T cell receptor (TCR) stimulation. *In vivo,* GADD 45γ-/- mice exhibit suppressed contact hypersensitivity, indicative of the significance of this protein in regulating immune function. Recently, we have generated GADD45β-/- mice and demonstrated that GADD45β is required for MAPK activation and signaling of both antigen receptor and inflammatory cytokines in T cells. GADD45 proteins also appear to regulate p38/JNK outside the immune system. GADD45α function was shown to be critical for the maintenance of sustained p38/JNK activities in UV-irradiated keratinocytes, and thus represents a key component protecting the skin against UV-induced tumors.

However, results from several other studies conflict with the notion that GADD45 proteins activate p38/JNK through MTK1/MEKK4. The

reason for the discrepancy between these studies is not clear, but is likely to reflect the multiple functions of GADD45 proteins in regulating intracellular processes. GADD45 proteins are known to interact with a number of nuclear proteins such as proliferating cell nuclear antigen (PCNA), p21, cdc2, and core histone protein, in addition to activating MTK1/MEKK4. Conceivably, in different cell types and/or stimulation conditions, GADD45 proteins could preferentially interact with one or several particular pathways, leading to the differential regulation of p38/JNK activities and other downstream events through distinct mechanisms.

Despite recent advances in our understanding of GADD45 and MTK1, a critical issue to be addressed is whether GADD45 and MTK1 are in the same genetic pathway *in vivo* and whether this pathway is physiologically important. Previous work using dominant-negative MTK1 is suggestive but has obvious limitations inherent with the use of the dominative-negative approach; for example, the overexpressed MTK1 could interfere with other pathways nonspecifically by interacting with upstream adaptors or downstream kinases not physiologically involved with MTK1, thus making the interpretation of the results difficult.

To elucidate the relationship between GADD 45 and MTK1/MEKK4 and the functional significance of the MTK1/MEKK4-dependent pathway, we created MEKK4-deficient mice to define the function and regulation of this pathway. CD4 T cells from MEKK4-/- mice have reduced p38 activity and defective IFNγ synthesis. Expression of GADD45β or GADD45γ promotes IFNγ production in MEKK4+/+ T cells, but not in MEKK4-/- cells or in cells treated with a p38 inhibitor. Thus, MEKK4 mediates the action of GADD45β and GADD45γ on p38 activation and IFNγ production. During Th1 differentiation, the GADD45β/GADD45γ/MEKK4 pathway appears to integrate upstream signals transduced by both T cell receptor and IL12/STAT4, leading to augmented IFNγ production in a process independent of STAT4. We thus obtained the first genetic evidence that GADD45 proteins and MTK1/MEKK4 are in the same pathway in CD4 T cells. Furthermore, our study has provided new insights into the regulation and function of this important pathway during Th1 cell differentiation. Our results suggest that this pathway integrates signals from TCR and IL12/STAT4 in developing Th1 cells and promotes STAT4-independent production of IFNγ.

3. Characterization of a novel low-molecular-mass dual-specificity phosphatase-3 (LDP-3) that enhances activation of JNK and p38.

Kentaro Takagaki[5], Takeshi Satoh[5], Nobuhiro Tamura[5], Kouhei Masuda[5], Mutsuhiro Takekawa, Hiroshi Shima[5], and Kunimi Kikuchi[5]: [5] Division of Biochemical Oncology and Immunology, Institute for Genetic Medicine, Hokkaido University

Reversible phosphorylation of tyrosine residues in proteins plays a critical role in regulating cellular responses such as growth, differentiation, metabolism, migration and malignant transformation. Protein tyrosine phosphorylation is controlled through the co-ordinate actions of protein tyrosine kinases and PTPs. The PTP family can be subdivided into two broad categories: (i) classical phosphotyrosine-specific enzymes, and (ii) DSPs (dual-specificity phosphatases), which dephosphorylate Ser/Thr as well as Tyr residues. The DSPs, which preserve the general catalytic mechanism of classical PTPs but display differences in the architecture of the active site, are thought to be involved in important signaling events, ranging from the control of MAPKs in cell proliferation to the regulation of cyclin-dependent kinases in the cell cycle. The DSP family includes: (i) MKPs (MAPK phosphatases), which negatively regulate MAPKs, (ii) members of the CDC25 family, which are positive regulators of G1/S and G2/M cell cycle transitions, (iii) members of the CDC14 family, which are indispensable for exit from mitosis, and (iv) other small DSPs whose function is currently unknown.

Activation of MAPK cascades plays a key role in transducing various extracellular signals to the nucleus. Three distinct MAPK families comprise ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38. The ERK pathway is activated by growth and differentiation factors, cell adhesion, phorbol esters and some oncogenes. The JNK and p38 pathways are activated by pro-inflammatory cytokines and a variety of environmental stresses, such as heat shock, UV light, irradiation, and osmotic and oxidative stresses. For full activation of these MAPKs, phosphorylation of both threonine and tyrosine residues in TXY (Thr-Xaa -Tyr) motifs found in the activation loop is required by dual-specificity kinases, which are members of the MKK (MAPK kinase) family. MEK1 (MAPK/ERK kinase 1) and MEK2 are specific kinases for ERK, MKK4 and MKK7 are specific for JNK, and MKK3 and MKK6 are kinases for p38.

Negative regulation of MAPKs is achieved by dephosphorylation of the TXY motif by phosphatases, such as MKPs. MKPs are composed

primarily of two domains, the DSP catalytic domain and the rhodanese domain, which is responsible for substrate binding and enables MKPs to specifically target individual MAPKs. It has been shown that small DSPs also negatively regulate MAPK activation. For example, VHR (VH1-related) is a small DSP that binds to and dephosphorylates ERK. We have shown that VHR dephosphorylates p38 and JNK as well as ERK. LDP-2 (low-molecular-mass dual-specificity phosphatase-2) was identified as a negative regulator of JNK, and LMW-DSP2 (low -molecular-weight DSP2) was shown to regulate JNK and p38. These results strongly suggest that not only MKPs, but also small DSPs, are important regulators of MAPKs.

This year, we isolated a mouse cDNA for a novel dual-specificity phosphatase designated LDP-3 (low-molecular-mass dual-specificity phosphatase-3). The 450 bp open reading frame encoded a protein of 150 amino acids with a predicted molecular mass of 16 kDa. Northern blot and reverse transcription-PCR analyses showed that LDP-3 transcripts were expressed in almost all mouse tissues examined. *In vitro* analyses using several substrates and inhibitors indicated that LDP-3 possessed intrinsic dual-specificity phosphatase activity. When expressed in mammalian cells, LDP-3 protein was localized mainly to the apical submembrane area. Forced expression of LDP-3 did not alter activation of ERK, but rather enhanced activation of JNK and p38 and their respective upstream kinases MKK4 and MKK6 in cells treated with 0.4 M sorbitol. By screening with a variety of stimuli, we found that LDP-3 specifically enhanced the osmotic stress-induced activation of JNK and p38.

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Department of Basic Medical Sciences

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

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

1. NMDA receptor phosphorylation and synaptic plasticity

Ayako M. Watabe, Shoji Komai[1], Fumiko Arima, Hiroko Izumi-Nakaseko, Takanobu Nakazawa[2], Tohru Tezuka[2], Tadashi Yamamoto[2], and Toshiya Manabe: [1]Division of Cell Biology and Neurophysiology, Department of Neuroscience, Faculty of Medicine, Kobe University, and [2]Division of Oncology, Department of Cancer Biology

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

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

2. Analysis of muscarinic acetylcholine receptor functions using knockout mice

Minoru Matsui, Shinji Kusakawa, Yuji Kiyama, Hideki Miwa, Toru Shinoe, Naoki Hirahara, Sayuri Inagaki, Naoko Numata, Shiho Sato, and Toshiya Manabe

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

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

We are investigating the significance of each subtype, employing molecular biology, electrophysiology, and behavioral experiments. During this year, we published eight original research articles (see the Publications section) and made nine presentations at scientific meetings.

3. Intracellular signaling in synaptic plasticity

Ayako M. Watabe, Sheng-Tian Li, Norikazu Katayama, Hideki Miwa, Saknan Bongsebandhu-phubhakdi, Thomas J. O'Dell[3], and Toshiya Manabe: [3]Department of Physiology, School of Medicine, University of California, Los Angeles, USA

The small GTPase Ras as well as Ras regulators and effectors are associated with NMDA-type glutamate receptors in the postsynaptic density of excitatory synapses. Although the role of Ras in NMDA receptor-mediated signaling has not been well characterized, several findings indicate that Ras signaling pathways have an important role in NMDA receptor-dependent forms of synaptic plasticity, such as long-term potentiation (LTP). For instance, mice with mutations affecting H-Ras or SynGAP (a synaptic Ras-GTPase-activating protein) have alterations in hippocampal LTP. Moreover, pharmacological inhibition of the Ras effectors phosphatidylinositol 3-kinase (PI3-kinase) and the p 44/42 MAPK (mitogen-activated protein kinase) pathway disrupts LTP. Although Ras-activated signaling pathways are clearly involved in LTP, the molecular details of how these pathways contribute to an enhancement of synaptic strength remain unclear. We therefore examined the role of PI3-kinase and ERK in LTP at excitatory synapses in the CA1 region of the mouse hippocampus. Consistent with the notion that PI3-kinase links NMDA receptors to the ERK pathway, PI3-kinase inhibitors significantly reduced both NMDA and high-frequency stimulation-induced increases in ERK2 phosphorylation. We found, however, that PI3-kinase inhibitors suppress LTP under conditions in which blocking ERK activation with MEK (MAP kinase kinase) inhibitors has no effect. Thus, although PI3-kinase contributes to NMDA receptor-mediated ERK activation, our results demonstrate that the induction of LTP is also dependent on PI3-kinase signaling through ERK-independent pathways.

We are currently extending these studies and have started new projects on a few signaling molecules.

4. Modulatory neurotransmitters and synaptic plasticity

Ayako M. Watabe, Shizuka Kobayashi, Hiroyuki Kato, Michiko Nakamura, Takayuki Morimoto, Akiko Moro, Thomas J. O'Dell[3], and Toshiya Manabe

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

tory 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.

We are currently extending these studies and have started new projects.

5. Age-dependent modulation of hippocampal LTP and spatial learning

a. Age-dependent enhancement of hippocampal LTP and impairment of spatial learning through the ROCK pathway in protein tyrosine phosphatase receptor type Z (Ptprz)-deficient mice

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Although protein tyrosine phosphatases are abundantly expressed in the brain, their roles in synaptic plasticity have not been well elucidated. In this study, we have examined the physiological functions of Ptprz, which is a receptor-type protein tyrosine phosphatase predominantly expressed in the brain as a chondroitin sulfate proteoglycan. We have examined phenotypes of mutant mice deficient in *Ptprz*, using electrophysiological, pharmacological and behavioral approaches. Mutant mice exhibit enhanced LTP in the CA1 region of hippocampal slices and impaired spatial learning abilities in an age-dependent manner: young adult (less than 10 weeks old) mutant mice show normal LTP and learning abilities in Morris water maze task, whereas adult (more than 13 weeks old) mutant mice exhibit enhanced LTP and impairment in the task. The enhanced LTP is specifically canceled out by the ROCK inhibitor Y-27632. These findings suggest that the lack of *Ptprz* leads to aberrant activation of ROCK, and resultantly to enhanced LTP in the slice and learning impairments in the animal.

b. Age-dependent enhancement of hippocampal long-term potentiation in knock-in mice expressing human apolipoprotein E4 instead of mouse apolipoprotein E

Hiroyuki W. Kitamura[6], Hiroki Hamanaka[6], Koji Wada[6], Chiharu Yamazaki[6], Nobuyuki Nukina[6], Masahiko Watanabe[7], Shinobu C. Fujita[8], and Toshiya Manabe: [6]Laboratory for Neurodegeneration Signal, RIKEN Brain Science Institute, [7]Department of Anatomy, Hokkaido University School of Medicine, and [8]Mitsubishi Kagaku Institute of Life Sciences

Human apolipoprotein E (apoE) comprises three isoforms, apoE2, apoE3 and apoE4, and apoE4 has been reported as a risk factor of Alzheimer's disease (AD). One of the clinical symptoms of AD is disorder of memory that has been suggested to be related with synaptic plasticity such as long-term potentiation (LTP). Here, we show the enhancement of hippocampal LTP at younger age in knock-in mice lacking mouse apoE, but instead expressing human apoE4. The enhancement of LTP in apoE4 knock-in mice is age dependent, and it disappears in adult apoE4 knock-in mice. In apoE3 knock-in mice LTP is unaltered, thus human apoE4, but not apoE3, specifically modulates synaptic plasticity at younger age. Since basal synaptic transmission and distribution of glutamate receptors, as well as presynaptic functions, are intact in apoE4 knock-in mice, postsynaptic functional modification of LTP through lipid homeostasis is suggested. ApoE4 knock-in mice would be a useful animal model of human apoE4 carriers, and our finding that LTP is enhanced in younger apoE4 knock-in mice is in accord with the previous report showing higher intelligence in young human apoE4 carriers.

6. Neurogenesis in the nervous system

Yoko Tabata[9], Yasuo Ouchi[9], Haruyuki Kamiya[1], Ken-ichi Arai[9], Sumiko Watanabe[9], Sheng-Tian Li, Noriko Takemura, Fumiko Arima, Ayako M. Watabe, and Toshiya Manabe: [9]Division of Molecular and Developmental Biology, Department of Basic Medical Sciences

With the goal of generating retinal cells from mouse embryonic stem (ES) cells by exogenous gene transfer, we introduced the RX/rax transcription factor, which is expressed in immature retinal cells, into feeder-free mouse ES cells, CCE. CCE cells expressing RX/rax as well as EGFP (CCE-RX/E cells) proliferated and remained in the undifferentiated state in the presence of leukemia inhibitory factor (LIF), as did parental ES cells. We made use of mouse embryo retinal explant cultures to address the differentiation ability of grafted ES cells. Dissociated embryo bodies were treated with retinoic acid for use as donor cells and co-cultured with retina explants for 2 weeks. In contrast to the parental CCE cells, which could not migrate into host retinal cultures, CCE-RX/E cells migrated

into the host retina and extended their process-like structures between the host retinal cells. Most of the grafted CCE-RX/E cells became located in the ganglion cell and inner plexiform layers and expressed ganglion and horizontal cell markers. Furthermore, these grafted cells had electrophysiological properties expected of ganglion cells. Our data thus suggest that subpopulations of retinal neurons can be generated in retinal explant cultures from the grafted mouse ES cell expressing ectopically the transcription factor Rx/rax.

We are currently extending these studies and have started new projects on adult neurogenesis.

7. The role of the AP-3 clathrin adaptor in the release of neurotransmitters

Noriko Kumazawa, Fubito Nakatsu[10], Arata Takeuchi[10], Takashi Saito[10], Hiroshi Ohno[10], Motohiro Okada[11], Hiroto Iwasa[11], Gang Zhu[11], Sunao Kaneko[11], Fumiaki Mori[12], Koichi Wakabayashi[12], Yasufumi Kasagi[13], Haruyuki Kamiya[1], Akihiro Harada[14], Kazuhiro Nishimura[15], Taisuke Miyazaki[7], Masahiko Watanabe[7], Shigeki Yuasa[16], and Toshiya Manabe: [10]RIKEN Research Center for Allergy and Immunology, [11]Department of Neuropsychiatry, Hirosaki University School of Medicine, [12]Department of Neuropathology, Institute of Brain Science, Hirosaki University School of Medicine, [13]Departments of Integrative Neurophysiology, Chiba University Graduate School of Medicine, [14]Laboratory of Cellular and Molecular Morphology, Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, [15]Department of Clinical Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, and [16]Department of Ultrastructural Research, National Institute of Neuroscience

AP-3 is a member of the adaptor protein (AP) complex family that regulates the vesicular transport of cargo proteins in the secretory and endocytic pathways. There are two isoforms of AP-3: the ubiquitously expressed AP-3A and the neuron-specific AP-3B. Although the physiological role of AP-3A has recently been elucidated, that of AP-3B remains unsolved. To address this question, we generated mice lacking μ3B, a subunit of AP-3B. $\mu3B^{-/-}$ mice suffered from spontaneous epileptic seizures. Morphological abnormalities were observed at synapses in these mice. Biochemical studies demonstrated the impairment of γ-aminobutyric acid (GABA) release because of, at least in part, the reduction of vesicular GABA transporter in $\mu3B^{-/-}$ mice. This facilitated the induction of long-term potentiation in the hippocampus and the abnormal propagation of neuronal excitability via the temporoammonic pathway. Thus, AP-3B plays a critical role in the normal formation and function of a subset of synaptic vesicles. This work adds a new aspect to the pathogenesis of epilepsy.

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Department of Basic Medical Sciences

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

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

教 授 医学博士 片 山 榮 作

Electron microscopy provides a useful and unique means to investigate the structure of biological materials including cells/tissues and purified macromolecules. If the specimens are properly prepared, we can preserve the instantaneous structure of functioning molecules not only in solution but also in live cells, and visualize their details with high contrast. Though the spatial resolution may not compete with X-ray crystallography, the real superior feature of the method is its almost unlimited applicability to those whose structure cannot otherwise be pursued. Our research activities are directed to two ways; one, development of new experimental tools, computer programs for the image analyses and several microscopic probes dedicated for our purpose to specifically label intracellular target proteins of interest, both to realize "Structural Biology of Single Molecules", and the other, their application to actual biological materials including the functioning molecules in situ in live cells.

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

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

We have been investigating the three-dimensional (3-D) architecture of various macromolecular assemblies that might play crucial roles in a number of cell motility and intracellular signal-transduction systems. Since a unique experimental approach, "single molecule physiology" was introduced to the field of molecular motor research about 20 years ago, it completely innovated the conventional knowledge on the intrinsic properties of various motor proteins and their interactions. The most important message from this new concept is that the behavior of individual protein molecules might be different from that of the others and that important information could be revealed by observation and separately measuring unaveraged properties of each single molecule. Thus, various experimental techniques to deal single molecules have already been established and used as the most powerful and indispensable tools in current biophysical sciences. On the other hand, conventional means of structural biology; *i.e.* X-ray crystallography or multi-dimensional NMR analysis, collects the data from a vast number of particles to be averaged both in time and space, and apparently is not compatible to "single molecule" matter. Electron microscopy is unique in terms that it has a potential to visualize the structure of individual macromolecules. In order to obtain the structural information of functioning actomyosin motor comparable to the results of single molecule physiology, we have been utilizing quick-freeze deep-etch replica electron microscopy with mica-flake technique to capture transient 3-D configuration of myosin cross-bridges supporting actin movement *in vitro*. In this technique, various molecular events exten-

sively studied under fluorescence microscope can be instantaneously arrested within one millisecond and the structure of individual protein molecules under well-characterized experimental conditions might be clearly visualized with a resolution that enables us to recognize subdomain arrangement of "individual" protein molecules, by high contrast metal shadowing. Since replica specimens are extremely tolerant to high-dose electron beam irradiation, it should be possible to take many micrographs of the same field, and to reconstruct the 3-D constitution of individual protein particles by a tomography technique, if "missing data-range problem" can be adequately avoided. As a matter of fact, this problem has been the biggest obstacle that hampered high-resolution 3-D electron microscopy of biological material by now. We devised a new method to overcome the problem (patent pending) and applied it successfully to obtain 3-D image of a single protein molecule from tilt-series micrographs. As a complementary approach to characterize the 3-D structure of the target particles, we have been attempting to simulate the replica images of protein molecules from their atomic coordinates. Since the image contrast of replica specimen arises by heavy metal shadowing, virtual model with the shape of protein particle was placed in cyber-space, and its image was rendered by ray-tracing computer software, as if it were illuminated by a number of surrounding light-sources from the appropriate elevation angles (patent pending). Until last year, we determined the best-matched face of the model by observer's visual inspection of the movable model images. We now devised a computer program to automatically extract the outer shape and characteristic feature patterns of the protein surface from real replica image and artificial model-image, then to compare two kinds of images by cross-correlation and finally find the best-matched face from thousands of model-images in various configurations and viewed from various directions. By that automated processing, we could objectively and reliably determine the orientation of given particles picked up from the replica images. Hence, we applied such strategies to examine 3-D structural features of myosin heads under a variety of conditions; free in solution with various bound nucleotides, and/or associated with actin filament. The question to be answered is whether "swinging of myosin lever-arm" actually occurs during actomyosin sliding movement. If so, how the intra- and/or intermolecular structural change is reflected to the sliding of actin filament. In order to examine that issue by the new analytical process, we examined, at first, the validity of the new program using three kinds of myosin head configurations as test samples; 1) rigor-form, 2) ADP/vanadate-form (Vi-form), which were well-characterized by X-ray crystallography, plus 3) new reversely-kinked configuration (pPDM-form) which we found last year in myosin head whose two reactive SH-groups were chemically cross-linked by bifunctional reagents. Since replica image for each configuration matched very nicely to respective simulated image, we compared actin-associated myosin configuration during sliding movement, with three kinds of structure as above and reconfirmed the last years conclusion that the lever-arm moiety of myosin head is not kinked as postulated in conventional "tilting lever-arm hypothesis" but is kinked rather oppositely. One prominent feature of such new configuration upon binding to actin is that the plane including upper- and lower-50K subdomains is parallel to the axis of actin, though the orientations of HMM particles were variable within that plane, as if heads were rocking within that plane. On the other hand, the same plane was almost perpendicular to actin under rigor condition. In order for pre-power stroke to eventually convert into rigor configuration, myosin motor-domain must somehow rotate around its main axis. Thus, we searched for the image of HMM particles that represents the configuration in between above two structures. Among many images of myosin heads supporting sliding actin-filament, we could pick up several particles taking the novel configuration as above, but the overall orientation is different from the others. There, the main-body of the motor domain was attached to actin only through the tip of the upper-50K domain, and has already rotated to give the appearance close to rigor, though the lever-arm moiety was still kinked to the opposite direction of Vi-form. Now, it is almost certain that lever-arm swinging takes place during sliding, but in quite an unexpected manner. The crossbridge in that configuration may extend to make strong rigor-binding, probably by a successive release of inorganic phosphate and ADP. From purely structural view, there are at least two candidate processes that may produce physiological active tension; one, the rocking motion among structures (in pPDM-form) observed very often during sliding, and the other the conversion from kinked pPDM-form to extended rigor-form. We have not yet any experimental evidences to decide which of them is crucial for tension development. In collaboration with Prof. Yanagida's team (Osaka Unv.), we are under way to determine which is the most important step responsible to the development of muscle tension. We expect that our approach "Structural biology of

Single Molecules" would find fruitful future applications in the other field and materials.

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

Single molecule imaging of G protein-coupled receptors in living cells

T. Tojo: In collaboration with Prof. T. Funatsu, Graduate School of Pharmaceutical Sciences

GPCRs (G Protein-Coupled Receptors) are thought to be the targets for 50～70% of the medicines for clinical treatments. GPCRs play critical rolls on maintenance of homeostasis as receptors for neurotransmitters, hormones, chemokines, etc.. GPCRs used to be considered to exist as a monomer on plasma membrane. In the last few years, however, increasing evidence using classical biochemical analyses implied that GPCRs form homo- and hetero- dimmers or oligomers. Even though classical biochemical analyses, such as SDS-PAGE, immunoprecipitation, etc., suggest the existence of GPCR oligomers, the number of molecules in each oligomer at the surface of living cells remains to be elucidated. The mechanism by which GPCR oligomerization participates in the activation of cellular responses by ligand stimuli is also unclear. Elucidation of the mechanism will contribute to pathological analyses and development of new medicines. For these purposes, using single molecule imaging techniques, FPR1, one of the chemoattractant receptors in the GPCR family, fused with EGFP at the C terminus of FPR1(FPR 1-EGFP) was expressed in CHO cells and observed using an objective type total internal reflection fluorescence microscope. Distributions of the fluorescent intensity of individual fluorescent spots indicated that the major population of FPR1-EGFP molecules are oligomers of various sizes. We confirmed that the oligomer formation of FPR1-EGFP is not attributable to the co-expression with EGFP. Hence, we conclude that FPR1 constitutively forms oligomers on the plasma membrane of a living cell and the oligomer formation of FPR1 is independent of ligand binding.

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Department of Basic Medical Sciences

Division of Structural Biology (2)
分子構造解析分野(2)

Research Associate Hiroshi Sagara, Ph.D.

助 手 医学博士 相 良 洋

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

1. Cell and developmental biology of the visual system

a. Vitamin A metabolism in vertebrates and invertebrates

Hiroshi Sagara and Emiko Suzuki[1]: [1]Gene Network Laboratory, Structural Biology Center, National Institute of Genetics, Research Organization of Information and Systems, SOKENDAI

RPE65 protein, which we previously identified in chick retinal pigment epithelial (RPE) cells, has high homology with beta-carotene dioxygenase in their amino-acid sequences. The beta-carotene dioxygenase is the enzyme that oxidatively cleaves the carbon double bond at the 15,15′ position of beta-carotene to yield two molecules of retinal (vitamin A). The amino-acid sequence of the RPE65 shows striking homology throughout vertebrates, more than 90% amino acid identities from fish to human, suggesting that this protein has essential roles in vitamin A metabolism in the eye. Although many researchers have studied this protein, its precise function in vitamin A metabolism is still obscure. For the purpose of clarifying this issue, we studied the homologue of RPE65 in Drosophila. The whole genome sequence of which has been clarified and the various molecular tools can be applicable. We found that Drosophila has only one homologue of the RPE65, DRPE65 with about 40 % identity in their amino-acid sequences. Sequence analysis indicated that DRPE65 protein is encoded by *ninaB*, one of the phototransduction genes. Recently, von Linting and Vogt showed that this protein has beta-carotene dioxygenase activity. When analyzed the localization of DRPE65 by immunohistochemistry, we found that it existed in eyes and a limited part of the digestive tracts. In eyes, it was localized to the cone cells that are thought to contain vitamin A metabolizing enzymes for visual cycle. In the digestive tract, the basal region of the epithelial cells in the oesophagus was immunopositive. These results indicate that in vertebrates, the RPE65 and the beta-carotene dioxygenase have distinct function in the eye and digestive tract respectively, but in Drosophila, the same molecule of DRPE65 has diverse functions not only in the visual cycle but also in vitamin A metabolism in the digestive system.

b. Development of the photoreceptor outer segments in vertebrates

Hiroshi Sagara, Yoko Tabata[2] and Sumiko

Watanabe[2]: [2]Division of Molecular and Developmental Biology, Department of Basic Medical Sciences

Photoreceptor cell outer segment consists of a stack of photo-receptive membrane discs, and is connected to the inner segment by a thin connecting cilium. Light energy is converted to electrical signals via the phototransduction cascade in the outer segment. During the morphogenetic process of the outer segment, the membrane discs are formed by the invagination of the plasma membrane, the constituents of which are synthesized in the inner segment. When the developing mouse retina is dissected out and cultured on the porous membrane, it has been known to develop into the normally layered retina. When the retinal explant culture was performed without retinal pigment epithelial (RPE) cells, even the cells in the outer nuclear layer formed well developed inner segments and the basal rootlet indicative of the connecting cilium, the outer segments were never formed in these cells. When they were cultured in the presence of RPE cells, although not so regularly arranged, some photoreceptor cells did formed the outer segments. This indicates that the outer segment promoting factor(s) are present on the surface of the RPE cells or that the factor(s) are secreted into the sub-retinal space by the RPE cells. Molecular nature of the factor(s) inducing the formation of outer segments are now under investigation.

2. Other collaborative research works

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

Takeshi Noda[3], Hiroshi Sagara and Yoshihiro Kawaoka[3]: [3]Division of Virology, Department of Microbiology and Immunology

b. Scanning electron microscopic analysis of the tooth epithelial cells cultured on the artificial scaffold.

Masaki Honda[4] and Hiroshi Sagara: [4]Division of Stem Cell Engineering (Tooth Regeneration)

c. Molecular and fine morphological analysis of the mechanisms involved in the bacterial infection processes.

Chihiro Sasakawa[5], Michinaga Ogawa[5], Tomoko Morita[5], Yosuke Iizumi[5] and Hiroshi Sagara: [5]Division of Bacterial Infection, Department of Microbiology and Immunology

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Department op Basic Medical Sciences

Division of Molecular Neurobiology (1)
脳神経発生・分化分野(1)

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Associate Professor	Takafumi Inoue, M.D., Ph.D.	助教授	医学博士	井　上　貴　文
Research Associate	Takayuki Michikawa, Ph.D.	助　手	医学博士	道　川　貴　章

Our research interest lies on molecular mechanisms of inositol polyphosphate/Ca^{2+} signaling and neurobiological activities. We have been studying on the structure and function of genes and their products involved in intracellular Ca^{2+} dynamics and neuronal development and plasticity.

1. Structure and function of IP_3 receptors

Takayuki Michikawa, Kozo Hamada,[2] Haruka Yamazaki,[2] Ray Yokoyama, Keiko Uchida, Toru Matsu-ura, Hideki Nakamura, Tadashi Shinohara, Miwako Iwai, Akiko Terauchi,[2] Ivan Bosanac,[3] Mitsuhiko Ikura,[3] Chikara Sato,[4] Toshihiko Ogura,[4] Atsuo Miyazawa,[5] Kenji Iwasaki,[6] Yoko Hiroaki,[7] Kazutoshi Tani,[7] Yoshinori Fujiyoshi[5,7,8] Mitsuharu Hattori,[9] and Katsuhiko Mikoshiba: [1]Laboratory for Developmental Neurobiology, Brain Science Institute, RIKEN; [2]Calcium Oscillation Project, ICORP, JST; [3]Division of Molecular and Structural Biology, University of Toronto; [4] Neuroscience Research Institute, BIRC, AIST; [5] RIKEN Harima Institute; [6]Research Center for Ultra-High Voltage Electron Microscopy, Osaka University; [7]Department of Biophysics, Faculty of Science, Kyoto University; [8]Japan Biological Information Research Centre; [9]Department of Biomedical Science, Graduate School of Pharmaceutical Sciences, Nagoya City University

We have been working on a protein (P_{400}) of which expression increases during development but is greatly decreased in the cerebellar mutant mice where Purkinje cells are deficient or spins of Purkinje cells are absent. We discovered that this developmentally regulated P_{400} protein is an inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R)/Ca^{2+} release channel. IP_3 is a second messenger produced through the phosphoinositide turnover in response to many extracellular stimuli (hormones, growth factors, neurotransmitters, neutrophines, odorants, light, etc.), and controls a variety of Ca^{2+}-dependent cell functions (cell proliferation, differentiation, fertilization, embryonic development, secretion, muscular contraction, immune responses, brain functions, chemical sense, light transduction, etc.) by inducing Ca^{2+} release from intracellular Ca^{2+} store sites, such as endoplasmic reticulum (ER). IP_3R is an IP_3-gated Ca^{2+} release channel and could be considered as a signal converter that exchanges IP_3 signals into Ca^{2+} signals. Our goal is to elucidate the structure-function relationship of the IP_3R and the physiological roles of IP_3R-mediated Ca^{2+} signaling in various cell-types.

We cloned three types of human and mouse IP_3R (IP_3R1, IP_3R2, and IP_3R3) and have analyzed the structure and function of each type of IP_3R by means of molecular biological, biochemical,

cell biological, physiological and histochemical approaches. We found that each type has different IP_3 binding (e.g., affinity, specificity, Ca^{2+} sensitivity) and modulation (e.g., phosphorylation, calmodulin binding) properties. The IP_3R is a polypeptide (~2,700 amino acids) with five major functionally distinct domains: (1) the amino-terminal IP_3-binding suppressor/coupling domain, (2) the IP_3-binding domain, (3) the central modulatory/coupling domain, (4) the channel-forming domain, and (5) the carboxy-terminal gate-keeper domain. Four IP_3R subunits assemble to form a functional IP_3-gated Ca^{2+} release channel and both homo- and hetero-tetrameric channels are detected. IP_3R possesses six transmembrane segments, suggesting that IP_3 R shares a basic design of the channel-forming domain with the voltage-gated and second messenger-gated ion channels on the plasma membrane. We analyzed the folding structure of the IP_3R channel by limited trypsin digestion and found that the IP_3R channel is an assembly of four subunits, each of which is constituted by non-covalent interactions of five major, well folded structural components. The IP_3-binding core, a minimum essential region for specific IP_3-binding, resides among residues 226-578 of mouse IP_3R1. The amino-terminal 220 residues directly preceding the IP_3-binding core domain play a key role in IP_3 binding suppression and partner protein interaction. Recently, we uncovered crystal structures of both the IP_3-binding core in complex with IP_3 and the suppressor domain of mouse IP_3R1 at 2.2 Å and 1.8 Å resolutions, respectively. The IP_3-binding core forms the asymmetric boomerang-like structure consists of an amino-terminal β-trefoil domain and a carboxy-terminal α-herical domain containing an armadillo repeat-like fold. The cleft formed by the two domains exposes a cluster of arginine and lysine residues that coordinate the three phosphoryl groups of IP_3. Displaying a shape akin to a hammer, the suppressor region contains a "head" subdomain forming the β-trefoil fold and an "arm" subdomain possessing a helix-turn-helix structure that protrudes from the globular head subdomain. Site-directed mutagenesis studies provide evidence for the involvement of a large conserved surface area on the head subdomain in the suppression of IP_3-binding to the IP_3-binding core domain. This conserved region is in close proximity to the previously proposed binding sites of Homer, RACK1, calmodulin, and CaBP1.

Ca^{2+} signaling often exhibits dynamic changes in time and space inside a cell (known as Ca^{2+} waves and Ca^{2+} oscillations). These complex spatiotemporal patterns are not produced by simple diffusion of cytoplasmic Ca^{2+}. The essential ingredients to generate repetitive Ca^{2+} spikes are positive feedback, cooperativity, deactivation (including negative feedback) and reactivation. The IP_3R1 is regulated by cytoplasmic Ca^{2+} in a biphasic manner with a maximal channel activity at 200-500 nM cytoplasmic Ca^{2+}. We found that the positive feedback regulation by cytoplasmic Ca^{2+} is an intrinsic property of the IP_3R 1, whereas the negative feedback regulation by Ca^{2+} is mediated by calmodulin, a ubiquitous and multifunctional Ca^{2+}-dependent regulator protein. Ca^{2+} induces marked structural changes in the tetrameric IP_3R1 purified from mouse cerebella. Electron microscopy of the IP_3R1 particles revealed two distinct structures with 4-fold symmetry: a windmill-like structure and a mushroom-like structure. Ca^{2+} reversibly promotes a transition from the mushroom-like structure to the windmill-like structure with relocations of four peripheral IP_3-binding domains. The effective concentration of Ca^{2+} for conformational changes in IP_3R1 is<100 nM. These data suggest that the Ca^{2+}-specific conformational change structurally regulates the IP_3-gated channel opening within IP_3R.

We analyzed the three-dimensional structure of the ligand free form of IP_3R1 purified from mouse cerebella based on single particle technique using an originally developed electron microscope equipped with a helium-cooled specimen stage and an automatic particle picking system. The shape of the density map obtained at 15 Å resolution is reminiscent of a hot air balloon, with the spherical cytoplasmic domain (diameter of 175 Å) representing the balloon and the square-shaped luminal domain (side length of 96 Å) representing the basket. The structure of the density map consists of two layers. The outer hot air balloon-shaped shell forms many holes and cavities, while the inner shell is composed of a continuous square-shaped tubular density. The inner tubular density is slightly rotated in consecutive sections, revealing the inner tubule to be twisted. There is a prominent vacant space over the inner tubular density. The double-layered structure is shared between IP_3R and the voltage-gated Na^+ channels.

2. Functional difference among three IP_3R types

Takayuki Michikawa, Mistuharu Hattori,[9] Takafumi Inoue, Takeshi Nakamura,[2] Akira Futatsugi,[2] Akinobu Z. Suzuki, Takayasu Higo, Miwako Iwai, and Katsuhiko Mikoshiba

We evaluated the IP_3 binding properties of all three types of recombinant mouse IP_3R expressed in Sf9 cells and found that each type of

receptor binds IP_3 in a different manner. The intrinsic association constants of IP_3R1, IP_3R2, and IP_3R3 are estimated to be 3.5_10^7, 1.7_10^8, and 3.4_10^6 (M^{-1}), respectively. In addition, we found that IP_3 binding to tetrameric IP_3R2 and IP_3R3 channels is not a random process. Nonlinear regression of the IP_3 binding data with the four-IP_3 binding site model showed that IP_3R2 exhibits both negative cooperativity and positive cooperativity, whereas IP_3R3 exhibits negative IP_3 binding cooperativity. This diversity of responsiveness to IP_3 observed among the three IP_3R types may contribute to the generation of the different degrees of IP_3 sensitivity of Ca^{2+} stores in mammalian cells.

Three subtypes of IP_3R share basic properties but differ in terms of regulation. To what extent they contribute to complex Ca^{2+} signaling, such as Ca^{2+} oscillations, remains largely unknown. We found that HeLa cells express comparable amounts of IP_3R1 and IP_3R3, but knockdown by RNA interference of each subtype results in dramatically distinct Ca^{2+} signaling patterns. Knockdown of IP_3R1 significantly decreases total Ca^{2+} signals and terminates Ca^{2+} oscillations. Conversely, knockdown of IP_3R3 leads to more robust and long lasting Ca^{2+} oscillations than in controls. Effects of IP_3R3 knockdown are surprisingly similar in COS-7 cells that predominantly (>90% of total IP_3R) express IP_3R3, suggesting that IP_3R3 functions as an anti-Ca^{2+}-oscillatory unit without contributing to peak amplitude of Ca^{2+} signals, irrespective of its relative expression level. Therefore, differential expression of the IP_3R subtype is critical for various forms of Ca^{2+} signaling, and, particularly, IP_3R1 and IP_3R 3 have opposite roles in generating Ca^{2+} oscillations.

3. IP_3R dynamics in the ER membrane

A) Lateral diffusion of IP_3R in the ER membrane

Takafumi Inoue, Hiroko Bannai,[1] Kazumi Fukatsu, Songbai Zhang,[2] Hideki Nakamura, and Katsuhiko Mikoshiba

We expressed green fluorescent protein (GFP)-tagged IP_3R1 in cultured rat hippocampal neurons and observed the lateral diffusion by the fluorescence recovery after photobleaching (FRAP) technique. IP_3R1 showed lateral diffusion with an effective diffusion constant of ~0.3 $\mu m^2/s$. Depletion of actin filaments increased the diffusion constant of IP_3R1, suggesting that the diffusion of IP_3R1 is regulated negatively through actin filaments. We also found that protein 4.1N, which binds to IP_3R1 and contains an actin-spectrin binding region, was responsible for this actin regulation of IP_3R1 diffusion constant. Overexpression of dominant-negative 4.1N and blockade of 4.1N binding to IP_3R1 increased the IP_3R1 diffusion constant. The diffusion of IP_3R3, one of the isoforms of IP_3Rs lacking the binding ability to 4.1N, was not dependent on actin filaments, but became to be dependent on actin filaments after the addition of a 4.1N binding sequence. These data suggest that 4.1N serves as a linker protein between IP_3R1 and actin filaments. This actin filament-dependent regulation of IP_3R1 diffusion may be important for the spatiotemporal regulation of intracellular Ca^{2+} signaling.

Although spatially restricted Ca^{2+} release from the ER through intracellular Ca^{2+} channels plays important roles in various neuronal activities, the accurate distribution and dynamics of ER in the dendrite of living neurons still remain unknown. To elucidate these, we expressed fluorescent protein-tagged ER proteins in cultured mouse hippocampal neurons, and monitored their movements using time-lapse microscopy. We found that a sub-compartment of ER forms in relatively large vesicles that are capable, similarly to the reticular ER, of taking up and releasing Ca^{2+}. The vesicular sub-compartment of ER moved rapidly along the dendrites in both anterograde and retrograde directions at a velocity of 0.2-0.3 μm/second. Depletion of microtubules, overexpression of dominant-negative kinesin and kinesin depletion by antisense DNA reduced the number and velocity of the moving vesicles, suggesting that kinesin may drive the transport of the vesicular sub-compartment of ER along microtubules in the dendrite. Rapid transport of the Ca^{2+} releasable sub-compartment of ER might contribute to rapid supply of fresh ER proteins to the distal part of the dendrite, or to the spatial regulation of intracellular Ca^{2+} signaling.

B) IP_3R clustering in the ER membrane

Takayuki Michikawa, Mitsuharu Hattori,[9] Hiroko Bannai,[1] Takeshi Nakamura,[2] Yoko Tateishi, Miwako Iwai, Takafumi Inoue, and Katsuhiko Mikoshiba

It was reported that IP_3R forms clusters on the ER when cytoplasmic Ca^{2+} concentrations is elevated. However, molecular mechanism of IP_3R clustering remains largely unknown. We found that the time course of clustering of green fluorescent protein-tagged IP_3R1 (GFP-IP_3R1), evoked by IP_3-generating agonists, did not correlate with cytoplasmic Ca^{2+} concentration but seemed compatible with cytoplasmic IP_3 concentration.

IP_3 production alone induced GFP- IP_3R1 clustering in the absence of a significant increase in Ca^{2+} concentration, but elevated Ca^{2+} concentration without IP_3 production did not. IP_3R1 mutants that do not undergo an IP_3-induced conformational change failed to form clusters. Thus, IP_3R clustering is induced by its IP_3-induced conformational change to the open state.

Stimulation with ATP or Ca^{2+} ionophore induced cluster formation by all three types of recombinant mouse IP_3R expressed in COS-7 cells. We found that the size and shape of stimulus-induced clusters differ among the three types of IP_3R, and IP_3R2 forms clusters even in resting cell. The average length of the major axis of the IP_3R1 clusters observed after 1 mM ATP stimulation was 2.3 μm (n=342). The average length of the IP_3R2 clusters in resting cells and stimulated cells was 0.34 (n=173) and 0.35 μm (n=177), respectively. After treatment with 1 mM ATP for 20 min, the size of mouse IP_3R3 clusters averaged 1.5 μm (n=251). When cells were treated with 1 μM Br-A23187, the average size of the IP_3R2 clusters and IP_3R3 clusters was 0.47 μm (n=111) and 2.3 μm (n=238), respectively. The difference may be attributable to the intrinsic nature of each type of IP_3R.

We found a novel alternative splicing segment, SI_{m2}, at 176-208 of IP_3R2. The long form (IP_3R2 SI^{+}_{m2}) was dominant, but the short form (IP_3R2 SI^{-}_{m2}) was detected in all tissues examined. IP_3R2 SI^{-}_{m2} has neither IP_3 binding activity nor Ca^{2+} releasing activity. IP_3R2 SI^{-}_{m2} does not form clusters in either resting or stimulated cells. Coexpression of IP_3R2 SI^{-}_{m2} prevents stimulus-induced IP_3R clustering, suggesting that IP_3R 2 SI^{-}_{m2} functions as a negative coordinator of stimulus-induced IP_3R clustering. Expression of IP_3R2 SI^{-}_{m2} in CHO-K1 cells significantly reduced ATP-induced Ca^{2+} entry, but not Ca^{2+} release, suggesting that the novel splice variant of IP_3R2 specifically influences the dynamics of the sustained phase of Ca^{2+} signals.

4. Identification and characterization of IP_3R binding proteins

A) IRBIT

Akihiro Mizutani,[2] Hideaki Ando,[1] Dai Tsuzurugi, Toru Matsu-ura, and Katsuhiko Mikoshiba

We found a novel protein, termed IRBIT (IP_3R binding protein released with inositol 1,4,5-trisphosphate), which interacts with IP_3R1 and was released upon IP_3 binding to IP_3R1. IRBIT was purified from a high salt extract of crude rat brain microsomes with IP_3 elution using an affinity column with the huge immobilized N-terminal cytoplasmic region of IP_3R1 (residues 1-2217). IRBIT, consisting of 530 amino acids, has a domain homologous to S-adenosylhomocysteine hydrolase in the C-terminal and in the N-terminal, a 104 amino acid appendage containing multiple potential phosphorylation sites. *In vitro* binding experiments showed the N-terminal region of IRBIT to be essential for interaction, and the IRBIT binding region of IP_3R1 was mapped to the IP_3 binding core. IP_3 dissociated IRBIT from IP3R1 with an EC_{50} of ~0.5 μM, i.e. it was 50 times more potent than other inositol polyphosphates. Moreover, alkaline phosphatase treatment abolished the interaction, suggesting that the interaction was dualistically regulated by IP_3 and phosphorylation. Immunohistochemical studies and co-immunoprecipitation assays showed the relevance of the interaction in a physiological context. These results suggest that IRBIT is released from activated IP_3 R, raising the possibility that IRBIT acts as a signaling molecule downstream from IP_3R.

B) 4.1N

Akihiro Mizutani,[2] Songbai Zhang,[2] Chihiro Hisatsune,[1] Takayasu Higo, Hiroko Bannai,[1] Tomohiro Nakayama, Mitsuharu Hattori,[9] Kazumi Fukatsu, Ayuko Kurokura, Takafumi Inoue, and Katsuhiko Mikoshiba

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

C) ERp44

Takayasu Higo, Mitsuharu Hattori,[9] Takeshi Nakamura,[2] Tohru Natsume,[10] Takayuki Michikawa, and Katsuhiko Mikoshiba: [10]Protein Network Team, Functional Genome Group, Japan Biological Information Research Center

It has been known that IP_3Rs are differentially regulated by a variety of cytosolic proteins, but their regulation by ER lumenal protein(s) remains largely unexplored. We found that ERp 44, an ER lumenal protein of the thioredoxin family, directly interacts with the third lumenal loop of IP_3R1 and that the interaction is dependent on pH, Ca^{2+} concentration, and redox state: the presence of free cysteine residues in the loop is required. Ca^{2+} imaging experiments and single-channel recording of IP_3R1 activity with a planar lipid bilayer system demonstrated that IP_3R1 is directly inhibited by ERp44. Thus, ERp 44 senses the environment in the ER lumen and modulates IP_3R1 activity accordingly, which should in turn contribute to regulating both intralumenal conditions and the complex patterns of cytosolic Ca^{2+} concentrations.

D) Na, K-ATPase

Ayako Miyakawa-Naitou,[11] Per Uhlen,[11] Mark Lal,[11] Oleg Aizman,[11] Katsuhiko Mikoshiba, Hjalmar Brismar,[11] Sergey Zelenin,[11] and Anita Aperia[11]: [11]Department of Woman and Children Health, Karolinska Institute

Recent studies indicate novel roles for the ubiquitous ion pump, Na,K-ATPase, in addition to its function as a key regulator of intracellular sodium and potassium concentration. We have previously demonstrated that ouabain, the endogenous ligand of Na,K-ATPase, can trigger intracellular Ca^{2+} oscillations, a versatile intracellular signal controlling a diverse range of cellular processes. Here we report that Na,K-ATPase and IP_3R form a cell signaling microdomain that, in the presence of ouabain, generates slow Ca^{2+} oscillations in renal cells. Using fluorescent resonance energy transfer (FRET) measurements, we detected a close spatial proximity between Na,K -ATPase and IP_3R. Ouabain significantly enhanced FRET between Na,K-ATPase and IP_3R. The FRET effect and ouabain-induced Ca^{2+} oscillations were not observed following disruption of the actin cytoskeleton. Partial truncation of the NH2 terminus of Na,K-ATPase catalytic 1-subunit abolished Ca^{2+} oscillations and downstream activation of NF-B. Ouabain-induced Ca^{2+} oscillations occurred in cells expressing an IP_3 sponge and were hence independent of IP_3 generation. Thus, we present a novel principle for a cell signaling microdomain where an ion pump serves as a receptor.

5. Regulation of IP_3R expression

A) SYNCRIP (heterogeneous nuclear ribonuclear protein Q1/NSAP1) is a component of mRNA granule transported with IP_3R1 mRNA in neuronal dendrites

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Recently we cloned an RNA-interacting protein, SYNCRIP (heterogeneous nuclear ribonuclear protein Q1/NSAP1), that is suggested to be important for the stabilization of mRNA. We found that SYNCRIP is a component of mRNA granules in rat hippocampal neurons. SYNCRIP was mainly found at cell bodies, but punctate expression patterns in the proximal dendrite were also seen. Time-lapse analysis in living neurons revealed that the granules labeled with fluorescent protein-tagged SYNCRIP were transported bi-directionally within the dendrite at 0.05 μm/s. Treatment of neurons with nocodazole significantly inhibited the movement of green fluorescent protein-SYNCRIP-positive granules, indicating that the transport of SYNCRIP containing granules is dependent on microtubules. The distribution of SYNCRIP- containing granules overlapped with that of dendritic RNAs and elongation factor 1. SYNCRIP was also found to be co-transported with green fluorescent protein- tagged human staufen1 and the 3 -untranslated region of IP_3R1 mRNA. These results suggest that SYNCRIP is transported within the dendrite as a component of mRNA granules and raise the possibility that mRNA turnover in mRNA granules and the regulation of local protein synthesis in neuronal dendrites may involve SYNCRIP.

B) Activity-dependent expression of IP_3R1 in hippocampal neurons

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There are several lines of evidence showing that synaptic activity regulates the level of expression of IP_3R1 in neurons. We examined the effect of chronic activity blockade on the localization and level of IP_3R1 expression in cultured hippocampal neurons. We found that chronic

blockade of NMDA receptors (NMDARs), one of the major Ca^{2+} permeable ion channels, increased the number of neurons that express a high level of IP_3R1 without any apparent changes in its intracellular localization. Interestingly, this up-regulation was time-dependent; there was no clear change in IP_3R1 expression level up to day 5 of the NMDAR blockade, but expression increased at day 6, and the increased expression level persisted for at least a week. The up-regulation of IP_3R1 depended on transcription and protein synthesis and required cAMP-dependent protein kinase activity. Moreover, although most of the control neurons did not respond to the metabotropic glutamate receptor (mGluR) stimulation, the 2-amino-5-phosphonopentanoic acid-treated neurons with high IP_3R1 expression became sensitive to mGluR stimulation. Furthermore, we also found that hippocampal neurons transiently overexpressing green fluorescent protein-tagged IP_3R1 released Ca^{2+} in response to mGluR and muscarinic acetylcholine receptor stimulation. These findings suggested that chronic NMDAR blockade increased the IP_3R1 expression and enhanced sensitivity to mGluR stimulation. The change in IP_3R1 expression level in response to alteration of synaptic activity may be an important determinant of the sensitivity of Ca^{2+} stores to G-protein-coupled receptor stimulation and would help to maintain intracellular Ca^{2+} homeostasis in hippocampal neurons.

6. Ca^{2+} dynamics in cerebellar Purkinje cells

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Ca^{2+} and Na^+ play important roles in neurons, such as in synaptic plasticity. Their concentrations in neurons change dynamically in response to synaptic inputs, but their kinetics have not been compared directly. We investigated the mechanisms and dynamics of Ca^{2+} and Na^+ transients by simultaneous monitoring in Purkinje cell dendrites in mouse cerebellar slices. High frequency parallel fibre stimulation (50Hz, 3-50-times) depolarized Purkinje cells, and Ca^{2+} transients were observed at the anatomically expected sites. The magnitude of the Ca^{2+} transients increased linearly with increasing numbers of parallel fibre inputs. With 50 stimuli, Ca^{2+} transients lasted for seconds, and the peak $[Ca^{2+}]$ reached 100μM, which was much higher than that reported previously, although it was still confined to a part of the dendrite. In contrast, Na^+ transients were sustained for tens of seconds and diffused away from the stimulated site. Pharmacological interventions revealed that Na^+ influx through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (A MPA) receptors and Ca^{2+} influx through P-type Ca channels were essential players, that AMPA receptors did not operate as a Ca^{2+} influx pathway and that Ca^{2+} release from intracellular stores through IP_3Rs or ryanodine receptors did not contribute greatly to the large Ca^{2+} transients.

7. Regulation of TRPC channels

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Various hormonal stimuli and growth factors activate the mammalian canonical transient receptor potential (TRPC) channel through phospholipase C (PLC) activation. However, the precise mechanism of the regulation of TRPC channel activity remains unknown. We provide the first evidence that direct tyrosine phosphorylation by Src family protein-tyrosine kinases (PTKs) is a novel mechanism for modulating TRPC6 channel activity. We found that TRPC6 is tyrosine-phosphorylated in COS-7 cells when coexpressed with Fyn, a member of the Src family PTKs. We also found that Fyn interacts with TRPC6 and that the interaction is mediated by the SH2 domain of Fyn and the N-terminal region of TRPC6 in a phosphorylation-independent manner. In addition, we demonstrated the physical association of TRPC6 with Fyn in the mammalian brain. Moreover, we showed that stimulation of the epidermal growth factor receptor induced rapid tyrosine phosphorylation of TRPC6 in COS-7 cells. This epidermal growth factor-induced tyrosine phosphorylation of TRPC 6 was significantly blocked by PP2, a specific inhibitor of Src family PTKs, and by a dominant negative form of Fyn, suggesting that the direct phosphorylation of TRPC6 by Src family PTKs could be caused by physiological stimulation. Furthermore, using single channel recording, we showed that Fyn modulates TRPC6 channel activity via tyrosine phosphorylation. Thus, our findings demonstrated that tyrosine phosphorylation by Src family PTKs is a novel regulatory mechanism of TRPC6 channel activity.

8. Role of the M3 muscarinic acetylcholine receptor in parasympathetic control of salivation in mice

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The M1 and M3 subtypes are the major muscarinic acetylcholine receptors in the salivary gland and M3 is reported to be more abundant. However, despite initial reports of salivation abnormalities in M3-knockout (M3KO) mice, it is still unclear which subtype is functionally relevant in physiological salivation. In the present study, salivary secretory function was examined using mice lacking specific subtype(s) of muscarinic receptor. The carbachol-induced $[Ca^{2+}]_i$ increase was markedly impaired in submandibular gland cells from M3KO mice and completely absent in those from M1/M3KO mice. This demonstrates that M3 and M1 play major and minor roles, respectively, in the cholinergically induced $[Ca^{2+}]_i$ increase. Two-dimensional Ca^{2+} imaging analysis revealed the patchy distribution of M1 in submandibular gland acini, in contrast to the ubiquitous distribution of M3. *In vivo* administration of a high dose of pilocarpine (10mg kg-1, S.C.) to M3KO mice caused salivation comparable to that in wild-type mice, while no salivation was induced in M1/M3KO mice, indicating that salivation in M3KO mice is caused by an M1-mediated $[Ca^{2+}]_i$ increase. In contrast, a lower dose of pilocarpine (1mg kg-1, S.C.) failed to induce salivation in M3KO mice, but induced abundant salivation in wild-type mice, indicating that M3-mediated salivation has a lower threshold than M1-mediated salivation. In addition, M3KO mice, but not M1KO mice, had difficulty in eating dry food, as shown by frequent drinking during feeding, suggesting that salivation during eating is mediated by M3 and that M1 plays no practical role in it. These results show that the M3 subtype is essential for parasympathetic control of salivation and a reasonable target for the drug treatment and gene therapy of xerostomia, including Sjögren's syndrome.

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

1. Full-length-enriched cDNA library of malaria parasites and Toxoplasma gondii

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Genome sequencing of parasitic protozoa and helminths is rapidly progressing. However, it has become evident that prediction of the exact structures of expressed genes is so difficult that less than a quarter of such structures are correctly predicted (see review). We have produced full-length-enriched cDNA libraries from the erythrocyte-stage parasites of the most important malaria parasite, *Plasmodium falciparum*. 5′ end-one-pass-sequencing of random clones provided important information which complements that obtained from genome sequencing projects. We have developed a mapping viewer that visualizes the sequences of cDNA clones on the determined genome. Furthermore, as a comparative biological approach, a full-length cDNA library of a murine malaria parasite, *Plasmodium yoelii*, was produced and sequenced. 5x draft genome sequences of *P. yoelii* and 5′ end sequences were aligned with the genome sequences of *P. falciparum*. Recently we have extended this database to *Toxoplasma gondii*, a close relative of malaria parasites that causes severe congenital disease in infants if a mother is infected during the early phase of pregnancy. A full-length cDNA library was produced from tachyzoites, and 5′-end-one-pass-sequences were mapped onto the genome sequences of *Toxoplasma gondii*.
The database "FULL-malaria" is now available at http://fullmal.ims.u-tokyo.ac.jp.

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

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Development of an effective malaria vaccine is imperative for the control of this disease. However, no really good vaccine candidate has been reported. Using a full-length cDNA library that was constructed using the RNA from erythrocyte

-stage parasites of lethal murine malaria, *P. berghei*, and an expression vector, we have started the screening of potential DNA vaccines in a murine malaria model. Immunization using pooled-vaccines caused significant prolongation of the survival after challenge infection, suggesting that this approach is promising. Efforts to identify the effective components and elucidation of effector mechanisms are underway.

3. Chaperone DnaJ homologues of malaria parasites

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DnaJ was first described by researchers at our institute as a gene that regulates phage replication in *E. coli*. In the genomic sequence of *Plasmodium falciparum*, 47 species of DnaJ homologues, which are characterized by a so-called J domain consisting of 70 conserved amino acids, have been identified. Though their ubiquitous existence in all organisms indicates the importance of these molecules, little is known about their functions. In addition, malaria parasites contain quite unique DnaJ homologues (RESA; ring-infected erythrocyte surface antigens). We have focused on Pfj2 (Pbj2 in murine malaria), which is localized in the ER and unique to Apicomplexa species. Pfj2 has a thioredoxin motif and is considered to function in protein folding in the ER. Gene knockout experiments suggest that Pfj2 is an essential gene. Systematic analyses will reveal the exact functions of these DnaJ homologues in parasitism.

4. Effects of cytosolic phospholipase A_2- and phospholipase A_2IIA enzyme in aggravation of severe malaria

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The mechanism of aggravation of malaria is still unknown. Based on the hypothesis that cytosolic phospholipase A_2- and phospholipase A_2 IIA enzyme are involved in this process, we infected mice deficient for these two enzymes with *Plasmodium berghei* ANKA, which causes severe malaria in CL57B/6 mice and leads to eventual death. Contrary to our expectation, the deficient mice died in a similar way as conventional mice. These observations suggest that these enzymes alone are not responsible for the aggravation of malaria.

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Review

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Department of Basic Medical Sciences

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

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

1. Translational Control and Protein-tRNA Molecular Mimicry

Koichi Ito, Miki Wada, Hanae Sato, Tomohiko Toyoda, Yuya Watanabe, Hiroyuki Kodama, Saori Murakami, Masayo Urata, Chunguang Kong[1], Martin A. Walsh[1], Yuying Liu[1], Sundramurthy Kumar[1], Haiwei Song[1], Zemfira N. Karamysheva[2], Andrey L. Karamyshev[2], and Yoshikazu Nakamura: [1]Laboratory of Macromolecular Structure, Institute of Molecular and Cell Biology, Singapore, and [2]Department of Biochemistry and Biophysics, Texas A&M University.

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. Crystal structure and functional analysis of the eukaryotic class II release factor eRF3 from *S. pombe*

Translation termination in eukaryotes is governed by two interacting release factors, eRF1 and eRF3. The crystal structure of the eEF1α-like region of eRF3 from *S. pombe* determined in three states (free protein, GDP- and GTP-bound forms) reveals an overall structure that is similar to EF-Tu, although with quite different domain arrangements. In contrast to EF-Tu, GDP/GTP binding to eRF3c does not induce dramatic conformational changes, and Mg^{2+} is not required

for GDP binding to eRF3c. Mg^{2+} at higher concentration accelerates GDP release, suggesting a novel mechanism for nucleotide exchange on eRF3 from that of other GTPases. Mapping sequence conservation onto the molecular surface, combined with mutagenesis analysis, identified the eRF1-binding region, and revealed an essential function for the C terminus of eRF3. The N-terminal extension, rich in acidic amino acids, blocks the proposed eRF1-binding site, potentially regulating eRF1 binding to eRF3 in a competitive manner.

b. Transient idling of posttermination ribosomes ready to reinitiate protein synthesis

The fate of ribosomes between termination and initiation during protein synthesis is very basic yet poorly understood. Here we found that translational reinitiation of the alkaline phosphatase gene occurs in *Escherichia coli* from an internal methionine codon when the authentic translation is prematurely terminated at a nonsense codon that is within seven codons upstream of the reinitiation codon (which we refer to as "reinitiation window"). Changing the reading frame downstream of the stop codon did not abolish the reinitiation, while inactivating the upstream initiation codon abolished the reinitiation. Moreover, depletion of the ribosome recycling factor, which disassembles posttermination ribosomes in conjunction with elongation factor G, did not influence the observed reinitiation. These findings suggest that posttermination ribosomes can undergo a transient idling state ready to reinitiate protein synthesis even in the absence of the Shine-Dalgarno sequence within the reinitiation window by evading disengagement from the mRNA.

2. Regulation of Ribosome Recycling

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

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

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

b. Heterologous expression of *A. aeolicus* ribosome recycling factor in *E. coli* is dominant lethal by forming a complex that lacks functional coordination for ribosome disassembly

Recycling the posttermination ribosomal complex requires the coordinated effort of the ribosome, RRF and elongation factor EF-G. Although *Aquifex aeolicus* RRF (aaRRF) binds *Escherichia coli* ribosomes as efficiently as *E. coli* RRF, the resulting complex is nonfunctional and dominant lethal in *E. coli*, even in the presence of homologous *A. aeolicus* EF-G. These findings suggest that the *E. coli* posttermination ribosomal complex with aaRRF lacks functional coordination with EF-G required for ribosome recycling. A chimeric EF-G (*E. coli* domains I-III, *A. aeolicus* domains IV/V) or an *A. aeolicus* EF-G with distinct mutations in the domain I-II interface could activate aaRRF. Furthermore, novel mutations that localize to one surface of the L-shape structure of aaRRF restored activity in *E. coli*. These aaRRF mutations are spatially distinct from mutations previously described and suggest a novel active center for coupling EF-G's G-domain motor action to ribosome disassembly.

3. Molecular Biology of Yeast Prions

Colin G. Crist, Hideyuki Hara, Hiroshi Kurahashi, Aiko Takahashi, Toru Nakayashiki[1], and Yoshikazu Nakamura: [1]Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, NIH-Bethesda, Maryland.

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. Conformational memory preserved in a weak-to-strong or strong-to-weak [*PSI*$^+$] conversion during transmission to Sup35 prion variants

The cytoplasmic [*PSI*$^+$] element of budding yeast represents the prion conformation of translation release factor Sup35. Much interest lies in understanding how prions are able to generate variation in isogenic strains. Recent observations suggest that a single prion domain, PrD, is able to adopt several conformations that account for prion strains. There are no reported mammalian or yeast PrD variants that strengthen the prion phenotype upon transmission at the molecular level. We report novel PrD variants of Sup35 that convert weak [*PSI*$^+$] to strong [*PSI*$^+$], and vice versa, upon transmission from wild-type Sup35. During the transmission from wild-type Sup35 to variant Sup35s, no conformational changes were detected by proteolytic fingerprinting and the original [*PSI*$^+$] strain was remembered upon return to wild-type Sup35. These findings suggest that during transmission to variant Sup35s, the [*PSI*$^+$] phenotype is variable while the original conformation is remembered. A mechanism of 'conformational memory' to remember specific [*PSI*$^+$] conformations during transmission is proposed.

4. Pharmaceutical RNA Design

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

The systematic evolution of ligands by exponential enrichment (SELEX) method is based on the *in vitro* selection of oligo-nucleotide ligands from large random-sequence libraries by repeated reactions of DNA transcription, RNA selection and RT-PCR amplification. The selected oligo-nucleotide ligands are called 'aptamer' which has high affinity and specificity to target molecules. We have initiated SELEX experiments using mammalian translation initiation factors including eIF4E, eIF4G, eIF1A and eIF4A provided by Dr. Nahum Sonenberg (McGill University, Canada) and Dr. John Hershey (UC-Davis, USA). eIF4G and eIF4A proteins are known to be crucial for catalyzing the initiation of protein synthesis by playing as a multipurpose ribosome adapter bridging eIF4E (cap-binding protein), eIF3 (40S subunit binding protein), eIF4A and Pab1p (poly-A binding protein), and an RNA unwinding helicase, respectively. Importantly, the abnormality in the protein level or the activity of either initiation factor is known to cause cell proliferation. We aim to test the possibility of developing anti-eIF RNA aptamers for novel diagnostic and therapeutic tools.

a. High affinity RNA for mammalian initiation factor 4E interferes with mRNA-cap binding and inhibits translation

The eukaryotic translation initiation factor 4F (eIF4F) consists of three polypeptides (eIF4A, eIF 4G and eIF4E) and is responsible for recruiting ribosomes to mRNA. eIF4E recognizes the mRNA 5′ cap structure (m^7GpppN where N is any nucleoside) and plays a pivotal role in control of translation initiation which is the rate-limiting step in translation. Overexpression of eIF4E has a dramatic effect on cell growth and leads to oncogenic transformation. Therefore, an inhibitory agent to eIF4E, if any, might serve as a novel therapeutic against malignancies that are caused by aberrant translational control. Along these lines, we developed two RNA aptamers, aptamer 1 and aptamer 2, with high affinity for mammalian eIF4E by *in vitro* RNA selection-amplification. Aptamer 1 inhibits the cap binding to eIF4E more efficiently than the cap analogue m7GpppN or aptamer 2. Consistently, aptamer 1 inhibits specifically cap-dependent *in vitro* translation while it does not inhibit cap-independent HCV IRES-directed translation initiation. The interaction between eIF4E and eIF4E-binding protein 1 (4E-BP1), however, was not inhibited by aptamer 1, hence the formation of heterotrimeric complex, aptamer · eIF4E · 4E-BP1 was detectable by a surface plasmon resonance assay. Aptamer 1 is composed of 86 nucleotides and the high affin-

ity to eIF4E is readily affected by even short deletions at both termini. Moreover, relatively large areas in the aptamer 1 fold are protected by eIF4E as determined by ribonuclease footprinting. These findings indicate that aptamers can achieve high affinity to a specific target protein via global conformational recognition. The genetic mutation and affinity study of variant eIF4E proteins suggests that aptamer 1 binds to eIF4E adjacent to the entrance of the cap-binding slot and blocks the cap binding pocket, thereby inhibiting translation initiation.

b. NMR structures of double loops of an RNA aptamer against mammalian initiation factor 4A

A high affinity RNA aptamer (APT58, 58 nucleotides long) against mammalian initiation factor 4A (eIF4A) requires its nearly entire nucleotide sequence for efficient binding. Since splitting either APT58 or eIF4A into two domains diminishes the affinity for each other, it is suggested that multiple interactions, or a global interaction between the two molecules accounts for the high affinity. To understand the structural basis of APT58's global recognition of eIF4A, we determined the solution structure of two essential nucleotide loops (AUCGCA and ACAUAGA) within the aptamer using nuclear magnetic resonance (NMR) spectroscopy. The AUCGCA loop is stabilized by a U-turn motif and contains a non-canonical A: A base pair (the single hydrogen bond mismatch: Hoogsteen/Sugar-edge). On the other hand, the ACAUAGA loop is stabilized by an AUA tri-nucleotide loop motif and contains the other type of A: A base pair (single hydrogen bond mismatch: Watson-Crick/Watson-Crick). Considering the known structural and functional properties of APT58, we propose that the AUCGCA loop is directly involved in the interaction with eIF4A, while the flexibility of the ACAUAGA loop is important to support this interaction. The Watson-Crick edges of C7 and C9 in the AUCGCA loop may directly interact with eIF4A.

c. RNA aptamers selected against the receptor activator of NF-kappaB acquire general affinity to proteins of the tumor necrosis factor receptor family

The receptor activator of NF-κB (RANK) is a member of the tumor-necrosis factor (TNF) receptor family and acts to cause osteoclastgenesis through the interaction with its ligand, RANKL. We isolated RNA aptamers with high affinity to human RANK by SELEX. Sequence and mutational analysis revealed that the selected RNAs form a G-quartet conformation that is crucial for binding to RANK. When the aptamer binding to RANK was challenged by RANKL, there was no competition between the aptamer and RANKL. Instead, the formation of a ternary complex, aptamer-RANK-RANKL, was detected by a spin down assay and by BIAcore surface plasmon resonance analysis. Moreover, the selected aptamer efficiently bound to other TNF receptor family proteins, such as TRAIL-R2, CD30, NGFR as well as osteoprotegerin (OPG), a decoy receptor for RANK. These results suggest that the selected aptamer recognizes, not the ligand-binding site, but rather a common structure conserved in the TNF receptor family proteins.

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Department of Basic Medical Sciences

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

Associate Professor Shinobu Imajoh-Ohmi, D. Sc.　助教授　理学博士　大　海　忍

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

1. Phagocytic differentiation and cell death

Taku Kuwabara and Shinobu Imajoh-Ohmi

Bacterial infection induces apoptotic cell death in human monoblastic U937 cells that have been pretreated with interferon_(U937IFN). Apoptosis occurs in a manner that is independent of bacterial virulence proteins. In the present study, we show that lipopolysaccharide (LPS), a membrane constituent of gram-negative bacteria, also induces apoptosis in U937IFN cells. LPS treatment led to the appearance of characteristic markers of apoptosis such as nuclear fragmentation and activation of caspases. While the caspase inhibitor Z-VAD-fmk prevented LPS-induced apoptosis as judged by its inhibition of nuclear fragmentation, it failed to inhibit cytochrome c release and loss of mitochondrial membrane potential. Transfection of peptides containing the BH4 (Bcl-2 homology 4) domain derived from the anti-apoptotic protein Bcl-XL blocked LPSinduced nuclear fragmentation and the limited digestion of PARP. These results suggest that LPS does not require caspase activation to induce mitochondrial dysfunction and that mitochondria play a crucial role in the regulation of LPS-mediated apoptosis in U937IFN cells.

2. Proteolysis and apoptotic 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. Furthermore, in some specific cells such as polymorphonuclear leukocytes, other proteases might be involved in cell death.

a. Cleavage of nonmuscle myosin heavy chain-A during apoptosis in human Jurkat T cells

Masahiko Kato, Hiroyuki Fukuda, Takashi

Nonaka and Shinobu Imajoh-Ohmi

We have previously reported that calpastatin, an endogenous inhibitory protein of calpain, is cleaved by a caspase-3-like protease during apoptosis in human Jurkat T cells. The caspase-catalyzed calpastatin loses the amino-terminal inhibitory unit, which undergoes further degradation in dying cells. We found here that non-muscle myosin heavy chain-A (NMHC-A) is cleaved during apoptosis in Jurkat cells by using a cleavage-site-directed antibody for calpastatin. The cleavage-site-directed antibody was raised against the amino-terminal fragment of calpastatin, and this antibody detected the in vitro cleaved calpastatin fragment. Although cleaved calpastatin was not detected, a 95-kDa polypeptide (p95) was detected in apoptotic cells by this antibody. This p95 was identified as the carboxyl-terminal fragment of NMHCA based on the results of peptide mass spectrometry fingerprinting and amino-terminal sequencing. Furthermore, two cleavage sites on NMHC-A, Asp-1153 and Asp-1948, were determined, and three cleaved fragments of NMHC-A, one cleaved at Asp-1153 and the other two cleaved at Asp-1948, were detected by cleavage-site-directed antibodies against each cleavage site. The results of confocal immunofluorescence microscopic analysis show that the cleavage at Asp -1948 occurs faster than that at Asp-1153 during apoptosis. In addition, the Asp-1153 cleaved fragment was distributed diffusely in the cytoplasm of apoptotic cells, whereas the Asp-1948 cleaved fragments were detected as condensed dots. In conclusion, our findings can be summarized as follows: (i) NMHC-A is cleaved at two sites during apoptosis, (ii) the timing of cleavage is different between these two cleavage sites, and (iii) the distribution of cleaved fragments is different in apoptotic cells.

b. Limited proteolysis of actin in polymorphonuclear leukocytes

Junko Ohmoto and Shinobu Imajoh-Ohmi

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

3. Proteome analysis of cancer cells treated with ITIIα-antisense oligonucleotide

Akira Nakanishi, Yoshiyasu Ohta, Koichi Kutsuzawa, Hiroyuki Fukuda and Shinobu Imajoh -Ohmi

Topoisomerase IIα is a ubiquitously expressed nuclear protein required for cell division in all organisms. This enzyme is the primary cellular target of several anticancer drugs. ITIIα (Inhibitor of Topoisomerase IIα) binds and inhibits the activity of topoisomerase IIα. We have found reduced expression of ITIIα correlates with topoisomerase IIα upregulation and the antisense oligonucleotide of ITIIα has been shown to cause growth arrest and apoptosis of cancer cells in vitro and in vivo. However, the signaling pathways leading to changes in cell growth are unclear. In order to investigate the protein expression alterations associated with the influence of the antisense oligonucleotide, we have comparatively analyzed the protein expression of HeLa cells transfected with antisense and control cells by two-dimensional gel electrophoresis. Image analysis of CBB stained 2-D gels revealed that nine protein spots showed significant expression changes in response to antisense treatment and the characterization of proteins is in progress by mass spectrometry. Among them, proteins involved in apoptosis, metabolism, components of cytoskeleton and heat shock protein were identified. We detected that the down-regulation of one of them targets, Hsp90, correlates with the down-regulation of ITIIα by the ITIIα-antisense oligonucleotide. The decrease of Hsp90 induced the down-regulation of Hsp90 client proteins, such as Raf-1 and Polo-like kinase. These results suggest that ITIIα-antisense affect the expression of other proteins as well as topoisomerase IIα and Hsp90 might be linked to processes involving apoptosis.

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. A novel method for hunting substrates of limited proteolysis

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

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

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

Jin Ling, Hiroyuki Fukuda and Shinobu Imajoh-Ohmi

E64c, [L-3-trans-carbonyloxirane-2-carbonyl]-L-leucine(3-methylbutyl)amide, is a synthetic inhibitor for cysteine proteases such as cathepsins B, H, L and calpain. To inhibit intracellular cysteine proteases E 64 d , [L-3-trans-ethoxycarbonyloxirane-2-carbonyl] -L-leucine (3-methylbutyl)amide, a membrane-permeable derivative of E64c is used instead of E64c. E64d penetrates into the cell where cellular esterases convert it to E64c that covalently binds to the SH group of active center in enzymes. Thus, anti-E64c antibody is a useful probe for in vivo analysis of cysteine proteases.

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

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

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Department of Basic Medical Sciences

Division of Molecular and Developmental Biology
染色体制御分野

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Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells as well as differentiated cells with emphasis on intracellular signal transduction. For this purpose, we are using systems ranging from zebrafish, chick, mouse and culture cells. The major research areas of interest are on: 1) development and regeneration of eye, 2) roles of cytokines and their receptors in hematopoietic stem cells, 3) Use of dendritic cells for cell therapy. 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.

The neural retina is a part of the central nervous system (CNS), and regeneration of the retina from retinal stem cells or other sources by transplantation is a critical issue from both clinical and neurobiological points of view. Although reports of successful regeneration of the CNS from neural stem cells (NSC) have appeared in the literature, such has not been the case for the vertebrate neural retina. Furthermore, the nature of retinal stem cells has not been clarified, making it difficult to attempt regeneration of the retina. Based on the technique and knowledge which we have accumulated through works of haematopoietic systems, we started to attempt to identify mammalian retinal stem cells and developmental process. For developmental biological analyses, we introduced zebrafish system in addition to mouse system as model animals. We are also continue to work on haematological projects, and bidirectional cooperative progress between neurological and haematological works is one of unique features of our laboratory. Projects which gave major findings during 2004 are as follows.

SSEA-1 marks regionally restricted immature subpopulations of embryonic retinal progenitor cells

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Identification and expansion of retinal progenitor cells are critical issues from both scientific and clinical aspects. Here, we identified SSEA-1 (CD15) as a novel surface antigen that can be used to define immature retinal progenitor cells. SSEA-1-expressing retinal cells were found in the peripheral region of the early embryonic mouse retina, and then their number dramatically disappeared along with retinal development. FACS analysis showed SSEA-1 strong positive cells co-expressed Ki67 proliferation antigen in all the examined developmental stages. The SSEA-1-expressing cells formed larger colonies than non-expressing ones in retinal re-aggregation cultures. Moreover, late onset of rhodopsin expression was observed in SSEA-1-positive progenitor cells, supporting the idea that these cells have an intrinsically immature

character. Differential expression of Wnt signal-related genes between SSEA-1 positive and negative subpopulations of retina cells was revealed, and the expression of constitutively active forms of Wnt signaling molecules resulted in a greater number of SSEA-1 positive cells. Taken together, we propose SSEA-1 as a surface marker to define regionally restricted immature subset of progenitor cells of mouse neural retina, and positive regulation of SSEA-1 cells by Wnt signals was suggested.

Negative regulation of retinal-neurite extension by Wnt-Lef-1 signalling pathway

Yasuo Ouchi, Yoko Tabata, Ken-ichi Arai[1], and Sumiko Watanabe

Although much data have accumulated regarding the regulation of neurite extension in the mouse brain, such mechanism in the neural retina has remained to be clarified. To delineate the role of Wnt signalling in retinal development, we expressed, by using a retrovirus vector mediated expression system, various mutants forms of Wnt signalling members in E17.5 mouse retinal explant cultures, which are an excellent system to examine retinal development *in vitro*. Expression of constitutively active b-catenin or constitutively active Lef-1 in the retinal cells resulted in failure of neurite extension.

Conversely, the neurite extension of retinal cells expressing dominant-negative Lef-1 was enhanced, suggesting that the b-catenin negatively regulates neurite extension in the retina through Lef-1 transcriptional activity. On the other hand, proliferation and differentiation of retinal cells into mature retinal cells such as rod-photoreceptor cells and Muller glia cells were not affected by perturbation of the Wnt-Lef-1 pathway. As in the retinal cells, activation of b-catenin-Lef-1 signalling inhibited NGF-induced neurite extension in PC12 cells without affecting their proliferation. Interestingly, the Wnt/Lef-1 signalling pathway suppressed the neurite extension without affecting Mek-1 signal activity, which is known to promote neurite extension. We found that the MAPK was activated during development in retinal explant cultures, but that perturbation of MAPK signals did not affect neurite extension. Taken together, our data suggest that the Wnt pathway functions for proper neurite extension by opposing positive signals for promotion of neurite extension that are distinct from those of the MAPK pathway.

Foxl 1 regulates midbrain formation through suppression of shh expression in zebrafish

Chisako Nakada, Yoko Tabata, Shinya Satoh, Ken-ichi Arai[1], and Sumiko Watanabe

We had identified a zebrafish forkhead transcription factor l1 (zfoxl1), as a gene strongly expressed in neural tissues, such as midbrain, hindbrain, and otic vesicle in early embryonic stage. Loss of the function of zebrafish foxl1 by MO resulted in failure of midbrain and eye development, and formation of pectral fin. Interestingly, ectopic expression of *shh* in the tectum and the elevation of *pax-2.1* expression in optic stalk were observed in the morphants. In contrast, expression of *pax-6* was suppressed in the pre-tectum and tectum regions, suggesting perturbation of shh signaling pathway. Injection of mRNA of *zfoxl1* into fertilized eggs led to various degree of defects of forebrain formation including deletion of the eye. Expression of *shh* and *krox-20* in 80-90% epiboly stage were reduced in the embryos injected with *zfoxl1* mRNA. Expression of zfoxl1-EnR rather than zfoxl1-VP16 showed similar phenotype with that induced by zfoxl1-mRNA, suggesting that zfoxl1 may act as a transcriptional repressor in zebrafish embryos. In fact, zfoxl1 suppressed isolated 2.3 kb *shh* promoter activity in PC12 cells but not in fibrobrasts, suggesting neural tissues specific transcriptional repression activity of zfoxl1. Taken together, we proposed zfoxl1 as a novel regulator of midbrain formation through suppression of *shh* expression.

Zebrafish numb homologue regulates left-right asymmetry by down-regulating the Notch signalling

Yuichi Niikura, Yoko Tabata, and Sumiko Watanabe

Mammalian Numb and Numb-like genes play important roles of neural development. We have cloned zebrafish Numb homologue (zNumb) by searching data base. zNumb contains several typical Numb specific features which are not observed in numb-like strongly suggested that the zNumb is orthologue of mammalian numb rather than numb-like. However, zNumb's chromosomal localization indicates colinearity with genes located around mouse and human Numb-like, suggesting occurrence of interchromosomal recombination in revolution. zNumb expressed in wide variety of adult tissues like mouse Numb gene. Expression of zNumb in zebrafish embryos by injection of zNumb-mRNA resulted in abnormal heart positioning such as reversed or no leftward shift of the heart. Roles of Notch for establishment of left side field is known in vertebrate from various experimental evidence.

Accordingly, as expected, we found that the activation of endogenous Notch by ectopic expression of *deltaD* resulted in the bilateral expression of left side-specific gene *lefty2* in the lateral plate mesoderm in zebrafish. Furthermore, no transcript of *lefty2* was observed in zNumb-expressing embryos, suggesting that zNumb perturbed the establishment of the left side field through down-regulation of endogenous Notch signaling.

Analysis of C-type lectin Ly49Q in murine plasmacytoid DC.

Yumiko Kamogawa, Jun Ohkawa, Jingtao Chen, Minkwon Cho, Kouji Ishida, Makiko Tohma and Sumiko Watanabe

Plasmacytoid Dendritic cell (pDC) or type I interferon-producing cells (IPCs) have been reported as immature (precursor) DCs and high producers of type I IFN in response to viral infection and other stimuli such as bacterial CpG in both humans and mice. pDCs control myeloid DCs and activate macrophages and NK cells through release of cytokines. In addition, upon viral infection, pDCs have the ability to differentiate into mature DCs, which are capable of inducing naive T-cell proliferation towards Th1 cells and expansion of IL-10-producing regulatory T cells. Thus, pDCs initiate an immune response by linking innate and acquired immunity upon activation. The frequency of pDCs in blood and secondary lymphoid organs is low in both humans and mice. It is therefore difficult to study the nature of this DC subset. BDCA-2 has been recently identified as a specific marker for pDCs in humans and it was suggested that this C-type lectin receptor may participate in antigen presentation. Two antibodies have been recently reported as specific to murine pDCs in mice. However, none of the antigens specific to murine pDCs have been molecularly cloned.

The function of DCs is mediated in part by the expression of specialized surface receptors. For example, DEC-205 expressed on the DC surface binds and internalizes carbohydrate-bearing antigens by receptor-mediated endocytosis, thus playing a role in antigen uptake and presentation.

In order to identify the molecules that control pDC functions, we generated a murine pDC-specific mAb, designated 2E6, and the molecular cloning of the cDNA encoding Ly49Q recognized by this newly generated mAb. Ly49Q encodes a type II C-type lectin membrane-associated polypeptide of 247 amino acids containing a single CRD motif at the COOH-terminal and an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic region. By using 2E6 Ab, we found that previously defined pDCs ($B220^+$, $CD11c^+$, $CD11b^-$) in bone marrow can be divided into two groups (Ly49Q + and Ly49Q−). While both subsets produced IFN-a upon CpG and herpes simplex virus stimulation, $Ly49Q^-$ pDCs responded poorly to influenza virus. In addition, $Ly49Q^-$ pDCs produced inflammatory cytokines such as IL-6, IL-12, and TNF-a upon stimulation at lower levels than those produced by $Ly49Q^+$ pDCs. In contrast to bone marrow, $Ly49Q^+$ pDCs were only found in peripheral blood, lymph node, and spleen. These results indicate that Ly49Q is a specific marker for peripheral pDCs and that expression of Ly49Q defines two subsets of pDCs in bone marrow.

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